Circulation Research Peer Review Report for DOI: 10.1161/CIRCRESAHA.121.320029

Title: Locus-Conserved Circular RNA cZNF292 Controls Endothelial Cell Flow Responses

In an effort to promote greater transparency in peer review, the authors and reviewers of this *Circulation Research* article have opted to post the original decision letter with reviewer comments to the authors and the authors' response to reviewers for each significant revision.

May 4, 2021

Dr. Stefanie Dimmeler
Goethe University Frankfurt
Institute for Cardiovascular Regeneration
Theodor Stern Kai 7, 60590 Frankfurt
Frankfurt 60590
Germany

RE: CIRCRES/2021/319402: Locus-conserved circular RNA cZNF292 controls endothelial cell flow responses

Dear Dr. Dimmeler:

Your manuscript has been carefully evaluated by 3 external reviewers and the editors as a Regular Article. We regret to inform you that the paper is not acceptable for publication in Circulation Research.

As you will gather from the reviews, the referees identified a number of substantive conceptual and methodological problems. The editors concur. Major issues include the need for further characterization of the knockout mouse and its use in disease models would be necessary. In addition, all reviewers requested further mechanistic studies of the interaction of cZNF292 and SDOS.

Given the nature of these concerns, which could not be adequately addressed without extensive new experimentation, the editors do not encourage revision. Nevertheless, if you feel that you can effectively address the reviewers' comments and are willing to perform the new experiments required, we would be willing to evaluate a resubmitted version on a de novo basis. The paper would be reviewed again, with no assurance of acceptance. Since the re-evaluation would be done de novo, the revised paper would be assigned a new number regardless of when it is resubmitted. One or more of the original reviewers would be re-consulted; the editors may also choose to obtain additional opinions from new reviewers. Please note that even after extensive modifications, we cannot guarantee that your manuscript will receive a priority sufficient for publication. Overall, fewer than 15% of all papers submitted to Circulation Research are eventually published.

As detailed in the reviewers' critiques, a responsive resubmission would require a substantial amount of new data. To read the comments to authors from the reviewers, please see below.

If you choose to resubmit, please include a detailed response to each of the referees' and editors' comments, providing each comment verbatim in bold followed by your response and giving the exact page number(s), paragraph(s), and line number(s) where each change was made. If you make substantive changes to the manuscript, please provide a clear description of what you did and where. If you insert important sentences, paragraphs, or sections in response to the comments, please also include them in your response. Please indicate clearly any deletions. Additionally, a marked up version of the resubmission with the changes highlighted or tracked should be uploaded as a supplemental file. If you do choose to resubmit, please do so online using the "Submit Resubmission" link available in your Author Tasks area or Post Decision Manuscripts folder.

Please ascertain that your resubmitted manuscript adheres to the Instructions to Authors as they appear online at https://www.ahajournals.org/res/author-instructions. Resubmission that do not conform to the current limits on numbers of words (8000 total) and display items (maximum of 8 tables and/or figures) will be returned to the authors for abbreviation. If you cannot reduce the overall word count, the editors may deem an extended print version appropriate; the authors should provide written assurance that they will cover the costs of the pages that are in excess of these limits. Note that paying for excess display items is not an option. Please refer to the Instructions to Authors for further details regarding our policy on page limits, articles with extended print versions, and related costs. No such limits apply to the online supplementary information, which can include supporting data and/or expanded text to offset the limits on the print version. Such online supplementary information can be cited in the print version as appropriate.

We know that you will be disappointed by this decision. Circulation Research currently receives approximately 2,000 manuscripts a year, of which fewer than 15% can be published; as a consequence, relative priorities must be considered in making the final decision.

Despite our decision, we wish to thank you for having submitted this manuscript to Circulation Research.

Sincerely,

Jane E. Freedman, MD
Editor-in-Chief
Circulation Research
An American Heart Association Journal

****

Reviewer comments to the Authors:

Reviewer #1:

In this manuscript, Heumülle et al study the role of a circular RNA (cZNF292) which results to be highly expressed in endothelial cells. They have previously identified the circular RNA as one of the most expressed in endothelial cells both in normoxic and hypoxic conditions. They also previously showed that this cRNA does not bind to Ago2 and therefore does not act as an inhibitor of microRNAs, in contrast to others.

In this manuscript, they show a mechanism through which this ncRNA acts, regulating endothelial cell morphology. In addition, they generate and characterize a KO mouse in which they delete the exon 1, altering the circular but not the linear mRNA form of the related gene.

In general, the authors should describe where the previous publication ends and where this one starts. The identification of human cRNAs from endothelial cells was previously conducted by the same authors (Circ. Res. 2015 Oct 23;117(10):884-90). In what the screening conducted here differs from the previous one is unclear. What is the circATLAS ID of the identified human circRNA? How conservation throughout species has been evaluated? Is there a conservation between mouse and human? Was locus conservation only accounted for (shared synteny)?

Is cZNF292 exclusively expressed by ECs? Is it expressed in other vascular cells, such as smooth muscle cells? This might be important also to understand the phenotype of KO animals.

In order to state that the strategy does not influence the expression of the host gene Zfp292, the authors should measure also the protein level and not only the RNA in the null mice generated by CRISPR/Cas9.

The authors made a significant effort in generating a KO mouse. Nonetheless, its phenotyping is limited. They elegantly showed that angiogenesis is not altered by the KO of cZNF292, using the neonatal retina as a model system. Have they tried other angiogenesis assays in vivo or ex vivo? How do they reconcile this result with their own previous results on the angiogenic effect of cZNF292 in vitro (Figure 4C and D of Circ. Res. 2015 Oct 23;117(10):884-90)?

Studying the EC properties from cells derived from the KO mouse, the authors stated that probably the phenotype might depend on the different level of cZNF292 expression among large and small vessels. If that is the case, could the authors design an experiment using ECs derived from small vessels and test, in a gain-of-function setting (in order to increase the level of cZNF292 close to EC-derived from large vessels), whether they can then behave similarly to the cells tested in figure 2I/J?

Authors conducted a very elegant analysis of protein partner identification through mass spectrometry. Out of the 75 putative partners, authors picked SDOS. Why? Is there a multiprotein interaction which involves SDOS and cZNF292?

It is unclear how the four aminoacid residues which are critical for cZNF292 binding to SDOS were identified.

SDOD recruitment is known to depend on the PKC pathway, but it also possible that the interaction between cZNF292/SDOS might modulate the SRC/FAK loop. Have the authors excluded this possibility?

The Authors nicely showed that the overexpression of the SDOD mutated protein on the putative cZNF292 binding aminoacids influences EC response to flow. Could the authors mutate the binding sequences on cZNF292 and determine whether its expression in ECs or KO H5V might induce a similar response to the flow?

Minor:
- Read count in figure 2k: was it normalized on library dimension?

- H26 --> H29?

Reviewer #2:
This manuscript by Huemueller leverages methodology to specifically edit introns retained in circRNA but not the corresponding linear mRNA to attempt to specifically study circRNA function without the confounder effects of altering expression of the linear mRNA in vivo. Methodology to specifically study circRNA function would have a high impact in the field, particularly in assessing their role in homeostasis and disease pathogenesis. The authors identify cZfp292 as a candidate for functional validation based on computational analysis. Their methodology does seem to work to decrease the circular and not linear form of the RNA, and the authors are able to identify interacting proteins. Silencing of these interacting proteins in cell culture systems, or mutation of the protein residues expected to bind to the circRNA phenocopies the effect of silencing the cZfp292. This is an elegant study, but not entirely novel, as the investigators had previously investigated the phenotype of cZfp292 in cultured cells (associated with sprouting), and this methodology to target retained introns has been described by other groups. Nonetheless the in vivo functional validation is a strength, but connecting this to disease pathogenesis (i.e. the relevance of this pathway in disease) would strengthen the overall story. My specific concerns are noted below:

1. The use of techniques to delete intronic sequences to specifically delete circRNAs without affecting the linear mRNA is not novel, and has been described by other groups (e.g. Zheng et al, Nature Comm 2016).
2. Additionally, the in vitro phenotype of cZNF292 was previously studied by the same group in 2015, although the phenotype in the models in this paper are different, attributed to higher levels in aortic endothelial cells; hence the functional assessment is not entirely novel, although the in vivo assessment is a significant advancement.

3. The phenotype is mostly explored in the cell culture system and points to altered morphology related to laminar flow. It is somewhat surprising that there is no exploration of how these defects affect disease pathogenesis in the murine models: the investigators are encouraged to explore if the mice are more prone to aortic pathologies (e.g. models of thoracic or aortic aneurysms or atherosclerosis that are well established in murine models). This would be a big advantage of developing the in vivo models.
4. The detailed study of the interacting proteins and the residues that are important is nicely described; can the authors explore how the binding of cZfn292 affect syndecan function? Does it change cellular localization or function? More detailed mechanism would be helpful.
5. Finally does this cZfp292 expression change in human or murine aortopathies? This should be examined in tissue samples.

Minor comments:
a. Did the authors verify that the splicing of the linear mRNA was not affected?
b. Fig 2A: Define what Rplp0 is (linear mRNA control?)
c. Fig 2A: Why is there higher level of cZfp292 with RNAse R digestion; would have expected same level if the primers are specific for the circRNA.
d. For Fig 2C it would be informative if the entire cZfp292 locus is shown with the consequence of the deletion (and the expected sizes).
e. Only 2 replicates were run for the EMSA assay; a minimum of three replicates should be assessed.
f. The figure legends are somewhat sparse; a little more detail on describing the figures should be provided.

Reviewer #3:
In the manuscript entitled 'Locus-conserved circular RNA cZNF292 controls endothelial cell flow responses' Heumuller et al. describe a novel role of cZNF292/cZfp292 in vascular biology, and in more detail, in the response of endothelial cells to flow. The current manuscript is a follow-up of the 2015 publication in Circulation Research by the same group. Here, the authors show that circular RNA cZNF292 interacts with SDOS and that this interaction drives the cytoskeletal organization upon induction of laminar flow. One particular highlight is that the authors were able to show locus conservation of cZNF292/ cZfp292 between human and mice by focusing on intronic elements that are retained across the two species - these same introns were then used for selective genetic removal, not affecting the (coding) mRNA of the host gene. The premise of the study is clear and could be of interest to the readers of Circulation Research, however several issues remain.

Major comments
- Figure 1A-E are described very briefly. If this reviewer understands well, most of the data is derived from publicly available data sets that were deposited by the research group themselves and then combined with the circATLAS database. More explanation, potentially with a graphic visualization of the pipeline, of how the authors got to cZNF292 and the locus-conserved cZfp292 would clear up the train of thought that was used here.
- The authors quickly go from 1158 candidates towards 1 prime candidate. Can the authors discuss other intronic circRNAs that are listed in Figure 3 D-E such as FOXP1 and RALY. Some of host genes listed have important functions in vascular biology and it will be exciting to follow up.
- What are the differences in nucleotide sequence between cZNF292 and cZfp292? It would be good to show potential synteny if we expected any functional conservation between the human and mouse orthologues.
- SDOS is a protein known to be expressed in the cytoplasm. What is the subcellular location of cZNF292 (and cZfp292)? Can the authors perform qPCR upon cell fractionation experiments or RNA-FISH. It should be possible to dissect linear versus circular transcript by using probes direct against exon1a, as this same exon is used for genetic knockdown as well as siRNA targeting in Figure 4.
- Figure 3D-F and Fig2D-E show strong evidence between cZNF292 and SDOS in a series of experiments where either cZNF292 or SDOS was overexpressed. Can the authors perform an IP on SDOS with endogenous expression?

Can the authors show RNA-FISH (with issue outlined in previous comment) in combination with IF for SDOS (and SDC4) to show that these molecules are both in the cytoplasm and colocalize.
- The CCCA motif is described as a preferential binding site for SDOS and several of them are found in cZNF292 and cZfp292 and EMSA is used to interrogate all of the three possibilities (of which one is CCCG and CCCA). Although EMSA is a powerful tool it uses oligonucleotides and as such neglects the 3D structure of the RNA in a cellular environment. To accompany the presented data the authors should complement these data with in silico RNA-protein binding data that can be derived by algorithms such as catRAPID (both pieces of evidence, with their flaws, will back-up the proposed mechanism).
- Along these lines, for the EMSA experiments only mutations in SDOS are being interrogated. Can the authors perform similar experiments by changing the CCCA sequences in the oligonucleotides used in the EMSA?
- If the authors want to show functional conservation for cZfp292 and cZNF292 alongside the locus-conservation, several experiments will need to be repeated using cZfp292 and murine SDOS.
- Can the authors elaborate on the functional consequence of the cZNF292, SDOS, SDC4 axis on cytoskeletal organization in endothelial cells? They comment that cZNF292 is required, but how? Does cZNF292 stabilize the protein complex?; does it act as a molecular 'shuttle'?; or does it provide binding between SDC4 and SDOS?

Minor comments
- Figure 1E shows three different isoforms of cZfp292 which one is followed up on?
- Can the authors list/discuss other potential cZfp292 binding partners? For example, is SDC4 in the mass-spec list and/or other proteins that could potentially bind to SDOS, such as CDCP1 and GPIC.
- The CCCA motif is listed as a known SDOS binding site. However, in Figure 5A, one the highlighted sequences in ZNF292 is CCCG.
- How do changes of potential RNA binding sites towards alanine change the whole protein structure of SDOS as this can have major implications on its ability to bind RNA independent of the CCCA sites?
- Can the authors provide the levels of linear ZNF292 after siRNA treatment.

Comments to the reviewers:

Reply to Reviewer #1:

In this manuscript, Heumüller et al study the role of a circular RNA (cZNF292) which results to be highly expressed in endothelial cells. They have previously identified the circular RNA as one of the most expressed in endothelial cells both in normoxic and hypoxic conditions. They also previously showed that this cRNA does not bind to Ago2 and therefore does not act as an inhibitor of microRNAs, in contrast to others.

In this manuscript, they show a mechanism through which this ncRNA acts, regulating endothelial cell morphology. In addition, they generate and characterize a KO mouse in which they delete the exon 1, altering the circular but not the linear mRNA form of the related gene.

In general, the authors should describe where the previous publication ends and where this one starts. The identification of human cRNAs from endothelial cells was previously conducted by the same authors (Circ. Res. 2015 Oct 23;117(10):884-90). In what the screening conducted here differs from the previous one is unclear. What is the circATLAS ID of the identified human circRNA? How conservation throughout species has been evaluated? Is there a conservation between mouse and human? Was locus conservation only accounted for (shared synteny)?

Answer: We thank the reviewer for the comment and have elaborated our screening approach and the conservation between human and mouse cZNF292 in more detail in the text of the revised manuscript. We additionally added Supplemental Figure 1 for illustration. Regarding the reviewer's question towards the conservation, our initial approach indeed aimed to identify potential locus-conserved circRNAs by synteny. Depending on the algorithm, pairwise sequence alignments show a similarity of ~65% (LALIGN), ~68% (Needleman-Wunsch) or ~75% (Smith-Waterman) between human cZNF292 and murine cZfp292.

We also clarified that in our previous study, a first screening to identify hypoxia-induced endothelial circRNAs was done in HUVEC, which led to the identification of cZNF292. cZNF292 mechanism of action and in vivo function was not further analysed. The present manuscript now starts by screening for conserved, and intronic spliced circRNAs. This new approach again led to cZNF292, which we now characterise in detail.

The revised text now reads (also see page 7, paragraph 2 and Fig.S1A):

"In order to identify locus-conserved endothelial intronic circRNA, we used published endothelial RNA-sequencing data with circRNAs listed in the circATLAS database (Fig. S1A). We first selected circRNAs commonly expressed between different types of human endothelial cells resulting in 1228 circRNAs from 868 host genes (Fig 1A). Further comparison of these circRNAs for their stability towards exonuclease digestion using an additional RNA sequencing dataset of RNase R-treated endothelial cells, showed that 1158 (~95%) can be considered true circRNA (Fig. 1B). However, only 29 of these were back-spliced to intronic cassettes (Fig. 1C). Importantly, 21 of the 29 candidates were also included in the top30 consistently detected human intronic circRNAs of the respective loci (Fig. 1D) consolidating their presence in human samples. When we additionally analysed the respective loci in mouse, several intronic circRNAs were commonly detected in circATLAS database (Fig. 1E; overlap of 13 host genes when comparing Fig. 1D and 1E), but only few circRNAs shared synteny. Of these candidates, we validated the expression and exonuclease resistance of the circRNAs cZNF292 (hsa-ZNF292_0014) and cFOXP1 (hsa-FOXP1_0045), which were both locus-conserved between human and mice (Fig. S1B-D). Although both were detectable, cZNF292 was expressed at higher levels. Therefore, we chose the

highly and commonly expressed cZNF292 and its locus-conserved mouse orthologue cZfp292 (mmu-Zfp292_0007) (Fig. 1F) as the prime candidate for functional validation."

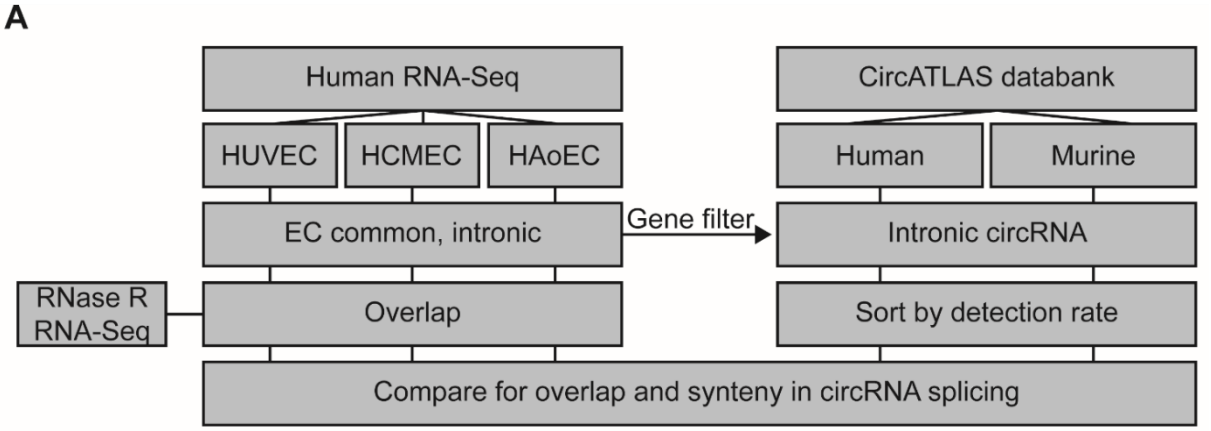
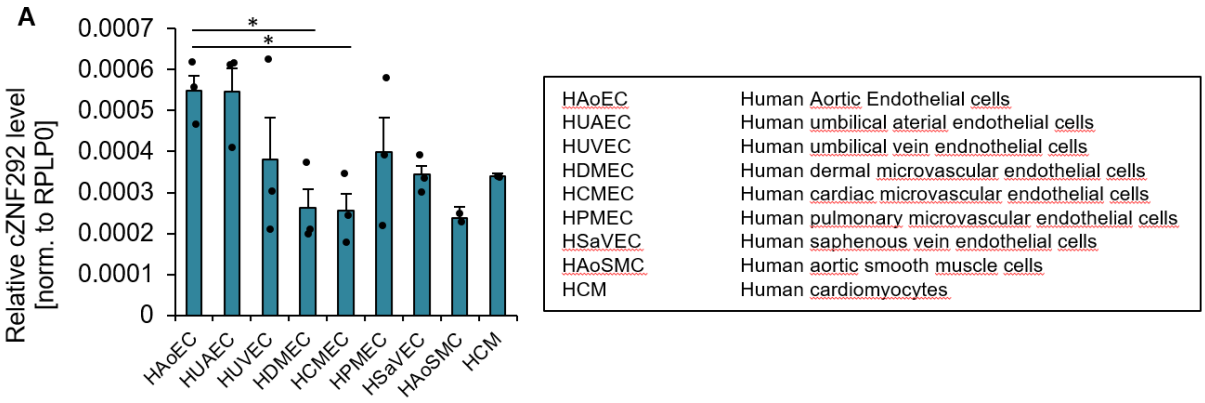


Fig. S1 Screening of locus conserved intronic circRNA. Refers to main figure 1. A Schematic representation of the screening process. Endothelial common intronic circRNA were identified from three different endothelial RNA-sequencing datasets and compared for checked for their resistance towards exonuclease digestion. CircRNA candidates were compared for the general detection rate among all intronic human or murine circRNA of the respective gene set as listed in the circATLAS database. Finally, circRNAs present in all datasets were analysed manually for their synteny in circRNA splicing

Is cZNF292 exclusively expressed by ECs? Is it expressed in other vascular cells, such as smooth muscle cells? This might be important also to understand the phenotype of KO animals.

Answer: To address the reviewer's question, we measured cZNF292 in various vascular cell types. cZNF292 is indeed expressed in various cell types including smooth muscle cells. However, its expression tends to be higher in aortic endothelial cells (see **Reviewer Figure 1** below). With this broad expression pattern, we cannot exclude effects in other vascular cells, besides endothelial cells. To address the specific function of cZNF292 in endothelial cells, however, floxed mice are required, which are currently not available.



Reviewer Figure 1. Expression of cZNF292 in different vascular beds. **A** Bar graph depicting the expression of cZNF292 in different human vascular cell types relative to the house-keeping gene RPLP0. RNA levels were determined by qRT-PCR. Data is shown as mean±SEM (n=2-3). *p<0.05 determined by two-tailed Student's t-test.

In order to state that the strategy does not influence the expression of the host gene Zfp292, the authors should measure also the protein level and not only the RNA in the null mice generated by CRISPR/Cas9.

Answer: We agree that this is an important control experiment and determined Znf292 protein levels in KO mice compared to wild type controls by Western blot. There was no difference in expression of the protein isoforms (**Fig. S2A**).

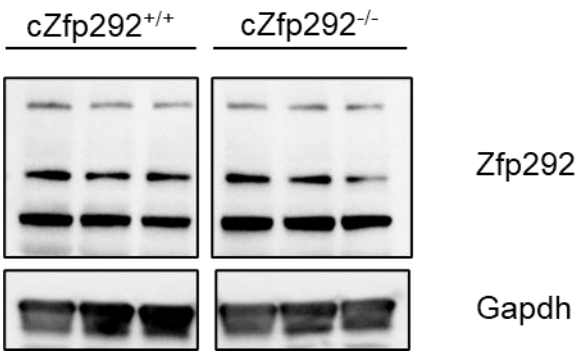


Fig S2A. Immunoblot analysis of Zfp292 protein levels in cZfp292+/+ and cZfp292-/- mice in muscle tissue. Gapdh levels are shown as normalization control.

The authors made a significant effort in generating a KO mouse. Nonetheless, its phenotyping is limited. They elegantly showed that angiogenesis is not altered by the KO of cZNF292, using the neonatal retina as a model system. Have they tried other angiogenesis assays in vivo or ex vivo? How do they reconcile this result with their own previous results on the angiogenic effect of cZNF292 in vitro (Figure 4C and D of Circ. Res. 2015 Oct 23;117(10):884-90)?

Answer: We thank the reviewer for the suggestion. We addressed the effect of cZfp292 deletion in other angiogenesis assays and indeed demonstrate that cZfp292 knock out reduces the outgrowth in aortic ring assays (**Figure 2I/J**). This demonstrates that angiogenic responses of aortic endothelial cells are diminished in the absence of cZfp292. This effect may reflect a higher expression of cZfp292 in aortic endothelial cells compared to other endothelial cells shown above and in **Figure 2 M/N**.

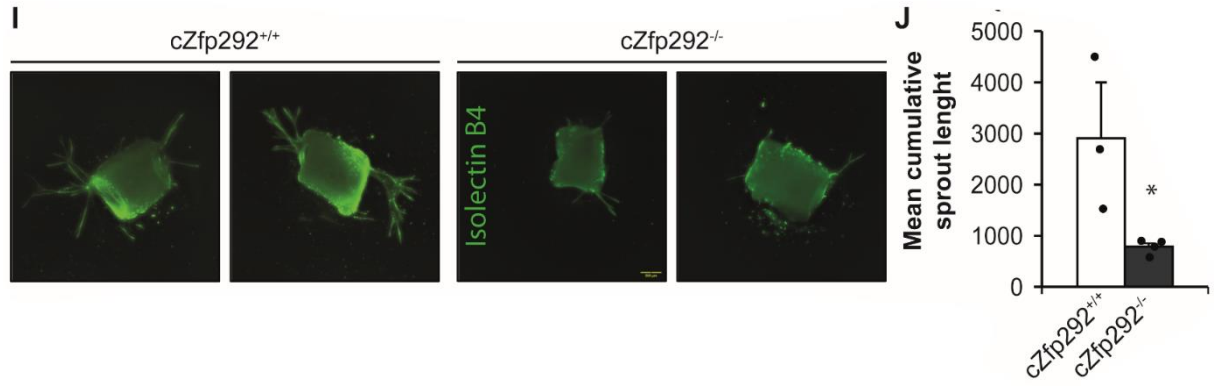


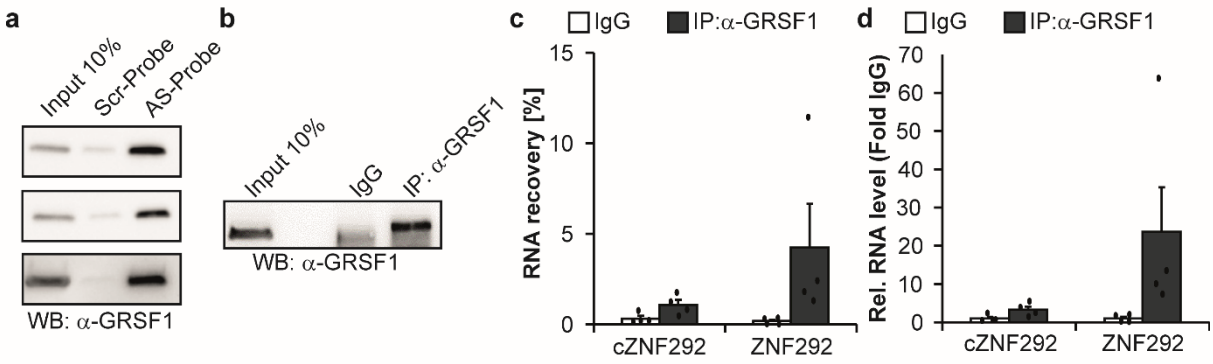
Figure 2I/J. Aortic ring assay. Representative images and quantification of sprout length of aortic rings of cZfp292 wildtype and mutant mice. Aortic rings were harvested and allowed to sprout in the presence of 30 ng/ml VEGF-A for 7 days before rings were fixed with 4% PFA and with Isolectin B4 (green). Mean values are representative of each 4 technical replicates and quantification is shown as mean±SEM. Statistical analysis was performed using two-twailed Student's t-test.

Studying the EC properties from cells derived from the KO mouse, the authors stated that probably the phenotype might depend on the different level of cZNF292 expression among large and small vessels. If that is the case, could the authors design an experiment using ECs derived from small vessels and test, in a gain-of-function setting (in order to increase the level of cZNF292 close to EC-derived from large vessels), whether they can then behave similarly to the cells tested in figure 2I/J?

Answer: As summarized above, we have shown that the outgrowth of aortic endothelial cells is diminished in cZfp292 deficient mice (**Figure 2I/J**). This supports the concept that aortic endothelial cells might be more dependent on cZfp292 expression as compared to e.g. retinal endothelial cells, where we did not see a significant reduction of vessel growth in knock out mice.

Authors conducted a very elegant analysis of protein partner identification through mass spectrometry. Out of the 75 putative partners, authors picked SDOS. Why? Is there a multiprotein interaction which involves SDOS and cZNF292?

Answer: We have picked SDOS because of the high enrichment. We also assessed some other proteins including the RNA binding protein GRSF-1, which is known to regulate RNA transport. Although we validated the binding to cZNF292, controls showed that it also binds the linear ZNF292 mRNA (**Reviewer Figure 2,** see below), suggesting that this is not a specific interaction partner.



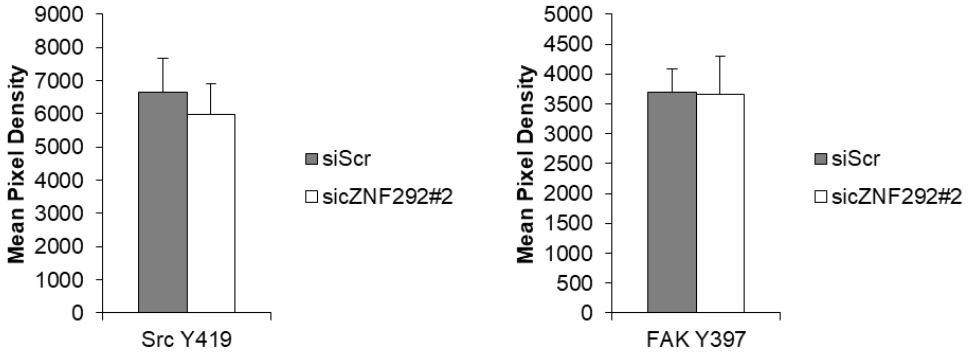
Reviewer Figure 2. Validation of the cZNF292/GRSF1 interaction. Immunoblot analysis of endogenous GRSF1 levels following **A** pulldown of cZNF292 using an antisense-probe under native conditions compared to a scrambled probe or **B** following immunoprecipitation of GRSF1 in HUVEC cell lysates. **C/D** Analysis of linear and circular ZNF292 levels following GRSF1 immunoprecipitation in HUVEC cell lysates depicted as either **C** RNA recovery relative to input levels or **D** as Fold IgG control (n=4). RNA levels were measured by qRT-PCR.

It is unclear how the four aminoacid residues which are critical for cZNF292 binding to SDOS were identified.

Answer: Potential amino acid residues of SDOS taking part in RNA binding were inferred by alignment to published RNA/protein interaction models of its close family member NUDT16.

SDOS recruitment is known to depend on the PKC pathway, but it also possible that the interaction between cZNF292/SDOS might modulate the SRC/FAK loop. Have the authors excluded this possibility?

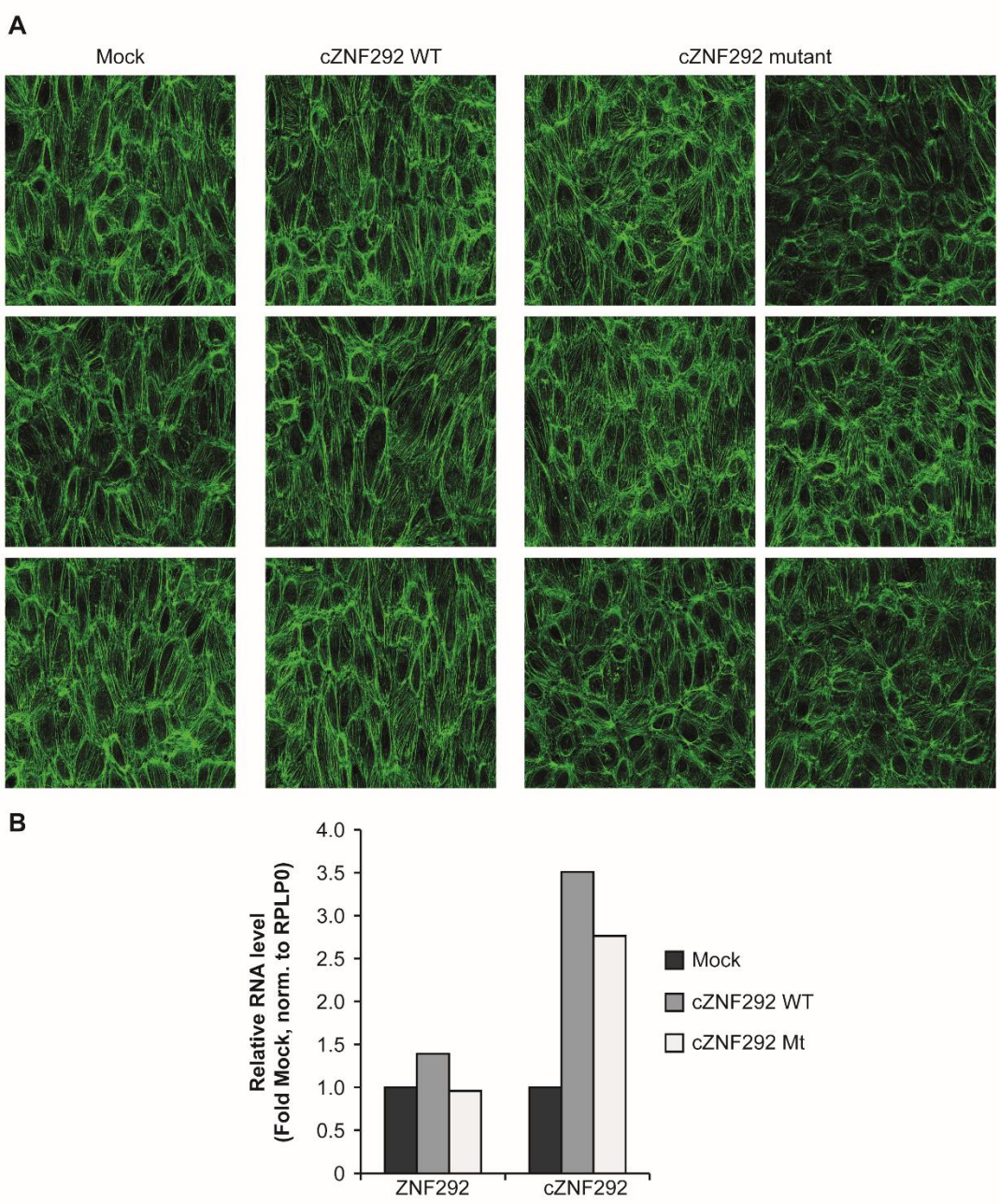
Answer: We addressed this question, however, did not detect a difference in Scr or FAK phosphorylation between cZNF292 silenced versus control endothelial cells (**Reviewer Fig. 3**).



Reviewer Figure 3. Analysis of **A** Src Y419 and **B** FAK Y397 phosphorylation 24h after silencing of cZNF292 in HUVECs. Phosphorylation was determined using the R&D human phosphokinase array kit (n=4). Data is shown as mean±SEM.

The Authors nicely showed that the overexpression of the SDOD mutated protein on the putative cZNF292 binding amino acids influences EC response to flow. Could the authors mutate the binding sequences on cZNF292 and determine whether its expression in ECs might induce a similar response to the flow?

Answer: To address the reviewer's suggestions, we have used an overexpression construct of cZNF292 with and without mutated SDOS binding motifs to analyse its influence under laminar flow. Overexpression of wildtype cZNF292 slightly aggravated endothelial flow response after exposure to 12 dyne laminar flow, whereas endothelial cells expressing cZNF292 with mutated SDOS motifs failed to align to flow (**Reviewer Figure 4**), demonstrating the importance of the SDOS interaction site within cZNF292.



Reviewer Figure 4. Lentiviral overexpression of cZNF292 variants under laminar flow. A, Immunofluorescence images of HUVECs stained with Phalloidin (green) for actin fibers. HUVECs were transduced with lentivirus carrying an overexpression construct for either wildtype cZNF292 or a cZNF292 variant with mutated SDOS binding sites. Transduced cells were reseeded to flow cells 48h after transduction and exposed to 12 dyne laminar flow for an additional 48h, after which cells were fixed (n=1). **B,** qPCR measurements of linear and circular ZNF292 levels in HUVECs 96h after lentiviral overexpression of cZNF292 variants compared to mock controls. RNA levels were normalized to RPLP0 (n=1).

Minor:

- Read count in figure 2k: was it normalized on library dimension?

Answer: Analysis of circRNAs from RNA-sequencing data was performed using the *circtools* DCC package, which indeed includes a normalization to sequencing depth.

- H26 --> H29?

Answer: We thank the reviewer for noticing this mistake and have accordingly corrected the manuscript text.

Reply to Reviewer #2:

This manuscript by Huemueller leverages methodology to specifically edit introns retained in circRNA but not the corresponding linear mRNA to attempt to specifically study circRNA function without the confounder effects of altering expression of the linear mRNA in vivo. Methodology to specifically study circRNA function would have a high impact in the field, particularly in assessing their role in homeostasis and disease pathogenesis. The authors identify cZfp292 as a candidate for functional validation based on computational analysis. Their methodology does seem to work to decrease the circular and not linear form of the RNA, and the authors are able to identify interacting proteins. Silencing of these interacting proteins in cell culture systems, or mutation of the protein residues expected to bind to the circRNA phenocopies the effect of silencing the cZfp292. This is an elegant study, but not entirely novel, as the investigators had previously investigated the phenotype of cZfp292 in cultured cells (associated with sprouting), and this methodology to target retained introns has been described by other groups. Nonetheless the in vivo functional validation is a strength, but connecting this to disease pathogenesis (i.e. the relevance of this pathway in disease) would strengthen the overall story. My specific concerns are noted below:

1. The use of techniques to delete intronic sequences to specifically delete circRNAs without affecting the linear mRNA is not novel, and has been described by other groups (e.g. Zheng et al, Nature Comm 2016).

Answer: We thank the reviewer for drawing out attention to this paper, which we have cited in the revised manuscript. Zheng et al indeed identified whole intron retaining circRNAs, but this manuscript did not further pursue deletion studies. Therefore, this study does not reduce the news value of our findings.

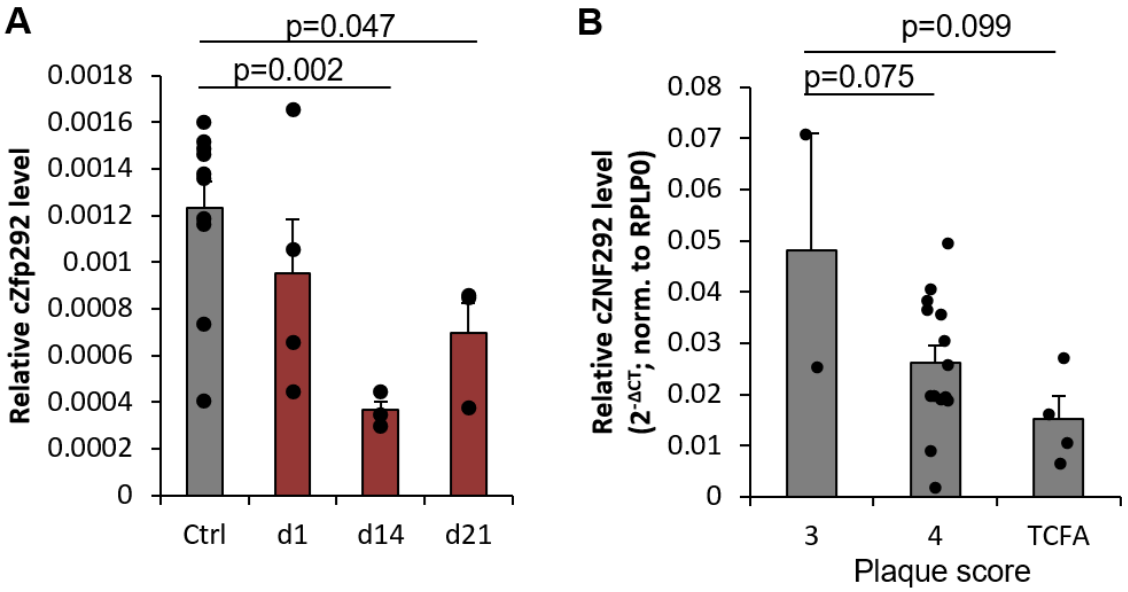
2. Additionally, the in vitro phenotype of cZNF292 was previously studied by the same group in 2015, although the phenotype in the models in this paper are different, attributed to higher levels in aortic endothelial cells; hence the functional assessment is not entirely novel, although the in vivo assessment is a significant advancement.

Answer: We respectfully disagree with the reviewer with respect to the news value of the present study. Our previous study was reporting a screen and used simple angiogenesis assays in vitro, which have very limited implications for vascular biology in vivo. We now report on the mechanism of action of cZNF292, which for the first time documents an effect on shear-dependent cytoskeletal re-arrangements. Finally, we would like to highlight that this is the first report demonstrating a functional relevance of a vascular circRNAs *in vivo*.

Arguing that the pure description of a new signalling molecule (in this case a circRNA) precludes any further high impact publications appears not appropriate given the many papers in Circulation Research on well-known endothelial signalling molecules which are known for decades (E.g. NOS3, Notch, Akt, transcriptional regulators etc).

3. The phenotype is mostly explored in the cell culture system and points to altered morphology related to laminar flow. It is somewhat surprising that there is no exploration of how these defects affect disease pathogenesis in the murine models: the investigators are encouraged to explore if the mice are more prone to aortic pathologies (e.g. models of thoracic or aortic aneurysms or atherosclerosis that are well established in murine models). This would be a big advantage of developing the in vivo models.

Answer: We appreciate the reviewers' comments and have meanwhile performed additional studies to determine the regulation of cZfp292 after wire injury and atherosclerosis. We show that cZNF292 expression was reduced after wire injury (see **Reviewer Figure 5A**). There was also a trend toward reduced expression of cZNF292 in human plaques with higher severity, however, the limited sample size available precluded further in-depth analysis (see **Reviewer Figure 5B**).



Reviewer Figure 5. cZNF292 in murine and human aortopathies. A, qRT-PCR analysis of cZfp292 levels during different stages after wire injury-induced vascular remodeling in the left carotid artery (n=3-4). **B,** qRT-PCR analysis of circular and linear ZNF292 in human aortic plaque samples (3: n=2, 4: n=14, TCFA: n=4, N/A: n=6). Plaques were scored according to (PMID: 23541627 and 10807742). Briefly an increase in plaque score indicates a worsening of the plaque towards the stage of thin cap fibroatheroma (TCFA). Data is depicted as mean±SEM; statistical analysis was performed using two-tailed Student's t-test.

To additionally explore the in vivo phenotype, we assessed endothelial cell outgrowth of aortic rings. We show that this is diminished in cZfp292-/- compared to control mice (**Figure 2I/J**). These data are included in the revised manuscript page 8, paragraph 1.

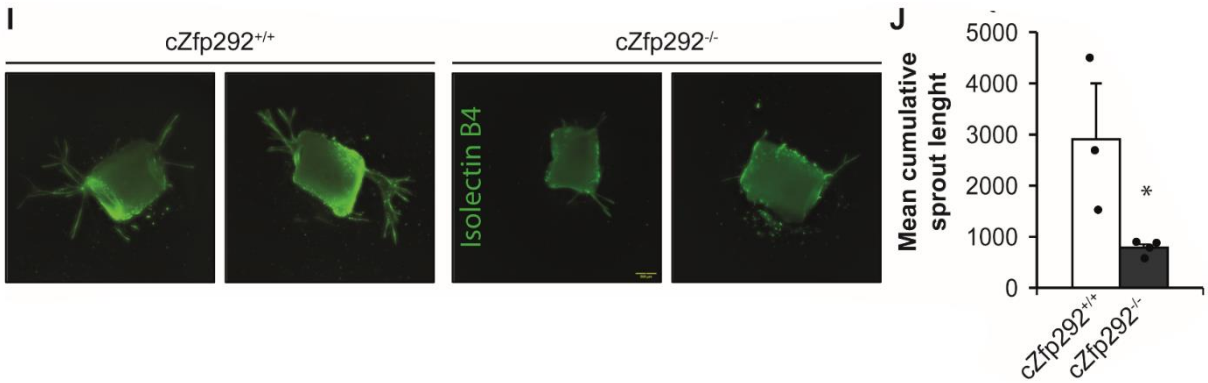


Figure 2I/J. Aortic ring assay. Representative images and quantification of sprout length of aortic rings of cZfp292 wildtype and mutant mice. Aortic rings were harvested and allowed to sprout in the presence of 30 ng/ml VEGF-A for 7 days before rings were fixed with 4% PFA and with Isolectin B4 (green). Mean values are representative of each 4 technical replicates and quantification is shown as mean±SEM. Statistical analysis was performed using two-tailed Student's t-test.

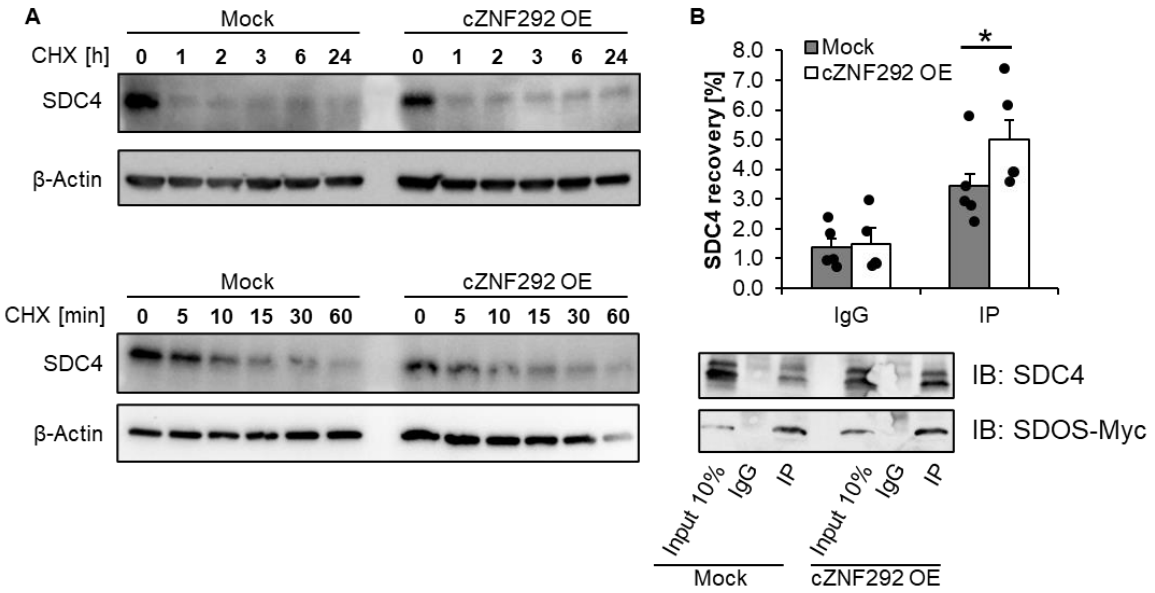
We agree with the reviewer that a further in-depth characterisation of cZNF292 in atherosclerosis or other pathologies is interesting and should be pursued. However, we would need many months to generate sufficient mice for the experiments, since the expansion of our colony has been halted during the COVID pandemic.

4. The detailed study of the interacting proteins and the residues that are important is nicely described; can the authors explore how the binding of cZfn292 affect syndecan function? Does it change cellular localization or function? More detailed mechanism would be helpful.

Answer: We thank the reviewer for this question and provide additional details regarding the effects of cZNF292 on Syndecan 4 (SDC4). Whereas the stability of SDC4 was not altered (**Reviewer Figure 6A**), we show that overexpression of cZNF292 increased the interaction of SDC4 with SDOS (**Reviewer Figure 6B** included as new **Main Figure 6I/J**), supporting our mechanism of action.

These results are included in page 11, paragraph 4 of the revised manuscript as follows:

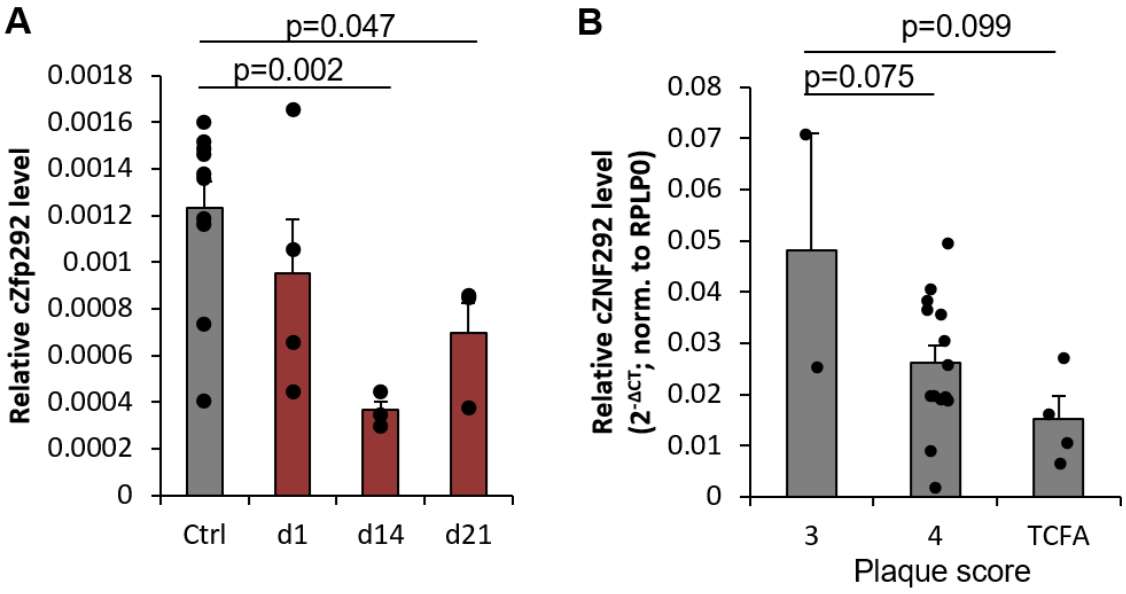
"These results suggest a model whereby cZNF292 enhances SDC4-SDOS interaction and subsequent signaling. To test this model, we determined the effect of cZNF292 overexpression of SDC4-SDOS protein interaction by co-immunoprecipitation. Overexpression of cZNF292 indeed increased the binding of SDC4 to SDOS (Fig. 6I/J)."



Reviewer Figure 6. cZNF292 influence on SDC4 protein. A, Timecourse-analysis of SDC4 protein stability by immunoblotting following treatment with cycloheximide in HeLa cells. HeLa were transfected with plasmids encoding for SDOS-Myc and a cZNF292 overexpression plasmid or respective mock controls for 24h after which cells were reseeded to new culture dishes and allowed to reattach for 24h. The next day, cells were treated with 300 µg/ml cycloheximide for the indicated timepoints, before cells were washed with ice-cold PBS and flash frozen in liquid nitrogen. Lysate were analyzed by Western blotting and probed for SDC4. β-actin was used as loading control (n=1). **B,** Representative image and quantification of SDC4 following immunoprecipitation of SDOS-Myc in HeLa cells (n=5). Membrane-associated fractions were extracted using a commercial kit 48h after overexpression of SDOS-Myc and SDC4-Flag in the presence of either a cZNF292 overexpression construct or a respective Mock control. Following SDOS-Myc was precipitated using antibodies targeting the Myc-tag. Immunoblots were stained for SDC4 (upper panel) and reprobed for SDOS-Myc.

5. Finally does this cZfp292 expression change in human or murine aortopathies? This should be examined in tissue samples.

Answer: As outlined above in response to the reviewers comment #3, we showed the regulation of cZfp292/cZNF292 expression in mouse and human aortopathies (**Reviewer Figure 5**).

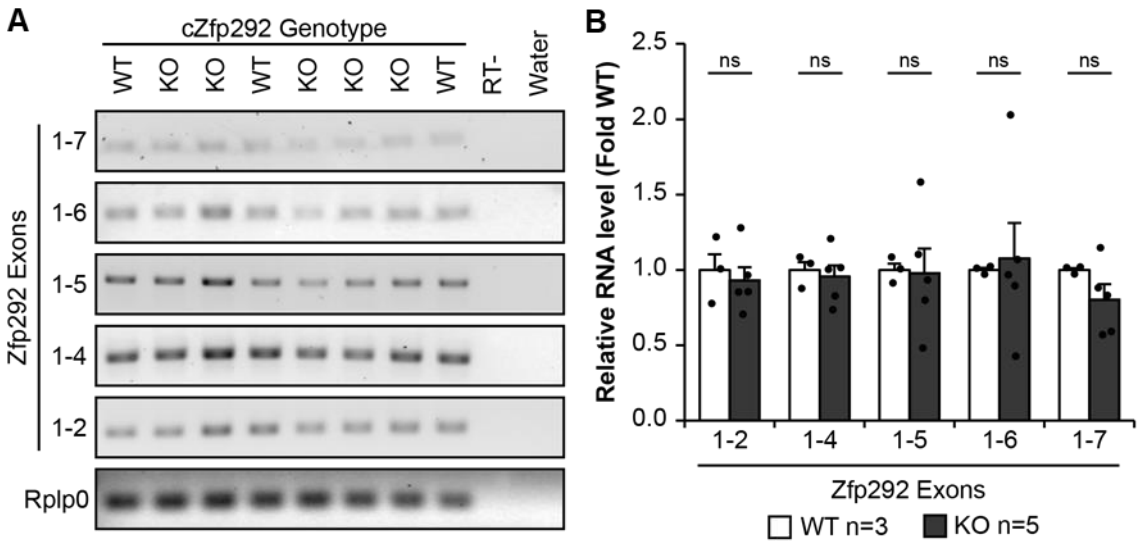


Reviewer Figure 5. cZNF292 in murine and human aortopathies. A, qRT-PCR analysis of cZfp292 levels during different stages after wire injury-induced vascular remodeling in the left carotid artery (n=3-4). **B,** qRT-PCR analysis of circular and linear ZNF292 in human aortic plaque samples (3: n=2, 4: n=14, TCFA: n=4, N/A: n=6). Plaques were scored according to (PMID: 23541627 and 10807742). Briefly an increase in plaque score indicates a worsening of the plaque towards the stage of thin cap fibroatheroma (TCFA). Data is depicted as mean±SEM; statistical analysis was performed using two-tailed Student's t-test.

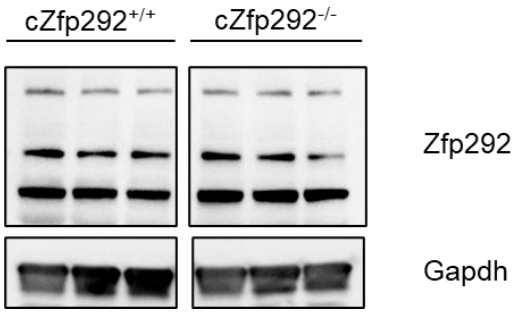
Minor comments:

a. Did the authors verify that the splicing of the linear mRNA was not affected?

Answer: We have previously validated that cZNF292 silencing did not alter expression of linear ZNF292 on pre-mRNA, mRNA or protein level (Boeckel et al CircRes 2015). To confirm these findings in the knock out mice, we have now included PCRs covering the entire mRNA documenting no effect of the deletion on linear Zfp292 mRNA (**Reviewer Figure 7**). In addition, we show a comparable expression of Zfp292 protein level in cZfp292+/+ and cZfp292-/- mice (see new **Fig. S2A** of the revised manuscript).



Reviewer Figure 7. Analysis of Zfp292 mRNA levels measured by **A** semi-quantitative PCR or **B** qRT-PCR in wildtype (n=3) or cZfp292 knockout (n=5) cells generated by CRISPR/Cas9-mediated deletion of cZfp292 exon1A in the immortalized murine endothelial cell line H5V. Data is shown as mean±SD; statistical analysis was performed using two-tailed student's t-test.



Supplementary Figure 2A. Immunoblot analysis of Zfp292 protein levels in cZfp292+/+ and cZfp292-/- mice in muscle tissue. Gapdh levels are shown as normalization control.

b. Fig 2A: Define what Rplp0 is (linear mRNA control?)

Answer: Indeed, Rplp0 served as a linear mRNA house-keeping control. The manuscript now states this more clearly.

c. **Fig 2A: Why is there higher level of cZfp292 with RNAse R digestion; would have expected same level if the primers are specific for the circRNA.**

Answer: This effect was observed in multiple studies. While this phenomenon has not been systematically been studied to our knowledge, it was discussed that this general effect might be due to a better saturation or less RNA/RNA interactions.

d. **For Fig 2C it would be informative if the entire cZfp292 locus is shown with the consequence of the deletion (and the expected sizes).**

Answer: We include the full locus in **Fig. S2B** of the revised manuscript**.**

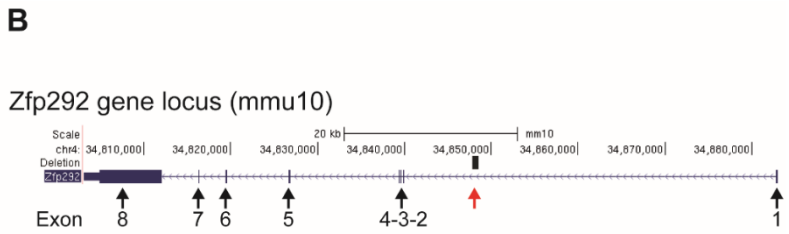
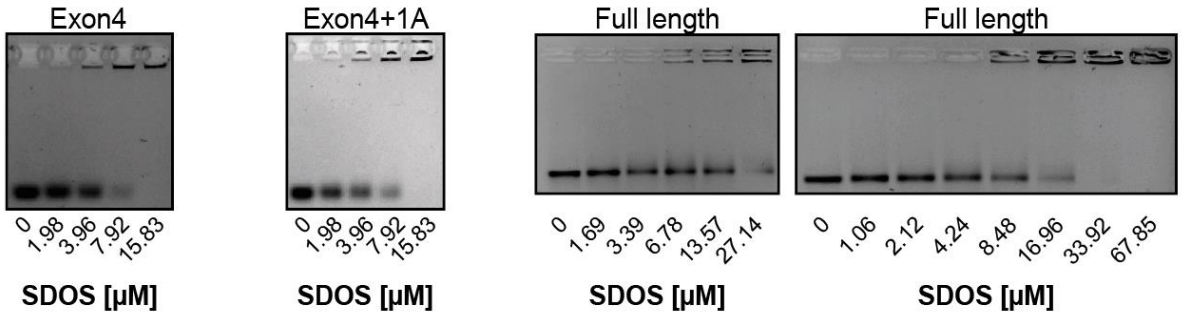


Fig. S2B. Representation of the Zfp292 gene locus (GRCm38/mm10 assembly) as well as the size of the region targeted for deletion by CRISPR/Cas9 (indicated by the red arrow). Image was generated using the UCSC genome browser**.**

e. Only 2 replicates were run for the EMSA assay; a minimum of three replicates should be assessed.

Answer: To substantiate our findings, we have additionally repeated the binding assay with a non-radioactive assay to validate the interaction between the RNA and SDOS (**Reviewer Figure 8**). These assays confirm the previous findings. Due to the current reconstruction of the radioactivity lab, we cannot repeat the radioactive assays shown in figure 5C, but the biological validation of the findings is n>3 (representative examples are shown below).



Reviewer Figure 8, Agarose-electrophoresis images of unlabeled RNA oligos comprising SDOS motifs of exon4, exon4+exon1A or the full length cZNF292 sequence.

f. The figure legends are somewhat sparse; a little more detail on describing the figures should be provided.

Answer: We thank the reviewer for this advice and provide more details in the figure legends.

Reply to Reviewer #3:

Comments to the authors:
In the manuscript entitled 'Locus-conserved circular RNA cZNF292 controls endothelial cell flow responses' Heumuller et al. describe a novel role of cZNF292/cZfp292 in vascular biology, and in more detail, in the response of endothelial cells to flow. The current manuscript is a follow-up of the 2015 publication in Circulation Research by the same group. Here, the authors show that circular RNA cZNF292 interacts with SDOS and that this interaction drives the cytoskeletal organization upon induction of laminar flow. One particular highlight is that the authors were able to show locus conservation of cZNF292/ cZfp292 between human and mice by focusing on intronic elements that are retained across the two species - these same introns were then used for selective genetic removal, not affecting the (coding) mRNA of the host gene. The premise of the study is clear and could be of interest to the readers of Circulation Research, however several issues remain.

Major comments
- Figure 1A-E are described very briefly. If this reviewer understands well, most of the data is derived from publicly available data sets that were deposited by the research group themselves and then combined with the circATLAS database. More explanation, potentially with a graphic visualization of the pipeline, of how the authors got to cZNF292 and the locus-conserved cZfp292 would clear up the train of thought that was used here.

Answer: We thank the reviewer for the comment and have elaborated the description of our screening approach in more detail in the text of the revised manuscript. We additionally added supplemental Figure 1 for illustration.

The revised text now reads (see page 7, para 2 and Suppl. Fig. 1):

"In order to identify locus-conserved endothelial intronic circRNA, we used published endothelial RNA-sequencing data with circRNAs listed in the circATLAS database (Fig. S1A). We first selected circRNAs commonly expressed between different types of human endothelial cells resulting in 1228 circRNAs from 868 host genes (Fig 1A). Further comparison of these circRNAs for their stability towards exonuclease digestion using an additional RNA sequencing dataset of RNase R-treated endothelial cells, showed that 1158 (~95%) can be considered true circRNA (Fig. 1B). However, only 29 of these were back-spliced to intronic cassettes (Fig. 1C). Importantly, 21 of the 29 candidates were also included in the top30 consistently detected human intronic circRNAs of the respective loci (Fig. 1D) consolidating their presence in human samples. When we additionally analysed the respective loci in mouse, several intronic circRNAs were commonly detected in circATLAS database (Fig. 1E; overlap of 13 host genes when comparing Fig. 1D and 1E), but only few circRNAs shared synteny. Of these candidates, we validated the expression and exonuclease resistance of the circRNAs cZNF292 (hsa-ZNF292_0014) and cFOXP1 (hsa-FOXP1_0045), which were both locus-conserved between human and mice (Fig. S1B-D). Although both were detectable, cZNF292 was expressed at higher levels. Therefore, we chose the highly and commonly expressed cZNF292 and its locus-conserved mouse orthologue cZfp292 (mmu-Zfp292_0007) (Fig. 1F) as the prime candidate for functional validation."

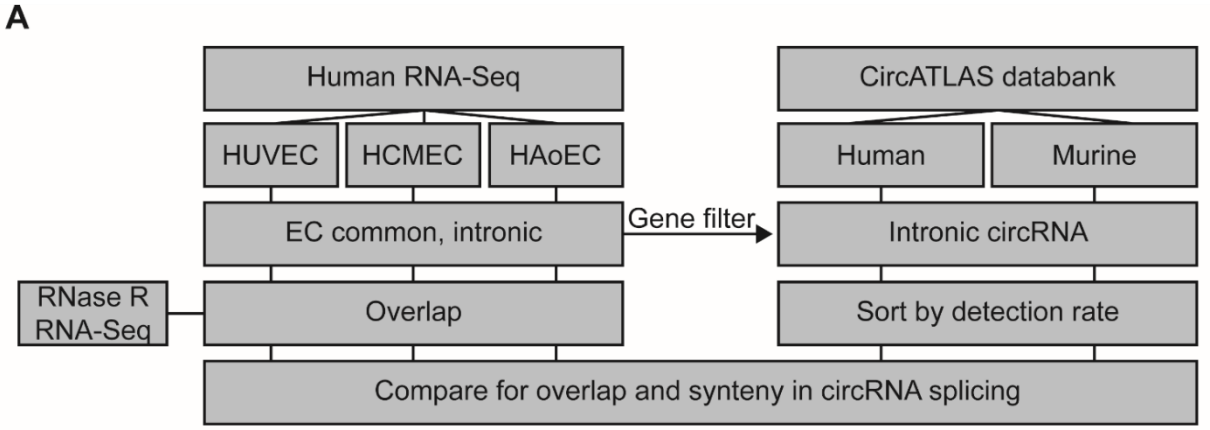
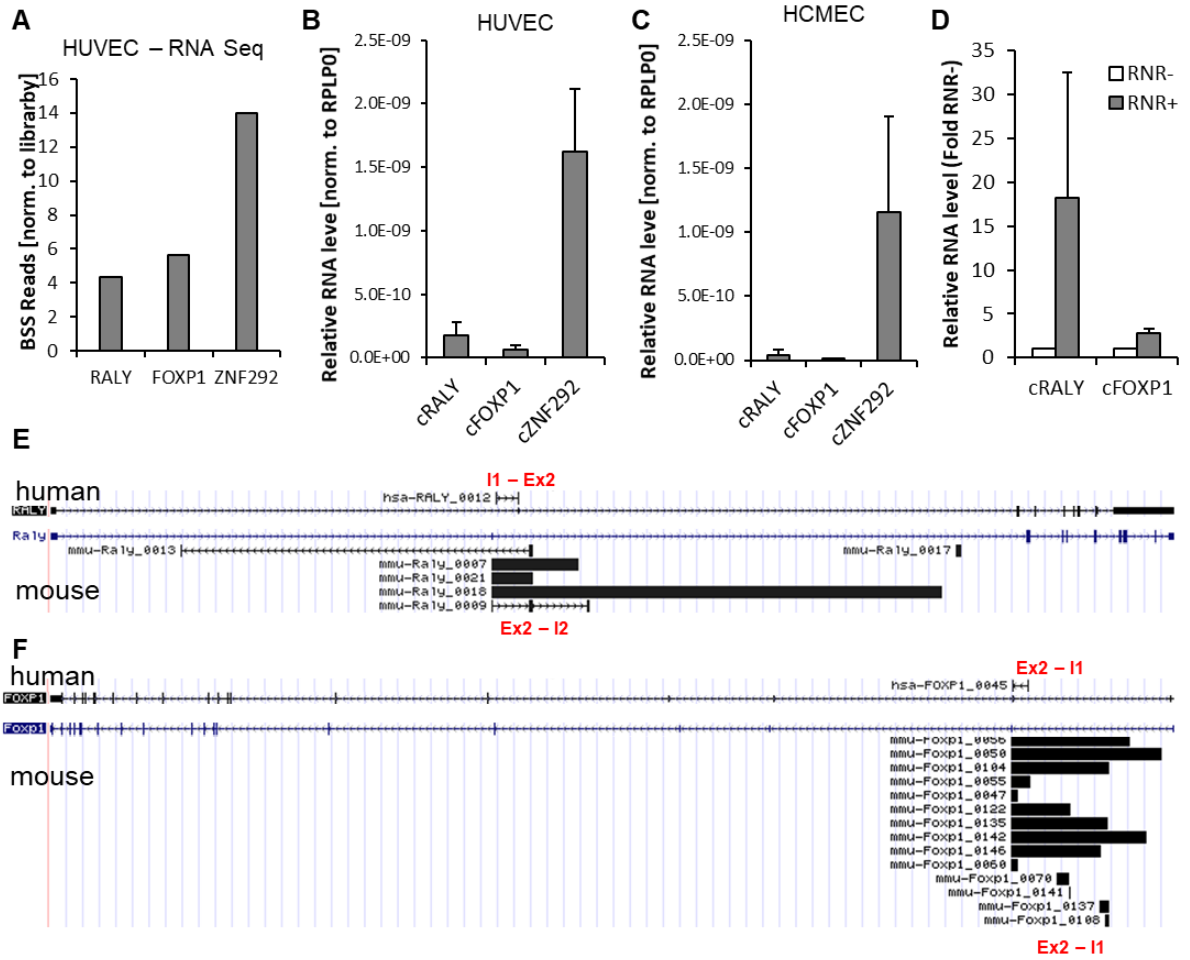


Fig. S1 Screening of locus conserved intronic circRNA. Refers to main figure 1. A Schematic representation of the screening process. Endothelial common intronic circRNA were identified from three different endothelial RNA-sequencing datasets and compared for checked for their resistance towards exonuclease digestion. CircRNA candidates were compared for the general detection rate among all intronic human or murine circRNA of the respective gene set as listed in the circATLAS database. Finally, circRNAs present in all datasets were analysed manually for their synteny in circRNA splicing

- The authors quickly go from 1158 candidates towards 1 prime candidate. Can the authors discuss other intronic circRNAs that are listed in Figure 3 D-E such as FOXP1 and RALY. Some of host genes listed have important functions in vascular biology and it will be exciting to follow up.

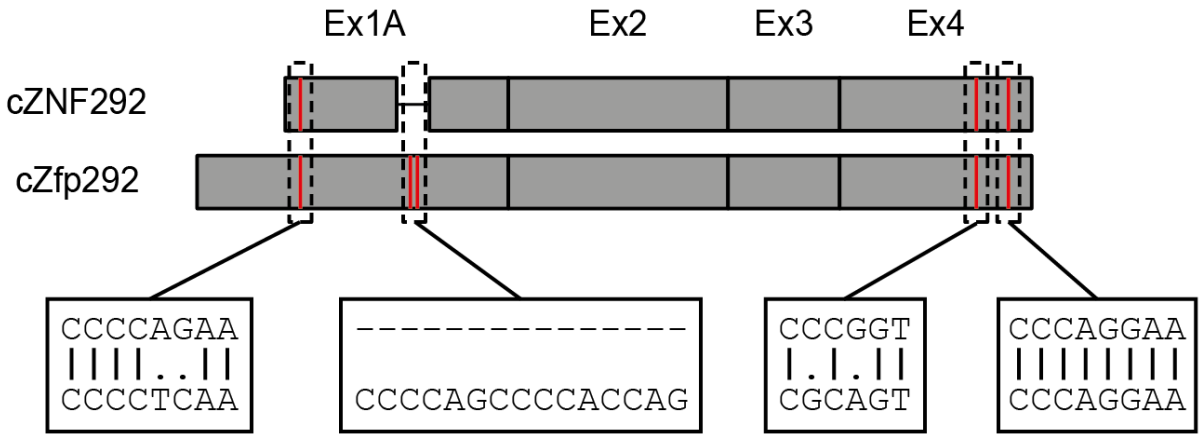
Answer: We additionally considered investigation of other circRNAs, including cFOXP1 and cRALY, but the expression of cFOXP1 and cRALY was quite low in human endothelial cells compared to cZNF292 (**Reviewer Figure 9, also Fig. S1B-D**). Furthermore, splicing of cRALY was not conserved in its synteny when compared to murine intronic circRNA of the RALY locus (**Reviewer Figure 9E**).



Reviewer Figure 9. Screening approach for cFOXP1 and cRALY. Analysis of the RNA levels of circular cRALY, cFOXP1 and cZNF292 in **A** RNA-sequencing data of HUVECs, **B** measured by qRT-PCR in HUVEC (n=3) or **C** in HCMEC (n=3). **D** Analysis on the stability of cRALY and cFOXP1 after exonuclease RNase R digestion of HUVEC RNA compared to mock treated controls (n=3). **E/F** Relative position of human cRALY and cFOXP1 within their respective gene locus as well as potential murine paralogue listed in the circATLAS database of the same region. Images were generated using the UCSC genome browser. All data ist depicted as mean±SEM.

- What are the differences in nucleotide sequence between cZNF292 and cZfp292? It would be good to show potential synteny if we expected any functional conservation between the human and mouse orthologues.

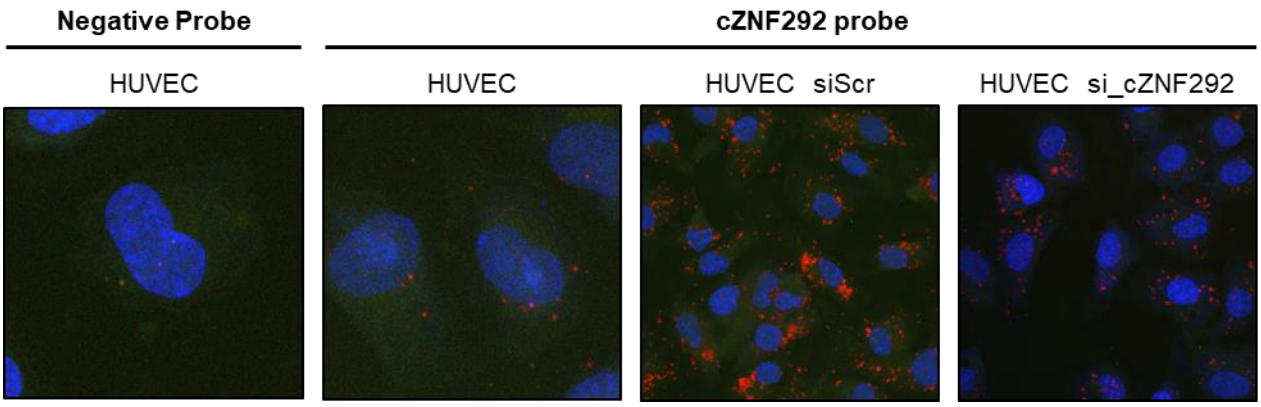
Answer: We thank the reviewer for his question and provide more details into the sequence conservation. Depending on the algorithm, pairwise sequence alignments show a similarity of ~65% (LALIGN), ~68% (Needleman-Wunsch) or ~75% (Smith-Waterman) between human cZNF292 and murine cZfp292. Importantly, while the human SDOS interaction sites are partially conserved in cZfp292, it also includes an additional RNA stretch including two SDOS binding motifs. Although sequence conservation is low between intronic elements, SDOS binding elements are conserved (Fig. 5B).



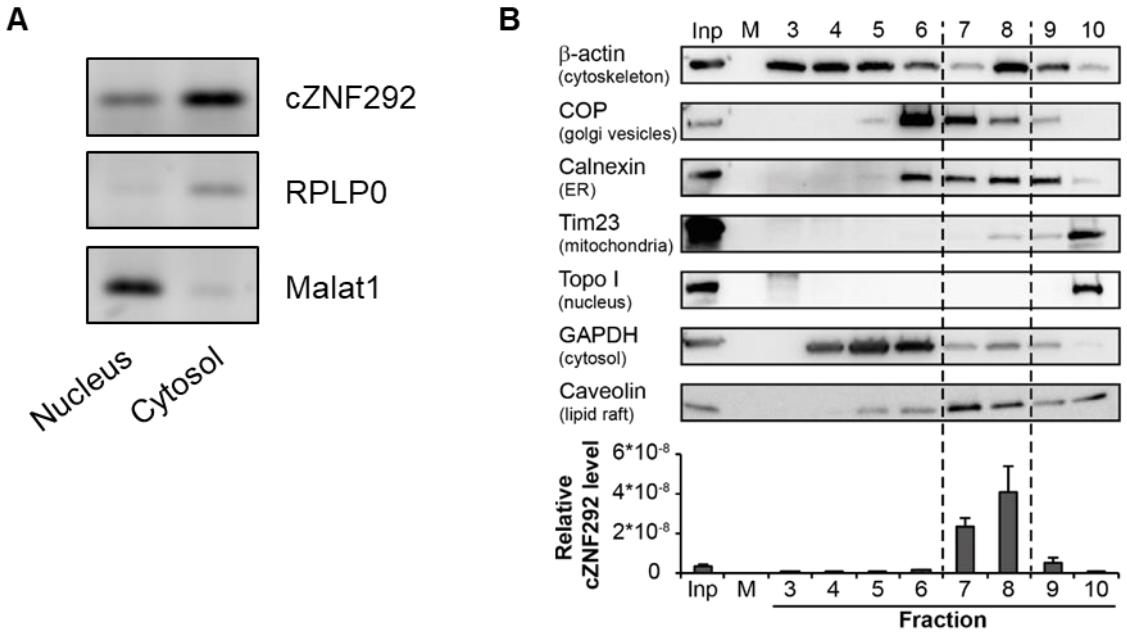
Main Fig. 5B. Schematic depiction of human and murine ZNF292 with potential SDOS binding sites highlighted in red. Sequence excerpts in the lower panel show conservation of the respective motifs by Smith-Waterman pair-wise sequence alignment

- SDOS is a protein known to be expressed in the cytoplasm. What is the subcellular location of cZNF292 (and cZfp292)? Can the authors perform qPCR upon cell fractionation experiments or RNA-FISH. It should be possible to dissect linear versus circular transcript by using probes direct against exon1a, as this same exon is used for genetic knockdown as well as siRNA targeting in Figure 4.

Answer: To address the question of the reviewer, we tried to perform RNA Fish experiments. Although we detected a specific signal in the cytoplasm compared to negative control, some staining remained in the siRNA cZNF292 treated endothelial cells, questioning the specificity of the approach (see **Reviewer Figure 10**). Since the available sequence to detect the circRNA is limited to the circRNA-specific sequences, we do not have the chance to improve this method. However, previous cell fractionation experiments confirmed that cZNF292 is detected in the cytoplasmic fraction (see **Reviewer Figure 11**). We additionally want to highlight that our pulldown assays and RNA immunoprecipitation experiments confirmed that SDOS interact with cZNF292 (see new **Figure 5 and Figure 6** of the revised manuscript).



Reviewer Figure 10. Detection of cZNF292 by FISH. Immunofluorescence staining of cZNF292 in HUVECs under basal conditions or in HUVECs 48h after silencing of cZNF292 compared to controls. Single probe FISH experiments were performed employing the View miRNA FISH Kit (Affymetrix). Left panels (siRNA experiments) derive from an independent experiment and are shown in lower magnification



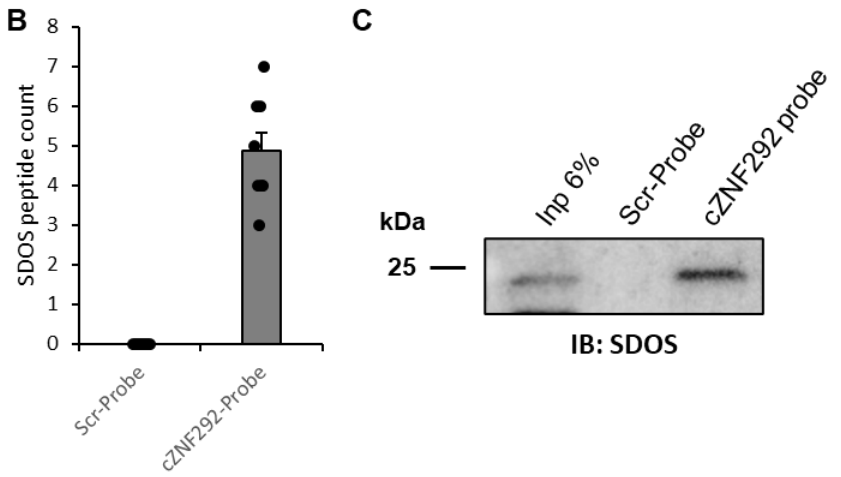
Reviewer Figure 11. cZNF292 is enriched in the cytoplasm. A, Subcellular fractionation of HUVECs differentiating between cytoplasmic and nuclear fractions. cZNF292 levels were measured by semi-quantitative qPCR. RPLP0 is shown as cytosolic control, Malat1 as nuclear control. **B,** Subcellular localization of cZNF292 in HUVECs after hypotonic cell lysis and sucrose density gradient centrifugation followed by fractionation. Image panels in the upper part show immunoblots of organelle-representative proteins. Quantification of cZNF292 levels in respective fractions was determined by qRT-PCR (n=3, mean±SEM). Input was measured in volumes equaling 10% of the lysates. (Abbreviations: COP: CARD Only Domain-Containing Protein 1, Tim23: Translocase of Inner Mitochondrial Membrane 23, Topo I: Topoisomerase I, GAPDH: Glyceraldehyde 3-phosphate dehydrogenase.

- Figure 3D-F and Fig2D-E show strong evidence between cZNF292 and SDOS in a series of experiments where either cZNF292 or SDOS was overexpressed. Can the authors perform an IP on SDOS with endogenous expression? Can the authors show RNA-FISH (with issue outlined in previous comment) in combination with IF for SDOS (and SDC4) to show that these molecules are both in the cytoplasm and colocalize.

Answer: To address the reviewers concern, we validated the protein interaction with endogenous proteins (**Fig. 3D, and see Reviewer Figure 12C below**). We also want to highlight that the interaction of cZNF292 with SDOS found by mass spectrometry was very clean (See **Reviewer Figure 12A,B**). We did find not peptides in scrambled controls, whereas the peptide count was high in all n=8 experiments.

A

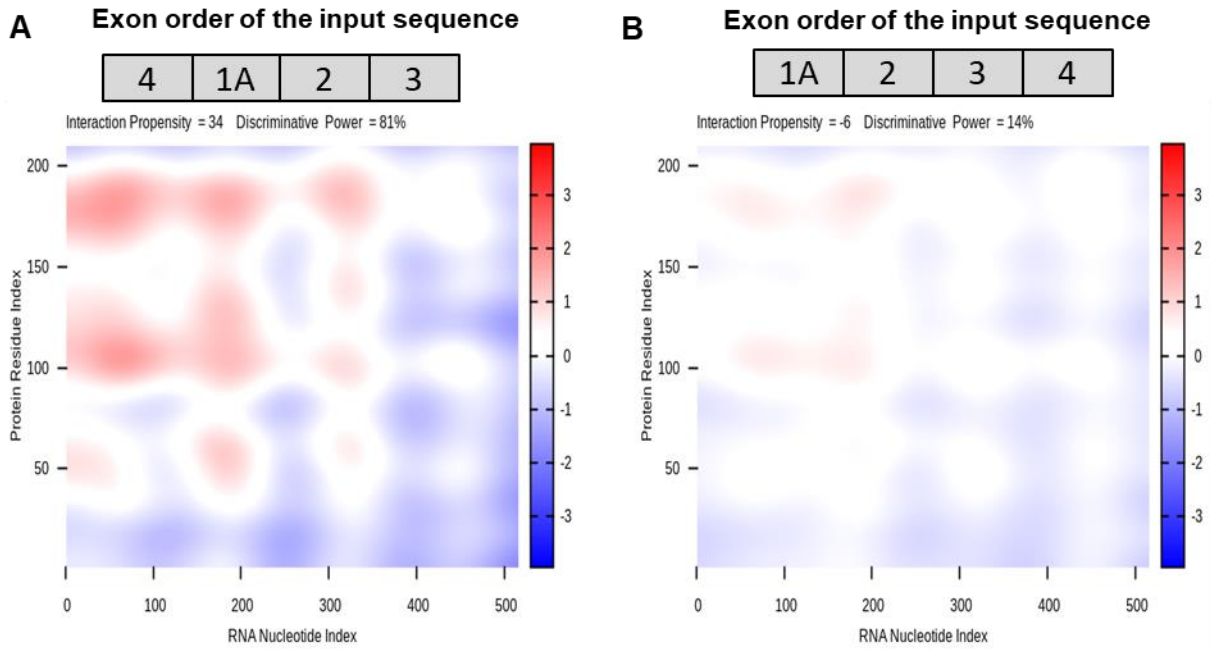
	SDOS -Peptide Count	
	Scr-Probe	cZNF292-Probe
n1	0	5
n2	0	6
n3	0	4
n4	0	3
n5	0	6
n6	0	4
n7	0	4
n8	0	7



Reviewer Figure 12. cZNF292 interaction with endogenous SDOS under native conditions. A/B Tabular and graphical representation of the SDOS peptide count identified by mass spectrometry following native pulldown of cZNF292 in HUVEC lysates compared to a scrambled control probe (n=8). **C** Immunoblot analysis of endogenous SDOS precipitated from HUVEC lysates using an cZNF292 antisense probe.

- The CCCA motif is described as a preferential binding site for SDOS and several of them are found in cZNF292 and cZfp292 and EMSA is used to interrogate all of the three possibilities (of which one is CCCG and CCCA). Although EMSA is a powerful tool it uses oligonucleotides and as such neglects the 3D structure of the RNA in a cellular environment. To accompany the presented data the authors should complement these data with in silico RNA-protein binding data that can be derived by algorithms such as catRAPID (both pieces of evidence, with their flaws, will back-up the proposed mechanism).

Answer: We thank the reviewer for this suggestion and perform in silico RNA-protein binding assessment by using catRAPID. However, the results have to be interpreted with caution given that the algorithm takes linear RNA as an input. Nevertheless, catRAPID suggested and interaction propensity of 34 of SDOS and cZNF292 when the input sequence of cZNF292 was shuffled to include the backsplice site (**Reviewer Figure 13A**), whereas the typical representation of the circRNA sequence (with linearization at the backsplice site) suggested an interaction propensity of -6 (**Reviewer Figure 13B**).



Reviewer Figure 13. Prediction of SDOS/cZNF292 interaction using CATRAPID. Analysis of the predicted SDOS/cZNF292 interaction strength using the catRAPID algorithm using the linear sequence of cZNF292 either A mimicking the backsplice site by shuffling the exon order or B without mimicking the backsplice site.

- Along these lines, for the EMSA experiments only mutations in SDOS are being interrogated. Can the authors perform similar experiments by changing the CCCA sequences in the oligonucleotides used in the EMSA?

Answer: Following the reviewers' suggestion we have further analysed the interaction between SDOS and cZNF292 after mutation of the SDOS motifs. Indeed, mutated oligos showed reduced interaction with SDOS in EMSA experiments (**new Fig. S5**). Additionally, we have also addressed the interaction between SDOS and short oligos carrying the different SDOS motifs of cZNF292 (**new Figure 5E**). Interestingly binding of SDOS to cZNF292 exon 4 and exon4/exon1A was still observed at the highest concentration of SDOS suggesting that other sequence motives and/or the secondary structure can still exhibit some binding activity. To exclude unspecific binding, we included unrelated oligos, which did not show any binding (**Figure 5E**, right panel).

These data have been included into the revised the main figure 5 and Fig. S5 of the manuscript.

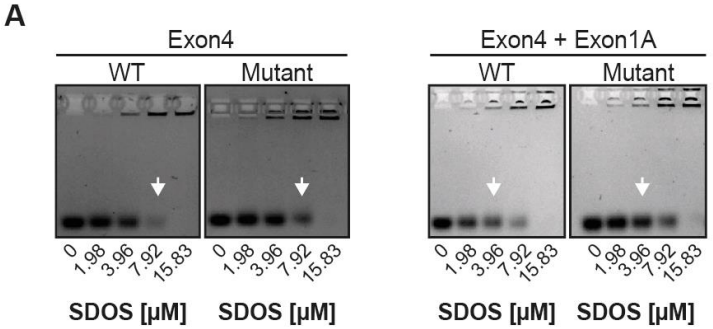


Fig. S5 Mutations in SDOS binding motifs reduced SDOS interaction. Refers to main figure 5. A, Representative agarose-gel electrophoresis images on the interaction between SDOS and unlabeled RNA oligos comprising wildtype or mutated SDOS motifs of the sequence excerpts of exon4 or exon4+exon1A as analyzed in Fig.5C (n=3). White arrow heads highlight bands with visible difference in SDOS binding between wildtype and mutant oligos.

- If the authors want to show functional conservation for cZfp292 and cZNF292 alongside the locus-conservation, several experiments will need to be repeated using cZfp292 and murine SDOS.

Answer: In addition to the demonstration of conservation of the SDOS binding site (**Fig. 5A)**, we also now show that on a functional level cZfp292 regulates aortic sprouting using the aortic ring assay in cZfp292-/- mice (**Fig. 2I/J**).

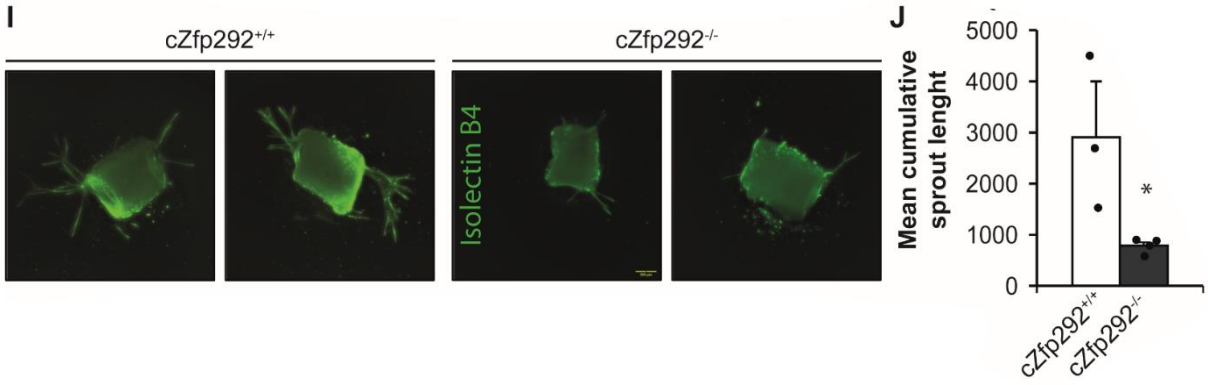


Figure 2I/J. Aortic ring assay. Representative images and quantification of sprout length of aortic rings of cZfp292 wildtype and mutant mice. Aortic rings were harvested and allowed to sprout in the presence of 30 ng/ml VEGF-A for 7 days before rings were fixed with 4% PFA and with Isolectin B4 (green). Mean values are representative of each 4 technical replicates and quantification is shown as mean±SEM. Statistical analysis was performed using two-tailed Student's t-test.

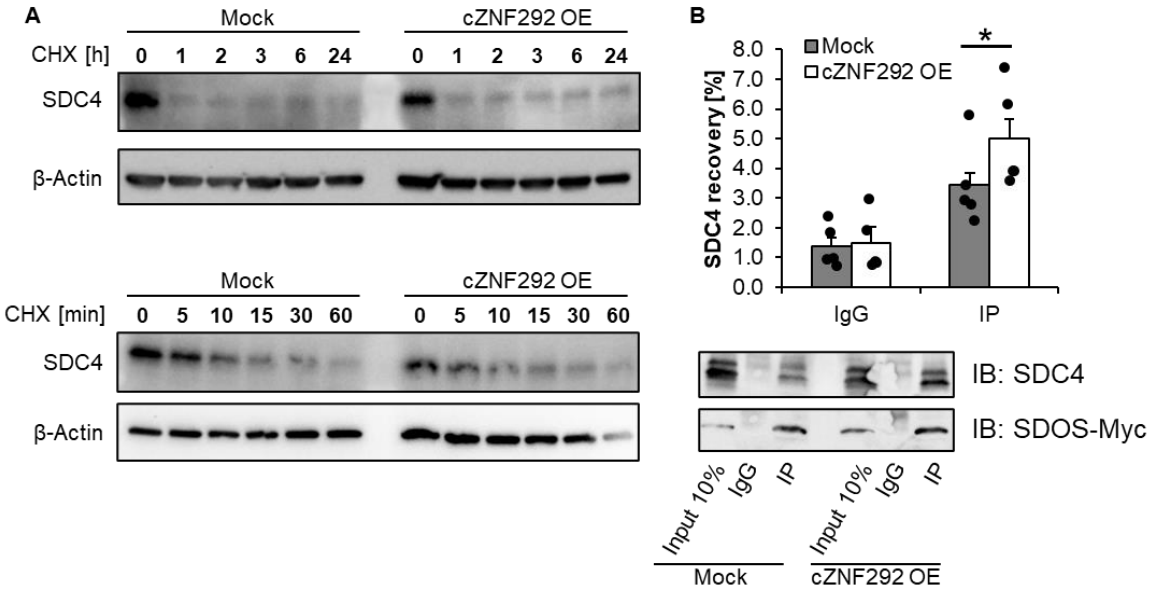
- Can the authors elaborate on the functional consequence of the cZNF292, SDOS, SDC4 axis on cytoskeletal organization in endothelial cells? They comment that cZNF292 is required, but how? Does cZNF292 stabilize the protein complex? Does it act as a molecular 'shuttle'?; or does it provide binding between SDC4 and SDOS?

Answer:

We thank the reviewer for this question and provide additional details regarding the effects of cZNF292 on Syndecan 4 (SDC4). Whereas the stability of SDC4 was not altered (**Reviewer Figure 6A**), we show that overexpression of cZNF292 increased the interaction of SDC4 with SDOS (**Reviewer Figure 6B** included as new **Main Figure 6I/J**), supporting our mechanism of action.

These results are included in page 11, para 4 of the revised manuscript as follows:

"These results suggest a model whereby cZNF292 enhances SDC4-SDOS interaction and subsequent signaling. To test this model, we determined the effect of cZNF292 overexpression of SDC4-SDOS protein interaction by co-immunoprecipitation. Overexpression of cZNF292 indeed increased the binding of SDC4 to SDOS (**Fig. 6I/J**)."



Reviewer Figure 6. cZNF292 influences SDOS/SDC4 interaction. A Immunoblot analysis of SDC4 protein stability in two different cycloheximide time courses in HeLa cells 24 after overexpression SDOS-Myc and transfection with either a mock control or a cZNF292 overexpression plasmid (n=1). β-Actin serves as a loading control. **B** Analysis of the interaction between SDOS and SDC4 by co-immunoprecipitation. HeLa cells were transfected with overexpression plasmids encoding Myc-tagged SDOS and Flag-tagged SDC4 as well as a mock control or a cZNF292 overexpression plasmid. 24h after transfection HeLa were harvested and membrane-associated fractions were isolated prior to immunoprecipitation of SDOS-Myc (n=4). Data is depicted as mean±SEM and statistical anaylsis was performed using two-tailed Student's t-test.

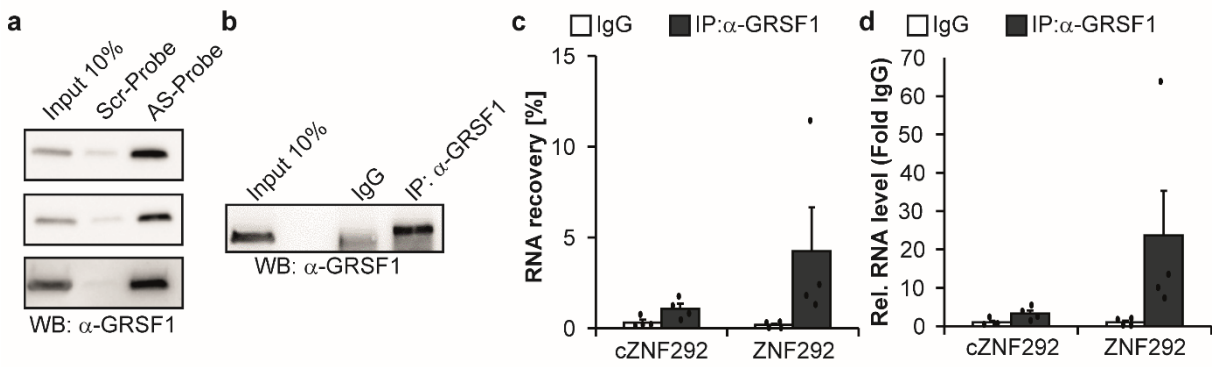
Minor comments

- Figure 1E shows three different isoforms of cZfp292 which one is followed up on?

Answer: We followed up on the isoform mmu-Zfp292_0007, which is also now outlined in the manuscript.

- Can the authors list/discuss other potential cZfp292 binding partners? For example, is SDC4 in the mass-spec list and/or other proteins that could potentially bind to SDOS, such as CDCP1 and GPIC.

Answer: We have further validated the interaction between cZNF292 and GRSF1 (**Reviewer Figure 2**), which we did not further investigate given that GRSF1 also strongly bound linear ZNF292 mRNA. Regarding the second part of the reviewers' comment the mass spectrometry analysis did not list further interaction partner of SDOS.



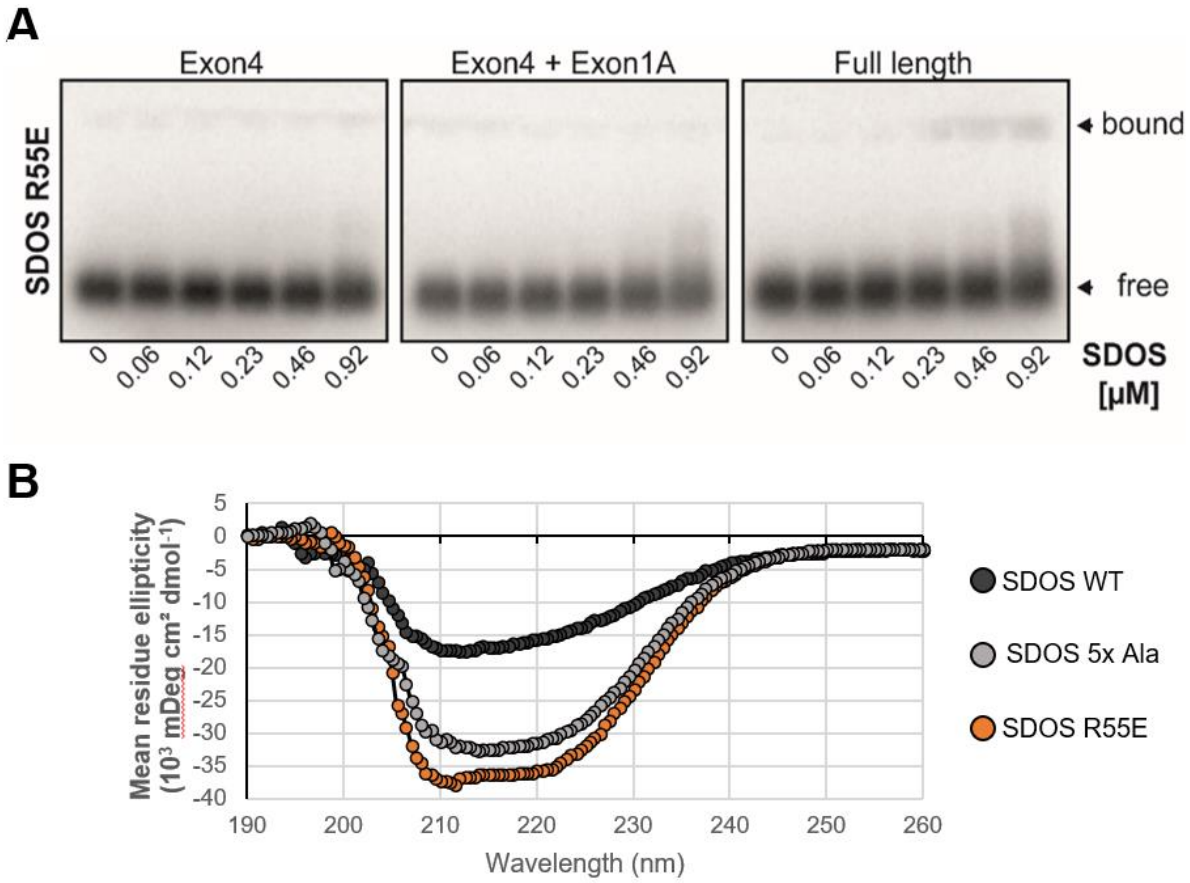
Reviewer Figure 2. Validation of the cZNF292/GRSF1 interaction. Immunoblot analysis of endogenous GRSF1 levels following **A** pulldown of cZNF292 using an antisense-probe under native conditions compared to a scrambled probe or **B** following immunoprecipitation of GRSF1 in HUVEC cell lysates. **C/D** Analysis of linear and circular ZNF292 levels following GRSF1 immunoprecipitation in HUVEC cell lysates depicted as either **C** RNA recovery relative to input levels or **D** as Fold IgG control (n=4). RNA levels were measured by qRT-PCR.

- **The CCCA motif is listed as a known SDOS binding site. However, in Figure 5A, one the highlighted sequences in ZNF292 is CCCG.**

Answer: We have further addressed the issue of SDOS binding motifs as outlined below and have revised Main Figure 5 to provide a more comprehensive presentation of the SDOS motifs in cZNF292. While the original publication of Avolio *et al.* indicates an CCCA core motif for SDOS binding, however the motif also includes a potential CCCG. The manuscript text now also indicates this.

- How do changes of potential RNA binding sites towards alanine change the whole protein structure of SDOS as this can have major implications on its ability to bind RNA independent of the CCCA sites?

Answer: To address the reviewers' concerns, we have additionally analysed a single charge reverse SDOS R55E mutant showing that it fails to bind to Exon4 and Exon4+Exon1 and it only weakly interacts with full length demonstrating that a single amino acid mutation is sufficient to reduce SDOS interaction to cZNF292. We additionally determined protein structure of the 5x Ala and R55E mutants by circular dichroism experiments. These experiments demonstrate that the mutations did not alter protein folding **(Reviewer Figure 14**).



Reviewer Figure 14. A, Analysis of the interaction between the charge reverse mutant SDOS R55E and RNA oligos comprising the binding sites of exon4, exon4+exon1A or the full-length sequence of cZNF292 (see also Main Figure 5). **B,** Circular dichroism experiment.

- Can the authors provide the levels of linear ZNF292 after siRNA treatment.

Answer: Linear RNA levels have now been included into the manuscript as **Supplementary Figure 3A.**

Circulation Research Peer Review Report for DOI: 10.1161/CIRCRESAHA.121.320029

October 4, 2021

Dr. Stefanie Dimmeler
Goethe University Frankfurt
Institute for Cardiovascular Regeneration
Theodor Stern Kai 7, 60590 Frankfurt
Frankfurt 60590
Germany

RE: CIRCRES/2021/320029D: Locus-conserved circular RNA cZNF292 controls endothelial cell flow responses

Dear Dr. Dimmeler:

Your manuscript has been carefully evaluated by 5 external reviewers and the editors as a Regular Article. While we are interested in your paper, further minor revision is required before we can accept the manuscript for publication in Circulation Research.

Please read the entire content of this letter and address carefully all of the comments in the reviewers' critiques and all of the formatting concerns described below. Specifically, reviewers 1 and 3 have indicated limitations of the current study that should be included in the discussion of the revised manuscript. Additionally, please address the concerns of the statistical and technical reviewers.

Please note that the paper cannot be accepted until you have addressed both the reviewers' critiques and all of the formatting issues. Please submit your revision at your earliest convenience.

Upon submission of revised manuscripts, authors reporting preclinical work will need to complete a checklist aimed at enhancing rigor, transparency, and reproducibility. Different checklists will be used for studies in animals and for studies in vitro.

All corresponding authors of articles accepted to AHA Journals are required to link an ORCID iD to their profile in the AHA Journal submission system. To register with ORCID or link your profile to your ORCID iD, please go to "Modify Profile/Password" on the submission site homepage (insert journal homepage link), and click the link in the "ORCID" section. Please note that upon resubmission, the corresponding author will be required to have their ORCID iD linked to their profile; processing of the revision will be held until the ORCID link is complete.

The Editors strongly encourage you to adhere to the journal's Statistical Reporting Recommendations in your revision, which can be found here: https://www.ahajournals.org/statistical-recommendations.

1. Reviewers' Critiques:

To read the comments to authors from the reviewers, please see below.

If you wish to respond to these suggestions, please include a detailed response to each of the referees' and editors' comments, providing each comment verbatim in bold followed by your response and giving the exact page number(s), paragraph(s), and line number(s) where each revision was made. If you make substantive changes to the manuscript, please provide a clear description of what you did and where. Additionally, a marked-up version of the revision with the changes highlighted or tracked should be uploaded as a supplemental file.

2. Formatting Issues:

Please ascertain that your revised manuscript adheres to the Instructions to Authors as they appear online at https://www.ahajournals.org/res/author-instructions. Accepted manuscripts are published online ahead of print, usually within 24 hours of acceptance. Therefore, when submitting the final files of the manuscript and figures, please ensure you have made any essential changes or corrections to content, grammar, and formatting. Please also ensure that author information provided in the online submission system is correct, including author order, proper names, and institutions. Once published ahead of print, you will be unable to make any revisions to the manuscript until you receive your author proofs from the publisher and any changes made to proofs will be reflected in the final print and online journal version of your article.

As your article may be published online immediately upon acceptance, neither the Editorial Office nor the AHA will be responsible for any consequences with regard to intellectual property rights. To safeguard their intellectual property, authors should ensure that appropriate reports of invention and patent applications have been filed before the manuscript is accepted. If you should need to delay publication of your article for any reason, please let the Editorial Office know as soon as possible.

Please provide/address the following areas:

Manuscript Text:
- Please be sure to provide your revised manuscript text in an editable Word Doc file containing all sections of the manuscript, including tables and figure legends. Tables should be embedded within the body of the text as they are mentioned to ensure proper ordering of references.
-Please move the supplemental figure legends to the online supplement.
-Please move the Transparency and Openness Promotion (TOP) statement so that it is the first sentence under the METHODS section of the main manuscript.
-Please include a Novelty and Significance section at the end of your Word file. Instructions for the Novelty and Significance section can be found at
https://www.ahajournals.org/res/revised-accepted-manuscripts.
- Provide a list of non-standard abbreviations and non-standard acronyms used in the manuscript text. The list should be included in the manuscript after the abstract and should be entitled "Non-standard Abbreviations and Acronyms." Circulation Research follows AMA style guidelines for standard and non-standard abbreviations.
- Please include a Disclosures section in the paper and state any financial, personal or professional relationships with other people or organizations that could reasonably be perceived as conflicts of interest or as potentially influencing or biasing this work. If no conflicts of interest exist, state "None."

Figures:
- The number of panels per figure in your manuscript is excessive, specifically for Figure #2. We ask that you reduce the number of panels to 6 or fewer. Create online figures from the remaining panels, if desired.
- Color figure charges are a flat per page rate of $653 per color page.
- Please note that color figures cannot be changed to black and white after the manuscript is accepted. Please make any color changes to your figures during the final revision.

Online Supplement:
- Upload the online data supplement as one complete PDF labeled "Supplemental Material" at the top of the first page.
- Rename the supplemental figures and/or table with Roman numerals (i.e., Online Figure I, Online Table I, Online Figure II, Online Table II, etc.). Ensure that this change is made on the display item itself, in the legend, and throughout the text.

-Supplementary Materials should not include a separate reference list and all citations in the Supplemental Materials should be included in the main reference list. In the manuscript text, following the Acknowledgments, Sources of Funding, & Disclosures section, please include a list of the supplemental materials with a callout to any references that are in the Supplemental Material only, for example:
Supplemental Materials
Expanded Materials & Methods
Online Figures I - IV
Online Video I
Data Set
References 34-39

Other Items:
- All persons acknowledged by name in the manuscript must send an email to CircRes@circresearch.org citing their permission to be acknowledged.
- A Graphical Abstract (to be uploaded as a separate supplemental file): The intent of the graphical abstract is to provide readers with a succinct summary of the study in a form that facilitates its dissemination in presentations. It should emphasize the new findings in the paper. Do not include data items; all content should be graphical. The graphical abstract should conform to the following format: A single figure panel, no more than a 15 cm square (15 cm x 15 cm); Font: prefer a san serif font that is no less than 12 point. Please upload as a graphic abstract file in JPG file format.
- All research materials listed in the Methods should be included in the Major Resources Table file, which will be posted online as a PDF with the article Supplemental Materials if the manuscript is accepted. A template Major Resources Table file (.docx) is available for download here: AHAJournals_MajorResourcesTable_2019.docx. Authors are encouraged to upload Major Resources Table PDF at first submission and will be required to do so at the revision stage. Authors should reference the PDF in their Methods as follows: "Please see the Major Resources Table in the Supplemental Materials."
- A supplement containing a short tweet that can be used to promote the article and a 1-2 line 'lay sentence' similar to those provided for NIH grants.
- Recent studies have shown that active engagement in social media is beneficial in advancing your science. Circulation Research encourages all authors to provide their twitter handles, if possible.

The Editors strongly encourage you to submit potential cover images. Appropriate figures should be both aesthetically beautiful and scientifically exciting. Potential cover images should be associated with the general topic of the paper, or may be altered/enhanced versions of an original figure within the manuscript. Potential cover figures should have a single panel, with no labels or text of any kind. The figure file should be supplied at exactly 8 1/8" width by 10 7/8" height. Please submit figure initially as a low-resolution PDF. Include a figure legend with the figure. If your figure is chosen, we will request a high-resolution version (minimum of 600 DPI, RGB color format, and TIF or EPS file format).

We look forward to receiving the final revised version of your manuscript as soon as possible. Thank you for contributing to Circulation Research.

Sincerely,

Jane E. Freedman, MD
Editor-in-Chief
Circulation Research
An American Heart Association Journal

****

REVIEWER COMMENTS FOR THE AUTHORS:

Reviewer #1:
The authors have answered to most of my concerns. Biochemistry and cellular biology are excellent.
The authors should discuss the limitations of their results more bluntly.
1. The expression of circular RNA cZNF292 in vascular cell types other than endothelial cells has to be highlighted.
2. The absence of a clear phenotype in vivo is a limit and needs to be clearly discussed. It could mean that the relevance of circular RNA cZNF292 in endothelial cells is limited or that authors have not used the right stimuli for unmasking a phenotype in vivo or it could be attributed to the limits of constitutive KO mouse models.
3. Since from the screening there are many other interacting targets, the possibility that circular RNA cZNF292 could interact with other proteins should be clearly stated.

Reviewer #2:
I am satisfied with the new experiments and the additions to the manuscript. My only suggestion is to incorporate some of the 'Reviewer Figures" into at least the supplemental figures, particularly for the in vivo models (accepting that the n was low due to COVID induced restrictions in breeding etc) as this lends some increased relevance to vascular diseases.

Reviewer #3:
The authors of 'Locus-conserved circular RNA cZNF292 controls endothelial cell flow responses' have done an excellent job in responding to this reviewers' comments. Furthermore, they have integrated a lot of the novel data presented to all three reviewers in the main manuscript and supplemental data, which results in a much clearer (and better) manuscript.

They authors included the sequence similarities and differences between cZNF292 and cZfp292 as well as shown more experiments underlining the angiogenic function of cZfp292, indicating a mechanistical conservation between the human and murine form. For many human lncRNA it is unique to have conserved counterpart in mice. Therefore, it is of relevance to show not only conserved function within similar biological pathway, but also conservation on molecular mechanistic level. However, no direct relation and/or physical interaction between murine SDOS and cZfp292 has been established by the authors in this manuscript. To prove this point and increase the relevance of this circRNA, an IP as shown in Figure 2I/J for murine SDOS and cZfp292 further would be needed to further confirm molecular mechanistic conservation between cZNF292 and cZfp292.

Statistical Reviewer:
The following gaps in statistical presentation and analysis must be addressed.

1. Please clarify the choice of a parametric test (t-test) versus a non-parametric test in the comparisons.

2. Please clarify whether a test for normality was used. If so, what statistical test was used? What threshold was assumed? How was the choice of a parametric test made?

3. Was multiple testing correction performed in the various analyses? What approach was used? It is not clear whether and how multiple testing adjustment was performed for the various analyses.

4. Please justify the use of 30 (versus some other number) out of 1404 intronic circRNAs (both human and murine in figure 1d and 1e). How was the threshold chosen? What statistical procedure was used?

5. How were "representative" images (e.g., of aortic ring sprout outgrowth after 7 days of culture in presence of VEGF-A) selected? What statistical procedure (and thresholds) was used?

6. Please provide a description of how the analysis of synteny in circRNA splicing was performed.

7. How were significant interacting proteins determined? Please clarify whether the parametric t-test is appropriate and then what threshold was used from the false discovery rate analysis. The sequence of tests must be clarified.

8. How were "commonly expressed" circRNAs determined from the different types of endothelial cells? What statistical approach was used? What thresholds if any were used?

9. How was the comparison of these circRNAs (see #8) using an additional RNA-Seq dataset done exactly? What statistical approach (including thresholds) was used to declare a circRNA a "true circRNA"? How were the circRNAs backspliced to intronic cassettes detected exactly? Please describe the method.

Technical Reviewer:
The current study was carefully evaluated for inclusion of guideline items present in the Circulation Research checklists for rigor, transparency, and reproducibility. The reviewer has identified a number of items that were either omitted or not adequately addressed in the text. Please see below for details:

In vitro checklist items:
1) Ensure that manufacturer catalog numbers are provided for all antibodies employed in experiments (including secondary antibodies, isotype controls, etc.). This information should be provided in both the methods section and "Major Resources Table."
2) Where immunostaining procedures are detailed, briefly describe controls employed to validate antibody specificity (isotype antibodies) and distinguish genuine target staining form background (secondary antibody only controls).
3) Please ensure that scale bar lengths are consistently reported for all micrographs (e.g., Fig. 6F scale bar size was not reported).
4) All presented immunoblots must contain markers (with units of measure in kDa) denoting the location of molecular weight standards electrophoretically resolved with experimental protein lysates.
5) All methods should of sufficient detail to allow replication, even for those procedures that may be considered routine. Referring to previously published procedures or manufacturer protocols is accepted; however, any deviations should be detailed in the text. Please carefully review for adherence to these guidelines. Some examples are shown below:
a. Cell culture: authors state that endothelial cells were purchased from Lonza and cultured as previously described. Please provide the appropriate citation.
b. PCR: Provide thermocycle conditions used in amplification (i.e., temperature and duration of denaturation, annealment, and extension).
c. Lentiviral overexpression: specify viral titers used in cell transduction.

In vivo checklist items:

1) Animal baseline characteristics: please specify the age, sex, and source of the animals used in the study.
2) Checklists indicate the use of animal randomization procedures; however, this was not evidenced in the text. Please address.
3) Blinding procedures must also be described in the text. Please discuss how they were performed and in what experiments were they used.
4) In the manuscript, briefly discuss how group sizes were determined.
5) It remains unclear as to whether sex was considered as a biological variable in the study (animal sex was not specified). If experiments do not include both male and female animals, written justification should be provided.

Other:
1) Per the Journal's requirements, please complete and submit a "Major Resources Table." Please refer to the website for formatting instructions.
2) A number of typographical errors were identified; please carefully review for errors and appropriate syntax.

Comments to the Authors on Research Guidelines and Reporting Authors indicate that RNA-sequencing data will be publicly available upon publication; please specify where said data will be provided.

****

REVIEWER COMMENTS FOR THE AUTHORS:

Reviewer #1:

The authors have answered to most of my concerns. Biochemistry and cellular biology are excellent. The authors should discuss the limitations of their results more bluntly.

Answer: We thank the reviewer for his comment. The respective limitations of our study have been addressed along the results part or in the discussion section as summarized below in response to the specific comments.

1. The expression of circular RNA cZNF292 in vascular cell types other than endothelial cells has to be highlighted.

Answer: The manuscript now mentions the expression in other cells of the vascular bed and the data has been accordingly added to the Online Figure II. It reads:

Results section (page 9, paragraph 1)

"Interestingly, RNA sequencing data and qPCR measurements indicated higher cZNF292 levels in arterial endothelial cells compared to microvascular cells and other cells of the vascular bed […]."

Discussion section (page 13, paragraph 1)

"Nevertheless, further studies will need to dissect to which extent the expression of cZfp292 in other cell types contributes to the observed phenotype."

2. The absence of a clear phenotype in vivo is a limit and needs to be clearly discussed. It could mean that the relevance of circular RNA cZNF292 in endothelial cells is limited or that authors have not used the right stimuli for unmasking a phenotype in vivo or it could be attributed to the limits of constitutive KO mouse models.

Answer: The manuscript now reads:

Discussion section (page 13, paragraph 1)

"Nevertheless, further studies will need to dissect to which extent the expression of cZfp292 in other cell types contributes to the observed phenotype. Our study is additionally limited by the sole observation of the phenotype under baseline conditions and the unknown function of cZNF292 in humans. It will be important to assess how cZfp292 may influence the morphology and function of endothelial cells in pro-atherosclerotic or under other stress conditions."

3. Since from the screening there are many other interacting targets, the possibility that circular RNA cZNF292 could interact with other proteins should be clearly stated.

Answer: The manuscript now reads:

Discussion section (page 14, paragraph 2)

"However, it should be noted that the protein interactions of cZNF292 might not be limited to SDOS given the enrichment of various other proteins following cZNF292 pulldown."

****

****

Reviewer #2:

I am satisfied with the new experiments and the additions to the manuscript. My only suggestion is to incorporate some of the 'Reviewer Figures" into at least the supplemental figures, particularly for the in vivo models (accepting that the n was low due to COVID induced restrictions in breeding etc) as this lends some increased relevance to vascular diseases.

Answer: As also pointed out to reviewer #3, we included additional reviewer figures in the main manuscript. Overall compared to our initial submission, the revision process has seen the inclusion of the figures **Fig. 3C, 3D, 4D, 6B, 6E, 8E, 8F** and the **Online Figures I A-D, II A, II B, II G, III E, V A-D** into the manuscript.

****

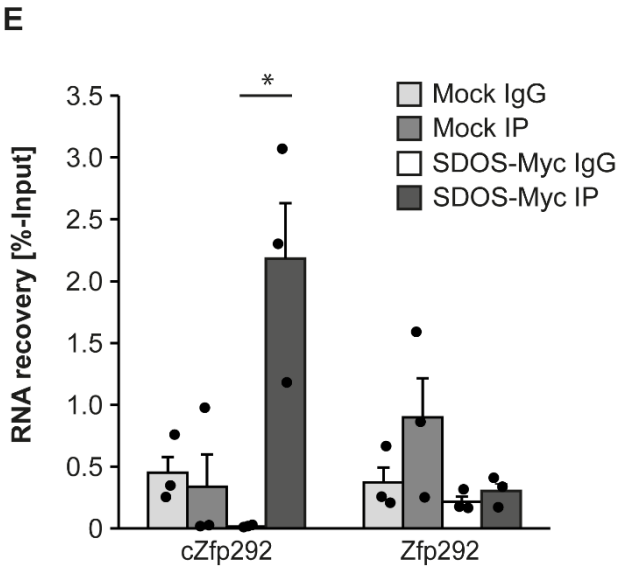
****

Reviewer #3:

The authors of 'Locus-conserved circular RNA cZNF292 controls endothelial cell flow responses' have done an excellent job in responding to this reviewers' comments. Furthermore, they have integrated a lot of the novel data presented to all three reviewers in the main manuscript and supplemental data, which results in a much clearer (and better) manuscript.

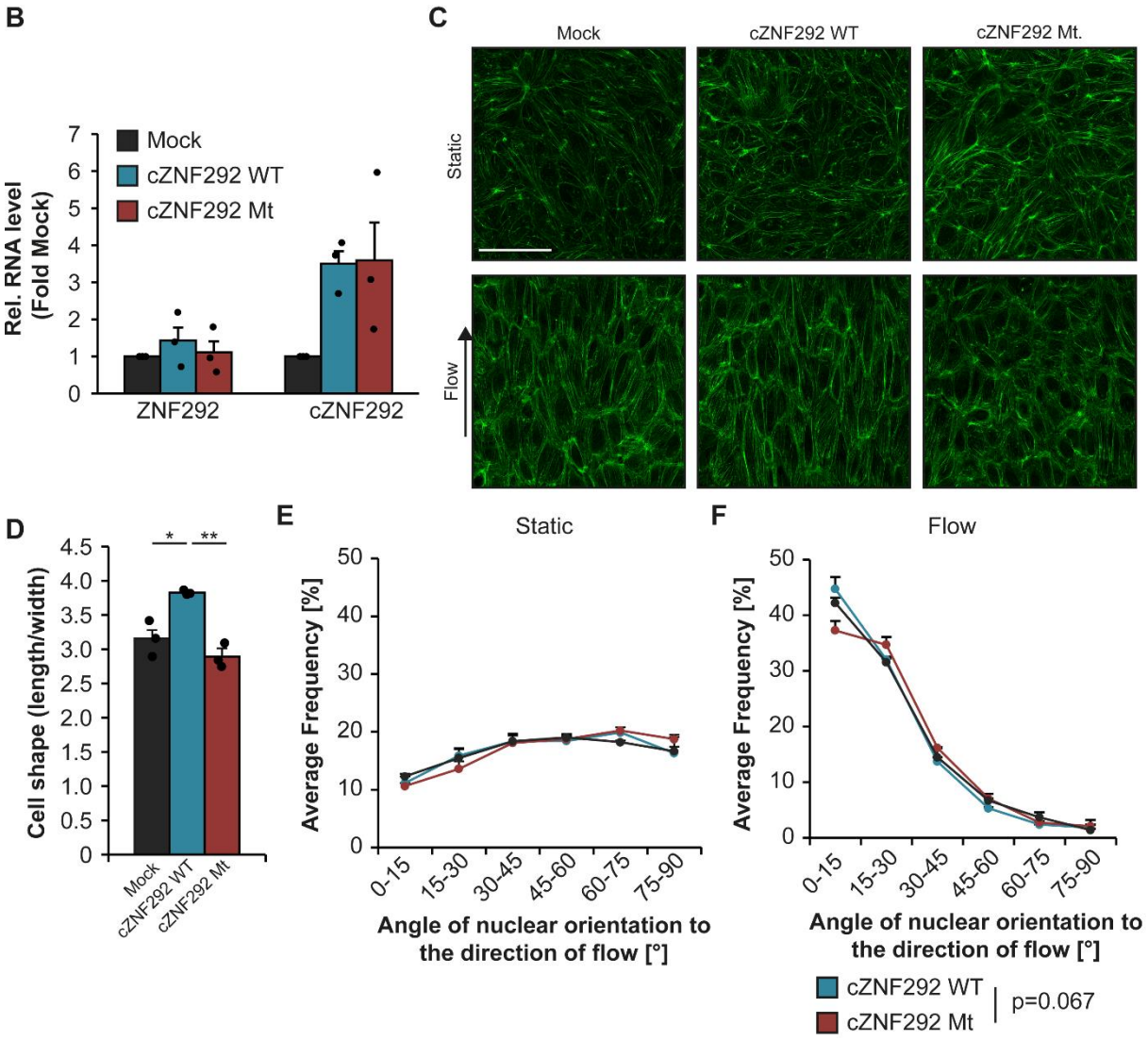
They authors included the sequence similarities and differences between cZNF292 and cZfp292 as well as shown more experiments underlining the angiogenic function of cZfp292, indicating a mechanistical conservation between the human and murine form. For many human lncRNA it is unique to have conserved counterpart in mice. Therefore, it is of relevance to show not only conserved function within similar biological pathway, but also conservation on molecular mechanistic level. However, no direct relation and/or **physical interaction between murine SDOS and cZfp292** has been established by the authors in this manuscript. To prove this point and increase the relevance of this circRNA, an IP as shown in Figure 2I/J for murine SDOS and cZfp292 further would be needed to further confirm molecular mechanistic conservation between cZNF292 and cZfp292.

Answer: We agree with the reviewer and have further addressed the interaction between SDOS and cZfp292 in murine cells. Given the lack of antibodies verified for the use of endogenous immunoprecipitation and the high conservation of SDOS between mouse and human (>95%) we performed immunoprecipitations following overexpression of human Myc-tagged SDOS in murine H5V endothelial cells. Using this approach, we could verify the interaction between SDOS and cZfp292 while we did not observe an enrichment of the respective linear host gene. Data has been included as **Online Figure III E.**



Online Figure III E. Quantification of murine RNA levels by qPCR following the immuno-precipitation of SDOS-Myc 48h after overexpressed human SDOS-Myc in murine H5V cells (n=3). Human and murine SDOS protein are highly conserved with an amino acid identity of >95%.

We have also provided supportive data showing the overexpression of cZNF292 lacking the SDOS binding sites, which influenced endothelial cell alignment and shape (**Online Figure V B-D**).



Online Figure V B, Quantification of RNA levels by qRT-PCR 72h after overexpression of wildtype or cZNF292 with mutated SDOS binding sites by lentiviral transduction compared to mock treated controls (n=3). **C,** Representative phalloidin stainings of HUVECs following lentiviral overexpression of the cZNF292 wildtype or mutant and exposure to 48h of 12 dynes laminar flow. Static controls were treated identically but where not exposed to flow. **D,** Analysis of the cell shape of HUVEC following cZNF292 overexpression and exposure to flow (as above). Cell shape was estimated by the ratio of the longitudinal cell axis towards the cell's width (n=3). **E,F** Distribution of the angle of orientation of nuclei in static controls slides (E) or after 48h exposure to 12 dyne laminar flow (n=3). Scale bar in white equals 100 µm.

****

****

Statistical Reviewer:

The following gaps in statistical presentation and analysis must be addressed.

1. Please clarify the choice of a parametric test (t-test) versus a non-parametric test in the comparisons.

Answer: The choice is based on assessing normal distribution of the data. The manuscript now states more clearly which statistical test was employed both in the figure legends and the method section.

2. Please clarify whether a test for normality was used. If so, what statistical test was used? What threshold was assumed? How was the choice of a parametric test made?

Answer: All data was checked for normality using Shapiro-Wilk Test at a significance level of $\alpha = 0.05$.

3. Was multiple testing correction performed in the various analyses? What approach was used? It is not clear whether and how multiple testing adjustment was performed for the various analyses.

Answer: Multiple testing correction was performed using the Bonferroni-Holm method. The manuscript now outlines the distinct use statistical test in more detailed.

4. Please justify the use of 30 (versus some other number) out of 1404 intronic circRNAs (both human and murine in figure 1d and 1e). How was the threshold chosen? What statistical procedure was used?

Answer: In our initial analysis, we chose to analyze these intronic circRNAs in more detail which were present in at least 25% of the RNA-Seq datasets stored on CircATLAS. This equaled a threshold of >60 samples for human RNA-Seq dataset and resulted in the respective 30 circRNAs outlined in Figure 1.

5. How were "representative" images (e.g., of aortic ring sprout outgrowth after 7 days of culture in presence of VEGF-A) selected? What statistical procedure (and thresholds) was used?

Answer: Representative images were selected out of the total available images to visualize the effects shown in the respective quantifications. Images were acquired in a blinded, randomized fashion and were all analyzed. No thresholds were used.

6. Please provide a description of how the analysis of synteny in circRNA splicing was performed.

Answer: The analysis on circRNA splicing synteny was performed manually by comparison of the back-splice site position to the overall composition of the gene locus (exon/intron count, transcript variants, sequence). Synteny was assumed if the splice pattern between mouse and human circRNA transcripts was shared.

7. How were significant interacting proteins determined? Please clarify whether the parametric t-test is appropriate and then what threshold was used from the false discovery rate analysis. The sequence of tests must be clarified.

Answer: We thank the reviewer for this comment and the “Extended Method Section” now elaborates this in more detail. It reads:

"To identify significant interacting proteins, LFQ intensities were logarithmized, and histograms were inspected to verify approximate normally distributed data. Data-set was reduced to proteins that were identified in at least 6 of 8 samples in one experimental group. Missing values were replaced by random values imputed from normal distribution according to the default options of Perseus (which shrink the distribution by a factor of "0.3" and shift it down by "1.8"). Data was quality checked by the analysis of the Pearson correlation of the LFQ intensities between samples and exceeded "0.7" in all comparisons and correlations within the Scr or AS-Oligo groups were higher than between the groups. Significant interacting proteins were then determined by an unpaired two-tailed Student's t-test, excluding results with a log2(Fold Change AS-Oligo/Scr) below 0.1, followed by an analysis for permutations-based FDR with 250 randomizations and a threshold of 0.05."

8. How were "commonly expressed" circRNAs determined from the different types of endothelial cells? What statistical approach was used? What thresholds if any were used?

Answer: We thank the reviewer for his comment. We chose common circRNAs, when reads for the respected circRNAs were detected in all used data sets. The threshold for counting the circRNA as expressed was set to the detection of at least two reads in at least two samples. This information has been added to the manuscript.

9. How was the comparison of these circRNAs (see #8) using an additional RNA-Seq dataset done exactly? What statistical approach (including thresholds) was used to declare a circRNA a "true circRNA"? How were the circRNAs backspliced to intronic cassettes detected exactly? Please describe the method.

Answer: The list of circRNAs detected among all three endothelial RNA-Seq datasets was further compared to a HUVEC RNA-Sequencing dataset comparing mock and RNase R treated samples. RNase R as an exonuclease digests linear RNA and is usually employed as a quality control for the detection of circRNAs. circRNAs were considered stable if the back-splice site read count was increased in at least one of the two RNase R treated replicates.

Intronic circRNAs were identified by annotation of the back-splice site position to known transcripts. CircRNAs spliced to intergenic regions were excluded from further analysis.

****

****

Technical Reviewer:

The current study was carefully evaluated for inclusion of guideline items present in the Circulation Research checklists for rigor, transparency, and reproducibility. The reviewer has identified a number of items that were either omitted or not adequately addressed in the text. Please see below for details:

In vitro checklist items:

1) Ensure that manufacturer catalog numbers are provided for all antibodies employed in experiments (including secondary antibodies, isotype controls, etc.). This information should be provided in both the methods section and "Major Resources Table."

Answer: The information on all specific and control antibodies has been included into the method section as well as into the "Major Resources Table".

2) Where immunostaining procedures are detailed, briefly describe controls employed to validate antibody specificity (isotype antibodies) and distinguish genuine target staining form background (secondary antibody only controls).

Answer: The method sections now outlines the respective controls used. Antibodies used for immunostaining procedures have been knockdown validated and are described repeatedly. Secondary antibody only controls were performed to evaluate and exclude background staining signal.

3) Please ensure that scale bar lengths are consistently reported for all micrographs (e.g., Fig. 6F scale bar size was not reported).

Answer: We thank the reviewer for finding this mistake and have reported the missing bar graph size accordingly.

4) All presented immunoblots must contain markers (with units of measure in kDa) denoting the location of molecular weight standards electrophoretically resolved with experimental protein lysates.

Answer: All immunoblots now contain a measurement in kDa.

5) All methods should of sufficient detail to allow replication, even for those procedures that may be considered routine. Referring to previously published procedures or manufacturer protocols is accepted; however, any deviations should be detailed in the text. Please carefully review for adherence to these guidelines. Some examples are shown below:

a. Cell culture: authors state that endothelial cells were purchased from Lonza and cultured as previously described. Please provide the appropriate citation.

b. PCR: Provide thermocycle conditions used in amplification (i.e., temperature and duration of denaturation, annealment, and extension).

c. Lentiviral overexpression: specify viral titers used in cell transduction.

Answer: We have addressed the issues pointed out in either the main method or the extended method section.

In vivo checklist items:

1) Animal baseline characteristics: please specify the age, sex, and source of the animals used in the study.

Answer: We have added the respective information in the method section.

2) Checklists indicate the use of animal randomization procedures; however, this was not evidenced in the text. Please address.

Answer: The method section now mentions how animal randomization and blinding procedures were performed. It reads:

"Animals were harvested and processed randomly by personal unaware of the respective genotype."

3) Blinding procedures must also be described in the text. Please discuss how they were performed and in what experiments were they used.

Answer: Is now stated in the method section. See comment above.

4) In the manuscript, briefly discuss how group sizes were determined.

Answer:

For mass spectrometry: We have based the sample sizes based on previous pull downs (Neumann et al., Nat Com, 2018). Since we noticed high variability and we decided to increase the number to 8 per sample group. Immunoprecipitation assays were performed each using biologically independent cell lysate and repeated for n>3. The analysis of the interaction between SDOS and cZNF292 employing radiolabeled RNA are representatives of two replicates, which we have further substantiated and validated by non-radioactive labelled assays (Online Figure V).

The choice of group sizes for cell biology studies is based on our experience with the variability of the assays. For the analysis of cell shape: We analyzed all cells which did not touch image borders in at least 3 images (technical replicates) to calculate the mean value, with at least n>3 biological replicates. For the analysis of focal adhesions: Focal adhesion count and size were determined in 5 adjacent cells of at least 3 images per biological replicate.

For in vivo studies we usually perform power calculations, however, in this specific case the phenotype was not seen before, therefore, we did not have estimates. Therefore, all available animals were all used.

5) It remains unclear as to whether sex was considered as a biological variable in the study (animal sex was not specified). If experiments do not include both male and female animals, written justification should be provided.

Answer: We thank the reviewer for his comment. The Animal experiments included both male and female animals. For more detail, animal baseline characteristics have been added to the manuscript. See comments above.

Other:

1) Per the Journal's requirements, please complete and submit a "Major Resources Table." Please refer to the website for formatting instructions.

Answer: The “Major Resources Table” has been added and is referenced in the method part of the main manuscript.

2) A number of typographical errors were identified; please carefully review for errors and appropriate syntax.

Answer: We have revised the manuscript and have corrected any mistakes found.

Comments to the Authors on Research Guidelines and Reporting Authors indicate that RNA-sequencing data will be publicly available upon publication; please specify where said data will be provided.

Answer: RNA sequencing data will be available from the GEO database. Mass spectrometry data will be available from the PRIDE databank, respectively.

****

November 17, 2021

Dr. Stefanie Dimmeler
Goethe University Frankfurt
Institute for Cardiovascular Regeneration
Theodor Stern Kai 7, 60590 Frankfurt
Frankfurt 60590
Germany

RE: CIRCRES/2021/320029DR1: Locus-conserved circular RNA cZNF292 controls endothelial cell flow responses

Dear Dr. Dimmeler:

We are pleased to inform you that we have accepted your revised manuscript for publication in Circulation Research as a Regular Article.

All original research articles are published in Circulation Research online ahead of print in PDF proof format within 24-36 hours acceptance, and are accessible through the Online First link at https://www.ahajournals.org/journal/res. Your paper will be published online first on THURSDAY, NOVEMBER 18 and will then appear in the JANUARY 14, 2022 print and online issue.

The corresponding author will receive the author proof after Online First publication of the manuscript. During the copyediting phase, there may be some changes in phrasing, but there will be no alterations of scientific content. When you receive your author proof, please read, correct, and return it immediately to avoid delay in publication. Please note that it is your responsibility to make yourself available to review the author proof whenever it arrives. If you are not available, publication of your manuscript will be delayed.

IMPORTANT: The Editors strongly encourage you to submit potential cover images. Appropriate figures should be both aesthetically beautiful and scientifically exciting. Potential cover images should be associated with the general topic of the paper, or may be altered/enhanced versions of an original figure within the manuscript. Potential cover figures should have a single panel, with no labels or text of any kind. The figure file should be supplied at exactly 8 1/8" width by 10 7/8" height. The figure file should be provided as a PDF. Higher resolution files will be requested if your figure is chosen.

Meet the First Author Page: Circulation Research encourages up to two first authors of original research articles to contribute a personal profile for our Meet the First Author page. First authors are invited to provide a photo (professional headshot preferred) and a bio for publication. Within 100 words, written in the third person, we ask that they briefly introduce themselves, provide educational background (from undergraduate to post-doctoral fellowship), and identify their main area of research interest. They may also discuss their plans for the future. Please also provide reason(s) for pursuing science in general and how they became involved in the current project as well as any obstacles they encountered as the story developed (i.e. any deviations from the initial central hypothesis). Please provide twitter handles, if available.

Optional information that may be included in the profile can include their experience in building an academic research career as a young scientist, the obstacles they might have faced or factors that might have contributed to their success, and any other interesting or unique information about themselves, either as scientists or in their lives outside of science. The Editors encourage first authors to use this opportunity to not only talk about their work but

also give the readers insight into the everyday lives of young scientists today! Please note that overly long profiles will not be published.
Please submit the first author profile(s) to circres@circresearch.org by DECEMBER 1.

You will be receiving an Open Access Publication Charge letter from the Journal's Publisher, Wolters Kluwer, when your manuscript is routed for copy editing. The letter will include instructions on how to submit any open access charges. The email will be from publicationservices@copyright.com with the subject line 'Please Submit Your Open Access Article Publication Charge(s)'. Please complete payment of the Open Access charges within 48 hours of receipt.

Although the Editorial Office will endeavor to notify authors of the anticipated online publication date, neither the Editorial Office nor the AHA will be responsible for any consequences of early online posting with regard to intellectual property rights. To safeguard their intellectual property, authors should ensure that appropriate reports of invention and patent applications have been filed before the manuscript is accepted.

As a service to authors, the publisher (Wolters Kluwer Health/Lippincott Williams & Wilkins) of Circulation Research will identify to PubMed Central articles that require depositing. The Copyright Transfer Agreement provides the primary mechanism for identifying such articles. The secondary mechanism is the "Public Access Policy Funding Disclosure" section of the manuscript metadata completed in the online submission process to Circulation Research. WKH/LWW will transmit the post-print of an article to PubMed Central, which is based on research funded in whole or in part by the US National Institutes of Health, the Howard Hughes Medical Institute, and/or the Wellcome Trust. On NIH request, it remains the legal responsibility of the author(s) to confirm with the PubMed Central the provenance of their manuscript for purposes of deposit. It is the policy of the AHA that author(s) will not deposit their articles themselves, nor alter the post-print already transmitted to PubMed Central. Author(s) may not authorize the display of the post-print prior to: (1) 12 months after publication of the final article, in the case of NIH, (2) 6 months after publication of the final article, in the case of HHMI and the Wellcome Trust. For more information on PubMed Central, please visit https://nihms.nih.gov.

Circulation Research is the official journal of the Council on Basic Cardiovascular Sciences of the American Heart Association. If you are not already a member of the Council, the Editors strongly encourage you and your coauthors to consider joining. Benefits include substantial discounts on registration at AHA-sponsored meetings and AHA journal subscriptions, early access to hotel reservations for Scientific Sessions, a subscription to the Council newsletter, and eligibility to submit late-breaking basic science abstracts for presentation at Sessions. Student/Trainee and Early Career members pay only $81. The highest level of membership, Premium Professional Plus, is $450 per year and includes online access to all eleven AHA journals. Premium Profession membership offers an extensive benefits package for $270/year, including online access to all eleven AHA journals. General Professional membership is also available at $85/year. Find out more about the benefits of AHA Membership at https://professional.heart.org/membership.

Congratulations on this outstanding work, and thank you for contributing to Circulation Research.
Sincerely,

Kathryn Moore, PhD
Associate Editor

Jane E. Freedman, MD
Editor-in-Chief
Circulation Research
An American Heart Association Journal