nature portfolio

Peer Review File

SRF SUMOylation modulates smooth muscle phenotypic switch and vascular remodeling

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

The manuscript titled "SRF SUMOylation Modulates Smooth Muscle Phenotypic Switch and Vascular Remodeling" by Xu et al explores the connection between SRF SUMOylation and vascular smooth muscle cells (VSMCs) phenotypic modulation using Senp1 deficiency mice in response to mouse carotid injury both in vivo and in vitro. The authors observed an increase in vascular remodeling in a VSMC-conditional Senp1-deficient mouse model. Their findings support the hypothesis that SUMOylation of SRF alters its binding partner from the contractile phenotype-responsive cofactor myocardin to the synthetic phenotype-responsive cofactor phosphorylated ELK1. This study builds upon existing knowledge regarding the role of SRF and SUMOylation in controlling VSMC phenotypic switching, providing a deeper understanding of the significance of SRF in this process. The manuscript is well-written and concise, and the conclusion is well-supported by the results. The experimental design is comprehensive, encompassing in vitro, in vivo, and patient samples. This study adds a new layer of understanding to the regulation of SRF in VSMC remodeling.

However, there is a limitation of the study. Although this study focuses on the effect of SRF SUMOylation on Elk phosphorylation and the interplay between SRF, Myocardin, and ELK, it does not investigate whether SRF SUMOylation impacts SRF phosphorylation given the influence of SRF phosphorylation on its nuclear localization and interaction with other transcription factors.

Here are specific comments:
1. Fig 2A: this is a beautiful experiment to show that carotid wire injury inhibits SMC differentiation. In carotid ligation injury (PMID: 11067866), SMC markers are down regulated in 1-2 weeks after carotid injury, but redifferentiation occurs at 2 weeks after injury. You may cite this study in discussion for future reference.

2. Fig 3H is confusing: please indicate the bands of SUMO1/2/3-SRF. There are so many bands with SUMO1 antibody: which band is Sumo1-SRF? Why the molecular weight of SUMO1-SRF in 3H, 3J and 3I is not the same?

3. It has been shown that SRF is SUMOylated at lysine 147 (Matsuzaki et al, 2003 BBRC). Is it the same lysine 143 you identified in this study?

4. Fig. 3F Myocardin is a SUMOylated protein. There is an upper band in MYCD blot. Is it SUMO-MYCD? Will longer exposure show SUMO-MYCD band?

5. Fig 3F: Since SRF is extensively phosphorylated and SRF phosphorylation affects SRF nuclear localization. Does SUMOylation affect SRF phosphorylation?

6. Fig 3I: Comparing the input in the panel of Fig 3I with that in 3H and 3J, should Sumo1 expression be higher in senp1KO than WT?

7. Fig 3J: it is interesting that AZD affects SRF SUMOylation and ELK phosphorylation. Does AZD affect SRF phosphorylation? Does SRF phosphorylation increase SRF-ELK interaction?

8. Fig. 4C Western blot: will reblotting with Sumo-1 antibody demonstrate the top band is SUMO-SRF? Are there SUMO-SRF antibodies to detect the changes of SRF with SUMOylation?

9. Fig 4H. Does 143 SUMOylation affect SRF phosphorylation? In literature, it is known that SRF phosphorylation affects SRF nuclear localization and interaction with other transcription factors? Is it possible that SRF SUMOylation increases SRF phosphorylation to promote SRF-ELK association?

10. Does AZD treatment affect SRF SUMOylation and phosphorylation?

Li Li

Reviewer #2 (Remarks to the Author):

Xu et al present a gigantic body of work examining the role of SUMOylation of the transcription factor SRF in vascular remodelling. They demonstrate that SENP1 knockout in vascular smooth muscle cells causes a phenotypic switch from a contractile to synthetic state, and leads to neointimal formation in mice. The further demonstrate that loss of SENP1 promotes SRF SUMOylation at K143, promoting its nuclear localisation and interaction with phospho-ELK1 to promote a synthetic phenotype. Importantly, they show that levels of SRF SUMOylation and phospho-ELK1 correlate with severity of cardiovascular disease in human patients. Furthermore, demonstrating the possible translational potential of their findings, they show that blocking ELK1 phosphorylation using AZD6244 promotes SRF-myocardin interaction, attenuating neointimal formation in SENP1 knockout mice.

Overall, the quality of data presented is outstanding. The conclusions drawn are fully supported by the data, and methodology sound.

In all honesty, I have no substantive comments other than to suggest that:

1. In the introduction the authors refer to SENP8 as a deSUMOylating enzyme - I believe it in fact is specific for NEDD8.

2. ELK1 has been previously reported to be itself a SUMO substrate - the authors may wish to discuss the potential implications of this to their work.

Other than that, I congratulate the authors on an outstanding piece of work and am fully supportive of publication of this manuscript.

Reviewer #3 (Remarks to the Author):

This study found that SRF SUMOylation modulates the VSMC responses to PDGF-BB in cultured cells and vascular injury in murine models. SENP1 deficiency in VSMCs accelerates injury-induced VSMC proliferation, migration, and phenotypic switch, promoting neointimal formation and vascular remodeling. This study aims to explore the causes of vascular remodeling and neointimal formation through a series of experiments. The research has some new ideas, but the following problems need to be solved.
1. This study lacks information in detail of the samples from patients with cardiovascular diseases (CVD), which cannot explain that neointimal formation, phenotypic transition of VSMCs, lipid deposition and macrophage infiltration, etc in patients with CAD is related to SRF SUMOylation.
2. KLF4 plays an important role in the phenotype of VSMCs. In this study, it was observed that SENP1 deficiency did not affect the SUMOylation of KLF4, which is inconsistent with some published articles. The author will briefly explain the reason. Furthermore, SRF is ubiquitously expressed, why researchers focus on SRF? Myocardin, another key factors in vascular remodeling, which selectively expressed in SMCs and cardiac muscle, also modulated by SUMOylation. Actually, the relationship between KLF4, myocardin, SRF and ELK1 is complex in vascular remodeling.Figure 3 showed the expression and SUMOylation of KLF4 and myocardin has little effect on SENP1 deficiency. How to explore the role and clinical significance of SUMOylation modification of such important factors and their complex relationship in vascular remodeling diseases?
3. Figure 3 showed the Senp1SMCKO mice exhibited a more rapid increase in phosphorylated ELK1 levels compared with WT mice. Why does SUMOylation modification affect phosphorylation? Mechanism? The author directly focuses on MEK, and ELK1, there are other signaling pathways involved? Reasons for excluding other pathway need to be added.
4. Some grammatical errors need to be corrected. The methods, data and analysis should be described in sufficient detail to be reproduced.

Reviewer #4 (Remarks to the Author):

General Comments:

The manuscript "SRF SUMOylation modulates smooth muscle phenotypic switch and vascular remodeling" by Xu et al. is a report that uses primarily Western blot and immunohistochemistry techniques to probe the functional significance of SRF SUMOylation in a mouse model of carotid

injury, as well as in cell culture and human coronary arteries. The scientific approach is reasonable, the blots are clean and the multiplex fluorescence immunohistochemistry is strikingly beautiful. However, the analytical methods for image analysis are not presented in adequate detail to enable others to reproduce the experiments. Furthermore, the "n" in terms of animal number is not always clear, leading to questions as to whether the findings have true statistical significance. The findings and conclusions drawn from these experiments represent a relatively modest advance over what has previously been published on the role of SUMOylation in the phenotypic switching of vascular smooth muscle cells (VSMCs), leading to reservations concerning potential scientific impact. Finally, the manuscript is well-organized, but there are multiple instances in which it could benefit from more careful editing and proofreading.

Specific Comments:

1) Page 2, line 11: In the abstract and throughout the manuscript, please correct "phosphor-ELK1" to "phospho-ELK1."

2) Page 4, line 21: The following sentence may be overstated given that it has been known for over 20 years that the SRF activity is modulated by SUMOylation (Reference 29): "In contrast to the relatively well-studied transcriptional control mechanisms, regulation of VSMC fate at the post-transcriptional level, such as SUMOylation, has not been well investigated."

3) Page 6, line 30: Recommend revising the last sentence on page 6. For example: "Despite the fact that
carotid artery morphometry at baseline was unaltered by VSMC-specific Senp1 deletion, …"

4) Page 7, line 24: Recommend clarifying what 1M refers to. For example: "(Fig.1J with quantifications in Fig.1M for EdU+ SMCs).

5) Page 8, line 10. The sentence in question could be simplified as follows: "Senp1 SMCKO mice exhibited a more rapid decrease in the four contractile markers and an increase in the two synthetic markers compared with WT mice (Fig.2A)."

6) Page 8, line 25: Recommend revising as follows: "… in normal carotid arteries and cultured aortic VSMCs, …"

7) Page 9, line 6: Recommend correcting "indued" to "induced".

8) Page 9, line 11: Recommend the following for clarity: "The results above prompted us to investigate the post-translational regulation of SRF."

9) Page 9, line 13: The following statement should be revised to reflect the distinction between "suggesting" something and "showing" it: "Previous report been suggested that SRF could be modified by SUMO1-mediated SUMOylation29." For example, the following sentence may be more accurate: "A previous report showed that SRF is modified by SUMO-1, chiefly at lysine147 within the DNA-binding domain."

10) Page 12, line 15: Suggest the following for clarity: "and most of these signals were co-localized with α-SMA-positive VSMCs (Fig.6C-D)."

11) Page 19, line 14: The last sentence reads: "DMSO served as vehicle control." However, Fig. 7A at top left of page 41 shows a timeline indicating that PBS was the vehicle control. Please resolve this contradiction.

12) Page 19, line 27: Please replace the word "sacrificed" with "euthanized" due to the negative connotation of the word "sacrifice".

13) Page 20, line 8: The following sentence does not address how many sections per patient: "…and images were obtained for one section per block at a final magnification of 40×." It appears that only one slide was analyzed per patient. If true, that it would be more accurate to state: "…and images were obtained from one slide per patient at a final magnification of 40×."

14) Page 20, line 10: The following sentence does not address how many specimens per patient: "The means of these parameters from four different areas for each specimen were calculated." It

appears that only one slide was analyzed per patient. If true, then it would be more accurate to state: "The means of these parameters from four different areas on each slide were calculated."

15) Page 22, line 9: Please indicate the mean number of cells counted per "randomely selected area". I.E. "These migrated VSMCs were quantified in four randomly selected fields at 100× magnification using Cellsens Dimention 1.15 software (Olympus, Tokyo, Japan), with each field containing a mean of XX cells."

16) Page 22, line 10: Recommend correcting "Dimention" to "Dimension".

17) Page 23, line 6: After the Methods section on: "Immunofluorescence staining," please insert a new section entitled "Quantification of Immunofluorescence Images". This new section should be used to disclose details of the images quantification methods shared in common between the images that were analyzed to yield quantitative results. If multiple approaches or analytical techniques were applied then it may be appropriate to list the techniques along with the Figure and Panel in which each technique was applied. The authors might use a review article such as PMID: 21209361 to identify which general strategy was employed, but if a software program such as Cellsens Dimension was used, then in addition the particular sub-program or analytical tool should be specified by the name used in the software package.

18) Page 23, line 9: The following sentence is a valid approach: "As the protein concentration from a single mouse carotid artery tissue was low, we mixed tissue samples from three to six individuals for protein extraction." However, from a statistical standpoint, a pooled sample containing 3 to 6 arteries is not the same as n=3-6 because separate measurements were not obtained from those 3 to 6 arteries.

19) Page 24, line 12: Recommend removing the following sentence unless its meaning is unclear: "All figures are representative of at least three experiments unless otherwise noted." This is unclear because what is meant by an "experiment" may change from on figure to the next. It is never clear from the figures that at least 3 separate experiments were conducted at 3 different times. In some cases, the authors may be equating an "experiment" with an artery, or a transfected well, or a lane in a Western blot. It is important to specify which of these possibilities apply in each Figure since they are not equivalent from a statistical perspective.

20) Page 24, line 15: Recommend revising the following sentence to identify the specific instances in which: "The normality and variance were not tested." This is because the sentence implies that normality and variance were never tested.

21) Page 29, line 3: Figure 1, Panels F and G: Nearly all lanes have ratios from densitometry beneath them. Please indicate this to the right of the densitometry ratios with something like "Ratio" or "Quant", then define that term more fully in the figure legend.

22) Page 30, line 3: Here and throughout the Figure Legends, please explicitly state precisely what n refers to. Take for example the following sentence: "Circumference of EEL, neointimal area, media area, and neointima/media ratio in carotid arteries were measured (n=12 per group)." Here, any of the following could be true: n=12 mice per group, n=12 carotid arteries per group, n=12 left carotid arteries per group or n=12 sections per group. In the case of 12 sections/group, it would also be important to specify how may sections per carotid.

23) Page 30, line 4: Again, please define "n=10 (what?) per group." In this case, it would be helpful to specify that only left carotid arteries were used, if that is true.

24) Page 30, line 11: The following sentence, or closely related sentences, occurs some 17 times in the Figure Legends: "Relative protein levels are presented by taking non-injured WT as 1.0 (n=3 per group)." When considered in combination with the statement from Page 23, line 9: "As the protein concentration from a single mouse carotid artery tissue was low, we mixed tissue samples from three to six individuals for protein extraction," this seems to imply that the Western blot shown contains only a single WT lane representing a pool of 3 WT mice. Please address this question, since that equates to "n=1" from a statistical perspective. If so, this needs to be clarified by revising the statement. For example: "Protein levels were calculated relative to protein extracted from a pool of 3 non-injured left carotids from WT mice." If instead 3 identical Western blots were performed using carotids from 9 different WT mice, then please include the additional blots within "Supplemental Fig.11: Uncut original gels."

25) Page 30, line 13: The following sentence refers to the graphs in Panel I: "% of Ki67+α-SMA+ VSMCs, MFI of MMP2 and MMP9 within the neointimal area was quantified (n=10 per group)." The first part of the sentence refers to the first graph, but the remainder appears unrelated to the 2nd and 3rd graphs. This is an example where the Methods used for image analysis need to be better defined, either individually here in the Figure legends or collectively in a new Methods section on "Quantification of Immunofluorescence Images". The problem with "MFI of MMP2 and MMP9 within the neointimal area was quantified" is that it inadequately describes the determination of Manders overlap, which is what appears to have been done here. Certainly, the Y-axes for the 2nd and 3rd graphs do not relate to mean fluorescence intensity (MFI). Further, as stated above, any reference to "n" needs to define n-what? In this case, this could mean: n=10 mice per group, n=10 sections per group, etc. Best practices would include stating not only how many left carotid arteries per group but also how many sections analyzed from each left carotid.

25) Page 30, line 14: Please enhance the following sentence to specify whether the VSMCs were isolated from carotid arteries, and if carotids were isolated from multiple mice please state how many carotids were pooled for each VSMC isolation.

26) Page 30, line 20: Previous concerns also relate to the following sentence: "(M) % Edu+α-SMA+ in (J), % wound closure in (K) and number of invaded VMSCs in (L) were quantified (n = 8 per group)." Here, n could refer to: n = 8 scratch assays per group, or n = 8 views per scratch assay. If the VSMCs were isolated from pooled carotids, that should also be clarified here.

27) Page 31, lines 307-374: As above, please clarify how: "MFI of each marker within the neointimal area was quantified (n=10 per group)" relates to the graphs presented in Panel C. If only MFI was determined, then how were individual cells identified to generate the Y-axes for each graph (e.g.: CNN1+ staining (%)? Note that all the immunostains in Panel B have the format: "Marker/alpha-SMA/DAPI" except for the panel at lower left which is labeled: "Marker/MYH11+/DAPI". It is unclear whether this was intended or not, but it contributes further to the uncertainly regarding whether MFI or Mander's overlap is being quantitated in the graphs.

28) Page 36, line 11: The meaning of the following statement is unclear: "GAPDH as the loading control (n = 3 per group)." It appears likely that this again refers to a pooled sample approach, where cell lysates were pooled from 3 different wells, or transfections, or experiments. Thus "n = 3 what? per group" needs to be defined.

29) Page 39, line 5: Please clarify whether the GAPDH blots were included only for visual confirmation of equivalent loading, or whether the numbers reported beneath each lane have been normalized relative to the intensity of the respective GAPDH bands.

30) Page 39, line 7: Please expand the following statement: "Five fields per section from each sample are analyzed" to include the minimum number of cells counted per field, and the number of sections analyzed per patient.

POINT-TO-POINT RESPONSE TO REVIEWERS' COMMENTS

RESPONSE TO REVIEWER #1

The manuscript titled "SRF SUMOylation Modulates Smooth Muscle Phenotypic Switch and Vascular Remodeling" by Xu et al explores the connection between SRF SUMOylation and vascular smooth muscle cells (VSMCs) phenotypic modulation using Senp1 deficiency mice in response to mouse carotid injury both in vivo and in vitro. The authors observed an increase in vascular remodeling in a VSMC-conditional Senp1-deficient mouse model. Their findings support the hypothesis that SUMOylation of SRF alters its binding partner from the contractile phenotype-responsive cofactor myocardin to the synthetic phenotype-responsive cofactor phosphorylated ELK1. This study builds upon existing knowledge regarding the role of SRF and SUMOylation in controlling VSMC phenotypic switching, providing a deeper understanding of the significance of SRF in this process. The manuscript is well-written and concise, and the conclusion is well-supported by the results. The experimental design is comprehensive, encompassing in vitro, in vivo, and patient samples. This study adds a new layer of understanding to the regulation of SRF in VSMC remodeling.

However, there is a limitation of the study. Although this study focuses on the effect of SRF SUMOylation on Elk phosphorylation and the interplay between SRF, Myocardin, and ELK, it does not investigate whether SRF SUMOylation impacts SRF phosphorylation given the influence of SRF phosphorylation on its nuclear localization and interaction with other transcription factors.

We appreciate your positive comments on our work. We also thank you for your constructive critiques and we have addressed your concerns as follows.

Specific comments:

1. Fig 2A: this is a beautiful experiment to show that carotid wire injury inhibits SMC differentiation. In carotid ligation injury (PMID: 11067866), SMC markers (SM22a, SMMHC and SMaA?) are down regulated in 1-2 weeks after carotid injury, but redifferentiation occurs at 2 weeks after injury. You may cite this study in discussion for future reference.

We thank the reviewer for this comment. According to the reviewer's suggestion, we have cited this study in the discussion.

2. Fig 3H is confusing: please indicate the bands of SUMO1/2/3-SRF. There are so many bands with SUMO1 antibody: which band is Sumo1-SRF? Why the molecular weight of SUMO1-SRF in 3H, 3J and 3I is not the same?

We thank the reviewer for this comment. SUMOylation is a transient post-translational modification with small-ubiquitin like modifiers (SUMO1, SUMO2 and SUMO3) covalently attached to their target-proteins via a multi-step enzymatic cascade. This modification adds an approximately 15 KD SUMO polypeptide to the ε-amino group of certain lysine residues.

In the input (left column of Fig 3I, J and K), Western blot with SUMO1 antibody could only detect the total SUMOylated proteins but not a specific SUMOylated protein (e.g., SUMO-SRF) in injured arteries (note: The strongest bands were not SUMO-SRF). Based on the molecular weight of SRF (~ 65KD), we could only estimate SUMOylated SRF at ~80KD. To further confirm SUMOylated SRF, we performed co-IP assays with anti-SRF antibody followed by Western blot with anti-SUMO1 (or anti-SUMO2/3) antibody (in right column of Fig 3I). Our Western blot result revealed a specific SUMO1-conjugated SRF band that was detected at 80KD (increased by ~15 KD) with anti-SRF antibody. However, SUMO2/3-conjugated SRF was not observed in the aortic lysates (Fig.3I). These results confirmed that Senp1 deficiency in VSMCs induced SUMO1-mediated SRF SUMOylation.

The molecular weight of SUMO1-SRF in all panels were the same. It was confused because we added the estimated molecular weight (not actual marker size) on the left. To avoid the confusing, we only label the actual molecular weight markers used for the immunoblotting.

3. It has been shown that SRF is SUMOylated at lysine 147 (Matsuzaki et al, 2003 BBRC). Is it the same lysine 143 you identified in this study?

We thank reviewer for the comment. The cell line in Matsuzaki' paper of BBRC is human cervical cancer HeLa cells. In our study, primary aortic VSMCs were isolated from mice and the MOVAS-1 cell line was derived from mouse aortic smooth muscle cells. In addition, there are 4-amino acid differences between homo sapiens and Mus musculus SRF protein sequence (Fig.S9). Thus, human SRF lysine 147 (Matsuzaki et al, 2003 BBRC) is equivalent to mouse SRF lysine 143.

4. Fig. 3F Myocardin is a SUMOylated protein. There is an upper band in MYCD blot. Is it SUMO-MYCD? Will longer exposure show SUMO-MYCD band?

We thank the reviewer for these questions. SUMOylated myocardin was detected in pluripotent fibroblasts (17101795) and smooth muscle cells upon SENP2 deletion (PMID 36293488). The datasheet on the rabbid polyclonal MYCD antibody (Sigma-Aldrich SAB4200539) indicated that

this antibody could detect several bands in Western blot. We performed co-IP assays with anti-MYCD antibody followed by Western blot with anti-SUMO1 or SUMO2/3 antibodies. However, SUMO1-conjugated MYCD was not observed in the aortic lysates. These results indicated that Senp1 deficiency in VSMCs might not induce Myocardin SUMOylation after wire injury. We have incorporated this into RESULTS (page 9).

5. Fig 3F: Since SRF is extensively phosphorylated and SRF phosphorylation affects SRF nuclear localization. Does SUMOylation affect SRF phosphorylation?

Thank you for pointing out this important issue, and we greatly appreciate your constructive suggestion. As suggested, we further evaluated whether Senp1 deficiency-mediated SRF SUMOylation in VSMCs affected SRF phosphorylation. The phosphorylated SRF level was low in normal carotid arteries, but progressively increased after wire injury. *Senp1*^{SMCKO} mice exhibited a more rapid increase in phosphorylated SRF level compared with WT mice (Fig. 3H). Our results indicated that Senp1 deficiency in VSMCs induced SUMO1-mediated SRF phosphorylation.

6. Fig 3I: Comparing the input in the panel of Fig 3I with that in 3H and 3J, should Sumo1 expression be higher in senp1KO than WT?

We thank the reviewer for this comment. Our previous reports suggested that SENP1 deletion could increased SUMO1 expression in endothelial cells (PMID 28760777) (PMID 28569748) and uterine stromal stem cells (PMID 31141693). In our present study, Western blot indicated that wire injury or PDGF-BB treatment indued an increase in SUMO1 expression in the carotid artery or VSMCs, which was significantly augmented by SENP1 deletion (Fig.3I-J). Consistently, immunostaining showed that SUMO1 expression was increased in α-SMA-positive VSMCs of the carotid artery in *Senp1*^{SMCKO} mice at basal condition (Fig.S6C-D), and more dramatically increased in the injured vessels.

7. Fig 3J: it is interesting that AZD affects SRF SUMOylation and ELK phosphorylation. Does AZD affect SRF phosphorylation? Does SRF phosphorylation increase SRF-ELK interaction?

Thank you for your insightful comments. Per the reviewer's suggestion, we performed Western blot to determine the effects of AZD6244 on SRF phosphorylation in WT and *Senp1*^{SMCKO} mice. We observed that AZD6244 treatment reduced the transient injury-induced increase of phospho-SRF expression at 7 days after wire injury (Fig 7C).

8. Fig. 4C Western blot: will reblotting with Sumo-1 antibody demonstrate the top band is SUMO-SRF? Are there SUMO-SRF antibodies to detect the changes of SRF with SUMOylation?

We thank the reviewer for this comment. As stated above, Western blot with SUMO1 antibody in the input could only detect the total SUMOylated proteins but not a specific SUMOylated protein (e.g., SUMO-SRF). SUMO1-conjugated SRF was confirmed in the previous figure (Fig.3) by co-IP assays with anti-SRF antibody followed by Western blot with anti-SUMO1. So far, there are no SUMO-SRF antibodies to directly detect the changes of SRF with SUMOylation.

9. Fig 4H. Does K143 SUMOylation affect SRF phosphorylation? In literature, it is known that SRF phosphorylation affects SRF nuclear localization and interaction with other transcription factors? Is it possible that SRF SUMOylation increases SRF phosphorylation to promote SRF-ELK association?

Thank you for your insightful comments. Per the reviewer's suggestion, we tested SRF phosphorylation in MOVAS-1 lines overexpressing a Flag-tagged SRF-WT, or SRF-K143R. Results showed that stable transfection of the SRFK143R mutation decreased phosphorylated SRF expression with PDGF-BB stimulation (Fig.4H). These results suggest that SRF SUMOylation at K143 promotes its phosphorylation and nuclear accumulation, and switches its binding preference from myocardin to ELK1 in VSMCs in response to PDGF-BB stimulation.

10. Does AZD treatment affect SRF SUMOylation and phosphorylation?

We thank the reviewer for this question. Per the reviewer's suggestion, we examined the effects of AZD6244 on the SRF phosphorylation in WT and *Senp1*^{SMCKO} mice by Western blotting. We observed that AZD6244 reduced SRF SUMOylation and phosphorylation (Fig.7C).

RESPONSE TO REVIEWER #2

Xu et al present a gigantic body of work examining the role of SUMOylation of the transcription factor SRF in vascular remodelling. They demonstrate that SENP1 knockout in vascular smooth muscle cells causes a phenotypic switch from a contractile to synthetic state, and leads to neointimal formation in mice. The further demonstrate that loss of SENP1 promotes SRF SUMOylation at K143, promoting its nuclear localisation and interaction with phospho-ELK1 to promote a synthetic phenotype. Importantly, they show that levels of SRF SUMOylation and phospho-ELK1 correlate with severity of cardiovascular disease in human patients. Furthermore, demonstrating the possible translational potential of their findings, they show that blocking ELK1 phosphorylation using AZD6244 promotes SRF-myocardin interaction, attenuating neointimal formation in SENP1 knockout mice. Overall, the quality of data presented is outstanding. The conclusions drawn are fully supported by the data, and methodology sound. In all honesty, I have no substantive comments other than to suggest that below. Other than that, I congratulate the authors on an outstanding piece of work and am fully supportive of publication of this manuscript.

We greatly appreciate your positive comments on our work.

1. In the introduction the authors refer to SENP8 as a deSUMOylating enzyme - I believe it in fact is specific for NEDD8.

We agree with the comment and have changed the statement regarding SENP8 in the introduction accordingly.

2. ELK1 has been previously reported to be itself a SUMO substrate - the authors may wish to discuss the potential implications of this to their work.

We agree with the comment and have provided the potential implications of ELK1 as a SUMO substrate in the discussion accordingly.

It is noteworthy that SUMOylation can keep ELK1 in an inactive state under basal conditions via the binding of a SUMO-histone deacetylase complex and the control of its nuclear-cytoplasmic shuttling (PMID: 14992729). The activation of ERK1/2 triggers de-SUMOylation of ELK1 and promotes the phosphorylation and activation of ELK1, leading to a transcriptionally active state (PMID: 14992729). In our study, phospho-ELK1 expression were increased in VSMCs after Senp1 deficiency. However, SUMO-conjugated ELK1 was not observed in the aortic lysates with or without Senp1 deficiency. We have incorporated this into RESULTS (page 9) and DISCUSSION (Pages 16-17).

RESPONSE TO REVIEWER #3

This study found that SRF SUMOylation modulates the VSMC responses to PDGF-BB in cultured cells and vascular injury in murine models. SENP1 deficiency in VSMCs accelerates injury-induced VSMC proliferation, migration, and phenotypic switch, promoting neointimal formation and vascular remodeling. This study aims to explore the causes of vascular remodeling and neointimal formation through a series of experiments. The research has some new ideas, but the following problems need to be solved.

We appreciate your positive comments on our work. We also thank you for your constructive critiques and we have addressed your concerns as follows.

1. This study lacks information in detail of the samples from patients with cardiovascular diseases (CVD), which cannot explain that neointimal formation, phenotypic transition of VSMCs, lipid deposition and macrophage infiltration, etc in patients with CAD is related to SRF SUMOylation.

We thank the reviewer for the constructive comments. We have added detailed information on the samples from patients with cardiovascular diseases (CVD) in Supplementary Table 1.

2. KLF4 plays an important role in the phenotype of VSMCs. In this study, it was observed that SENP1 deficiency did not affect the SUMOylation of KLF4, which is inconsistent with some published articles. The author will briefly explain the reason.

Thank you for this insightful comment. Previous studies found that Ubc9 interacted with and promoted the SUMOylation of KLF4, which allowed the recruitment of transcriptional co-repressors, such as p21, and enhanced PDGF-BB induced VSMC proliferation. (PMID：26945917; PMID：25791170). They used Ubc9 siRNA or ginkgolic acid (a SUMOylation inhibitor) to suppresses KLF4 SUMOylation. In our study, SUMOylation was induced by SENP1 deficiency. However, KLF4 protein expression and nuclear localization were not altered in VSMCs after SENP1 deficiency.

Furthermore, SRF is ubiquitously expressed, why researchers focus on SRF? Myocardin, another key factors in vascular remodeling, which selectively expressed in SMCs and cardiac muscle, also modulated by SUMOylation.

We thank the reviewer for this question. In our study, firstly, we found that SENP1 deficiency in VSMCs accelerates injury-induced neointimal formation and vascular remodeling. Secondly, we performed whole-transcriptome analysis by bulk RNA-seq to investigate its mechanisms, and

found that Senp1 deletion in VSMCs significantly enriched physiological processes related to tissue remodeling, VSMC proliferation, contraction, migration, differentiation and ERK1/2 cascade. KLF4, myocardin, SRF and ELK1 are all indeed key regulating factors that control phenotypic switching in VSMCs during vascular remodeling. Even more interesting is that they all have been previously reported to be themselves SUMO substrates. However, we found that SENP1 deficiency in VSMCs had profound effects on SRF SUMOylation. In contrast, SENP1 deficiency in VSMCs had little effect on the SUMOylation of KLF4, myocardin, or ELK1 in our system. The SUMOylation of KLF4, myocardin and ELK1 could be regulated by other SENP members such as SENP2 which need more investigations. We have incorporated this into DISCUSSION (Pages 16-17).

Actually, the relationship between KLF4, myocardin, SRF and ELK1 is complex in vascular remodeling. Figure 3 showed the expression and SUMOylation of KLF4 and myocardin has little effect on SENP1 deficiency. How to explore the role and clinical significance of SUMOylation modification of such important factors and their complex relationship in vascular remodeling diseases?

Thank you for this constructive comment. Our analyses of human CAD specimens reveal that SRF, SUMO1, and phospho-ELK1 levels were higher in luminal VSMCs from CAD groups compared with the normal coronary arteries, progressively increasing with the disease severity. Accordingly, VSMCs in human intimal hyperplasia exhibited phenotypic switches (with gain of synthetic markers and loss of contractile markers) and correlated with the expression of SRF, SUMO1, and phospho-ELK1 and the I/M ratios. These results reveal the role and clinical significance of SRF SUMOylation and its complex relationship with ELK1 and MYCD in vascular remodeling diseasest.

3. Figure 3 showed the *Senp1*^{SMCKO} mice exhibited a more rapid increase in phosphorylated ELK1 levels compared with WT mice. Why does SUMOylation modification affect phosphorylation? Mechanism?

We thank the reviewer for this clarifying question. In our study, we found that SENP1 deficiency in VSMCs accelerates injury-induced neointimal formation, reflected by enlarged intima areas, decreased media areas, and increased intima/media ratios (Fig.1A). Immunohistochemical staining results revealed a significant increase in the number of phospho-ELK1-positive cells in the neointima of *Senp1*^{SMCKO} mice compared with that of WT mice 28 days after injury; most of these were co-localized with α-SMA-positive VSMCs. These results indicated that SENP1

deficiency accelerated injury-induced neointimal areas, accompanied by increased phospho-ELK1 in the neointima.

The author directly focuses on MEK, and ELK1, there are other signaling pathways involved? Reasons for excluding other pathway need to be added.

We thank the reviewer for their constructive comments. It has been demonstrated that the sustained activation of multiple signal pathways, such as NF-κB, P38-MAPK, AKT and STAT3, regulates injury-induced neointimal formation. Through Western blot, we found the protein levels of Phospho-NF-κB-P65, Phospho-P38, Phospho-AKT and Phospho-STAT3 progressively increased in carotid arteries from 1 day after injury, peaked at 3-7 days, and dropped thereafter (Fig.S6). However, no obvious phosphorylated alterations of these proteins were observed between WT and *Senp1*^{SMCKO} mice.

4. Some grammatical errors need to be corrected. The methods, data and analysis should be described in sufficient detail to be reproduced.

We apologize for the grammatical errors and problems with methods, data and analysis. Per the reviewer's suggestion, the manuscript has been revised by professional scientific editors to improve the clarity of this manuscript and the reproducibility of our results.

RESPONSE TO REVIEWER #4

General Comments: The manuscript "SRF SUMOylation modulates smooth muscle phenotypic switch and vascular remodeling" by Xu et al. is a report that uses primarily Western blot and immunohistochemistry techniques to probe the functional significance of SRF SUMOylation in a mouse model of carotid injury, as well as in cell culture and human coronary arteries. The scientific approach is reasonable, the blots are clean and the multiplex fluorescence immunohistochemistry is strikingly beautiful.

We appreciate your positive comments on our work. We also thank you for your constructive critiques and we have addressed your concerns as follows.

However, the analytical methods for image analysis are not presented in adequate detail to enable others to reproduce the experiments.

We apologize for the problems with our description of image analysis. According to the reviewer's suggestion, we have added detailed information regarding image analysis in ONLINE METHODS.

Furthermore, the "n" in terms of animal number is not always clear, leading to questions as to whether the findings have true statistical significance. The findings and conclusions drawn from these experiments represent a relatively modest advance over what has previously been published on the role of SUMOylation in the phenotypic switching of vascular smooth muscle cells (VSMCs), leading to reservations concerning potential scientific impact.

Thank you very much for pointing out this problem and your valuable suggestions. According to the reviewer's suggestion, we have made the animal number more clear in FIG.LEGENDS.

Finally, the manuscript is well-organized, but there are multiple instances in which it could benefit from more careful editing and proofreading.

We apologize for the grammatical errors and problems with sentence structure. Per the reviewer's suggestion, the manuscript has been revised by professional scientific editors to improve the clarity of this manuscript.

Specific Comments:

1) Page 2, line 11: In the abstract and throughout the manuscript, please correct "phosphor-ELK1" to "phospho-ELK1."

Thank you for pointing out this error and we have corrected related content in the revised manuscript.

2) Page 4, line 21: The following sentence may be overstated given that it has been known for over 20 years that the SRF activity is modulated by SUMOylation (Reference 29): "In contrast to

the relatively well-studied transcriptional control mechanisms, regulation of VSMC fate at the post-transcriptional level, such as SUMOylation, has not been well investigated."

Thank you for your pointing this out. We have revised the sentence to "Thus, investigations into the molecular mechanisms underlying SRF-mediated VSMC phenotypic switch would improve our understanding of the intricate mechanisms of vascular remodeling and neointima formation and hopefully inspire new strategies for treating CVD".

3) Page 6, line 30: Recommend revising the last sentence on page 6. For example: "Despite the fact that carotid artery morphometry at baseline was unaltered by VSMC-specific Senp1 deletion, …"

Thank you for your advice. We have revised the sentence to "Despite the fact that carotid artery morphometry at baseline was unaltered by VSMC-specific Senp1 deletion".

4) Page 7, line 24: Recommend clarifying what 1M refers to. For example: "(Fig.1J with quantifications in Fig.1M for EdU+ SMCs).

Thank you for your suggestion. We have revised these sentences in the RESULTS accordingly.

5) Page 8, line 10. The sentence in question could be simplified as follows: "Senp1 SMCKO mice exhibited a more rapid decrease in the four contractile markers and an increase in the two synthetic markers compared with WT mice (Fig.2A)."

Thank you for this suggestion. We have revised the sentence to "*Senp1*^{SMCKO} mice exhibited a more rapid decrease in the four contractile markers and an increase in the two synthetic markers compared with WT mice (Fig.2A)".

6) Page 8, line 25: Recommend revising as follows: "… in normal carotid arteries and cultured aortic VSMCs, …"

Thank you for your edits. We have corrected this line in the revised manuscript.

7) Page 9, line 6: Recommend correcting "indued" to "induced".

Thank you, and we have edited the word in accordance with your suggestion.

8) Page 9, line 11: Recommend the following for clarity: "The results above prompted us to investigate the post-translational regulation of SRF."

Thank you for your suggestion. We have corrected this line in the revised manuscript.

9) Page 9, line 13: The following statement should be revised to reflect the distinction between "suggesting" something and "showing" it: "Previous report been suggested that SRF could be modified by SUMO1-mediated SUMOylation29." For example, the following sentence may be more accurate: "A previous report showed that SRF is modified by SUMO-1, chiefly at lysine147 within the DNA-binding domain."

Thank you for your underlining this distinction. We have corrected this line in the revised manuscript.

10) Page 12, line 15: Suggest the following for clarity: “and most of these signals were co-localized with α-SMA-positive VSMCs (Fig.6C-D).”

Thank you. We have made this edit in the revised manuscript.

11) Page 19, line 14: The last sentence reads: “DMSO served as vehicle control.” However, Fig. 7A at top left of page 41 shows a timeline indicating that PBS was the vehicle control. Please resolve this contradiction.

We apologize for this contradiction. We further confirmed that PBS served as vehicle control and corrected the description in the revised manuscript.

12) Page 19, line 27: Please replace the word ”sacrificed” with “euthanized” due to the negative connotation of the word “sacrifice”.

Thank you for pointing this out. We have edited the word in accordance with your suggestion.

13) Page 20, line 8: The following sentence does not address how many sections per patient: “…and images were obtained for one section per block at a final magnification of 40×.” It appears that only one slide was analyzed per patient. If true, that it would be more accurate to state: “…and images were obtained from one slide per patient at a final magnification of 40×.”

Thank you for indicating this point of confusion. According to your suggestion, we have corrected the relevant content in the revised manuscript.

14) Page 20, line 10: The following sentence does not address how many specimens per patient: “The means of these parameters from four different areas for each specimen were calculated.” It appears that only one slide was analyzed per patient. If true, then it would be more accurate to state: “The means of these parameters from four different areas on each slide were calculated.”

Thank you for pointing out this ambiguity. According to your suggestion, we have made appropriate edits in the revised manuscript.

15) Page 22, line 9: Please indicate the mean number of cells counted per “randomely selected area”. I.E. “These migrated VSMCs were quantified in four randomly selected fields at 100× magnification using Cellsens Dimention 1.15 software (Olympus, Tokyo, Japan), with each field containing a mean of XX cells.”

Thank you for this suggestion. In our study, different groups contain different mean numbers of cells in each field, which have been shown in the Excel file of Source Data.

16) Page 22, line 10: Recommend correcting “Dimention” to “Dimension”.

Thank you for your highlighting this spelling error. We have corrected it in the revised manuscript.

17) Page 23, line 6: After the Methods section on: “Immunofluorescence staining,” please insert a new section entitled “Quantification of Immunofluorescence Images”. This new section should be used to disclose details of the images quantification methods shared in common between the images that were analyzed to yield quantitative results. If multiple approaches or analytical techniques were applied then it may be appropriate to list the techniques along with the Figure and Panel in which each technique was applied. The authors might use a review article such as PMID: 21209361 to identify which general strategy was employed, but if a software program such as Cellsens Dimension was used, then in addition the particular sub-program or analytical tool should be specified by the name used in the software package.

Thank you for your advice. We have inserted a new section entitled “Quantification of Immunofluorescence Images” in the ONLINE METHODS and disclosed details of the image quantification methods accordingly (Page 22).

Quantification of Immunofluorescence Images: Immunofluorescence images were acquired using an LSM880 laser confocal microscope (Carl Zeiss, Germany) equipped with a ×40 objective. Image-Pro Plus 6.0 software (NIH, Bethesda, MD, USA) was used for quantitative analyses. The neointimal area was determined by subtraction of the lumen area from the area circumscribed by the internal elastic lamina traced on stained sections (Fig.S). The medial area was defined as the area between an external elastic lamina and internal elastic lamina (Fig.S). Measurements were obtained from four discontinuous sections per sample. Two different fields per murine section and four different fields for human samples and cultured VSMCs are analyzed, the minimum number of cells counted per field is fifty in human and murine samples and thirty in cultured VSMCs. For quantitative analysis of MMP2, MMP9, CNN1, α-SMA, SM22α, MYH11, MYH10 or OPN staining, the positively stained areas were measured. The neointimal area in human left main coronary arteries, carotid artery after wire injury or media area in un-injury carotid artery was shown as 100%. For quantitative analysis of Ki67, p-ELK1, SRF, cleaved caspase-3 or SUMO1 staining, the number of positively stained cells was calculated. The α-SMA- positive VSMCs in human left main coronary arteries, neointimal area of carotid artery after wire injury or media area in un-injury carotid artery was shown as 100%. For quantitative analysis of Edu staining, the number of Edu-positive stained VSMCs were calculated. The number of α-SMA- positive VSMCs was shown as 100%.

18) Page 23, line 9: The following sentence is a valid approach: “As the protein concentration from a single mouse carotid artery tissue was low, we mixed tissue samples from three to six individuals for protein extraction.” However, from a statistical standpoint, a pooled sample

containing 3 to 6 arteries is not the same as n=3-6 because separate measurements were not obtained from those 3 to 6 arteries.

Thank you for this comment. We have removed the “n=3 per group” in the Western blots of FIG.LEGENDS according to your suggestion.

19) Page 24, line 12: Recommend removing the following sentence unless its meaning is unclear: “All figures are representative of at least three experiments unless otherwise noted.” This is unclear because what is meant by an “experiment” may change from on figure to the next. It is never clear from the figures that at least 3 separate experiments were conducted at 3 different times. In some cases, the authors may be equating an “experiment” with an artery, or a transfected well, or a lane in a Western blot. It is important to specify which of these possibilities apply in each Figure since they are not equivalent from a statistical perspective.

Thank you for pointing out this error, and we have removed the sentence in the revised manuscript.

20) Page 24, line 15: Recommend revising the following sentence to identify the specific instances in which: “The normality and variance were not tested.” This is because the sentence implies that normality and variance were never tested.

Thank you for pointing out this error, and we have revised the sentence to “The Shapiro-Wilk test was used to check normality, and Bartlett’s test was used to check equal variance”.

21) Page 29, line 3: Figure 1, Panels F and G: Nearly all lanes have ratios from densitometry beneath them. Please indicate this to the right of the densitometry ratios with something like “Ratio” or “Quant”, then define that term more fully in the figure legend.

Thank you for your advice. According to your suggestion, we have added “Ratio” to the right of the densitometry ratios, then define it more fully in the figure legend.

22) Page 30, line 3: Here and throughout the Figure Legends, please explicitly state precisely what n refers to. Take for example the following sentence: “Circumference of EEL, neointimal area, media area, and neointima/media ratio in carotid arteries were measured (n=12 per group).” Here, any of the following could be true: n=12 mice per group, n=12 carotid arteries per group, n=12 left carotid arteries per group or n=12 sections per group. In the case of 12 sections/group, it would also be important to specify how many sections per carotid.

Thank you for your advice. According to your suggestion, we have stated precisely what n refers to in the Figure Legends, then define it more fully in the ONLINE METHODS.

23) Page 30, line 4: Again, please define “n=10 (what?) per group.” In this case, it would be helpful to specify that only left carotid arteries were used, if that is true.

Thank you for your advice. According to your suggestion, we have stated precisely what n refers to in the Figure Legends.

24) Page 30, line 11: The following sentence, or closely related sentences, occurs some 17 times in the Figure Legends: “Relative protein levels are presented by taking non-injured WT as 1.0 (n=3 per group).” When considered in combination with the statement from Page 23, line 9: “As the protein concentration from a single mouse carotid artery tissue was low, we mixed tissue samples from three to six individuals for protein extraction,” this seems to imply that the Western blot shown contains only a single WT lane representing a pool of 3 WT mice. Please address this question, since that equates to “n=1” from a statistical perspective. If so, this needs to be clarified by revising the statement. For example: “Protein levels were calculated relative to protein extracted from a pool of 3 non-injured left carotids from WT mice.” If instead 3 identical Western blots were performed using carotids from 9 different WT mice, then please include the additional blots within “Supplementary Fig.12: Uncut original gels.”

Thank you for your advice. In our study, we mixed carotids from three individuals for protein extraction. According to your suggestion, we have stated “n=1 per group” in the Figure Legends.

25) Page 30, line 13: The following sentence refers to the graphs in Panel I: “% of Ki67+α-SMA+ VSMCs, MFI of MMP2 and MMP9 within the neointimal area was quantified (n=10 per group).” The first part of the sentence refers to the first graph, but the remainder appears unrelated to the 2nd and 3rd graphs. This is an example where the Methods used for image analysis need to be better defined, either individually here in the Figure legends or collectively in a new Methods section on “Quantification of Immunofluorescence Images”. The problem with “MFI of MMP2 and MMP9 within the neointimal area was quantified” is that it inadequately describes the determination of Manders overlap, which is what appears to have been done here. Certainly, the Y-axes for the 2nd and 3rd graphs do not relate to mean fluorescence intensity (MFI). Further, as stated above, any reference to “n” needs to define n-what? In this case, this could mean: n=10 mice per group, n=10 sections per group, etc. Best practices would include stating not only how many left carotid arteries per group but also how many sections analyzed from each left carotid.

Thank you for your advice. We inserted a new section entitled “Quantification of Immunofluorescence Images” in the ONLINE METHODS and disclosed details of the image quantification methods accordingly (Page 22).

25) Page 30, line 14: Please enhance the following sentence to specify whether the VSMCs were isolated from carotid arteries, and if carotids were isolated from multiple mice please state how many carotids were pooled for each VSMC isolation.

Thank you for your advice. In the ONLINE METHODS section on “VSMCs isolation and culture”, we stated that the VSMCs were isolated from thoracic aortas, and six thoracic aortas were pooled for each group for VSMC isolation.

26) Page 30, line 20: Previous concerns also relate to the following sentence: “(M) % Edu+α-SMA+ in (J), % wound closure in (K) and number of invaded VMSCs in (L) were quantified (n = 8 per group).” Here, n could refer to: n = 8 scratch assays per group, or n = 8 views per scratch assay. If the VSMCs were isolated from pooled carotids, that should also be clarified here.

Thank you for your advice. According to your suggestion, we have stated precisely “n = 8 views per group” and “Six thoracic aortas were pooled for each group for VSMC isolation” in the Figure Legends.

27) Page 31, lines 307-374: As above, please clarify how: ”MFI of each marker within the neointimal area was quantified (n=10 per group)” relates to the graphs presented in Panel C. If only MFI was determined, then how were individual cells identified to generate the Y-axes for each graph (e.g.: CNN1+ staining (%)? Note that all the immunostains in Panel B have the format: “Marker/alpha-SMA/DAPI” except for the panel at lower left which is labeled: “Marker/MYH11+/DAPI”. It is unclear whether this was intended or not, but it contributes further to the uncertainly regarding whether MFI or Mander’s overlap is being quantitated in the graphs.

Thank you for highlighting this point of confusion. For quantitative analysis of CNN1, α-SMA, SM22α, MYH11, MYH10 or OPN staining, these markers positive stained areas were measured. The neointimal area in the left carotid artery after wire injury or media area in the un-injured carotid artery was shown as 100%. We have changed the labels of Y-axes for each graph.

28) Page 36, line 11: The meaning of the following statement is unclear: “GAPDH as the loading control (n = 3 per group).” It appears likely that this again refers to a pooled sample approach, where cell lysates were pooled from 3 different wells, or transfections, or experiments. Thus “n = 3 what? per group” needs to be defined.

Thank you for your advice. According to your suggestion, we have stated “n=1 per group” in the Figure Legends.

29) Page 39, line 5: Please clarify whether the GAPDH blots were included only for visual confirmation of equivalent loading, or whether the numbers reported beneath each lane have been normalized relative to the intensity of the respective GAPDH bands.

Thank you for your advice. According to your suggestion, we have confirmed that the numbers reported beneath each lane have been normalized relative to the intensity of the respective GAPDH bands.

30) Page 39, line 7: Please expand the following statement: “Five fields per section from each sample are analyzed” to include the minimum number of cells counted per field, and the number of sections analyzed per patient.

Thank you for your advice. According to your suggestion, we have stated precisely, “Measurements were obtained from two discontinuous sections per sample. Four different fields for human samples were analyzed, and the minimum number of cells counted per field was fifty in human samples.” in the ONLINE METHODS.

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

The authors have addressed my comments satisfactorily. The manuscripts has improved significantly. Here are minor comments: (1) The interplay of SRF-MYOCD-ELK1 in controlling SMC phenotypic modulation was first defined by Wang et.al (2004, PMID 15014501). This fundamental paper in VSMC phenotypic modulation could be cited here. "Moreover, vascular injury or PDGF-BB stimulation induces MEK-extracellular signal-related kinases (ERK)-dependent phosphorylation of Ets-like transcription factor-1 (ELK1), which displaces phospho-SRF from the myocardin complex to form the SRF-ELK1 complex." (2) To increase the impact of the paper, could the mechanism be summarized by a graphic abstract? (3) Myocardin is mostly known as MYOCD although MYCD is acceptable.

Reviewer #2 (Remarks to the Author):

The authors have fully addressed my two very minor points, and I now fully support publication of their manuscript. I congratulate them on an outstanding body of work.

Reviewer #3 (Remarks to the Author):

Dear editor

Thanks for the trust again. I have carefully reviewed the author's revision suggestions and we have reached a basic agreement. I recommend that the magazine accept the submission.

Reviewer #4 (Remarks to the Author):

General Comments:

The manuscript “SRF SUMOylation modulates smooth muscle phenotypic switch and vascular remodeling” by Xu et al. is a report that uses primarily Western blot and immunohistochemistry techniques to probe the functional significance of SRF SUMOylation in a mouse model of carotid injury, as well as in cell culture and human coronary arteries. The scientific approach is reasonable, the blots are clean and the multiplex fluorescence immunohistochemistry is strikingly beautiful. In the current revision, the authors have added a new paragraph to the Methods on the “Quantification of Immunofluorescence Images”. While this is a welcome addition, the presentation of these Methods in the figure legends continues to be inconsistent and makes it difficult to follow the analytical methods used for image analysis. Similarly, the “n” in terms of animal numbers has been clarified in many of the figure legends, but many of the findings still claim to be “significant” when there are no p-values available to support those claims. The findings and conclusions drawn from these experiments represent a relatively modest advance over what has previously been published on the role of SUMOylation in the phenotypic switching of vascular smooth muscle cells (VSMCs), leading to reservations concerning overall scientific impact. Finally, the manuscript is well-organized, but there remain inconsistencies between what is actually shown in the figures and what is claimed in the text.

Specific Comments:

As mentioned above, the manuscript has been improved by the addition of a new paragraph to the Methods on the “Quantification of Immunofluorescence Images”. This new section helps distinguish between the two general strategies employed for quantitative analysis: area analysis and co-localization analysis. However, this clarification remains to be applied consistently throughout the text and figure legends. Specific examples follow, but it was not possible to identify all such problems in the text.

Page 29, line 18: The reference to “MFI of MMP2 and MMP9” is misleading because the new paragraph on image analysis identifies this as an area measurement, not a measurement of “Mean Fluorescence Intensity”. Perhaps MFI was used to set the threshold for automated area analysis, but the methods do not specify whether the areas were determined manually, or by thresholding.

Regardless, any such method of image segmentation is error-prone and fraught with difficulty, particularly if painstaking methods are not carefully applied to avoid common pitfalls (PMID: 29187165).

Page 30, line 7: The reference to "(B). MFI of each marker within the neointimal area…" is misleading for the same reason. The figure has been revised to read "Area", so the Figure legend should be revised accordingly.

Page 30, line 19: The reference to "C) MFI of p-ELK within the neointimal areas…" is puzzling for several reasons. The y-axis in Figure 3C reads "p-ELK1+/CD31+ ECs in neointimal area (%)," so this is probably a co-localization measurement. If so, the y-axis should instead read "p-ELK1+/CD31+ ECs (%) within the neointima." A similar correction should be applied to "p-ELK1+/a-SMA+ VSMCs in neointimal area (%)," and any reference to MFI in the figure legend should be removed.

Page 30, line 32: The reference to "C) MFI of SRF within the neointimal areas…" is puzzling for the several reasons. The y-axis in Figure 3E reads "SRF+/CD31+ ECs in neointimal area (%)," so this is probably a co-localization measurement. If so, the y-axis should read "SRF+/CD31+ ECs (%) within the neointima." A similar correction should be applied to "SRF+/a-SMA+ VSMCs in neointimal area (%)," and any reference to MFI in the figure legend should be removed.

Page 34, line 9: The reference to "(H). MFI of each marker within the neointimal areas…" is misleading. The figure has been revised to read "Area", so the figure legend should be revised accordingly.

Page 34, line 12: The reference to MFI in the figure legend for (J) is misleading. Please re-word to something like: "Ki67+α-SMA+ VSMCs, and fractional areas of the neointima occupied by MMP2+ or MMP9+ cells were quantified…"

As mentioned above under "General Comments", the manuscript has been improved by clarifying what "n" refers to in many of the figure legends. However, many of the findings are characterized as "significant" in the absence of p-values to support those claims. The authors are cautioned to reserve the words "significant" or "significantly" for specific instances that also quote a p-value <0.05.

In a related matter, the authors are encouraged to revisit their revisions that refer to pools of carotid arteries as "n=1". The previous review contained a comment that pools of 3-6 arteries equate to "n=1" from a statistical perspective, but that comment was not intended to suggest that "n=1" needed to be repeated throughout the manuscript. Consistent with the Methods section on "western blot analysis," a more even-handed approach would be to replace the multiple instances of "by taking non-injured WT as 1.0 (n=1 per group)" with "by defining as 1.0 the signal from each pool of 3-6 non-injured WT carotid arteries per lane". This may be a lengthy explanation, but it more faithfully portrays the experiment.

With regard to the caveat against claiming "significance" in the absence of a p-value, the following instances serve as examples where significance was claimed in the absence of $p<0.05$. It is recommended that the words "significant" or "significantly" be deleted from the following sentences unless p-values can be provided.

Page 7, line 6: "significantly higher cyclin D1 and PCNA levels were detected in Senp1SMCKO mice than that in WT mice (Fig.1F)."

Page 7, line 15: "with significantly higher levels in Senp1SMCKO mice than WT mice (Fig.1F-G)."

Page 8, line 15: "Senp1 deficiency in VSMCs caused significant repression of CNN1, α-SMA, SM22α, and MYH11, but promotion of MYH10 and OPN (Fig.2D-E) in a time dependent manner compared to the VSMCs from WT mice."

Page 9, line 11: "Western blot indicated that injury induced a time-dependent increase of total SRF (Fig.3F) and phospho-SRF (Fig.3H) expression in the carotid artery which was significantly augmented by SENP1 deletion."

Page 10, line 18: "PDGF-BB stimulation significantly increased SRF expression in both lysosomes

and nuclei, where SRF was co-localized with lysosome marker LAMP2 and nuclear marker DAPI, respectively."

Page 10, line 23: "We confirmed the SRF localization in SENP1-deficient VSMCs by a cellular fractionation assay; PDGF-BB stimulation significantly increased total SRF and SUMOylated SRF levels in nuclear and cytoplasm factions,"

Page 10 line 26: "Cycloheximide assays indicated that SENP1 deficiency significantly sustained the levels of both SUMOylated and total SRF proteins in cultured aortic VSMCs (Fig.4C)."

Page 11, line 10: "However, SRFK143R mutant significantly decreased phospho-SRF expression (Fig.4H)."

Page 11, line 15: "Coimmunoprecipitation assays revealed that the non-SUMOylated mutant SRFK143R significantly increased SRF binding to myocardin concomitant with reduced binding to ELK1 in the untreated and PDGF-BB-treated VSMCs (Fig.4J)."

Page 13, line 24: "…AZD6244 significantly reduced the expression of SRF-ELK1-mediated synthetic markers (MYH10 and OPN) but increased that of SRF/myocardin-mediated contractile markers (CNN1, α-SMA, SM22α, and MYH11) (Fig.7D)."

Page 13, line 26: "Moreover, AZD6244 treatment significantly suppressed the expression of proliferation markers (PCNA and CyclinD1), and increased the activities of migratory proteins (MMP2 and MMP9), as evident by their cleavage in injured arteries (Fig.7D)."

Page 14, line 10: "Moreover, AZD6244 treatment in injured arteries significantly suppressed PCNA and cyclin D1 expression from day 7 to day 28 post-injury,"

The following specific comments serve as examples of how the data presented in the Figures do not always fully support the conclusions that are drawn.

Page 8, line 25: The following statement is only partially supported by data: "Immunohistochemical staining revealed a significant increase in phospho-ELK1-positive cell number in the neointima of Senp1SMCKO mice compared with that of WT mice 28 days after injury; most of these were co-localized with αSMA-positive VSMC and little with the CD31-positive ECs (Fig.3B with quantifications in Fig.3C)." The problem here is that the raw data for the number of phospho-ELK1-positive cells in the neointima is never presented. The available data only shows the percentage of CD31+ or αSMA+ cells that were also phosph-ELK1+. A more accurate summary of the results might be: "Immunohistochemical staining revealed a significant increase in the percentage of phospho-ELK1-positive, αSMA+ VSMCs in the neointima of Senp1SMCKO mice as compared to that of WT mice 28 days after injury. In contrast, no statistical difference was detected in the percentage of phospho-ELK1-positive, CD31-positive ECs in the neointima of Senp1SMCKO mice as compared to that of WT mice 28 days after injury."

Page 13, line 28: The following statement is only partially true: "AZD6244 treatment significantly inhibited injury-induced expression of Col15a1, Col5a2, Col6a2, Timp1, Mmp14 and Col1a1 at 28 days after wire injury as determined by qRT-PCR (Supplementary Fig.11)." A more accurate alternative might be: "AZD6244 treatment significantly inhibited injury-induced expression of Col15a1, Col5a2, Col6a2, Timp1, and Mmp14 at 28 days after wire injury in either WT or Senp1SMCKO mice (or both) as determined by qRT-PCR (Supplementary Fig.11)."

Page14, line 13: The following statement is only partially true: "Consistent with the decreased MMP activity detected by western blot, immunohistochemical staining showed significantly decreased MMP2 and MMP9 protein levels in both WT and Senp1SMCKO mice after AZD6244 treatment, but no significant differences in fluorescence intensities between Senp1SMCKO mice and WT mice (Fig.7I-J)." A more accurate alternative might be: "Consistent with the decrease in active MMPs detected by western blot, immunohistochemical staining showed a significant decrease in the percent of neointimal area that stained positive for MMP2 or MMP9 in both WT and Senp1SMCKO mice after AZD6244 treatment, but no significant differences in the percent of neointimal area stained positive for MMP2 or MMP9 between WT and Senp1SMCKO mice treated with AZD6244 (Fig.7I-J)."

POINT-TO-POINT RESPONSE TO REVIEWERS' COMMENTS

RESPONSE TO REVIEWER #1

The authors have addressed my comments satisfactorily. Here are minor comments: (1) The interplay of SRF-MYOCD-ELK1 in controlling SMC phenotypic modulation was first defined by Wang et.al (2004, PMID 15014501). This fundamental paper in VSMC phenotypic modulation could be cited here. "Moreover, vascular injury or PDGF-BB stimulation induces MEK-extracellular signal-related kinases (ERK)-dependent phosphorylation of Ets-like transcription factor-1 (ELK1), which displaces phospho-SRF from the myocardin complex to form the SRF-ELK1 complex."

We appreciate your positive comments on our work. We have cited the work in this section (ref #6).

To increase the impact of the paper, could the mechanism be summarized by a graphic abstract?

Thank you for your great suggestion. We have provided a graphic abstract.

Myocardin is mostly known as MYOCD although MYCD is acceptable.

Thank you for your great suggestion. We have changed from MYCD to MYOCD throughout the text and figures.

RESPONSE TO REVIEWER #4

General Comments: The manuscript "SRF SUMOylation modulates smooth muscle phenotypic switch and vascular remodeling" by Xu et al. is a report that uses primarily Western blot and immunohistochemistry techniques to probe the functional significance of SRF SUMOylation in a mouse model of carotid injury, as well as in cell culture and human coronary arteries. The scientific approach is reasonable, the blots are clean and the multiplex fluorescence immunohistochemistry is strikingly beautiful. In the current revision, the authors have added a new paragraph to the Methods on the "Quantification of Immunofluorescence Images".

While this is a welcome addition, the presentation of these Methods in the figure legends continues to be inconsistent and makes it difficult to follow the analytical methods used for image analysis.

Similarly, the "n" in terms of animal numbers has been clarified in many of the figure legends, but many of the findings still claim to be "significant" when there are no p-values available to support those claims. The findings and conclusions drawn from these experiments represent a relatively modest advance over what has previously been published on the role of SUMOylation in the phenotypic switching of vascular smooth muscle cells (VSMCs), leading to reservations concerning overall scientific impact.

Finally, the manuscript is well-organized, but there remain inconsistencies between what is actually shown in the figures and what is claimed in the text.

We appreciate your positive comments on our work. We also thank you for your constructive critiques and we have addressed your concerns as follows.

Specific Comments:

As mentioned above, the manuscript has been improved by the addition of a new paragraph to the Methods on the "Quantification of Immunofluorescence Images". This new section helps distinguish between the two general strategies employed for quantitative analysis: area analysis and co-localization analysis. However, this clarification remains to be applied consistently throughout the text and figure legends. Specific examples follow, but it was not possible to identify all such problems in the text.

Thank you very much for pointing out this problem and your valuable suggestions. According to the reviewer's suggestion, we have fully checked and edited the relevant content in the revised manuscript. Specifically, we have clarified it as "the number of cells with positively stained or each marker was manually calculated in a blind fashion".

Page 29, line 18: The reference to "MFI of MMP2 and MMP9" is misleading because the new paragraph on image analysis identifies this as an area measurement, not a measurement of "Mean Fluorescence Intensity". Perhaps MFI was used to set the threshold for automated area analysis, but the methods do not specify whether the areas were determined manually, or by thresholding. Regardless, any such method of image segmentation is error-prone and fraught with difficulty, particularly if painstaking methods are not carefully applied to avoid common pitfalls (PMID: 29187165).

Thank you for indicating this point of confusion. According to your suggestion, we have corrected the relevant content in the revised manuscript. Specifically, we have clarified it "$Ki67^+$α-SMA^+ VSMCs, and fractional areas of the neointima occupied by $MMP2^+$ or $MMP9^+$ cells were quantified (n=10 left carotid arteries per group)."

Page 30, line 7: The reference to "(B). MFI of each marker within the neointimal area…" is misleading for the same reason. The figure has been revised to read "Area", so the Figure legend should be revised accordingly.

Thank you for indicating this point of confusion. According to your suggestion, we have corrected the relevant content in the revised manuscript. We have revised those as "Fractional number of p-ELK^+ cells within the neointimal areas or ECs were quantified (n =10 left carotid arteries per group)".

Page 30, line 19: The reference to "C) MFI of p-ELK within the neointimal areas…" is puzzling for several reasons. The y-axis in Figure 3C reads "p-ELK1+/CD31+ ECs in neointimal area (%)," so this is probably a co-localization measurement. If so, the y-axis should instead read "p-ELK1+/CD31+ ECs (%) within the neointima." A similar correction should be applied to "p-ELK1+/a-SMA+ VSMCs in neointimal area (%)," and any reference to MFI in the figure legend should be removed.

Thank you for indicating this point of confusion. According to your suggestion, we have corrected the relevant content in the Figure 3 and Figure Legends of revised manuscript as follows: "Ratio in (A) means relative protein levels, which are presented by defining as 1.0 the signal from each pool of 3-6 non-injured WT carotid arteries per lane".

Page 30, line 22: The reference to "C) MFI of SRF within the neointimal areas…" is puzzling

for the several reasons. The y-axis in Figure 3E reads “SRF+/CD31+ ECs in neointimal area (%),” so this is probably a co-localization measurement. If so, the y-axis should read “SRF+/CD31+ ECs (%) within the neointima.” A similar correction should be applied to “SRF+/a-SMA+ VSMCs in neointimal area (%),” and any reference to MFI in the figure legend should be removed.

Thank you for indicating this point of confusion. According to your suggestion, we have corrected the relevant content in the Figure 3 and Figure Legends of revised manuscript as follows: “(C) Fractional number of p-ELK^{+} cells within the neointimal areas or ECs were quantified (n =10 left carotid arteries per group”.

Page 34, line 9: The reference to “(H). MFI of each marker within the neointimal areas…” is misleading. The figure has been revised to read “Area”, so the figure legend should be revised accordingly.

Thank you for indicating this point of confusion. According to your suggestion, we have corrected the relevant content in the Figure Legends of revised manuscript as follows: “(H) Fractional areas of the neointima occupied by each marker were quantified (n = 10 left carotid arteries per group)”.

Page 34, line 12: The reference to MFI in the figure legend for (J) is misleading. Please re-word to something like: “Ki67+α-SMA+ VSMCs, and fractional areas of the neointima

Thank you for indicating this point of confusion. According to your suggestion, we have corrected the relevant content in the Figure Legends of revised manuscript as follows: “(J) Ki67^{+}α-SMA^{+} VSMCs, and fractional areas of the neointima occupied by MMP2^{+} or MMP9^{+} cells were quantified (n = 10 left carotid arteries per group)”.

As mentioned above under “General Comments”, the manuscript has been improved by clarifying what “n” refers to in many of the figure legends. However, many of the findings are characterized as “significant” in the absence of p-values to support those claims. The authors are cautioned to reserve the words “significant” or “significantly” for specific instances that also quote a p-value <0.05.

In a related matter, the authors are encouraged to revisit their revisions that refer to pools of carotid arteries as “n=1”. The previous review contained a comment that pools of 3-6 arteries equate to “n=1” from a statistical perspective, but that comment was not intended to suggest that “n=1” needed to be repeated throughout the manuscript. Consistent with the Methods section on “western blot analysis,” a more even-handed approach would be to replace the multiple instances of “by taking non-injured WT as 1.0 (n=1 per group)” with “by defining as 1.0 the signal from each pool of 3-6 non-injured WT carotid arteries per lane”. This may be a lengthy explanation, but it more faithfully portrays the experiment.

Thank you for pointing out this ambiguity. According to your suggestion, we have made appropriate edits in the revised manuscript.

With regard to the caveat against claiming “significance” in the absence of a p-value, the following instances serve as examples where significance was claimed in the absence of p<0.05. It is recommended that the words “significant” or “significantly” be deleted from the

following sentences unless p-values can be provided.
Page 7, line 6: “significantly higher cyclin D1 and PCNA levels were detected in Senp1SMCKO mice than that in WT mice (Fig.1F).”
Page 7, line 15: “with significantly higher levels in Senp1SMCKO mice than WT mice (Fig.1F-G).”
Page 8, line 15: “Senp1 deficiency in VSMCs caused significant repression of CNN1, α-SMA, SM22α, and MYH11, but promotion of MYH10 and OPN (Fig.2D-E) in a time dependent manner compared to the VSMCs from WT mice.”
Page 9, line 11: “Western blot indicated that injury induced a time-dependent increase of total SRF (Fig.3F) and phospho-SRF (Fig.3H) expression in the carotid artery which was significantly augmented by SENP1 deletion.”
Page 10, line 18: “PDGF-BB stimulation significantly increased SRF expression in both lysosomes and nuclei, where SRF was co-localized with lysosome marker LAMP2 and nuclear marker DAPI, respectively.”
Page 10, line 23: “We confirmed the SRF localization in SENP1-deficient VSMCs by a cellular fractionation assay; PDGF-BB stimulation significantly increased total SRF and SUMOylated SRF levels in nuclear and cytoplasm factions,”
Page 10 line 26: “Cycloheximide assays indicated that SENP1 deficiency significantly sustained the levels of both SUMOylated and total SRF proteins in cultured aortic VSMCs (Fig.4C).”
Page 11, line 10: “However, SRFK143R mutant significantly decreased phospho-SRF expression (Fig.4H).”
Page 11, line 15: “Coimmunoprecipitation assays revealed that the non-SUMOylated mutant SRFK143R significantly increased SRF binding to myocardin concomitant with reduced binding to ELK1 in the untreated and PDGF-BB-treated VSMCs (Fig.4J).”
Page 13, line 24: “…AZD6244 significantly reduced the expression of SRF-ELK1-mediated synthetic markers (MYH10 and OPN) but increased that of SRF/myocardin-mediated contractile markers (CNN1, α-SMA, SM22α, and MYH11) (Fig.7D).”
Page 13, line 26: “Moreover, AZD6244 treatment significantly suppressed the expression of proliferation markers (PCNA and CyclinD1), and increased the activities of migratory proteins (MMP2 and MMP9), as evident by their cleavage in injured arteries (Fig.7D).”
Page 14, line 10: “Moreover, AZD6244 treatment in injured arteries significantly suppressed PCNA and cyclin D1 expression from day 7 to day 28 post-injury,”
Thank you for your advice. According to your suggestion, we have removed the words “significant” or “significantly” in the revised manuscript.

The following specific comments serve as examples of how the data presented in the Figures do not always fully support the conclusions that are drawn.
Page 8, line 25: The following statement is only partially supported by data: “Immunohistochemical staining revealed a significant increase in phospho-ELK1-positive cell number in the neointima of Senp1SMCKO mice compared with that of WT mice 28 days after injury; most of these were co-localized with αSMA-positive VSMC and little with the CD31-positive ECs (Fig.3B with quantifications in Fig.3C).” The problem here is that the raw data for the number of phospho-ELK1-positive cells in the neointima is never presented. The

available data only shows the percentage of CD31+ or αSMA+ cells that were also phosph-ELK1+. A more accurate summary of the results might be: “Immunohistochemical staining revealed a significant increase in the percentage of phospho-ELK1-positive, αSMA+ VSMCs in the neointima of Senp1SMCKO mice as compared to that of WT mice 28 days after injury. In contrast, no statistical difference was detected in the percentage of phospho-ELK1-positive, CD31-positive ECs in the neointima of Senp1SMCKO mice as compared to that of WT mice 28 days after injury.”
We have made this change as you suggested.

Page 13, line 28: The following statement is only partially true: “AZD6244 treatment significantly inhibited injury-induced expression of Col15a1, Col5a2, Col6a2, Timp1, Mmp14 and Col1a1 at 28 days after wire injury as determined by qRT-PCR (Supplementary Fig.11).” A more accurate alternative might be: “AZD6244 treatment significantly inhibited injury-induced expression of Col15a1, Col5a2, Col6a2, Timp1, and Mmp14 at 28 days after wire injury in either WT or Senp1SMCKO mice (or both) as determined by qRT-PCR (Supplementary Fig.11).”
We have made this change as you suggested (page 14).

Page14, line 13: The following statement is only partially true: “Consistent with the decreased MMP activity detected by western blot, immunohistochemical staining showed significantly decreased MMP2 and MMP9 protein levels in both WT and Senp1SMCKO mice after AZD6244 treatment, but no significant differences in fluorescence intensities between Senp1SMCKO mice and WT mice (Fig.7I-J).” A more accurate alternative might be: “Consistent with the decrease in active MMPs detected by western blot, immunohistochemical staining showed a significant decrease in the percent of neointimal area that stained positive for MMP2 or MMP9 in both WT and Senp1SMCKO mice after AZD6244 treatment, but no significant differences in the percent of neointimal area stained positive for MMP2 or MMP9 between WT and Senp1SMCKO mice treated with AZD6244 (Fig.7I-J).”
We have made this change as you suggested (page 14).

REVIEWER COMMENTS

Reviewer #4 (Remarks to the Author):

General Comments:

The manuscript "SRF SUMOylation modulates smooth muscle phenotypic switch and vascular remodeling" by Xu et al. is a report that uses primarily Western blot and immunohistochemistry techniques to probe the functional significance of SRF SUMOylation in a mouse model of carotid injury, as well as in cell culture and human coronary arteries. The scientific approach is reasonable, the blots are clean and the multiplex fluorescence immunohistochemistry is strikingly beautiful. In the current revision, the authors have made a good faith effort toward implementing the requested revisions. While the authors have been responsive to review, the previous weaknesses of the manuscript are noted.

1) The findings and conclusions drawn from these experiments represent a relatively modest advance over what has previously been published on the role of SUMOylation in the phenotypic switching of vascular smooth muscle cells (VSMCs), leading to reservations concerning overall scientific impact.

2) In the first revision, the authors acknowledged that their technique or pooling 3-6 non-injured WT carotid arteries for a single lane in nearly every Western blot unfortunately equates to "n=1" from a statistical perspective. As it is impossible to apply statistics to a comparison involving "n=1", the authors are unable to make any statements regarding significant changes relative to baseline based upon ANY of their Western blots. Similarly, the single lane representing each timepoint in the Western blots consists of pooled samples. Thus, although the time course Westerns often suggest interesting trends over time, no statistics can be applied due to the effective n=1 for each timepoint.

3) In the second revision, the authors revised three statements the reviewer identified as incompletely supported by the data (on Page 8, line 25; Page 13, line 28 and Page 14, line 13). In so doing, the authors acknowledged having mis-interpreted their own data, and as a result made unsupported claims.

4) After numerous rounds of review, some figures are still incompletely labeled and therefore difficult to interpret. For example, all five rows of Fig 4A are labeled "SRF/LAMP2/DAPI." This implies that all 5 rows consist of a merge of all 3 colors (green, red and blue), yet it appears that only the fourth and fifth rows show merges of all 3 channels. Fig. 4H is similarly problematic.

POINT-TO-POINT RESPONSE TO REVIEWERS' COMMENTS

RESPONSE TO REVIEWER #4

General Comments: The manuscript "SRF SUMOylation modulates smooth muscle phenotypic switch and vascular remodeling" by Xu et al. is a report that uses primarily Western blot and immunohistochemistry techniques to probe the functional significance of SRF SUMOylation in a mouse model of carotid injury, as well as in cell culture and human coronary arteries. The scientific approach is reasonable, the blots are clean and the multiplex fluorescence immunohistochemistry is strikingly beautiful. In the current revision, the authors have made a good faith effort toward implementing the requested revisions. While the authors have been responsive to review, the previous weaknesses of the manuscript are noted.

We appreciate your positive comments on our work. We also thank you for your constructive critiques and we have addressed your concerns as follows.

Specific Comments:

1) The findings and conclusions drawn from these experiments represent a relatively modest advance over what has previously been published on the role of SUMOylation in the phenotypic switching of vascular smooth muscle cells (VSMCs), leading to reservations concerning overall scientific impact. In addition, please thoroughly describe the advances the current manuscript brings in comparison to the referenced literature, of which the key findings should also be mentioned.

Your suggestion is highly appreciated. SRF SUMOylation was only described in an overexpression system in Hela cells (Matsuzaki K et al 2003 BBRC; ref #31). Our current study has clearly advanced our understanding of SENP1 and SRF SUMOylation in VSMC function and vascular remodeling. We have described the advances of our work compared to the previous references thorough in the text. Please find the listed paragraphs:

Abstract section (Page 2): However, it is not known how post-translational SUMOylation regulates the SRF activity in CVD.

Our new findings: Mechanistically, SENP1 deficiency in VSMCs increased SRF SUMOylation at lysine 143, which reduced its lysosomal localization concomitant with increased nuclear accumulation. SUMOylation of SRF promoted its phosphorylation and switched its binding with the contractile phenotype-responsive cofactor myocardin to binding with the synthetic phenotype-responsive cofactor phosphorylated ELK1. Both SUMOylated SRF and phospho-ELK1 were increased in VSMCs from coronary arteries of CVD patients.

Introduction section (page 5): However, the role of SENP1-mediated SUMOylation in VSMCs is unclear. The present study aimed to identify a direct association between SENP1-mediated SUMOylation and VSMC fates in vascular remodeling and human CVD.

Result section:

Page 6 on SENP1 function in VSMC: These results indicate that *Senp*1 deficiency in VSMCs accelerates injury-induced neointimal formation. This is in sharp contrast to our previous observation in *Senp1*^{ECKO} mice where EC-specific deletion attenuated vascular remodeling in several models, suggesting that SENP1 has distinct regulatory functions in VSMCs and aortic ECs.
Page 9 on SRF SUMOylation: A previous report showed that in a Hela cell overexpression system SRF was modified by SUMO-1, chiefly at lysine147 within the DNA-binding domain[31]. To further investigate if endogenous SRF was modified by SUMOylation in aorta tissues, carotid artery extracts obtained…..
Page 10: As vascular injury reportedly displaces SRF from the SRF-myocardin complex to form the SRF-ELK1 complex, resulting in VSMC phenotypic switch[4-7,10], we investigated whether SRF SUMOylation affected its binding to myocardin and ELK1.
Page 11 on a novel regulation of SRF by SUMOylation: Although SUMO1-mediated SUMOylation has been previously reported for SRF in an overexpression system, its role in SRF regulation remains unclear[31].
These results suggest that SRF SUMOylation at K143 induces its nuclear accumulation and switches its binding preference from myocardin to ELK1 in VSMCs in response to PDGF-BB stimulation.
Page 11-14: All our results described are novel and have not reported previously. Specifically, we how that SRF SUMOylation regulates SRF-ELK1 complex formation; SRF SUMOylation and SRF-ELK complex regulate VSMC proliferation, migration, and phenotypic switch; the SRF SUMOylation and phospho-ELK were upregulated in human intimal hyperplasia; blocking shift from SRF-myocardin to SRF-ELK complex by AZD6244 inhibits injury-induced neointimal formation.

Discussion section (Page 16-17): We have discussed potential SUMOylation of other SRF factors in our system as follows:
In our study, KLF4 protein expression and nuclear localization were not altered by SENP1 deficiency. It is unclear if SENP1 regulates KLF4 complex formation in VSMCs. Alternatively, KLF4 SUMOylation is regulated by other SENPs.
Further studies are needed to examine whether SENP1-mediated deSUMOylation regulates PKD2 or Runx2 in VSMCs during vascular remodeling.
It is noteworthy that SUMOylation can keep ELK1 in an inactive state under basal conditions via the binding of a SUMO-histone deacetylase complex and the control of its nuclear-cytoplasmic shuttling [34]. In our study, phospho-ELK1 expression were increased in VSMCs after Senp1 deficiency. However, SUMO-conjugated ELK1 was not observed in the aortic lysates with or without Senp1 deficiency.
Similarly, SENP1 deficiency in VSMCs had little effect on the SUMOylation of KLF4 or myocardin although they have been previously reported to be themselves SUMO substrates. However, we found that SENP1 deficiency in VSMCs had profound effects on SRF SUMOylation.

2) In the first revision, the authors acknowledged that their technique or pooling 3-6 non-injured WT carotid arteries for a single lane in nearly every Western blot unfortunately

equates to “n=1” from a statistical perspective. As it is impossible to apply statistics to a comparison involving “n=1”, the authors are unable to make any statements regarding significant changes relative to baseline based upon ANY of their Western blots. Similarly, the single lane representing each timepoint in the Western blots consists of pooled samples. Thus, although the time course Westerns often suggest interesting trends over time, no statistics can be applied due to the effective n=1 for each timepoint.

We agree with the reviewer that we are unable to make any statements regarding significant changes and therefore we have removed “significance” from the text describing Western blots.

We thank you for your great advice. We have essentially repeated Western blotting for all in vivo samples using newly prepared mouse aortas which were different from the previous blots; Fig.1, Fig.2, Fig.3, Fig.7, Supplemental Fig.4 and Supplemental Fig.6). We have quantified bots presented in Fig.6 (see Revised Fig.6B-D). The new data are presented as 2nd set in both Supplemental Figure 12 and the Source data. Accordingly, we have revised the text in the legends as follows: “Protein bands were quantified by densitometry and fold changes are presented by taking non-injured WT carotid arteries as 1.0 (n=2)”. We are unable to repeat the in vitro experiments with VSMC (Fig.4 and Fig.5) and kept those legends as ““Protein bands were quantified by densitometry and fold changes are presented by taking untreated VSMC as 1.0 (n=1)”.

3) In the second revision, the authors revised three statements the reviewer identified as incompletely supported by the data (on Page 8, line 25; Page 13, line 28 and Page 14, line 13). In so doing, the authors acknowledged having mis-interpreted their own data, and as a result made unsupported claims.

While we thank reviewer’s comments, we don’t agree that we had mis-interpreted our own data. In the last revision we rephrased several sentences based on revised quantification methods as requested by the Reviewers. Please find the comparisons between the reworded paragraphs.

Page 8 line 25:

Original: Immunohistochemical staining revealed a significant increase in phosphor-ELK1-positive cell number in the neointima of Senp1SMCKO mice compared with that of WT mice 28 days after injury; most of these were co-localized with α-SMA-positive VSMCs, and little with CD31-positive ECs.

Reworded in revision 1: Immunohistochemical staining revealed a significant increase in the percentage of phospho-ELK1-positive, α-SMA-positive VSMCs in the neointima of Senp1SMCKO mice as compared to that of WT mice 28 days after injury. In contrast, no statistical difference was detected in the percentage of phospho-ELK1-positive, CD31-positive ECs in the neointima of Senp1SMCKO mice as compared to that of WT mice 28 days after injury (Fig.3B with quantifications in Fig.3C.

Reason for rewording: We have quantified “the percentage of positive cells” instead of “total positive cell number” in the figure as requested. We agree that quantification of percentage of positive cells is more accurate.

Page 13 line 28:

Original: AZD6244 treatment significantly inhibited injury-induced expression of Col15a1, Col5a2, Col6a2, Timp1, Mmp14 and Col1a1 at 28 days after wire injury as determined by qRT-PCR.
Reworded in revision 1: AZD6244 treatment significantly inhibited injury-induced expression of Col15a1, Col5a2, Col6a2, Timp1, and Mmp14 at 28 days after wire injury in either WT or Senp1SMCKO mice as determined by qRT-PCR (Fig.S11).
Reason for rewording: We clarify that AZD6244 treatment also had effects in both WT and Senp1SMCKO mice.

Page 14, line 13:
Original: Consistent with the decrease in active MMPs detected by western blot, immunohistochemical staining showed a significant decrease in the percent of neointimal area that stained positive for MMP2 or MMP9 in both WT and Senp1SMCKO mice after AZD6244 treatment, but no significant differences in the percent of neointimal area stained positive for MMP2 or MMP9 between WT and Senp1SMCKO mice treated with AZD6244 (Fig.7I-J).
Reworded in revision 1: Consistent with the decreased MMP activity detected by western blot, immunohistochemical staining showed significantly decreased MMP2 and MMP9 protein levels in both WT and Senp1SMCKO mice after AZD6244 treatment, but no significant differences in fluorescence intensities between Senp1SMCKO mice and WT mice (Fig.7I-J).
Reason for rewording: We have quantified "the percent of neointimal area that stained positive for MMP2 or MMP9" instead of "MMP2 and MMP9 protein levels" in the figure as requested. We agree that quantification the percent of MMP positive areas in neointimal is more accurate.

4) After numerous rounds of review, some figures are still incompletely labeled and therefore difficult to interpret. For example, all five rows of Fig 4A are labeled "SRF/LAMP2/DAPI." This implies that all 5 rows consist of a merge of all 3 colors (green, red and blue), yet it appears that only the fourth and fifth rows show merges of all 3 channels. Fig. 4H is similarly problematic.
We apologize for the confusion. The initial purpose using combined labeling was due to the limited space in the figure. We used green, red, blue colored word to label the respective split channel images. We appreciate your suggestions and now we have labeled each split channel image individually in Figure 4 and other figures (Fig. 1, Fig.2, Fig.6, Fig.7, Supplemental Fig.S5, S7, S8 and S10).

REVIEWER COMMENTS

Reviewer #4 (Remarks to the Author):

The manuscript "SRF SUMOylation modulates smooth muscle phenotypic switch and vascular remodeling" by Xu et al. is a report that uses primarily Western blot and immunohistochemistry techniques to probe the functional significance of SRF SUMOylation in a mouse model of carotid injury, as well as in cell culture and human coronary arteries. The scientific approach is reasonable, the blots are clean and the multiplex fluorescence immunohistochemistry is strikingly beautiful. In the current revision, the authors have been responsive to review and have further improved the manuscript, although a number of previous weaknesses remain.

1) The additions made in blue text are indeed helpful in framing the context of this work relative to previous publications, although reservations remain concerning overall scientific impact.

2) The repeats of the Western blots of normal mouse aortas represents a good faith effort towards establishing reproducibility. However, it is not entirely clear that the changes from n=1 to n=2 were fully implemented in the "ratios" appearing under the blots in Figs 1, 3 and 7. Regardless, Supplementary Fig.12 might benefit from a figure legend explaining that the "2nd set" figures were performed at a later date to establish reproducibility. The reader may otherwise find the "2nd set" to be confusing.

3) In the current revision, the revisions on Page 8, line 25; Page 13, line 28 and Page 14, line 13 have improved the accuracy of the relevant statements.

4) The revisions made to the figures now better help orient the readers to the antibody used in each row of figures. However, some figures are still incorrectly labeled and therefore difficult to interpret. For example, the 3rd columns in both Fig. 5J and 5K are labeled "WT" when it is almost certain they should be labeled "Senp1SMCKO". Similarly, the legend to Fig 6 should mention that the IF for the "correlations of α-SMA and OPN in VSMCs with neointimal formation in human specimens" can be found in Supp. Fig. 10. Otherwise, there is no connection between Fig 6I and the source data in Supp. Fig. 10.

5) In addition, the reviewer was unable to find any IF source data for Fig 6J. It is clear that many double and triple-immunostains were performed, but no double-IF for SRF and p-ELK can be found in human coronaries. Nor can any examples of double-IF for SRF and SUMO1 be found. Finally, in the recent update to Fig 6, the Y-axis of the second panel in Fig 6J was inadvertently deleted. It previously read "SRF+ SMCs (%)."

POINT-TO-POINT RESPONSE TO REVIEWERS' COMMENTS

RESPONSE TO REVIEWER #4

The manuscript "SRF SUMOylation modulates smooth muscle phenotypic switch and vascular remodeling" by Xu et al. is a report that uses primarily Western blot and immunohistochemistry techniques to probe the functional significance of SRF SUMOylation in a mouse model of carotid injury, as well as in cell culture and human coronary arteries. The scientific approach is reasonable, the blots are clean and the multiplex fluorescence immunohistochemistry is strikingly beautiful. In the current revision, the authors have been responsive to review and have further improved the manuscript, although a number of previous weaknesses remain.

We appreciate your positive comments on our work. We also thank you for your constructive critiques and we have addressed your concerns as follows.

1) The additions made in blue text are indeed helpful in framing the context of this work relative to previous publications, although reservations remain concerning overall scientific impact.

As you appreciate it, we have described the advances of our work compared to the previous references thorough in the text. Please note that SRF SUMOylation was only described in an overexpression system in Hela cells (Matsuzaki K et al 2003 BBRC; ref #31). Our current study has clearly advanced our understanding of SENP1 and SRF SUMOylation in VSMC function and vascular remodeling.

2) The repeats of the Western blots of normal mouse aortas represents a good faith effort towards establishing reproducibility. However, it is not entirely clear that the changes from n=1 to n=2 were fully implemented in the "ratios" appearing under the blots in Figs 1, 3 and 7. Regardless, Supplementary Fig.12 might benefit from a figure legend explaining that the "2nd set" figures were performed at a later date to establish reproducibility. The reader may otherwise find the "2nd set" to be confusing.

We appreciate your suggestions. The ratios under blots in Figs. 1, 2, 3 and 7 are specific to the blots presented. However, we have provided additional repetition of the in vivo Western blotting in Supplemental Fig.12.

We have stated in the legends that "Each tissue sample was pooled from three individual aortas and protein bands were quantified by densitometry and fold changes are presented by taking non-injured WT carotid arteries as 1.0. (Additional two experiments were performed with different biological repeats presented in Supplemental Fig.12).

We have added the following description to the Supplementary Fig.12 legend:

Supplementary Fig.12: **Uncut original gels**. Each tissue sample was pooled from three individual aortas and protein bands were quantified by densitometry and fold changes are presented by taking non-injured WT carotid arteries as 1.0. 2nd and 3rd sets of Western blots were performed at a later date to establish reproducibility.

3) In the current revision, the revisions on Page 8, line 25; Page 13, line 28 and Page 14, line 13 have improved the accuracy of the relevant statements.

We appreciate your positive comments.

4) The revisions made to the figures now better help orient the readers to the antibody used in each row of figures.

However, some figures are still incorrectly labeled and therefore difficult to interpret. For example, the 3rd columns in both Fig. 5J and 5K are labeled “WT” when it is almost certain they should be labeled “Senp1SMCKO“. Similarly, the legend to Fig 6 should mention that the IF for the “correlations of α-SMA and OPN in VSMCs with neointimal formation in human specimens” can be found in Supp. Fig. 10. Otherwise, there is no connection between Fig 6I and the source data in Supp. Fig. 10.

We apologize for the mislabeling and we have corrected the labeling of Senp1SMCKO in Fig.5J and 5K.

In Fig.6, we have also mention that “The immunofluorescence staining for the correlations of α-SMA and OPN in VSMCs with neointimal formation in human specimens can be found in Supp. Fig. 10”.

5) In addition, the reviewer was unable to find any IF source data for Fig 6J. It is clear that many double and triple-immunostains were performed, but no double-IF for SRF and p-ELK can be found in human coronaries. Nor can any examples of double-IF for SRF and SUMO1 be found. Finally, in the recent update to Fig 6, the Y-axis of the second panel in Fig 6J was inadvertently deleted. It previously read “SRF+ SMCs (%).”

We appreciate your comments. Double-IF for SRF and p-ELK were not performed due to the same species of the available antibodies. We performed double-IF staining for SRF with α-SMA and p-ELK with α-SMA as presented in Fig.6E, and the correlations of SRF and p-ELK in VSMCs with neointimal formation in human specimens were analyzed in 6J. We have clarified this in the legend “Based on immunofluorescence stainings in 6E, the corresponding Spearman’s correlation coefficient (r) between SRF-, SUMO1- or p-ELK1-positive VSMCs and the P value are calculated”.

In addition, we have added “SRF+ SMCs (%)” to the Y-axis of the second panel in Fig 6J.

REVIEWER COMMENTS

Reviewer #4 (Remarks to the Author):

The manuscript "SRF SUMOylation modulates smooth muscle phenotypic switch and vascular remodeling" by Xu et al. is a report that uses primarily Western blot and immunohistochemistry techniques to probe the functional significance of SRF SUMOylation in a mouse model of carotid injury, as well as in cell culture and human coronary arteries. The scientific approach is reasonable, the blots are clean and the multiplex fluorescence immunohistochemistry is strikingly beautiful. In the current revision, the authors have been responsive to review and have further improved the manuscript, although a serious problem in experimental design remains.
In the previous review, the reviewer noted that no immunofluorescence source data was provided for Fig 6J. IE, no double- immunofluorescence (IF) for SRF and p-ELK was provided. Similarly, no double-IF for SRF and SUMO1 was provided. In their rebuttal, the authors responded as follows:

*Double-IF for SRF and p-ELK were not performed due to the same species of the available antibodies. We performed double-IF staining for SRF with α-SMA and p-ELK with α-SMA as presented in Fig.6E, and the correlations of SRF and p-ELK in VSMCs with neointimal formation in human specimens were analyzed in 6J. We have clarified this in the legend "Based on immunofluorescence stainings in 6E, the corresponding Spearman's correlation coefficient (r) between SRF-, SUMO1- or p-ELK1-positive VSMCs
and the P value are calculated".*

The critical passage in this reply is "Double-IF for SRF and p-ELK were not performed due to the same species of the available antibodies." One can only assume that the same holds true for the combination of SRF and SUMO-1, and that double-IF was not performed for SRF and SUMO-1 either. If this rebuttal statement is true, then it is not possible to generate plots of SRF vs. p-ELK and SRF vs SUMO-1 (Fig. 6J) without combining data from different fields on different slides. This would be consistent with the sentence that was added to the legend, but it is not scientifically valid to graph correlations based on different fields from different slides.

Upon close examination of Fig 6, another concern arises regarding Fig 6H. In this paper, the authors define SMCs as those cells expressing a-SMA. While this is common practice, it raises the question of what the authors intended to convey when they plotted "a-SMA+ SMC" on the X-axes of Fig. 6H. If SMCs are defined as being a-SMA+, then 100% of SMCs should be a-SMA+. However, that is not what is graphed in Fig. 6H. Perhaps the Y-axes of the top 3 panels in Fig 6H were intended to read "SRF+ cells (%)", "SUMO1+ cells (%)" and "p-ELK-1+ cells (%)", respectively; while the X-axes were all intended to read "a-SMA+ cells (%)." However, if this is indeed the case, it raises the question of whether the X- and Y-axes in Fig. 6I also require similar revision.

POINT-TO-POINT RESPONSE TO REVIEWERS' COMMENTS

RESPONSE TO REVIEWER #4

The manuscript "SRF SUMOylation modulates smooth muscle phenotypic switch and vascular remodeling" by Xu et al. is a report that uses primarily Western blot and immunohistochemistry techniques to probe the functional significance of SRF SUMOylation in a mouse model of carotid injury, as well as in cell culture and human coronary arteries. The scientific approach is reasonable, the blots are clean and the multiplex fluorescence immunohistochemistry is strikingly beautiful. In the current revision, the authors have been responsive to review and have further improved the manuscript, although a serious problem in experimental design remains.

We thank you very much for your positive response and appreciate your instructive suggestions. We have addressed your concerns as follows.

In the previous review, the reviewer noted that no immunofluorescence source data was provided for Fig 6J. IE, no double- immunofluorescence (IF) for SRF and p-ELK was provided. Similarly, no double-IF for SRF and SUMO1 was provided. In their rebuttal, the authors responded as follows:

Double-IF for SRF and p-ELK were not performed due to the same species of the available antibodies. We performed double-IF staining for SRF with α-SMA and p-ELK with α-SMA as presented in Fig.6E, and the correlations of SRF and p-ELK in VSMCs with neointimal formation in human specimens were analyzed in 6J. We have clarified this in the legend "Based on immunofluorescence stainings in 6E, the corresponding Spearman's correlation coefficient (r) between SRF-, SUMO1- or p-ELK1-positive VSMCs and the P value are calculated".

The critical passage in this reply is "Double-IF for SRF and p-ELK were not performed due to the same species of the available antibodies." One can only assume that the same holds true for the combination of SRF and SUMO-1, and that double-IF was not performed for SRF and SUMO-1 either. If this rebuttal statement is true, then it is not possible to generate plots of SRF vs. p-ELK and SRF vs SUMO-1 (Fig. 6J) without combining data from different fields on different slides. This would be consistent with the sentence that was added to the legend, but it is not scientifically valid to graph correlations based on different fields from different slides.

Thank you very much for pointing out this problem and appreciate your valuable suggestions. Our initial correlation analyses among various markers (SRF, SUMO1, p-ELK1, and OPN) were based on immunostainings from adjacent sections. However, we fully agree with you that it is not scientifically valid to graph correlations based on different fields from different slides since we didn't perform double-IF for SRF with OPN, SUMO1 with OPN, p-ELK1-eith OPN, SRF with p-ELK1, or SRF with SUMO-1. Based on your suggestions, we have removed the graphs presented in original Fig.6I and 6J. For clarity, we also removed data on SMA, and focused on OPN presented in Supplemental Fig.10. We have revised the legends for Fig.6 and Supplemental Fig.10 accordingly.

It is worth mentioning that removal of Fig.6I and 6J doesn't change our conclusion that these observations establish an association between the SRF SUMOylation, ELK1 phosphorylation, and CAD severity, as well as VSMC phenotypic switch in human CAD samples.

We have revised our results as follows (Pages 12-13):

In the same samples, immunohistochemical staining showed that the expression of SRF, SUMO1, and p-ELK1 progressively increased with disease severity, and most of these signals were co-localized with α-SMA-positive VSMCs (Fig.6E-F). A strong positive linear relationship between the intima/media (I/M) ratio and number of VSMCs expressing SRF (r=0.7779, P<0.0001), SUMO1 (r=0.8639, P<0.0001) or p-ELK1 (r=0.5972, P=0.0001) was observed (Fig.6G). Furthermore, number of cells expressing the contractile marker a-SMA inversely correlated with numbers of cells expressing SRF (r=-0.4681, P=0.0040), SUMO1 (r=-0.6361, P<0.0001), p-ELK1 (r=-0.3321, P=0.0478) (Fig.6H). We also examined the VSMC synthetic marker OPN expression in this patient cohort. Immunostaining revealed that luminal VSMCs exhibited increasing OPN expression with increase in CAD severity (Supplementary Fig.10A, B), and that OPN expression positively correlated with I/M ratio (r=0.6644, P<0.0001) (Supplementary Fig.10C). These observations establish an association between the SRF SUMOylation, ELK1 phosphorylation, and CAD severity, as well as VSMC phenotypic switch in human CAD samples.

Upon close examination of Fig 6, another concern arises regarding Fig 6H. In this paper, the authors define SMCs as those cells expressing a-SMA. While this is common practice, it raises the question of what the authors intended to convey when they plotted "a-SMA+ SMC" on the X-axes of Fig. 6H. If SMCs are defined as being a-SMA+, then 100% of SMCs should be a-SMA+. However, that is not what is graphed in Fig. 6H. Perhaps the Y-axes of the top 3 panels in Fig 6H were intended to read "SRF+ cells (%)", "SUMO1+ cells (%)" and "p-ELK-1+ cells (%)", respectively; while the X-axes were all intended to read "a-SMA+ cells (%)." However, if this is indeed the case, it raises the question of whether the X- and Y-axes in Fig. 6I also require similar revision.

Thank you for your great suggestions. We have revised Fig.6H as you suggested, i.e. the Y-axes have been changed to "SRF^+ cells (%)", "$SUMO1^+$ cells (%)" and "p-$ELK-1^+$ cells (%)", respectively; while the X-axes have been changed to "$a\text{-}SMA^+$ cells (%)."

We have revised our results accordingly as follows (Page 12):

Furthermore, number of cells expressing the contractile marker a-SMA inversely correlated with numbers of cells expressing SRF (r=-0.4681, P=0.0040), SUMO1 (r=-0.6361, P<0.0001), p-ELK1 (r=-0.3321, P=0.0478) (Fig.6H).

As stated above, we have deleted the original Fig.6I and 6J, and correction or source data are no longer relevant.

REVIEWERS' COMMENTS

Reviewer #1 (Remarks to the Author):

I appreciate the logical thinking and thorough evaluation to identify the problem by reviewer #4. I agree with the reviewer that it is not scientifically valid to graph correlations based on different fields from different slides. The authors also concur with this point. Unfortunately, due to the limitation of available antibodies, they were unable to perform the corresponding double-IF assays for subsequent quantitation. Consequently, they removed the quantitation of IF assays.

In the Manuscript, the authors extensively used co-immunoprecipitation (co-IP) to demonstrate the presence of protein-protein interaction or with modifications within the same complex. While immunofluorescence (IF) assays can provide supportive evidence, the limited availability of antibodies prevented the authors from conducting corresponding double-IF assays. I agree with the authors that without the quantitation of the double-IF assays should not change the conclusion.
The revised results section (Pages 12-13) properly describes the findings, aligning with the results shown in Fig 6E-6G.

Note: Generally, co-IP assays are considered more stringent for detecting protein-protein interactions than co-IF assays. Interestingly, the SRF-ELK1 interaction, which competes with SRF-Myocd interaction, was initially determined using co-IP (PMID: 15014501).

Here are minor comments:
1. The authors like to use lumen for vessel wall: for example, in the legends of Fig 6F and Supplementary Figure 10B. In fact, the lumen is the space inside the vessel, while the vessel wall comprises the layers surrounding it. The authors should check the MS to make necessary corrections.
2. Figure10C, Y axis: If the OPN signal originates from all cells, then labeling them as “OPN+ cells” is appropriate. If the OPN signal originates from SMA+cells, then the Y axis labeling should be “OPN+ VSMC cells”.
3. The method of Quantification of Immunofluorescence Images is described with enough details to follow. Could the authors cite a couple of well-accepted papers to support the validity of this quantitation method?

POINT-TO-POINT RESPONSE TO REVIEWERS' COMMENTS

RESPONSE TO REVIEWER #1

I appreciate the logical thinking and thorough evaluation to identify the problem by reviewer #4. I agree with the reviewer that it is not scientifically valid to graph correlations based on different fields from different slides. The authors also concur with this point. Unfortunately, due to the limitation of available antibodies, they were unable to perform the corresponding double-IF assays for subsequent quantitation. Consequently, they removed the quantitation of IF assays.

We thank you very much for your positive response and appreciate your instructive suggestions. We have addressed your concerns as follows.

In the Manuscript, the authors extensively used co-immunoprecipitation (co-IP) to demonstrate the presence of protein-protein interaction or with modifications within the same complex. While immunofluorescence (IF) assays can provide supportive evidence, the limited availability of antibodies prevented the authors from conducting corresponding double-IF assays. I agree with the authors that without the quantitation of the double-IF assays should not change the conclusion.

The revised results section (Pages 12-13) properly describes the findings, aligning with the results shown in Fig 6E-6G.

We thank you very much for your agreements.

Note: Generally, co-IP assays are considered more stringent for detecting protein-protein interactions than co-IF assays. Interestingly, the SRF-ELK1 interaction, which competes with SRF-Myocd interaction, was initially determined using co-IP (PMID: 15014501).

We thank you very much for your comments.

Here are minor comments:

1. The authors like to use lumen for vessel wall: for example, in the legends of Fig 6F and Supplementary Figure 10B. In fact, the lumen is the space inside the vessel, while the vessel wall comprises the layers surrounding it. The authors should check the MS to make necessary corrections.

We have changed to "the vessel wall."

2. Figure10C, Y axis: If the OPN signal originates from all cells, then labeling them as "OPN+ cells" is appropriate. If the OPN signal originates from SMA+cells, then the Y axis labeling should be "OPN+ VSMC cells".

They were from all cells so we keep "OPN+ cells".

3. The method of Quantification of Immunofluorescence Images is described with enough details to follow. Could the authors cite a couple of well-accepted papers to support the validity of this quantitation method?

We have cited our previous publications. Quantification of Immunofluorescence Images in the aortas were performed as we previously described (Yu, 2011 *Cir Res*; Qin, 2014 JACC} [1,2].

1. Yu, L., et al. AIP1 prevents graft arteriosclerosis by inhibiting interferon-gamma-dependent smooth muscle cell proliferation and intimal expansion. Circ Res 109, 418-427 (2011).
2. Qin, L., et al. SOCS1 prevents graft arteriosclerosis by preserving endothelial cell function. J Am Coll Cardiol 63, 21-29 (2014).