Reviewers' comments:

Reviewer #1 (Remarks to the Author):

This manuscript by Zhou et al investigated the role of VEGFR SUMOylation in angiogenesis. The authors found reduced angiogenesis in endothelial specific SENP1 knock out (SENP1-ECKO) mice. The authors also found that VEGFR K1270 SUMOylation kept VEGFR in the Golgi, and reduced surface expression, which inhibited VEGFR-dependent angiogenic signaling. SENP1 expression was decreased by hyperglycemia, lead to VEGFR SUMOylation, and subsequently decrease VEGFR2-dependent angiogenic signaling. Lastly, the authors found that overexpression of VEGFR2 SUMOylation site inhibited angiogenesis. The data is potentially interesting, but the potential role of SENP1 in angiogenesis has been reported1. Therefore, to clarify the role of VEGFR2 SUMOylation would be critical.

Major
1. The authors stated that "However, the role of SENP1 in endothelial cells and angiogenesis has not been determined" in the introduction, but this is misleading. Xu et al have reported that "induction of SENP1 in endothelial cells contributes to hypoxia-derived VEGF expression and angiogenesis". Xu et al showed that the depletion of SENP1 decreases VEGF expression via destabilizing HIF1a and angiogenetic activity in endothelial cells1. In addition, Xu et al showed the reduction of angiogenesis in embryonic renal glomeruli in SENP1-/- than wild type littermates.
2. Therefore, the novelty of this study is to determine the crucial role of VEGFR2 SUMOylation in angiogenesis. The authors stated that they could not find any decrease in HIF1a protein expression and VEGF mRNA level in ischemic hindlimb model in SENP1-EKO mice. As the authors stated that this might be due to the ischemia in the less angiogenic tissue, it may be difficult to exclude the possibility of the HIF-1a-VEGF-mediated effect on the depletion of SENP1-mediated reduction of angiogenesis. If the authors repeated these experiments in vitro and did not find any difference of HIF1a and VEGF expression under ischemia, it is necessary to discuss this controversy in details.
3. It is possible that VEGFR-SUMO chimera inhibited and VEGFR K1270R mutant increased VEGFR tyrosine phosphorylation, which is independent on SUMOylation because of structural change. First, it is necessary to overexpress VEGFR-WT and VEGFR K1270R mutant and compare the VEGFR tyrosine phosphorylation after VEGF stimulation (without siSENP21 transfection). Is VEGFR tyrosine phosphorylation higher in VEGFR K1270R mutant than VEGFR-WT? Next, the authors also need to perform these studies in wound healing assay and compare VEGFR-WT and VEGFR-K1270R mutant in control (without siSENP1 condition).
4. Previously, the authors reported that SENP1 causes apoptosis induced by HIPK1-ASK1 signaling in endothelial cells and fibroblasts. In this study, the authors showed that the depletion of SENP1 inhibits Akt activation, which may increase apoptotic responses. This can be contradicted. Please check HIPK1-ASK1 signaling in ischemic hindlimb model and subsequent endothelial apoptosis.
5. The authors stated that SENP1 expression was decreased in diabetes. Please show the pathological role of VEGFR SUMOylation in diabetes. This would be done by Ad-VEGF with Ad-VEGFR2-WT and Ad-VEGFR2-K1270R in non-diabetic and diabetic mice as shown in Fig.6.
6. Previously the authors reported that inflammation induced SENP1, and promotes the de-SUMOylation of GATA2 and IkB in endothelial cells, and increases NF-kB activity2. Since it is well known that diabetes and hyperglycemia can increase NF-kB activation, please study why hyperglycemia decrease, but inflammatory stimuli can increase SENP1 expression. Does this mean that the depletion of SENP1 inhibits both angiogenesis and inflammation?
6. The authors previously reported that SENP1-mediated NEMO deSUMOylation and inhibits NF-kB activation3. This can be contradictory to the data of GATA2 and IkB. Since the strong relationship between angiogenesis and inflammation has been well established, please clarify the role of VEGFR SUMOylation in regulating GATA2, NEMO, IkB SUMoylation in SENP1 EKO system.

Ref
1. Xu, Y., et al. Induction of SENP1 in endothelial cells contributes to hypoxia-driven VEGF expression and angiogenesis. J Biol Chem 285, 36682-36688 (2010).

2. Qiu, C., et al. The critical role of SENP1-mediated GATA2 deSUMOylation in promoting endothelial activation in graft arteriosclerosis. Nat Commun 8, 15426 (2017).
3. Shao, L., et al. SENP1-mediated NEMO deSUMOylation in adipocytes limits inflammatory responses and type-1 diabetes progression. Nat Commun 6, 8917 (2015).

Reviewer #2 (Remarks to the Author):

Summary:
The manuscript by Zhou et al. shows that deletion of the SUMO endopetidase SENP1 in endothelial cells of mice results in altered VEGFR2 signaling and in reduced VEGF-induced and pathological angiogenesis. It is shown that deletion of SENP1 in EC results in increased SUMOylation of VEGFR2, which results in retention of the receptor in the Golgi. The authors identify K1270 on VEGFR2 as the potential SUMOylation site that would be responsible for attenuating VEGFR2 signaling. Finally, hyperglycemia and diabetes in mice provoke downregulation of SENP1 that could contribute to increased VEGFR2 SUMOylation and reduced angiogenesis in this pathological context.

Comments:
General
The demonstrations that VEGFR2 is SUMOylated and that SUMOylation results in altered trafficking of the receptor are convincing and interesting. The consequence on pathological angiogenesis of SENP1 deletion in EC highlights the potential implication of this novel post-translational modification of VEGFR2 on signaling in normal and pathological settings. However, the functional role of SENP1 in VEGFR2 signaling in EC remains obscure. It is not clear if SENP1 or an unidentified SUMO E3 ligase is the principal regulator of normal VEGFR2 signaling by SUMOylation. The fact that deletion of a de-SUMOylating enzyme in EC results in altered VEGFR2 signaling and in increased SUMOylation is somewhat an indirect demonstration that SUMOylation is a regulator of VEGFR2-dependent angiogenesis. It would be worthwhile to demonstrate that VEGF-dependent VEGFR2 SUMOylation is actively regulated both by a SUMO E3 ligase and a SUMO endopeptidase, which contribute to VEGFR2-mediated effects in EC.

Specific
It is not clear if the consequences of SENP1 deletion in EC are all due to increased VEGFR2 SUMOylation. The specificity of these effects could be investigated further. For instance, VEGFR2 levels appear to be increased in ECKO (Figure 1J) and in HUVEC transfected with SENP1 siRNA (Figure 3F); are the levels of other EC-specific proteins, such VE-cadherin, affected as well? How about other known SUMOylated proteins in EC?

The reduction in p-VEGFR2 levels in HUVEC transfected with siSENP1 are marginal. Statistics and quantification on multiple experiments should be provided. Does this slight reduction in VEGFR2 activation explain all the effects of SENP1 downregulation?

There is no demonstration that SUMOylation of VEGFR2 is agonist dependent. Does SUMOylation of VEGFR2 occur at the plasma membrane or in the Golgi in response to exposure of EC with VEGF? Could a possible SUMO ligase responsible for this be identified?

Figure 4F shows that expression of VEGFR2-K1270F or of the TKR mutant abolishes the presence of HA-SUMO1 in the Golgi. This suggests that VEGFR2 is the only sumoylated protein in this organelle, which would be surprising. The specificity of this assay is questionable.

It is mentioned in the text that VEGFR2 co-localize with Golgi marker following glucose treatment. However, no Golgi marker staining is presented in Figure 7B (only VEGFR2 and nuclei).

Minor comments:

This phrase in the abstract should be re-written:
SENP1-ECKO mice survive after birth with normal development and growth, but exhibit reduced pathological angiogenesis and tissue repair in ischemic hindlimb; VEGF-induced cornea, retina and ear angiogenesis.

Title of the second paragraph on page 6 should be corrected: VEGF-induced neovascularization was greatly /augmented/REDUCED/ in SENP1-ECKO mice.

Third paragraph on page 7 - describing Figure 4E - is difficult to understand.

Reviewer #3 (Remarks to the Author):

The manuscript entitled "SUMOylation of VEGFR2 regulates its intracellular trafficking and pathological angiogenesis" demonstrates that VEGFR2 signaling is regulated by deSUMOylation in pathological conditions. This study uses an endothelial cell-specific deletion of SENP1 as well as several cell culture models to demonstrate that SENP1 decreases SUMOylation of VEGFR2 and increases VEGFR2 signaling. Furthermore, the authors demonstrate that SUMOylated VEGFR2 accumulates in the Golgi resulting in reduced VEGFR2 surface expression and signaling. However, the manuscript is plagued by the lack of mass spectrometry data on SUMOylation of VEGFR2 in vivo. The primary support for the SUMOylation sites determined in this manuscript is based on molecular modeling. Mass spectrometry performed using endothelial cells to confirm these SUMOylation sites on VEGFR2 would be absolutely crucial for this manuscript to substantiate its claimed significance and impact in the field. Additionally, there are numerous concerns regarding missing proper controls, quality of figures, and concepts put forward.

1. Given the claimed importance of SUMOylation on the function of VEGFR2, it seems highly unusual that the SENP1-ECKO mice have no obvious developmental defects. Importantly, expression of the SENP1 in endothelial cells during different developmental stages has never been examined.

2. As a foundation for the elaborated studies presented in the manuscript, the SENP1-ECKO endothelial cells should exhibit decreased cell surface levels of VEGFR2; however, this is never demonstrated. Without this data, the foundation of this study is very weak. Given that augmented levels of total VEGFR2 are prominent in endothelial cells lacking SENP1, surface levels of VEGFR2 in SENP1-ECKO versus WT primary endothelial cells should be firmly determined.

3. In the text, HUVECs transfected with vector control are compared to HUVECs expressing VEFR2 construct in the context of Figure 6A; however, there is no vector control in Figure 6A. This should be included to the figure that compares HUVECs expressing VEGFR2 construct to HUVECs expressing VEGFR2-SUMO construct. More importantly, experiments utilizing endothelial cells expressing an endogenous VEGFR2-SUMO fusion protein using gene-editing technique comprising CRISPR-Cas9 would be much needed to solidify their claim that SUMOylation hinders VEGFR2 cell surface distribution.

4. The experiments in Figures 4F, 5B, 5C, 5D, 5E, and Supplemental 5B should be performed in HUVECs or primary mouse endothelial cells rather than COS-7 cells to avoid artifacts observed in non-endothelial cells which intrinsically do not express and process any VEGFR2. The processing machineries in endothelial cells involved in VEGFR2 expression would be considerably distinct from those in cells that have never seen VEGFR2 expression.

5. All of the immunoprecipitations shown in figures 1J, 4C, and 7A are lacking a loading control. The bands for the immunoprecipitated SUMO1 should be shown to demonstrate that the IP itself worked correctly.

6. Supplemental Figure 3C is missing a WT control for 0, 5, and 15 minutes of VEGF treatment.

7. The quality of the immunofluorescent images is lacking. Figures 4A, 4E, 4F, 7B, 7E, and Supplemental 1B would strongly benefit with higher resolution images. Figures 4E and 5C should be shown as split channel images. Furthermore, several panels in Figures 6F and Supplemental 6B are either fuzzy and out of focus or extremely overexposed making it difficult to analyze.

8. The model depicted in Figure 8 includes KIF3B and T-SNARE in the context of exocytosis. However, no experiments looking at the function of these two proteins are performed. This should be analyzed to strengthen this claim.

9. It would be beneficial to show confirmation of Type I diabetes induced by STZ treatment by showing the blood glucose levels over the course of STZ treatment at least in the supplemental figures.

10. Numerous grammar errors and incorrect or convoluted sentence structure throughout the manuscript make it somewhat difficult to read.

11. It is indicated in the text and figure legend that a Golgi marker was used in Figures 7B and 7E. This is not indicated in the figure; the figure itself is lacking labels for what is stained. If a Golgi marker was used here, a panel for this should be included. For consistency, labels to indicate Golgi VEGFR2 and cytosolic/membrane VEGFR2 as used in Supplemental Figure 4 would make Figure 7 clearer. Furthermore, adding a Golgi marker to Supplemental Figure 4 would also make this figure clearer.

12. The text claims that TNFR2 surface localization is not affected by SENP1 deletion, which is supported by Figure 4B. However, Supplemental Figure 4B appears to show Golgi localization of TNFR2. This is not addressed at all. Thus, the Golgi retention of plasma membrane proteins owing to SENP1 deficiency appears not specific to VEGFR2.

Response to Reviewers' comments:

RESPONSE TO REVIEWER #1

1. The authors stated that "However, the role of SENP1 in endothelial cells and angiogenesis has not been determined" in the introduction, but this is misleading. Xu et al have reported that "induction of SENP1 in endothelial cells contributes to hypoxia-derived VEGF expression and angiogenesis". Xu et al showed that the depletion of SENP1 decreases VEGF expression via destabilizing HIF1a and angiogenetic activity in endothelial cells [1]. In addition, Xu et al showed the reduction of angiogenesis in embryonic renal glomeruli in SENP1-/- than wild type littermates.

- We agree with the reviewer and we have modified the sentence: "The role of SENP1 in endothelial cells and angiogenesis has been examined. Specifically, it is reported that induction of SENP1 in endothelial cells contributes to hypoxia-derived VEGF expression and angiogenesis in vitro *cells* [1]. Endothelial SENP1 also suppresses Notch overactivation and contributes to normal retinal sprouting at neonatal stages [2]. Retinal sprouting in SENP1-ECKO is similar to WT mice after day 9 (Supplemental Fig.S2). However, the role of SENP1 in pathological angiogenesis has not been investigated.
In this regard, our study to define the role of SENP1-mediated VEGFR2 SUMOylation in pathological angiogenesis is innovative and significant.

2. Therefore, the novelty of this study is to determine the crucial role of VEGFR2 SUMOylation in angiogenesis. The authors stated that they could not find any decrease in HIF1a protein expression and VEGF mRNA level in ischemic hindlimb model in SENP1-ECKO mice. As the authors stated that this might be due to the ischemia in the less angiogenic tissue, it may be difficult to exclude the possibility of the HIF-1a-VEGF-mediated effect on the depletion of SENP1-mediated reduction of angiogenesis. If the authors repeated these experiments in vitro and did not find any difference of HIF1a and VEGF expression under ischemia, it is necessary to discuss this controversy in details.

- We have performed the experiments that Reviewer suggested. Indeed, we observed a decrease in HIF1α protein and VEGF-A mRNA under hypoxia in SENP1 knockdown ECs compared to WT ECs (Supplemental Fig.S3A-B), consistent with a previous report by Xu et al. We have discussed potential reasons for less changes in HIF1α protein expression and VEGF mRNA level in ischemic hindlimb model in SENP1-ECKO mice.
"SENP1 have been shown to stabilize HIF1α and HIF1α-dependent VEGF transcription [3]. Indeed, we observed a decrease in HIF1α protein and VEGF-A mRNA under hypoxia in SENP1 knockdown ECs compared to WT ECs (Supplemental Fig.S3A-B). We examined SENP1 and HIF1α protein as well as VEGF mRNA levels in the ischemic hindlimb tissues of WT and SENP1-ECKO. SENP1 protein was upregulated in WT mice in response to ischemia and only weakly detected in the SENP1-ECKO muscle tissues (**Fig.1H**), suggesting that the SENP1 protein was induced by ischemia primarily in the vascular endothelium. This was further confirmed for SENP1 expression in ischemic hindlimb by immunohistochemistry (Supplemental Fig.S3C). We did not observe significant differences between the two groups in HIF1α protein and VEGF mRNA levels (**Fig.1H-I**). This suggest that other cell types such as myocyte rather than EC primarily contributes to HIF1a and VEGF-A expression in ischemic hindlimb".

3. It is possible that VEGFR-SUMO chimera inhibited and VEGFR K1270R mutant increased VEGFR tyrosine phosphorylation, which is independent on SUMOylation because of structural change. First, it is necessary to overexpress VEGFR-WT and VEGFR K1270R mutant and compare the VEGFR tyrosine phosphorylation after VEGF stimulation (without siSENP1 transfection). Is VEGFR tyrosine phosphorylation higher in VEGFR K1270R mutant than VEGFR-WT? Next, the authors also need to perform these studies in wound healing assay and compare VEGFR-WT and VEGFR-K1270R mutant in control (without siSENP1 condition).

- We have performed the experiment that Reviewer suggested. To determine if the aberrant compartmentalization of VEGFR2 reduces VEGFR2 activity and angiogenic signaling, we first examined effects of SUMO1 conjugation on VEGFR2 activity. VEGFR2-WT, VEGFR2 K1270R (a form that cannot be SUMOylated) or VEGFR2-SUMO1 (a form that is constitutively SUMOylated) was expressed in HUVEC by lentivirus infection, and VEGF-induced p-VEGFR2 was examined by Western blotting. Compared to the vector control, VEGFR2 expression in HUVEC induced autophosphorylaton of VEGFR2-WT. While VEGFR2 K1270R

showed slightly stronger than WT in VEGF-induced VEGFR2 tyrosine phosphorylation, SUMO1 fusion to VEGFR2 diminished its autophoshorylation (**Fig.5C-D**). Function of VEGFR2 SUMOylation on EC migration in a monolayer "wound injury" assay was determined by expressing VEGFR2-WT, VEGFR2-SUMO1 or VEGFR2-K1270R mutant into HUVEC. Expression of VEGFR2-WT or VEGFR2-KR augmented VEGF-induced EC migration compared to the vector control cells. However, VEGFR2-SUMO1 significantly blunted VEGF-induced EC migration (**Fig.5E-F)**. These data suggest that KR mutation does not induces structural changes of VEGFR2.

4. Previously, the authors reported that SENP1 causes apoptosis induced by HIPK1-ASK1 signaling in endothelial cells and fibroblasts. In this study, the authors showed that the depletion of SENP1 inhibits Akt activation, which may increase apoptotic responses. This can be contradicted. Please check HIPK1-ASK1 signaling in ischemic hindlimb model and subsequent endothelial apoptosis.

- We believe these data are not contradicted to each other if we consider signaling pathways in the biological context.

1) The previous study on HIPK1-ASK1 is in TNF signaling while current study is in VEGF signaling (not specifically on Akt as Reviewer mentioned). TNF alone elicits multiple signaling, including activations of ASK1/JNK/p38, NF-kB and Akt while VEGF induces activations of Akt, PLC-γ and p38 pathways. Despite TNF a prototype inflammatory cytokine whereas VEGF is a proangiogenic factor, they do share some common downstream signaling. Particularly, they both induce vascular endothelial permeability and inflammation.

2) We have examined ASK1 activity in ischemic hindlimb as Reviewer suggested. Consistent with the role of SENP1 in ASK1 activation [4], SENP1 deletion also reduced ischemia-induced ASK1 activation (p-ASK1), which is known to mediate inflammation and cellular apoptosis [5]. Consistently, we observed reduced inflammation (macrophage infiltration staining) (Supplemental Fig.S3D-E) as well as cell death (TUNEL assay) in ischemic hindlimb of SENP1ecKO (Supplemental Fig.S3F-G).

- Therefore, reduced inflammation may contribute to impaired angiogenesis observed in SENP1-ECKO mice.

5. The authors stated that SENP1 expression was decreased in diabetes. Please show the pathological role of VEGFR SUMOylation in diabetes. This would be done by Ad-VEGF with Ad-VEGFR2-WT and Ad-VEGFR2-K1270R in non-diabetic and diabetic mice as shown in Fig.6.

- We have performed the studies. The role of VEGFR2 SUMOylation has been examined in both non-diabetic mice (revised Fig.5G-H) and diabetic mice (Fig.7).

6. Previously the authors reported that inflammation induced SENP1, and promotes the de-SUMOylation of GATA2 and IkB in endothelial cells, and increases NF-kB activity (Qiu et al . Since it is well known that diabetes and hyperglycemia can increase NF-kB activation, please study why hyperglycemia decrease, but inflammatory stimuli can increase SENP1 expression. Does this mean that the depletion of SENP1 inhibits both angiogenesis and inflammation? The authors previously reported that SENP1-mediated NEMO deSUMOylation and inhibits NF-kB activation (Shao et al). This can be contradictory to the data of GATA2 and IkB. Since the strong relationship between angiogenesis and inflammation has been well established, please clarify the role of VEGFR SUMOylation in regulating GATA2, NEMO, IkB SUMoylation in SENP1EKO system.

- We thank Reviewer for these great questions.

1) Why hyperglycemia decrease, but inflammatory stimuli can increase SENP1 expression? First, inflammation (e.g., cytokines) and hyperglycemia do not always have the same effects. Cytokines utilize their receptor induce specific downstream signaling cascades. Among cytokines, they have synergistic or antagonizing effects on many downstream targets. The exact mechanism by which SENP1 regulation by cytokines is currently unclear. On the other hand, hyperglycemia induced effects are largely attributed to ROS which may not always the same as cytokines.

Specifically, our data that SENP1 is upregulated in ischemia hindlimb is in agreement with the published work by Xu et al [1]. We have cited the paper and discussed in Discussion: "……the Senp1 gene promoter contains

hypoxia response element (HRE) and a mutation on the Senp1 promoter abolishes its transactivation in response to hypoxia [1]. Therefore, the induction of SENP1 in ischemic hindlimb is likely at a transcriptional level driven by HIF1". Contrasting to hypoxia-induced SENP1 expression, our data indicate that hyperglycemia attenuates SENP1 expression in EC. Hyperglycemia in EC could result in ROS generation [6,7], which is known to modulate SENPs expression and activity [8,9]. For example, ROS induce SENP3 thiol modifications and subsequent ubiquitination and degradation. ROS could also modify SENP1 at cysteine residues and attenuate SENP1 activity to increase protein SUMOylation. However, the exact mechanism for SENP1 downregulation by hyperglycemia needs more investigations.

2) Does this mean that the depletion of SENP1 inhibits both angiogenesis and inflammation? Yes, depletion of SENP1 indeed inhibits both angiogenesis and inflammation as we discussed in Response #4.

3) SENP1-mediated NEMO deSUMOylation and inhibition on NF-κB activation was observed in adipocytes [10]. SENP1 promotes the deSUMOylation of GATA2 and IκBα in endothelial cells, resulting in increased GATA2 stability, promoter-binding capability, and NF-κB activity [11]. SUMOylation can modify many targets, even signaling molecules in the same pathway (e.g., IκB and NF-κB) with opposite function. It is possible that SENP1 selects its substrates in a cell type-dependent manner. We believe that examining effects of VEGFR2 SUMOylation on GATA2, NEMO, IκB SUMOylation is out of the scope in our current study, and these studies will be further investigated in the future.

As the role of SENP1 in regulation of VEGFR2 has not been explored, we have therefore focused on SENP1-VEGFR2 in our current studies.

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1. Xu, Y.*, et al.* Induction of SENP1 in endothelial cells contributes to hypoxia-driven VEGF expression and angiogenesis. *J Biol Chem* **285**, 36682-36688 (2010).
2. Zhu, X.*, et al.* SUMOylation Negatively Regulates Angiogenesis by Targeting Endothelial NOTCH Signaling. *Circ Res* **121**, 636-649 (2017).
3. Cheng, J., Kang, X., Zhang, S. & Yeh, E.T. SUMO-specific protease 1 is essential for stabilization of HIF1alpha during hypoxia. *Cell* **131**, 584-595 (2007).
4. Li, X.*, et al.* SENP1 mediates TNF-induced desumoylation and cytoplasmic translocation of HIPK1 to enhance ASK1-dependent apoptosis. *Cell Death Differ* (2008).
5. Yin, M.*, et al.* ASK1-dependent endothelial cell activation is critical in ovarian cancer growth and metastasis. *JCI Insight* **2**(2017).
6. Dokun, A.O., Chen, L., Lanjewar, S.S., Lye, R.J. & Annex, B.H. Glycaemic control improves perfusion recovery and VEGFR2 protein expression in diabetic mice following experimental PAD. *Cardiovasc Res* **101**, 364-372 (2014).
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8. Chhunchha, B., Fatma, N., Kubo, E. & Singh, D.P. Aberrant sumoylation signaling evoked by reactive oxygen species impairs protective function of Prdx6 by destabilization and repression of its transcription. *FEBS J* **281**, 3357-3381 (2014).
9. Yan, S.*, et al.* Redox regulation of the stability of the SUMO protease SENP3 via interactions with CHIP and Hsp90. *EMBO J* **29**, 3773-3786 (2010).
10. Shao, L.*, et al.* SENP1-mediated NEMO deSUMOylation in adipocytes limits inflammatory responses and type-1 diabetes progression. *Nature communications* **6**, 8917 (2015).
11. Qiu, C.*, et al.* The critical role of SENP1-mediated GATA2 deSUMOylation in promoting endothelial activation in graft arteriosclerosis. *Nature communications* **8**, 15426 (2017).

RESPONSE TO REVIEWER #2

1. The demonstrations that VEGFR2 is SUMOylated and that SUMOylation results in altered trafficking of the receptor are convincing and interesting. The consequence on pathological angiogenesis of SENP1 deletion in EC highlights the potential implication of this novel post-translational modification of VEGFR2 on signaling in normal and pathological settings. However, the functional role of SENP1 in VEGFR2 signaling in EC remains obscure. It is not clear if SENP1 or an unidentified SUMO E3 ligase is the principal regulator of normal VEGFR2 signaling by SUMOylation. The fact that deletion of a de-SUMOylating enzyme in EC results in altered VEGFR2 signaling and in increased SUMOylation is somewhat an indirect demonstration that SUMOylation is a regulator of VEGFR2-dependent angiogenesis. It would be worthwhile to demonstrate that VEGF-dependent VEGFR2 SUMOylation is actively regulated both by a SUMO E3 ligase and a SUMO endopeptidase, which contribute to VEGFR2-mediated effects in EC.

- Thank you for your excellent suggestions. We agree with the Editors, identifying E3 SUMO ligases and other SUMO endopeptidase are not essential for the current work and would be investigated in our future studies. We have incorporated into Discussion.
"In addition, the E3 ligase responsible for VEGFR2 SUMOylation has not been identified. Therefore, additional mechanisms for hyperglycemia-induced VEGFR2 SUMOylation need further investigation".

2. It is not clear if the consequences of SENP1 deletion in EC are all due to increased VEGFR2 SUMOylation. The specificity of these effects could be investigated further. For instance, VEGFR2 levels appear to be increased in ECKO (Figure 1J) and in HUVEC transfected with SENP1 siRNA (Figure 3F); are the levels of other EC-specific proteins, such as VE-cadherin, affected as well? How about other known SUMOylated proteins in EC?

- We have examined another EC surface marker VE-cadherin which was not affected by SENP1 deletion in tissues (Fig.1J, 3G, 6D). GATA2 was slightly reduced by SENP1-deletion in EC (Fig.3G, 6D), consistent with our previous observation that SENP1 stabilizes GATA2 in EC [11]. SENP1-mediated NEMO deSUMOylation and inhibition on NF-κB activation was observed in adipocytes [10].

3. The reduction in p-VEGFR2 levels in HUVEC transfected with siSENP1 are marginal. Statistics and quantification on multiple experiments should be provided. Does this slight reduction in VEGFR2 activation explain all the effects of SENP1 downregulation?

- We have performed multiple experiments and we have obtained better knockdown of SENP1 therefore stronger inhibition on VEGFR2 signaling. We have added new figure with statistical analyses (Revised Fig.3G-H).

There is no demonstration that SUMOylation of VEGFR2 is agonist dependent. Does SUMOylation of VEGFR2 occur at the plasma membrane or in the Golgi in response to exposure of EC with VEGF? Could a possible SUMO ligase responsible for this be identified?

- Thank you for your suggestions. We observed VEGF-induced VEGFR2 SUMOylation and Golgi accumulation in a time-dependent manner, peaking at 15 min upon VEGF engagement (Fig.4E-F). With regards to SUMO E3 ligase, please see response #1.

Figure 4F shows that expression of VEGFR2-K1270F or of the TKR mutant abolishes the presence of HA-SUMO1 in the Golgi. This suggests that VEGFR2 is the only sumoylated protein in this organelle, which would be surprising. The specificity of this assay is questionable.

- We agree with Reviewer that overexpression of SUMO1 might cause artificial SUMOylation of target proteins. Therefore, we have eliminated the original Fig.4F in Cos7 cells. Instead, we present new data using HUVECs (revised Fig.4).

4. It is mentioned in the text that VEGFR2 co-localize with Golgi marker following glucose treatment. However, no Golgi marker staining is presented in Figure 7B (only VEGFR2 and nuclei).

- We have added the Golgi marker (revised Fig.7B).

Minor comments:

This phrase in the abstract should be re-written:
SENP1-ECKO mice survive after birth with normal development and growth, but exhibit reduced pathological angiogenesis and tissue repair in ischemic hindlimb; VEGF-induced cornea, retina and ear angiogenesis.
- We have revised the sentence: We show that SENP1-ECKO mice exhibit reduced pathological angiogenesis and tissue repair in ischemic hindlimb, and in VEGF-induced cornea, retina and ear angiogenesis models.

Title of the second paragraph on page 6 should be corrected: VEGF-induced neovascularization was greatly /augmented/REDUCED/ in SENP1-ECKO mice.
- We have corrected the title: VEGF-induced neovascularization was greatly reduced in SENP1-ECKO mice.

Third paragraph on page 7 - describing Figure 4E (revised 4G) - is difficult to understand.
- We have revised the sentence: VEGFR2-WT was distributed on plasma membrane in normal ECs. However, SENP1 deletion induced a Golgi accumulation. Interesting, the mutation at K1270 (K1270R or TKR) diminished the accumulation of VEGFR2 at the Golgi in ECs (**Fig.4G**; Supplemental Fig.S6C-D for split channel images). These results suggest that a potential modification of VEGFR2 at K1270 induces its Golgi accumulation in SENP1-deficient ECs.

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1. Xu, Y.*, et al.* Induction of SENP1 in endothelial cells contributes to hypoxia-driven VEGF expression and angiogenesis. *J Biol Chem* **285**, 36682-36688 (2010).
2. Zhu, X.*, et al.* SUMOylation Negatively Regulates Angiogenesis by Targeting Endothelial NOTCH Signaling. *Circ Res* **121**, 636-649 (2017).
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11. Qiu, C.*, et al.* The critical role of SENP1-mediated GATA2 deSUMOylation in promoting endothelial activation in graft arteriosclerosis. *Nature communications* **8**, 15426 (2017).

RESPONSE TO REVIEWER #3

…. However, the manuscript is plagued by the lack of mass spectrometry data on SUMOylation of VEGFR2 in vivo. The primary support for the SUMOylation sites determined in this manuscript is based on molecular modeling. Mass spectrometry performed using endothelial cells to confirm these SUMOylation sites on VEGFR2 would be absolutely crucial for this manuscript to substantiate its claimed significance and impact in the field.

- We have performed mass-spectrometry analyses at W.M. Keck Foundation, Proteomics service, Yale University School of Medicine. Specifically, SUMOylation of VEGFR2 was analyzed in HUVEC after expression His-tagged SUMO-1 followed by pulldown by His-purification column. The sample was then subjected to a tryptic digestion followed by injection the digested sample via LC-MS/MS on an LTQ Orbitrap MS instrument. VEGFR2 SUMOylation was analyzed by database search for SUMOylated peptides. The process of tryptic digestion on a SUMOylated protein leaves behind a Gly-Gly (mass shift of +114.042927, monoisotopic) residue from SUMO that is bound to the Lys of the modified protein. We have detected modifications of Lys1270 by post-translational modifications. However, we cannot be sure this modification is a SUMOylation. We have discussed with Cell Signaling Technology to map VEGFR2 SUMOylation in SENP1-knockdown cells using their newly established SUMOScan platform. This is based on a specific cleavage by newly identified WaLP (wild-type α-lytic protease) after T of the SUMO conjugation …..TGG-K but not after R of the ubiquitin conjugation… RGG-K. An antibody specific for KGG-peptides will be used to enrich peptides from SUMOylated sites that will be then identified by mass spectrometry (see Lumpkin et al 2017) [12]. This study is ongoing and still needs to solve some technical issues.

1. Given the claimed importance of SUMOylation on the function of VEGFR2, it seems highly unusual that the SENP1-ECKO mice have no obvious developmental defects. Importantly, expression of the SENP1 in endothelial cells during different developmental stages has never been examined.

- We have recently reported that retina angiogenesis in SENP1-ECKO is delayed at neonatal pups at P4- P6 due to increased Notch signaling [2]. However, retinal sprouting in SENP1-ECKO is similar to WT mice after day 9 (Supplemental Fig.S2). SENP1-ECKO did not exhibit obvious vascular defects with normal breeding and growth compared to $SENP1^{lox/lox}$ or normal C57BL/6 mice in adulthood (see basal groups presented for hindlimb, ear skin and retina). We have discussed that certain genes that play more important roles in pathological (e.g., inflammation and ischemia) are not involved in physiological angiogenesis.

2. As a foundation for the elaborated studies presented in the manuscript, the SENP1-ECKO endothelial cells should exhibit decreased cell surface levels of VEGFR2; however, this is never demonstrated. Without this data, the foundation of this study is very weak. Given that augmented levels of total VEGFR2 are prominent in endothelial cells lacking SENP1, surface levels of VEGFR2 in SENP1-ECKO versus WT primary endothelial cells should be firmly determined.

- We have shown that VEGFR2 is accumulated at the Golgi in SENP1-deficient ECs. Furthermore, the reduced surface expression of VEGFR2 in SENP1-deficient ECs was confirmed by a cell-surface biotinylation assay as we previously described [13] (**Fig.4C**). As observed for overexpressed VEGFR2-SUMO1, endogenous VEGFR2-SUMO1 in KDR-SUMO1 ECs was also exhibited a Golgi accumulation (**Fig.6B**) with reduced surface expression as confirmed by a cell-surface biotinylation assay (**Fig.6C**).

3. In the text, HUVECs transfected with vector control are compared to HUVECs expressing VEGFR2 construct in the context of Figure 6A; however, there is no vector control in Figure 6A. This should be included to the figure that compares HUVECs expressing VEGFR2 construct to HUVECs expressing VEGFR2-SUMO construct.
More importantly, experiments utilizing endothelial cells expressing an endogenous VEGFR2-SUMO fusion protein using gene-editing technique comprising CRISPR-Cas9 would be much needed to solidify their claim that SUMOylation hinders VEGFR2 cell surface distribution.

- We have included the vector control group to compare VEGFR2 (revised **Fig.5C**).
More importantly, we established endothelial cells expressing an endogenous VEGFR2-SUMO fusion protein using Clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9

nuclease (Cas9)-mediated gene editing. Specifically, we designed a specific single-guide RNA (sgRNA) targeting the vicinity of the stop codon of KDR gene encoding VEGFR2 and a repair template containing targeting arms with exon 30 as 5' arm and the 3'UTR of the KDR gene as 3' arm flanking the SUMO1 cDNA. Upon CRISPR/Cas9-mediated DNA double-strand breaks were repaired through homologous-directed repair [14], the SUMO1 cDNA was integrated into the KDR locus just before the termination signal in endothelial colony forming cell-derived ECs so that engineered KDR-SUMO1 EC expresses VEGFR2-SUMO1 fusion protein (**Fig.6A**; Supplemental Fig.S8). We examined cellular localization and angiogenic activity of VEGFR2-SUMO1 fusion protein in engineered ECs. As observed for overexpressed VEGFR2-SUMO1, endogenous VEGFR2-SUMO1 in KDR-SUMO1 ECs was also exhibited a Golgi accumulation (**Fig.6B**) with reduced surface expression as confirmed by a cell-surface biotinylation assay (**Fig.6C**). Accordingly, VEGF-induced autophosphorylaton of VEGFR2-SUMO1 was abrogated (**Fig.6D-E**). For functional analyses, we performed a 3D spheroid sprouting assay using normal (WT) and KDR-SUMO1 knockin human EC. Quantitative analyses indicated that the number of sprout formation by KDR-SUMO1 ECs was drastically reduced compared to normal ECs (**Fig.6F-G**). Taken together, our results indicate that SUMOlation of VEGFR2 hinders VEGFR2 cell surface distribution and its angiogenic signaling.

4. The experiments in Figures 4F, 5B, 5C, 5D, 5E, and Supplemental 5B should be performed in HUVECs or primary mouse endothelial cells rather than COS-7 cells to avoid artifacts observed in non-endothelial cells which intrinsically do not express and process any VEGFR2. The processing machineries in endothelial cells involved in VEGFR2 expression would be considerably distinct from those in cells that have never seen VEGFR2 expression.

- We have performed immunostaining of VEGFR2 localization in HUVEC or primary mouse endothelial cells. Specifically, we detected localization of endogenous VEGFR2 and VEGFR2 mutants in WT and SENP1ecKO mouse EC (revised Fig.4A, 4F, 4G), localization of VEGFR2 truncates in HUVEC (Supplemental Fig.S6B), localization of VEGFR2-SUMO1 fusion in HUVEC (revised Fig.5B), and endogenous VEGFR2-SUMO1 in CRISPR-Cas9-edited KDR-SUMO1 ECs (revised Fig.6B).

5. All of the immunoprecipitations shown in figures 1J, 4C, and 7A are lacking a loading control. The bands for the immunoprecipitated SUMO1 should be shown to demonstrate that the IP itself worked correctly.

- We have added protein loading controls for each figure and also added immunoprecipitated SUMO1 (Fig.1J, 4D and 7A).

6. Supplemental Figure 3C is missing a WT control for 0, 5, and 15 minutes of VEGF treatment.

- We have added WT to compare ECKO and ECKO/lenti-SENP1 rescue group (revised Supplemental Fig.S4).

7. The quality of the immunofluorescent images is lacking. Figures 4A, 4E, 4F, 7B, 7E, and Supplemental 1B would strongly benefit with higher resolution images. Figures 4E and 5C should be shown as split channel images. Furthermore, several panels in Figures 6F and Supplemental 6B are either fuzzy and out of focus or extremely overexposed making it difficult to analyze.

- We have provided split channel images with better qualities (Supplemental Fig.S1B, revised Fig.4A, 4F, 4H with Supplemental Figure S6C-D, 5B, 7B). We also provided confocal images for retinal angiogenesis (revised Fig.4G).

8. The model depicted in Figure 8 includes KIF13B and T-SNARE in the context of exocytosis. However, no experiments looking at the function of these two proteins are performed. This should be analyzed to strengthen this claim.

- We have examined effect of KIF13B on VEGFR2 localization and have added the data to Supplemental Fig.S5B).

9. It would be beneficial to show confirmation of Type I diabetes induced by STZ treatment by showing the blood glucose levels over the course of STZ treatment at least in the supplemental figures.

- Yes, we have presented the data in Supplemental Fig.S9.

10. Numerous grammar errors and incorrect or convoluted sentence structure throughout the manuscript make it somewhat difficult to read.

- We have edited the manuscript.

11. It is indicated in the text and figure legend that a Golgi marker was used in Figures 7B and 7E. This is not indicated in the figure; the figure itself is lacking labels for what is stained. If a Golgi marker was used here, a panel for this should be included. For consistency, labels to indicate Golgi VEGFR2 and cytosolic/membrane VEGFR2 as used in Supplemental Figure 4 would make Figure 7 clearer. Furthermore, adding a Golgi marker to Supplemental Figure 4 would also make this figure clearer.

- We have performed co-staining experiments with a Golgi marker. We have labeled * and arrowheads to indicate Golgi VEGFR2 and cytosolic/membrane VEGFR2 in all images.

12. The text claims that TNFR2 surface localization is not affected by SENP1 deletion, which is supported by Figure 4B. However, Supplemental Figure 4B appears to show Golgi localization of TNFR2. This is not addressed at all. Thus, the Golgi retention of plasma membrane proteins owing to SENP1 deficiency appears not specific to VEGFR2.

- TNFR2 is synthesized and secreted to membrane. It should be partly detected in the Golgi. In contrast, the majority of VEGFR2 is accumulated in the Golgi with reduction on cell surface.

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REVIEWERS' COMMENTS:

Reviewer #1 (Remarks to the Author):

NA

Reviewer #2 (Remarks to the Author):

The authors now allude to the involvement of a SUMO E3 ligase.
All other queries have been addressed. I have no further comments.

Reviewer #3 (Remarks to the Author):

The authors have addressed the majority of concerns adequately. However, the manuscript is still plagues by a lack of mass spectrometry data on the SUMOylation of VEGFR2 and relies primarily on molecular modeling to support the notion that VEGFR2 is SUMOylated. This experiment should be performed as it is absolutely crucial for this manuscript to substantiate its claimed significance and impact in the field.

Editorial note: This manuscript was sent back to reviewers #1 and #2 for a third round of review following an editorial request for additional evidence for the reproducibility of some of the western blot data

REVIEWERS' COMMENTS:

Reviewer #1 (Remarks to the Author):

Since the authors stated that they performed n =3 for each experiment, it will be important to show every replicates. Furthermore, the quantification cannot be done by duplicates. Please show all the n=3 replicates and redo the statistics based on n = 3.

Reviewer #2 (Remarks to the Author):

The authors provided new data sets that do not concord anymore with the original text and previous conclusions. I have some concerns on the interpretation of these new data. As a whole, I believe the major conclusions of the study are still valid but the text for the modified data needs to be corrected and some of the interpretations revised.

Specific concerns:
It is indicated that there the levels of HIF1a in the hindlimb muscles are not different in WT and in SENP1-ECKO following ischemia. However, the blot presented in Fig. 1h and the quantifications indicate a marked and significant decrease in HIF1a at day 3 post-ischemia. The text must be corrected. Also, it is concluded that “this suggestS that other cell types such as myocyte rather than EC primarily contributes to HIF1a and VEGF-A expression in ischemic hindlimb.” Is this affirmation still valid since reduced expression of HIF1a in non-EC could contribute to the reduced vascularization following ischemia in SENP1-ECKO?

It is written in the text that SENP1 deletion reduces ischemia-induced ASK1 activation (p-ASK1) (line 361), however, in Fig. 1j shows that the levels of p-ASK1 are not reduced significantly, from 2.4 to 2.2 pASK1/ASK1 ratio; p> 0.05. This needs to be corrected and the interpretation revised.

The blots provided for Fig. 3g suggest a decrease in GATA2 levels in siSENP1 cells, which differs from the text mentioning the SENP1 depletion had no effect. In addition, the quantifications provided confirm a significant reduction of GATA2 in siSENP1 transfected EC. These new results are more in line with the recent study from the authors that GATA2 is de-sumoylated by SENP1 (Qiu et al. Nat Commun, 2017). The text should now reflect this.

Line 423, Fig. 4h should be corrected for Fig. 4g.

For figure 7c, it is mentioned that SENP1 expression was significantly reduced in whole brain lysates from STZ mice. However, the blot provided shows a clear increase in SENP1 expression levels in the brain following STZ treatment (from 1 to 1.5). Also, the interpretation that hyperglycemia affects more profoundly the brain vasculature should be revised.

A point-by-point response to Reviewers

Reviewer #1 (Remarks to the Author):

Since the authors stated that they performed n =3 for each experiment, it will be important to show every replicates. Furthermore, the quantification cannot be done by duplicates. Please show all the n=3 replicates and redo the statistics based on n = 3.

- We thank you for your critical comments and instructive suggestions. Since we cannot identify some of our original films due to lab relocation, we can only provide duplicates for most of Western blots. Therefore, we have revised our text by stating the figure legends and methods section that blots are representative of two experiments. We have provided quantifications and statistical analyses based on n=2 in the data source (Supplementary Data 1).

Reviewer #2 (Remarks to the Author):

The authors provided new data sets that do not concord anymore with the original text and previous conclusions. I have some concerns on the interpretation of these new data. As a whole, I believe the major conclusions of the study are still valid but the text for the modified data needs to be corrected and some of the interpretations revised.

- We thank you for your critical comments and instructive suggestions.

Specific concerns:

a. It is indicated that there the levels of HIF1a in the hindlimb muscles are not different in WT and in SENP1-ECKO following ischemia. However, the blot presented in Fig. 1h and the quantifications indicate a marked and significant decrease in HIF1a at day 3 post-ischemia. The text must be corrected. Also, it is concluded that "this suggests that other cell types such as myocyte rather than EC primarily contributes to HIF1a and VEGF-A expression in ischemic hindlimb." Is this affirmation still valid since reduced expression of HIF1a in non-EC could contribute to the reduced vascularization following ischemia in SENP1-ECKO?

- We have revised the text as follows (Page 6):

Despite that HIF1α protein was markedly decreased at day 3 post-ischemia in SENP1-ECKO mice, we did not observe significant differences between the two groups in VEGF mRNA levels (Fig.1h-i). This suggest that other cell types such as myocyte rather than EC primarily contributes to VEGF-A expression in ischemic hindlimb.

b. It is written in the text that SENP1 deletion reduces ischemia-induced ASK1 activation (p-ASK1) (line 361), however, in Fig. 1j shows that the levels of p-ASK1 are not reduced significantly, from 2.4 to 2.2 pASK1/ASK1 ratio; p> 0.05. This needs to be corrected and the interpretation revised.

- - We have revised the text as follows (Page 6):

SENP1-mediated ASK1 activation (p-ASK1), which is known to mediate inflammation and cellular apoptosis[36,37], was not significantly attenuated by SENP1 deletion in ischemic muscle. These data suggest that ASK1 signaling did not contribute to reduced macrophage infiltration (Supplementary Fig.3d-e) and cell death (TUNEL assay) in ischemic hindlimb of SENP1-ECKO (Supplementary Fig.3f-g).

c. The blots provided for Fig. 3g suggest a decrease in GATA2 levels in siSENP1 cells, which differs from the text mentioning the SENP1 depletion had no effect. In addition, the quantifications provided confirm a significant reduction of GATA2 in siSENP1 transfected EC. These new results are more in line with the recent study from the authors that GATA2 is de-sumoylated by SENP1 (Qiu et al. Nat Commun, 2017). The text should now reflect this.

- We have revised the text as follows (Page 7):

In line with our recent study [38], we observed a significant reduction of GATA2 in siSENP1 transfected EC. While SENP1 knockdown by siRNA had no effects on VE-cadherin, VEGF-induced phosphor-VEGFR2 and its downstream Akt were reduced by SENP1 siRNAs in HUVEC (Fig.3g-h).

d. Line 423, Fig. 4h should be corrected for Fig. 4g.

- We have corrected (Page 8).

e. For figure 7c, it is mentioned that SENP1 expression was significantly reduced in whole brain lysates from STZ mice. However, the

blot provided shows a clear increase in SENP1 expression levels in the brain following STZ treatment (from 1 to 1.5). Also, the interpretation that hyperglycemia affects more profoundly the brain vasculature should be revised.

- We have revised the text as follows (Page 10):

Although SENP1 expression was weakly increased in whole brain lysates, it was significantly reduced in the vascular layers of retina and ear skin from STZ mice compared to normal groups (Fig.7c), suggesting that hyperglycemia effects on SENP1 expression is more profound in the vasculature.