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p66Shc Mediates SUMO2-induced Endothelial Dysfunction

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eLife Assessment

This study offers **valuable** insights into the role of post-translational modifiers, specifically SUMO2ylation at K81 in p66Shc, and its impact on endothelial function through reactive oxygen species. A series of **compelling** experiments demonstrated that lysine 81 of p66Shc is the site of SUMO2 conjugation, which is crucial for mitochondrial localization and essential for S36 phosphorylation, leading to specific pathological effects. The combination of cell overexpression and animal studies provides **solid** data supporting this mechanistic link.

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Abstract

Background Sumoylation is a post-translational modification that can regulate different physiological functions. Increased sumoylation, specifically conjugation of SUMO2/3 (small ubiquitin-like modifier 2/3), is detrimental to vascular health. However, the molecular mechanism mediating this effect is poorly understood.

Methods We used cell-based assays and mass spectrometry to show that p66Shc is a direct target of SUMO2 and SUMO2 regulates p66Shc function via lysine-81 modification. To determine the effects of SUMO2-p66ShcK81 on vascular function, we generated p66ShcK81R knockin mice and crossbred to LDLr$^{-/-}$ mice to induce hyperlipidemia. Next, to determine p66ShcK81-SUMO2ylation-induced changes in endothelial cell signaling, we performed mass spectrometry followed by Ingenuity Pathway Analysis.

Results Our data reveal that p66Shc mediates the effects of SUMO2 on endothelial cells. Mass spectrometry identified that SUMO2 modified lysine-81 in the unique collagen homology-2 domain of p66Shc. SUMO2ylation of p66Shc increased phosphorylation at serine-36, causing it to translocate to the mitochondria, a step critical for oxidative function of p66Shc. Notably, sumoylation-deficient p66Shc (p66ShcK81R) was resistant to SUMO2-induced p66ShcS36 phosphorylation and mitochondrial translocation. P66ShcK81R knockin mice were resistant to endothelial dysfunction induced by SUMO2ylation and hyperlipidemia. Ingenuity Pathway Analysis revealed multiple signaling pathways regulated by p66ShcK81-SUMO2ylation in endothelial cells, highlighting JAK-STAT as a major pathway affected by SUMO2-p66ShcK81.

Conclusions Collectively, our work reveals SUMO2-p66Shc signaling as a fundamental regulator of vascular endothelial function. We discovered that p66ShcK81 is an upstream modification regulating p66Shc signaling and mediates hyperlipidemia-induced endothelial dysfunction and oxidative stress.

Introduction

Endothelial cells play an important role in regulating vascular health. One of the primary functions of endothelial cells is to maintain vascular tone and regulate blood flow in response to physiological changes. In the context of disease, endothelial cells either fail to produce enough nitric oxide (NO) or the available NO is quenched by reactive oxygen species (ROS). Consequently, this leads to endothelial dysfunction (ED), characterized by the reduced availability of NO and impaired vasorelaxation.[1] ED is associated with various conditions such as diabetes, hyperlipidemia, hyperglycemia, obesity, and aging.[2] There is a strong correlation between ED and atherosclerotic changes in vessel walls.[3] Therefore, ED is one of the prime targets to prevent pathologies including myocardial infarction and stroke.

Sumoylation is a dynamic post-translational modification involved in many pathologies including vascular disease.[4] Increased sumoylation promotes ED and subsequent development of atherosclerosis.[5, 6] Sumoylation occurs by conjugation of **s**mall **u**biquitin-like **mo**difier**s** (SUMOs) to the lysine moiety of the target protein, which may change its fate and/or function. SUMO conjugation is predicted to occur at a specific motif, ΨKXE, where Ψ represents an aliphatic-branched amino acid, X a random amino acid, and E glutamic acid, although other non-canonical sumoylation sites have also been reported.[4, 7, 8] There are four SUMO isoforms (SUMO1, SUMO2, SUMO3 and SUMO4) and among them SUMO2 is the most abundantly expressed. Unlike SUMO1 and SUMO3, genetic deletion of SUMO2 is embryonically lethal.[9, 10] While sumoylation is known to promote cardiovascular diseases, the effect of SUMO isoforms on the vasculature is not well studied. Recently, we reported that endothelial-specific overexpression of SUMO2 impairs vascular endothelial function in mice.[11] However, the molecular mechanisms mediating the endothelial effects of SUMO2 are not known.

p66Shc is a member of the ShcA family of adaptor proteins. Among the ShcA protein family (p66Shc, p52Shc, and p46Shc), p66Shc is the largest protein due to an additional collagen homology domain (CH2) at its N-terminus. Posttranslational modifications in the CH2 domain regulate the oxidative function of p66Shc;[12-15] whereas phosphorylation in the CH1 domain leads to mitogenic signaling.[16, 17] p66Shc is abundantly expressed in endothelium and mediates ED due to hyperlipidemia, hyperglycemia, and aging.[18] Correspondingly, mice with a genetic deletion of p66Shc have a longer life span and are protected against ED due to metabolic disorders.[19, 20] Here, we examined whether p66Shc mediates the effects of SUMO2 on endothelial cells. Using multiple techniques, we show that SUMO2 directly modifies p66Shc at lysine-81 in its CH2 domain, which is essential for SUMO2-induced ROS production and ED. Thus, our study uncovers a previously unknown posttranslational modification that regulates p66Shc function in the endothelium and defines a novel molecular mechanism by which increased SUMO2ylation induces ROS, leading to vascular ED.

Results

p66Shc mediates SUMO2-induced ROS production in endothelial cells

To address whether p66Shc mediates SUMO2-induced ROS production, we overexpressed SUMO2 in human umbilical vein endothelial cells (HUVECs) with and without knockdown of p66Shc and used MitoSOX to detect mitochondrial level of ROS, a prominent function of p66Shc. As expected, overexpression of SUMO2 led to a robust increase in levels of ROS (Fig. 1A and B). This SUMO2-induced increase in ROS was significantly blunted in HUVECs with p66Shc knockdown (Fig. 1A and C). In a complementary approach, we used H_2DCFDA to determine cellular ROS level and noted a similar increase in ROS level with overexpression of SUMO2 which was significantly reduced by the knockdown of p66Shc (Fig. 1D).This suggests that SUMO2 is targeting p66Shc for ROS production in endothelial cells.

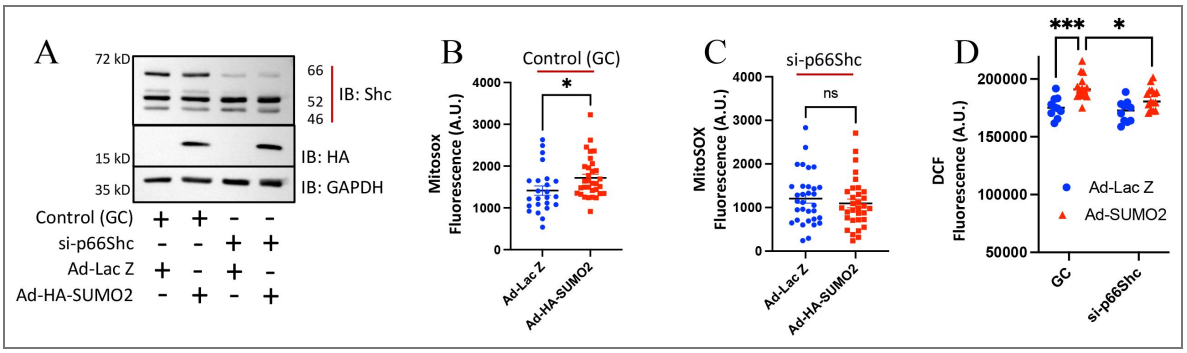

Figure 1. p66Shc mediates SUMO2-induced ROS production and inflammation in endothelial cells.

(A) Immunoblot for Shc and SUMO2 expression in HUVECs following p66Shc knockdown (si-p66Shc) and SUMO2 overexpression (Ad-HA-SUMO2). (B and C) Quantification of ROS (MitoSOX fluorescence) in HUVECs from A. *P<0.05; n=24-32; student's t-test. (D) Quantification of ROS in HUVECs following p66Shc knockdown (si-p66Shc) and SUMO2 overexpression (Ad-HA-SUMO2). ***P<0.001, P<0.05; n=10-15; One-way ANOVA. Data represents mean ±SEM. Immunoblots are representative of at least three independent experiments. GC, scrambled siRNA used as control; HUVECs, human umbilical vein endothelial cells.

p66Shc is directly modified by SUMO2

We next asked whether p66Shc is a direct target for SUMO2. We overexpressed p66Shc in HEK-293 cells with and without SUMO2. Immunoprecipitation of p66Shc showed many slower migrating protein bands when SUMO2 and p66Shc were co-expressed, suggesting that p66Shc may be SUMO2ylated (Fig. 2A). To confirm that the slower migrating bands were due to sumoylation, we overexpressed SUMO-ligase (Ubc9) to facilitate sumoylation, and a deSUMOylating enzyme (SENP1) which removes SUMO proteins. Overexpression of Ubc9 increased the signal of slower migrating bands whereas SENP1 reduced the signal suggesting the slower migrating bands were due to sumoylation (Fig. 2B). Next, we asked whether the SUMO2-p66Shc interaction exists in endothelial cells by overexpressing SUMO2 and p66Shc in HUVECs. Immunoprecipitation of flag-tagged p66Shc revealed sumoylation (Fig. 2C) which consistently increased in intensity with increasing SUMO2 expression (Fig. S1). To verify that p66Shc undergoes SUMO2ylation in HUVECs we treated cells with a pharmacological inhibitor of sumoylation (anacardic acid). This reduced sumoylation of p66Shc (Fig. 2D &E), demonstrating that indeed p66Shc undergoes SUMO2ylation in endothelial cells. We also performed an *in vitro* sumoylation of p66Shc using recombinant His-tagged p66Shc protein and a commercially available kit (Enzo, Farmingdale, NY), which showed that recombinant p66Shc is modified by SUMO2 (Fig. 2F). Collectively, these findings indicate that p66Shc is a direct target of SUMO2.

SUMO2 modifies p66Shc via lysine-81

The CH2 domain of p66Shc is highly conserved and involved in oxidative function of p66Shc. Within this region there are several conserved lysine residues. Among these is lysine 81, which is part of the ΨKXE motif required for sumoylation (Fig. 3A) Therefore, to examine if K81 is sumoylated, we took advantage of SUMO2ylated recombinant p66Shc and resolved it on SDS-PAGE. Based on the approximate location of modified protein bands seen in immunoblotting, we cut out the gel piece and subjected it to mass spectrometry analysis (Fig. S3). This demonstrated that lysine-81 (K81) in p66Shc was SUMO2ylated, identified as lysine conjugated with the QTGG motif present at c-terminal of SUMO2 (Fig. 3B). To confirm that SUMO2 modifies p66Shc at K81, we mutated this residue to Arginine (p66ShcK81R) and overexpressed it in HEK-293 cells with SUMO2 to determine if sumoylation still occurred. Immunoprecipitation showed that p66ShcK81R is resistant to SUMO2ylation, whereas it still occurred with the wild-type (WT) form (Fig. 3C). Similar results were also observed in HUVECs (Fig. 3D). We next performed an *in vitro* SUMO2ylation of recombinant p66ShcWT and p66ShcK81R. As anticipated, immunoblotting showed that, unlike p66ShcWT, p66ShcK81R is resistant to SUMO2ylation (Fig. 3E). Next, to determine the endogenous SUMO2-p66Shc, we generate a custom antibody against the branched peptide, mimicking the region of SUMO2 attachment (SUMO2-K81) to p66Shc using a commercial source (YenZym, CA). Using this SUMO2-p66ShcK81 antibody, we performed immunoprecipitation and immunoblotting in HUVECs treated with o-LDL (200 μg/ml) for 48 hours and noted p66Shc-SUMO2ylation at endogenous level (Fig. 3F). Thus, our findings demonstrate that SUMO2 modifies p66Shc at K81.

SUMO2 promotes serine36 phosphorylation and mitochondrial translocation of p66Shc

In response to pro-oxidant stimuli, p66Shc becomes phosphorylated at serine36 (S36) and translocates to mitochondria. Once there it oxidizes cytochrome C to produce ROS [21, 22], which is a central mechanism to activate the oxidative function of p66Shc. Since the K81 and S36 residues of p66Shc are both present in its conserved CH2 domain, we asked whether promoting SUMO2ylation also facilitates phosphorylation of the S36 residue of p66Shc. In HEK-293 cells as well as in HUVECs, we noted increased levels of p66ShcS36 phosphorylation when SUMO2 and p66Shc were co-expressed (Fig. 4A-D). To determine whether SUMO2-induced S36 phosphorylation is mediated via K81 sumoylation, we evaluated p66ShcS36 phosphorylation in HUVECs overexpressing p66ShcK81R and SUMO2. SUMO2 overexpression significantly increased S36 phosphorylation in cells expressing p66ShcWT whereas this response was markedly attenuated in

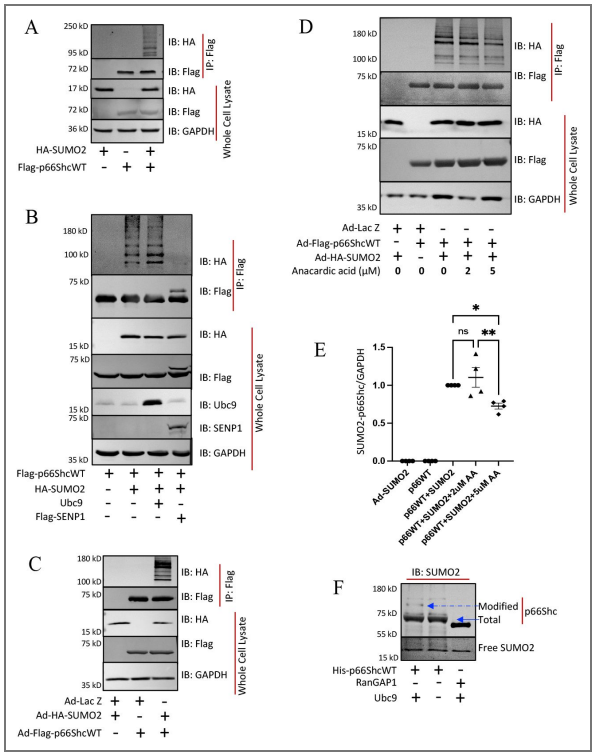

Figure 2. SUMO2 directly modifies p66Shc.

(A) Immunoblot for sumoylated p66Shc in HEK-293 cells overexpressing SUMO2 (HA-SUMO2) and p66Shc (Flag-p66Shc). (B) Immunoblot for p66Shc sumoylation in HEK-293 cells overexpressing SUMO2 (HA-SUMO2) and p66Shc (Flag-p66Shc) with or without overexpression of Ubc9 (HA-Ubc9) and SENP1 (Flag-SENP1). (C) Immunoblot for sumoylation of p66Shc in HUVECs overexpressing SUMO2 (Ad-HA-SUMO2) or p66Shc (Ad-Flag-p66Shc). (D) Immunoblot for p66Shc sumoylation in HUVEC cells overexpressing SUMO2 (Ad-HA-SUMO2) or p66Shc (Ad-Flag-p66Shc) in the presence or absence of anacardic acid. Quantification of SUMO2-p66Shc from immunoblot of D. One-way ANOVA, followed by Tukey's post-hoc analysis, n=4 *P<0.05, **P<0.01. (F) Immunoblot for sumoylated recombinant p66Shc in the presence of absence of Ubc9. Immunoblots are representative of at least three independent experiments. Data represents mean ±SEM. Ad-Lac Z was used as adenoviral control to compensate for the difference in MOI among the groups.

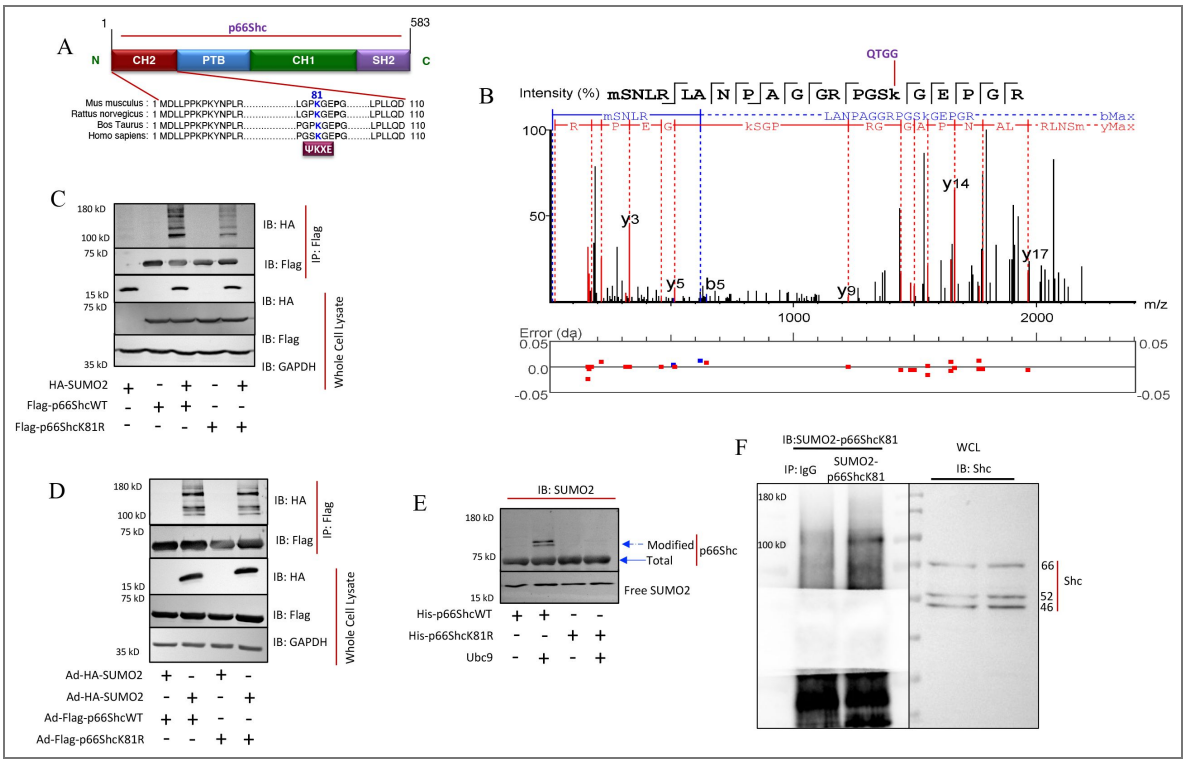

Figure 3. SUMO2 modifies p66Shc at lysine-81.

(A) Modular structure of p66Shc showing conserved lysine-81 in the CH2 domain. (B) Tandem mass spectrometry of *in vitro SUMO*2ylated recombinant p66Shc. (C) Immunoblot showing sumoylation of p66Shc in HEK-293 cells overexpressing p66ShcWT (Flag-p66ShcWT) or p66ShcK81R (Flag-p66ShcK81R) with and without SUMO2 (HA-SUMO2). (D) Immunoblot showing sumoylation of p66Shc in HUVECs overexpressing p66ShcWT (Ad-Flag-p66ShcWT) or p66ShcK81R (Ad-p66ShcK81R) with and without SUMO2 (Ad-HA-SUMO2). (E) Immunoblot for sumoylation of recombinant p66ShcWT or p66ShcK81R. (F) Immunoprecipitation of SUMO2-p66Shc using SUMO2-p66ShcK81 antibody or the matching IgG control in HUVECs followed by immunoblotting with SUMO2-p66ShcK81 antibody showing endogenous SUMO2ylation of p66Shc (left-side panel). Immunoblotting with Shc antibody in whole cell lysate (right-side panel). Immunoblots are representative of at least three independent experiments. CH2, collagen homology 2; PTB, phosphotyrosine binding; CH1, Collagen homology 1; SH2, Src homology 2.

cells expressing p66ShcK81R (Fig. 4E and F), indicating that K81 SUMOylation promotes, but is not strictly required for, S36 phosphorylation. As p66ShcS36 phosphorylation increases p66Shc level in mitochondria, we evaluated whether SUMO2 affects mitochondrial levels of p66Shc. Levels of p66Shc in the mitochondria of HUVECs increased in a dose-dependent manner upon SUMO2 overexpression (Fig. 5A and B). However, mitochondrial expression of p66Shc was significantly lower in HUVECs expressing p66ShcK81R relative to p66ShcWT (Fig. 5C and D). Collectively, these findings support a model in which SUMO2ylation at K81 facilitates S36 phosphorylation and enhances mitochondrial translocation of p66Shc, thereby promoting its oxidative signaling function.

Mice expressing non-sumoylatable p66ShcK81R are protected against SUMO2-induced ED

To determine whether SUMO2ylation of p66Shc at lysine 81 modulates endothelial control of vascular tone, we generated a knock-in mouse model in which endogenous p66Shc was replaced with a SUMOylation-deficient mutant (p66ShcK81R). This model was generated using CRISPR-Cas9-mediated genome editing to substitute arginine for lysine at position 81 of p66Shc (Fig. 6A and B). These mice are viable and there was no obvious physiological difference from wild type mice, except that the endothelium-independent relaxation of aortic rings was more pronounced in p66ShcK81R knockin mice (Fig. S4). To examine the effect of SUMO2 on endothelial function in these mice, we used adenoviruses to express SUMO2 in aortic rings from WT and p66ShcK81R knockin mice (Fig. 6C and D). Subsequently, the aortic rings were evaluated for endothelium-dependent relaxation (induced by acetylcholine) and endothelium-independent relaxation (induced by sodium nitroprusside). In aortic rings from WT mice, endothelium-dependent relaxation was significantly delayed by SUMO2 overexpression (Fig. 6E) whereas it was unchanged in p66ShcK81R mice (Fig. 6F). In contrast, endothelium-independent relaxation was not affected by SUMO2 overexpression (Fig. 6G and H). This suggests that the effect of SUMO2 on endothelial function is primarily mediated via p66ShcK81.

P66ShcK81R knockin mice are protected against hyperlipidemia-induced endothelial dysfunction

To examine the physiological significance of p66Shc-SUMO2, we chose hyperlipidemia as a suitable stimulus as it has been shown to promote sumoylation.[23] To confirm hyperlipidemia-induced activation of sumoylation and involvement of SUMO2 specifically, we knocked down SUMO2 in HUVECs and treated them with oxidized low-density lipoprotein (o-LDL). Immunoblotting with anti-SUMO2/3 antibody showed a robust increase in sumoylation with o-LDL and the majority of sumoylation was due to SUMO2 (Fig. 7A). Next, we crossbred p66ShcK81R knockin mice with low-density lipoprotein receptor knockout ($LDLr^{-/-}$) mice to generate $LDLr^{-/-}$Xp66ShcK81R KI mice and fed them high fat diet (HFD) for 4 weeks to induce hyperlipidemia. There was no difference in serum cholesterol level between the $LDLr^{-/-}$ and $LDLr^{-/-}$Xp66ShcK81R KI mice on HFD (Fig. 7B). We performed vascular reactivity assay on the aortic rings of WT mice, $LDLr^{-/-}$ mice on normal diet and $LDLr^{-/-}$ on HFD and noted to a significant impairment of endothelium-dependent relaxation of aortic rings from $LDLr^{-/-}$ mice compared to aortic rings from wild type mice and $LDLr^{-/-}$Xp66ShcK81R KI (Fig. 7C). However, the endothelium-independent relaxations were similar among the groups (Fig. 7D). Further we evaluated the level of oxidative stress in aortas of $LDLr^{-/-}$ and $LDLr^{-/-}$Xp66ShcK81R KI mice on HFD by determiening the level of 8-OHdG (8-hydroxy deoxyguansine), a marker of oxidative damage of DNA. We noticed that $LDLr^{-/-}$ Xp66ShcK81R KI mice have significantly less oxidative damage to aortic endothelium compared to $LDLr^{-/-}$ mice (Fig. 7E and F). Collectively, our study shows that p66Shc mediates the effects of SUMO2 on vasculature.

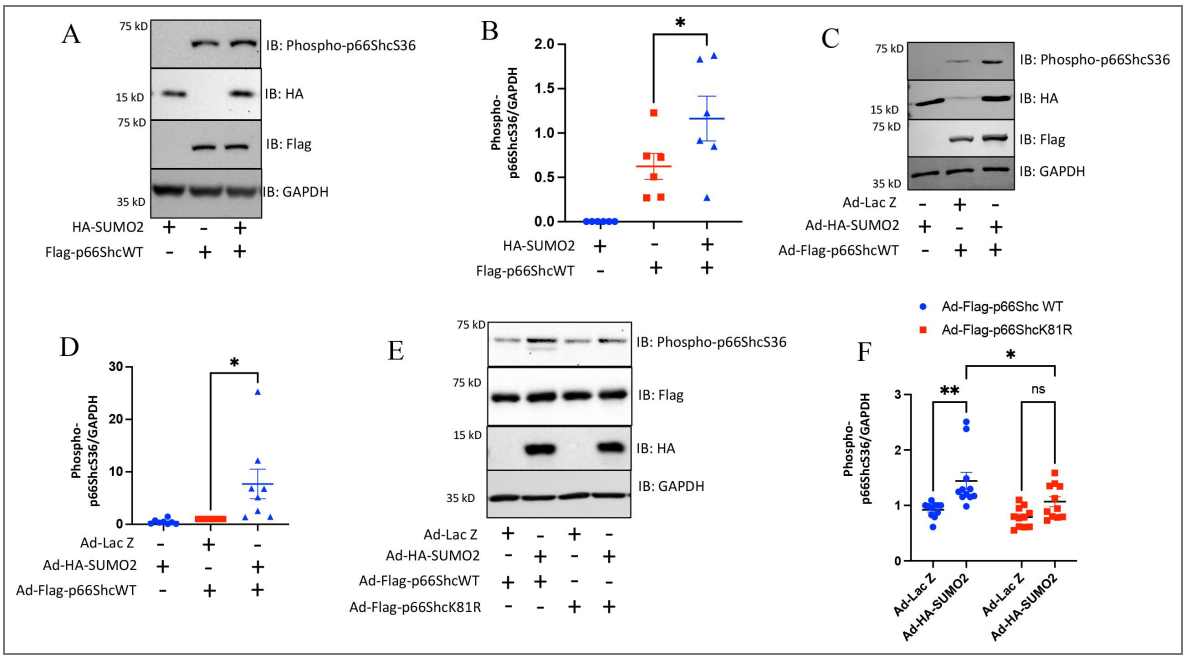

Figure 4. SUMO2 promotes p66ShcS36 phosphorylation by sumoylation of K81.

(A) Immunoblot for p66ShcS36 phosphorylation in HEK-293 cells overexpressing SUMO2 (HA-SUMO2) and p66ShcWT (Flag-p66ShcWT). (B) Quantification of p66ShcS36 phosphorylation from A. *P<0.05; n=6; one-way ANOVA. (C) Immunoblot for p66ShcS36 phosphorylation in HUVECs overexpressing SUMO2 (Ad-HA-SUMO2) or p66ShcWT (Ad-Flag-p66ShcWT). (D) Quantification of p66ShcS36 phosphorylation from C. *P<0.05; n=8; one-way ANOVA. (E) Immunoblot for p66ShcS36 phosphorylation in HUVECs expressing SUMO2 (Ad-HA-SUMO2) and p66ShcWT (Ad-Flag-p66ShcWT) or p66ShcK81R (Ad-Flag-p66ShcK81R). (F) Quantification of p66ShcS36 phosphorylation from E. *P<0.05, **P<0.01; n=11; two-way ANOVA. Immunoblots were quantified with iBright image analysis software. Data represents mean ±SEM. Immunoblots are representative of at least three independent experiments.

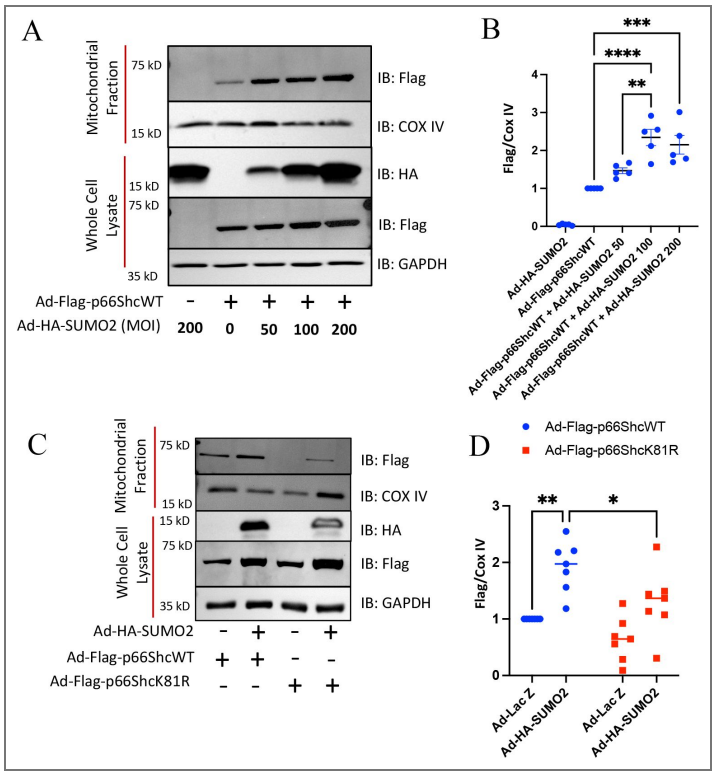

Figure 5. p66ShcK81 SUMO2ylation of K81 in p66Shc promotes its translocation to the mitochondria.

(A) Immunoblot for p66ShcWT in whole cell lysates or the mitochondrial fraction of HUVECs ectopically expressing SUMO2 (Ad-HA-SUMO2) and p66ShcWT (Ad-Flag-p66ShcWT). (B) Quantification of the relative abundance of p66Shc in the mitochondria from A. **P<0.01, ****P<0.0001; n=5; one-way ANOVA. (C) Immunoblot for p66Shc in whole cell lysates or the mitochondrial fraction of HUVECs ectopically expressing SUMO2 (Ad-HA-SUMO2) and p66ShcWT (Ad-Flag-p66ShcWT or p66ShcK81R (Ad-Flag-p66ShcK81R). (D) Quantification of relative abundance of p66Shc in the mitochondria from C. *P<0.05, **P<0.01; n=7; two-way ANOVA. Data represents mean ±SEM. Immunoblots are representative of at least three independent experiments.

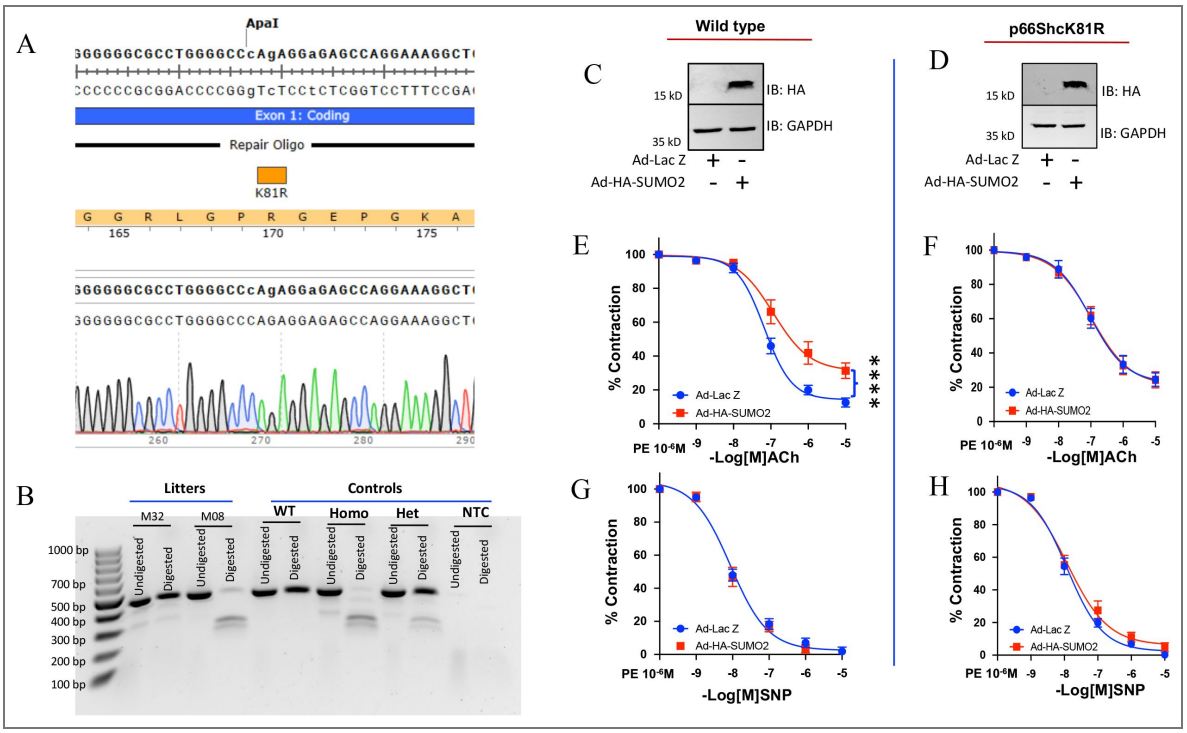

Figure 6. p66ShcK81R knockin (KI) are resistant to SUMO2-induced endothelial dysfunction.

(A) DNA sequencing data from a p66ShcK81R knockin (KI) mouse showing a point mutation (A>G) resulting in an amino acid change (K→R), and insertion of silent mutation to create a digestion site for ApaI endonuclease for genotyping. (B) DNA gel electrophoresis of a PCR-amplified and ApaI-digested amplicon identifying the p66ShcK81R KI allele. (C and D) Representative immunoblots showing SUMO2 expression in aortic rings from wild type and p66ShcK81R KI mice (1.67×10^8 pfu/ring). (E and F) Graphs showing % contraction following induction of endothelium-dependent relaxation in aortic rings from (E) wild-type mice, n=14-16 from 4 mice) and (F) p66ShcK81R KI mice (n=15-16 rings from 4 mice) expressing a control vector (Ad-Lac Z) or overexpressing SUMO2 (Ad-HA-SUMO2). (G and H) Graphs showing % contraction following induction of endothelium-independent relaxation in aortic rings from (G) wild type (n=14-16 from 4 mice) and (H) p66ShcK81R KI mice (n=14-15 from 4 mice) expressing a control vector (Ad-Lac Z) or overexpressing SUMO2 (Ad-HA-SUMO2). Curves were compared for nonlinear fit. ****P<0.0001. Ach-acetylcholine, SNP-sodium nitroprusside, PE-Phenylephrine, Homo-homozygous, Het-heterozygous, and NTC-No template control. Data represents mean ±SEM.

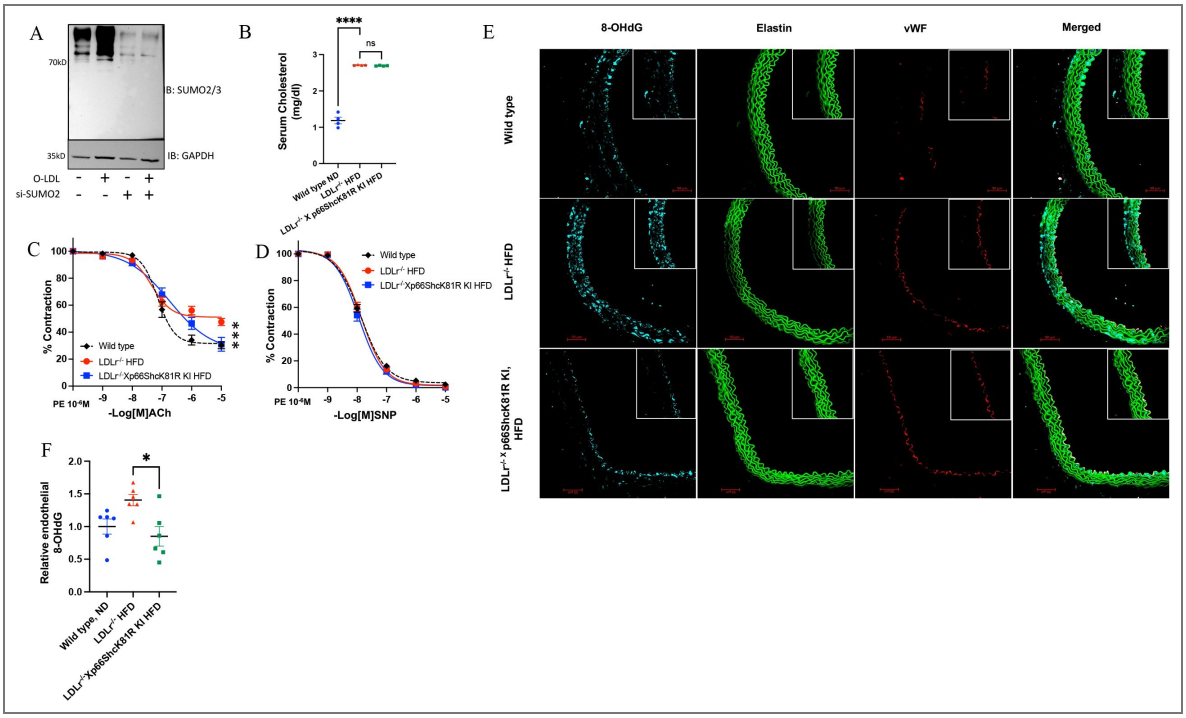

Figure 7. p66ShcK81R knockin (KI) prevents hyperlipidemia-induced endothelial dysfunction and oxidative stress.

(A) Immunoblot showing oxidized-LDL (o-LDL)-induced changes in SUMO2/3-conjugated proteins in HUVECs with and without SUMO2. (B) Graph showing serum cholesterol-level in wild type, LDLr$^{-/-}$ mice on high fat diet, and p66ShcK81R KI mice on high fat diet (n=4/group). One-way ANOVA, ****P<0.0001. Graphs showing % contraction following induction of endothelium-dependent relaxation (C) and endothelium-independent relaxation (D) in aortic rings from male wild-type mice, (n=14 rings from 5 mice), LDLr$^{-/-}$ mice on high fat diet (n=18 rings from 6 mice) and LDLr$^{-/-}$ Xp66ShcK81R KI mice on high fat diet (n=12 rings from 6 mice). Two-way ANOVA. ***P<0.001. (E) Representative images showing the level of 8-OHdG in aortic section of LDLr$^{-/-}$ mice on high fat diet (n=6 mice) and LDLr$^{-/-}$ Xp66ShcK81R KI mice on high fat diet (n=6 mice) and their (F) quantification. One-way ANOVA. *P<0.05. HFD-high fat diet Ach-acetylcholine, SNP-sodium nitroprusside, PE-Phenylephrine, 8-OHdG-8 hydroxy deoxyguanosine. Data represents mean ±SEM.

p66ShcK81-SUMO2ylation affects multiple signaling pathways in endothelial cells

Given that SUMO2 modifies p66Shc at K81 and causes endothelial dysfunction, we next asked how p66ShcK81-SUMO2 affects endothelial cells. We performed global proteomics profiling of HUVECs overexpressing SUMO2 and p66ShcWT or p66ShcK81R using mass spectrometry. Comparative analysis of protein expression was performed against control (HUVECs infected with an equal number of adenovirsues expressing LacZ) (Fig. S5A). Principal component analysis showed a distinct protein expression profile for cells expressing p66ShcWT and p66ShcK81R (Fig. 8A and B). To understand the effect of p66Shc K81 SUMOylation, we examined pathways altered in cells expressing p66ShcWT and p66ShcK81R. The majority of pathways were downregulated, with particularly strong suppression of JAK–STAT signaling. This observation is consistent with the known role of Shc proteins in mediating growth factor receptor signaling. Unlike p52Shc and p46Shc, p66Shc is reported to negatively regulate growth receptor signaling. Supporting this, our mass spectrometry data revealed a greater than 15-fold downregulation of JAK-STAT signaling in cells expressing p66ShcWT in the presence of SUMO2 (Fig. 8C and D). In contrast, this effect was attenuated in cells expressing p66ShcK81R with SUMO2. It is important to note that endogenous p66ShcWT was present in both conditions, which may have competed with the overexpressed p66ShcK81R. Together, these findings suggest that K81 SUMOylation plays a critical role in regulating p66Shc function.

Discussion

Although SUMO2 and p66Shc are expressed in endothelium and are associated with ED, the mechanisms by which this occurs are unclear. Here we identified SUMO2 modification of p66Shc as a novel molecular signal regulating vascular endothelial function. SUMO2 modifies p66Shc at K81, which increases phosphorylation of p66Shc at S36, resulting in its translocation to the mitochondria. These steps are critical for p66Shc-dependent ROS production. Notably, this phenomenon was evident in non-endothelial cells as well, suggesting that SUMO2-p66Shc may also regulate other physiologic functions. Mice expressing a non-sumoylatable form of p66Shc (p66ShcK81R KI) were protected against SUMO2 and hyperlipidemia-induced ED and oxidative stress, which further supports the role of SUMO2-p66ShcK81 in regulating endothelium function.

Multiple studies suggest that sumoylation promotes ED.[24] Preventing sumoylation by downregulating the SUMO ligases, Ubc9 (E2 ligase) and PIAS1 (E3 ligase), rescued oxidative stress and advanced glycation end product-mediated endothelial inflammation and dysfunction.[25] Conversely, partial genetic deletion of the de-sumoylation enzyme, sentrin/SUMO-specific protease 2 (SENP2), significantly increased endothelial apoptosis, inflammation, and dysfunction, which promoted atherosclerotic plaque formation.[5, 6] However, the isoform-specific effects of SUMOs on endothelial function have not been well studied. We previously reported that endothelial expression of SUMO2 impairs vasorelaxation.[11] By showing that mice having p66ShcK81R knockin are resistant to endothelial effects of SUMO2 and hyperlipidemia, this study explains the prior findings that the effects of SUMO2 on vasculature are likely mediated by p66Shc.

The adapter protein p66Shc mediates receptor tyrosine kinase signaling in a variety of cell types. Among them, the effects of p66Shc on endothelial cells are well studied and pronounced.[18] In particular, endothelial function in mice with genetic deletion of p66Shc are protected against a variety of stimuli such as diabetes, aging, and hyperlipidemia.[19, 20, 26] p66Shc produces ROS by oxidation of cytochrome C in the mitochondria as well as by activating Rac1.[13, 22] Phosphorylation at S36 in the CH2 domain of p66Shc is one of the earliest discovered posttranslational modifications that govern its oxidative function.[12] Crosstalk among different posttranslational modifications including phosphorylation, acetylation, and sumoylation is well known.[27-30] Our finding that SUMO2 increases S36 phosphorylation in p66Shc suggests the existence of such a crosstalk in regulating the oxidative function of p66Shc. The molecular weight of the phosphorylated p66ShcS36 protein band (close to 70kD) suggests that the pool of SUMO2-induced phosphorylated p66ShcS36 is not SUMO2ylated. Therefore, it is likely that K81-SUMOylated and

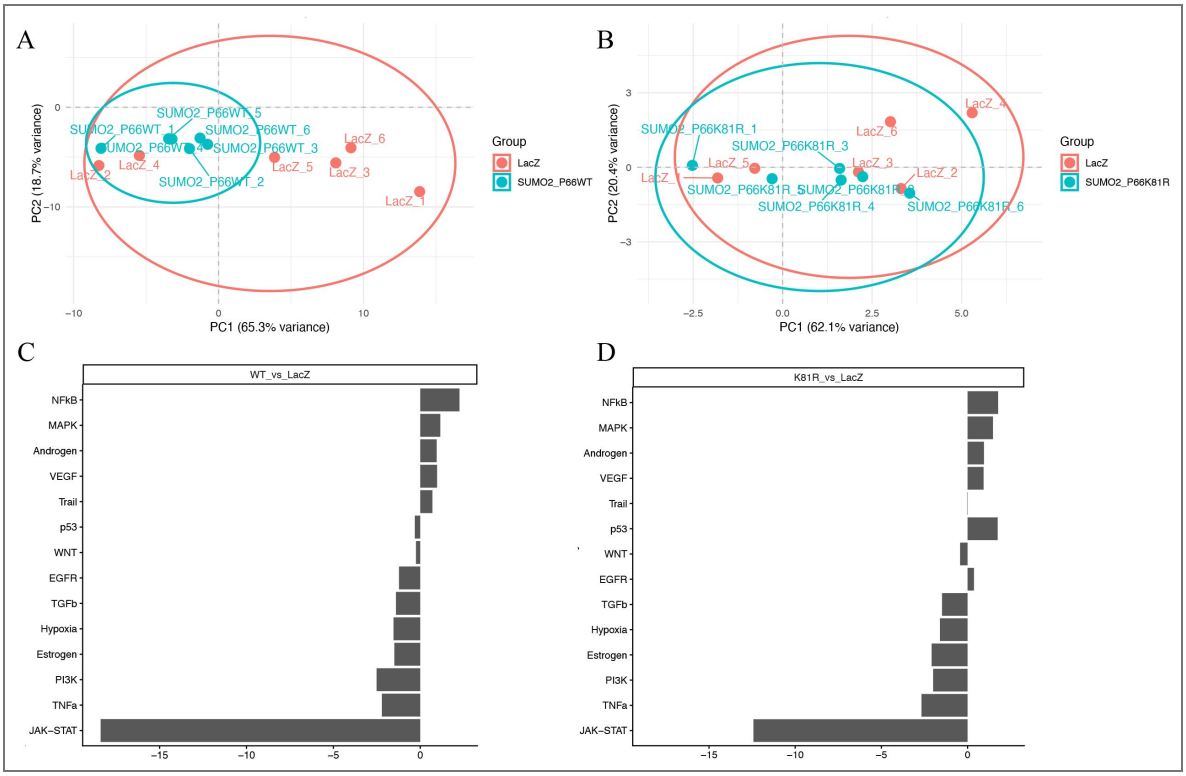

Figure 8. p66ShcK81 sumoylation affects multiple key regulatory pathways in endothelial cells.

(A&B) Principal component analysis (PCA) of proteins expressed in endothelial cells expressing SUMO2+p66ShcWT and SUMO2+p66ShcK81R. (C&D) Bar chart presentation of key regulatory pathways differentially affected by SUMO2-p66ShcWT (C) and SUMO2-p66ShcK81R (D) in endothelial cells. The protein expression data (fold change) was obtained from HUVECs expressing SUMO2 with p66ShcWT or p66ShcK81R compared to those expressing Ad-Lac Z. Pathway enrichment analysis was performed using R-based bioinformatics tools, including PROGENy/decoupleR pathway activity inference and visualization packages. Data were derived from a sample size of n=6.

S36-phosphorylated forms of p66Shc coexist in the same pool of protein, but that p66Shc is not SUMOylated and phosphorylated at the same time. Future studies will be needed to establish the hierarchy of this phenomena.

Based on mass spectrometry analysis and *in vitro* sumoylation of recombinant p66Shc, it appears that p66ShcK81 is a primary target of SUMO2. However, the multiple protein bands higher than 66kD that were present in the immunoprecipitation assay suggest the possibility that additional sumoylation sites are present in p66Shc. Technical challenges limited our ability to determine the nature of these protein bands. Yet, in silico analysis using an available sumoylation prediction tool (Cuckoo workgroup) indicates 3 possible sumoylation motifs present in p66Shc, namely K81, K462, and K582, which can non-specifically be modified by SUMO1/2/3/4. Importantly, among the possible sumoylation motifs only K81 is present in the CH2 domain, which is unique structural feature of p66Shc (i.e., not present in other isoforms: p52Shc and p46Shc) that has a regulatory effect on its function. Therefore, even though other lysines are sumoylated, it is likely that the effect of p66Shc-sumoylation is mediated through the K81 modification and has also been supported by proteomics analysis.

The primary function of the ShcA family of adaptor proteins is to mediate growth factor receptor signaling [18]. However, p66Shc is unique within this family, as it deviates from the classical role of Shc proteins and instead promotes oxidative stress. Our global mass spectrometry data suggest that enhancing p66ShcK81 SUMO2ylation inhibits JAK-STAT signaling, a key downstream pathway of growth factor receptor signaling. The negative regulation of growth receptor signaling by p66Shc, through competition with p52Shc for Grb2 binding, has been previously reported [31]. In addition, a recent study demonstrated that p66Shc can mediate EGFR-ERK signaling [32]. Therefore, beyond its established role in ROS regulation, p66ShcK81-SUMO2ylation may have additional functional roles in endothelial cell physiology.

One of the limitations of this study is that we did not perform gain-of-function experiments to ascertain if SUMO2-modified p66ShcK81 regulates p66Shc function *in vivo*, which is technically too challenging. Additionally, we were unable to examine the behavior of the phosphomimetic double mutant (S36D/K81R) to directly assess the interplay between K81 SUMO2ylation and S36 phosphorylation in mitochondrial localization. Prior studies have shown that K81 of p66Shc undergoes acetylation.[14] and that a specific lysine can undergo sumoylation as well as acetylation.[29] Although p66ShcK81R disrupts acetylation and sumoylation that occurs from acetyl transferase and SUMOylating enzymes, it is possible that the same lysine is sumoylated and acetylated in a context dependent manner. Importantly, the extent of post translational modification is so small that a competition between acetylation and sumoylation for the same target residue is highly unlikely. The fact that p66ShcK81R KI mice are protected against SUMO2-induced ED supports the hypothesis that p66ShcK81 mediates the endothelial effects of SUMO2.

Collectively, our study identified p66Shc SUMO2ylation as a unique cellular signaling mechanism specifically regulating vascular endothelial function. As such, this signaling mechanism may also affect advanced pathologies such as atherosclerosis, where both increased SUMOylation and expression of p66Shc has been reported. Nevertheless, given its potential to regulate endothelial function, SUMO2ylation of p66Shc presents a novel therapeutic target for pathologies associated with ED.

Acknowledgements

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mouse was generated at the University of Iowa Genome Editing Core Facility, supported in part by grants from the NIH and from the University of Iowa Carver College of Medicine. We wish to thank Norma Sinclair, Patricia Yarolem, Joanne Schwarting and Rongbin Guan for their technical expertise in generating transgenic mice. We gratefully acknowledge Kris Griener of the Design Center for language editing and Dr. Jennifer Y. Barr of the Scientific Editing and Research Communication Core at the University of Iowa Carver College of Medicine for critical reading of the manuscript and suggestions toward improving the clarity of the manuscript.

Additional files

Supplement file

Additional information

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Peer reviews

Reviewer #2 (Public review):

Summary:

The manuscript titled "p66Shc Mediates SUMO2-induced Endothelial Dysfunction" by Kumar et al. builds upon established literature demonstrating that both p66Shc and SUMOylation are essential players in nitric oxide (NO)-mediated endothelial vascular homeostasis and development (PMID: 10580504, 28760777, and 35187108).

In this study, the authors uncover a novel mechanism showing how the SUMO2ylation of p66Shc drives reactive oxygen species (ROS) production in endothelial cells. Specifically, they identify Lysine 81 (K81) as the critical residue on p66Shc conjugated to SUMO2, proving it is essential for the protein's mitochondrial localization.

The authors convincingly demonstrate that:

p66Shc is actively SUMO2ylated at the K81 site in cellular models.

Phosphorylation at Serine 36 (S36) is significantly reduced upon the loss of this critical SUMOylation site.

Conclusion:

Overall, this study provides strong evidence for a novel regulatory axis in endothelial cells. It successfully opens the door for further dissection of the complex mechanistic crosstalk between three key post-translational modifications on p66Shc: S36 phosphorylation, K81 SUMO2ylation, and acetylation.

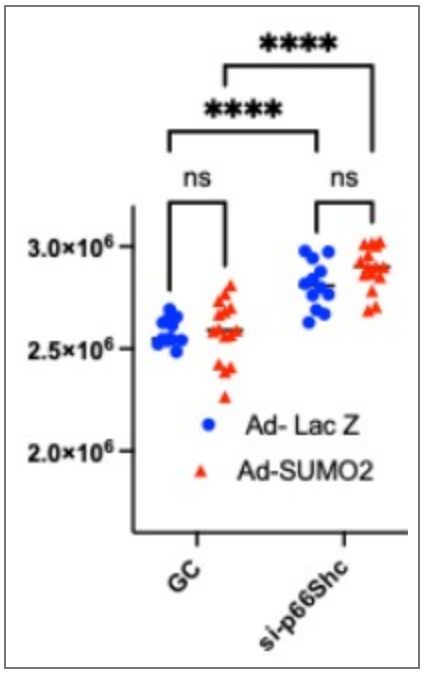
https://doi.org/10.7554/eLife.106344.2.sa1

Author response:

The following is the authors' response to the original reviews.

> ***Public Reviews:***
>
> ***Reviewer #1 (Public review):***
>
> *Summary:*
>
> *The authors describe a role of sumoylation at K81 in p66Shc which affects endothelial dysfunction. This explores a new mechanism for understanding the role of PTMs in cellular processes.*
>
> *Strengths:*
>
> *The experiments are well planned and the results are well represented.*
>
> *Vascular tonality experiments were carried out nicely, given the amount of time and effort one needs to put in to get clean results from these experiments.*
>
> *Weaknesses:*
>
> *(1) The production of ROS has been measured in a very superficial way.*
>
> *The term "ROS" confers a plethora of chemical species which exerts different physiological effects on different cells and situations.*
>
> *Mitochondria through one of the source, but not the only source of ROS production. Only measuring ROS with mitosox do not reflect the cellular condition of ROS in a specific condition. I would suggest authors consider doing IF of oxidative stress specific markers, carbonyl group and also, maybe, Amplex red for determining average oxidative stress and ros production in the cells.*

As suggested, we employed an additional ROS-sensitive probe, H_2DCFDA, which revealed an overall increase in intracellular ROS levels upon SUMO2 overexpression; this effect was reversed by knockdown of p66Shc. In addition, we performed the Amplex Red assay on conditioned media to assess extracellular ROS release. SUMO2 overexpression did not alter ROS levels detected in the media, whereas a paradoxical increase was observed following p66Shc knockdown.



Author response image 1. Amplex Red assay performed in HUVECs with and without knockdown of p66Shc expressing SUMO2 (Ad-SUMO2) or a control virus (Ad-LacZ).

Amplex Red predominantly detects hydrogen peroxide (H_2O_2), which may originate from NADPH oxidases or be generated through the dismutation of superoxide. In contrast to superoxide, H_2O_2 is relatively stable, membrane-permeable, and well recognized as a second messenger in endothelial signaling, where it modulates kinase and phosphatase activity and supports physiological vascular functions. Thus, the elevated H_2O_2 detected following p66Shc knockdown may represent a signaling-competent redox state rather than a pathological increase in oxidative stress. Moreover, the SUMO2-p66Shc-mediated increase in mitochondrial ROS may be efficiently buffered by cellular antioxidant defense mechanisms, thereby limiting detectable extracellular ROS release.

> *(2) 8-OHG signal seems very confusing in Figure 7E. 8-ohg is supposed to be mainly in the nucleus and to some extent in mitochondria. The signal is very diffused in the images. I would suggest a higher magnification and better resolution images for 8-ohg. Also, the VWF signal is pretty weak whereas it should be strong given the staining is in aorta. Authors should redo the experiments.*

We have provided a better image for figure 7E. We repeated the staining with another antibody for vWF which showed stronger signal for vWF.

> *(3) PCA analysis is quite not clear. Why is there a convergence among the plots? Authors should explain. Also, I would suggest that the authors do the analysis done in Figure 8B again with R based packages. IPA, though being user-friendly, mostly does not yield meaningful results and the statistics carried out is not accurate. Authors should redo the analysis in R or Python whichever is suitable for them.*

We thank the reviewer for their valuable feedback and insightful suggestions.

Regarding the PCA analysis, the observed convergence among the data points reflects the underlying biological similarity between the samples within each group. Given the relatively low abundance and limited number of quantified peptides in our dataset, the variance captured by PCA is modest, leading to partial overlap between groups. This convergence is

therefore likely due to shared biological characteristics and inherent sample variability, rather than technical issues.

In response to the reviewer's suggestion on pathway analysis, we have re-performed the analysis using R-based approaches. Specifically, we utilized established pipelines PROGENy-based signaling pathway activity inference. These methods provide statistically robust and reproducible results. The updated analyses and corresponding figures have been included in the revised manuscript, replacing the previous IPA-based results (Fig. 8). We believe these additions strengthen the interpretation of signaling pathway alterations in our dataset.

> *(4) The MS analysis part seems pretty vague in methods. Please rewrite.*

We have revised the methodology for MS.

> ***Reviewer #2 (Public review):***
>
> *Summary:*
>
> *The article builds on the earlier work that both p66Shc and SUMOylation are essential nitric oxide (NO) based development of endothelial vasculature (PMID: 10580504; 28760777 and 35187108). The current manuscript brings forward a finding of how SUMO2ylation of p66Shc mediated ROS production which is essential for endothelial cells. They further identify that lysine 81 of p66Shc is the residue which is conjugated to SUMO2 and is crucial for mitochondrial localization. They further show that K81 SUMO2ylation is essential for S36 phosphorylation.*
>
> *Strengths:*
>
> *Convincingly shows that p66Shc is SUMO2ylated on lysine 81 in cells and also shows that the phosphorylation (serine 36) reduces upon loss of this critical SUMOylation site.*
>
> *Weaknesses:*
>
> *All the experiments performed here are in overexpression background therefore, it would be crucial to show that p66Shc is SUMO2ylated at physiological levels.*

As detecting endogenous SUMO2-p66Shc is technically challenging considering the almost 92% homology between SUMO2 and SUMO3 and the absence of a specific antibody to detect p66Shc, we generated a custom-made antibody which can detect SUMO2-p66Shc (YenZym, CA). Using this antibody, we performed immunoprecipitation which showed endogenous SUMO2-p66Shc at a molecular weight higher than p66Shc suggesting the SUMO2 modification of p66Shc at physiological level.

> ***Reviewer #3 (Public review):***
>
> *Summary:*
>
> *The authors set out to determine how SUMO2 impairs endothelial function through direct modification of the protein p66Shc. p66Shc is known to promote reactive oxygen species production, and here the authors demonstrate that SUMO2 modifies p66Shc at lysine-81, resulting in increased phosphorylation, mitochondrial translocation. These are prosed to mediate the detrimental effects of SUMO2 in a mouse model of hyperlipidemia.*
>
> *Strengths:*
>
> *A major strength of this work is the multi-pronged approach combining biochemical assays, proteomic analyses, and a genetically modified mouse model expressing a SUMOylation resistant mutant of p66Shc. These experiments comprehensively illustrate*

> *that lysine-81 SUMOylation of p66Shc is necessary for the observed endothelial dysfunction in hyperlipidemic conditions.*
>
> *Weaknesses:*
>
> *One notable weakness is that the link between the observed cellular changes and the ultimate in vivo phenotype remains only partially explored. While the authors successfully show that p66ShcK81R knockin mice are protected from endothelial dysfunction in a hyperlipidemic context, additional experiments characterizing the broader tissue-specific roles, or examining further endothelial assays in vivo, would strengthen the mechanistic conclusions. It would also be beneficial to see more direct evaluations of p66Shc subcellular localization in the protective knockin mice to complement the proteomic findings.*

We agree with the reviewer's suggestion. However, due to limited resources, we could not pursue additional studies.

Despite these gaps, the data broadly support the authors' main conclusions. The authors lay out a plausible mechanistic pathway for how hyperlipidemia and increased global SUMOylation can converge on the oxidative stress pathway to provoke vascular dysfunction.

The likely impact of this work on the field is noteworthy. Beyond clarifying how a single post-translational modification event can influence the pathophysiology of endothelial cells, the study provides a model for investigating broader roles of SUMO2 in other cardiovascular conditions and highlights the importance of identifying additional SUMOylation sites and their downstream impact.

In conclusion, by demonstrating the direct SUMOylation of p66Shc at lysine-81 and linking that modification to endothelial dysfunction in a hyperlipidemic mouse model, this paper offers valuable insights into how broadly acting post-translational modifiers can evoke specific pathological effects.

> ***Recommendations for the authors:***
>
> ***Reviewer #1 (Recommendations for the authors):***
>
> *(1) Please rearrange the figures. It is very hard to follow.*

We rearranged some of the figures for a better presentation.

> ***Reviewer #2 (Recommendations for the authors):***
>
> ***Please note that I am not an expert of mouse work therefore I will not be able to comment on the mouse work (Figure 7).*
>
> *The following suggested changes major concerns will strengthen the work, and the minor concerns will increase the paper's accessibility to eLife's broad scientific community.*
>
> *Major concerns.*
>
> *(1) All the work done here is based on overexpression studies, therefore it will be very useful to show that p66Shc gets SUMO2ylated under physiological conditions using SUMO-Trap beads or using tandem SUMO-Interacting Motifs (as shown in Silva-Ferrada et al., 2013: https://doi.org/10.1038/srep01690).*

We have demonstrated endogenous SUMO2 conjugation of p66Shc under physiological conditions by immunoprecipitation using a custom-generated antibody specific for SUMO2-p66Shc. This approach allows detection of SUMO2ylated p66Shc without reliance on

overexpression systems, thereby confirming that SUMO2 modification of p66Shc occurs endogenously.

> *(2) The authors claim that K81 is the only site of SUMO2ylation and based on the HUVEC experiments (Fig 3C, D) it appears that p66Shc K81R mutant still gets SUMO2ylated indicating that there are other residues which can get SUMO2ylated. Do you see the other sites getting SUMO2ylated in your mass spec data?*

Our mass spectrometry analysis did not identify SUMO2 modification on lysine residues other than K81. However, we acknowledge that SUMOylation detected *in vitro* on recombinant protein may differ from SUMOylation occurring in a cellular context, where protein conformation, interacting partners, and local enzyme availability can influence modification patterns. These differences may account for the appearance of multiple SUMOylated p66Shc species in cell lysates despite the absence of additional SUMOylation sites in the mass spectrometry dataset. Our emphasis on K81 is based on its localization within the CH2 domain of p66Shc, a region unique to the p66 isoform. Previous studies have demonstrated that post-translational modifications within the CH2 domain, such as phosphorylation, are critical for activation of the oxidative and pro-apoptotic functions of p66Shc. Accordingly, we focused our mechanistic analyses on SUMO2ylation at K81. Nevertheless, we do not exclude the possibility that additional lysine residues within the PTB or CH1 domains of p66Shc may also undergo SUMOylation in cells. Importantly, our functional data support the conclusion that SUMO2ylation at K81 is a key regulatory modification driving the oxidative activity of p66Shc.

> *(3) In Figure 4 the authors claim that SUMO2ylation at K81 is a prerequisite for S36 phosphorylation, however the phosphorylation changes are marginal (Figure 4 A, E) and why do they not see the higher molecular weight bands in the western blots.*

We appreciate the critique and agree with the reviewer that our data do not provide direct evidence that K81 SUMO2ylation and S36 phosphorylation occur simultaneously on the same p66Shc molecule. Rather, our findings suggest that SUMO2ylation at K81 may facilitate or enhance S36 phosphorylation, but is not an absolute requirement for this modification. Accordingly, we have revised the wording in the main text to avoid implying a strict prerequisite relationship and to more accurately reflect the magnitude of the observed changes in S36 phosphorylation.

> *(4) The authors further claim that the SUMO2ylation at K81 effects S36 phosphorylation which has consequences for mitochondrial localization. However, K81R still translocate to mitochondria which is indicative of SUMO2ylation is important but not necessary (Fig 5A, C).*

We agree with the reviewer's observation and have revised the main text accordingly, as described in our prior response, to clarify that K81 SUMO2ylation facilitates, but is not essential for, S36 phosphorylation and mitochondrial translocation.

> *(5) To know if the K81 SUMO2ylation had any effect on phosphorylation it would be interesting to see how the phosphomimic (S36D) mutant along with/without K18R will behave in mitochondrial localization experiments.*

We agree that examining the double mutant (S36D/K81R) would provide valuable mechanistic insight into the relationship between K81 SUMO2ylation and S36 phosphorylation in regulating mitochondrial localization. Although we are currently unable to perform these experiments due to constraints in manpower and resources, we have acknowledged this as a limitation in the Discussion and plan to pursue these studies in future work to further validate the mechanism.

> *Minor Concerns*

> *(a) In Figure 1A the authors claim that SUMO2ylation of p66Shc promotes ROS production however they do not include any well-known ROS induced proteins in the western blots such as KEAP1 or NRF2 or any other marker.*

We agree that NRF2 is a well-established transcriptional regulator of antioxidant responses. However, the primary aim of Figure 1A was to directly examine the effect of SUMO2ylation on p66Shc-mediated ROS production. While NRF2 and other ROS-responsive proteins reflect downstream adaptive responses, they do not provide a direct measure of ROS generation. Therefore, we focused on measuring SUMO2-induced changes in cellular ROS levels. To further strengthen this conclusion, we have performed additional complementary ROS assays, which are now included in the revised manuscript.

> *(b) In Figure 2D, the concentration of Anacardic acid used is low and therefore the SUMO2ylation is not completely inhibited. It would be advisable if the authors go to concentration of complete inhibition.*

We acknowledge that some residual SUMO2ylation is visible in the immunoblots following anacardic acid treatment. However, the assay demonstrates a clear, dose-dependent reduction in SUMO2ylation levels, which is sufficient to interpret the effect of SUMO2 inhibition on p66Shc function. Increasing the concentration further could introduce off-target effects, and the current assay provides a reliable and physiologically relevant readout.

> *(c) The figure panels need uniform fonts and also the size/resolution of the western blots needs to be improved*

We thank the reviewer for this suggestion. All figures have been updated to ensure uniform fonts, and the western blot images have been improved for size and resolution in the revised manuscript.

> *(d) Supplemental figures are very low resolution.*

High-resolution images have been provided.

> *(e) Please introduce abbreviations before using them (like LDLr and ND).*

The abbreviations have been explained.

> *(f) Figure 6A seems like data that belongs in the SI rather than the main text.*

We kept it in main figure as the knock-in mouse was generated for this study.

> *(a) Figure 7B needs a WT HFD control.*

It is a valid suggestion. However, it is known in the literature (we have prior experience as well) that wild-type mice do not exhibit a drastic increase in serum lipid level with high-fat diet feeding.

> *(b) The differences in Figure 7E are not profound and immediately obvious. Is there a better way to show the data, potentially with quantification?*

We agree, the difference is not that huge, we have provided the qualification as Fig. 7F.

> *(h) Figure panels 6E-H could use some labels to differentiate the data from WT vs p66ShcK81R mutant mice within the figure panel.*

We mentioned that on the top and have inserted a vertical line to separate the groups.

> *(i) Lines 165-168, 182-185, 214-218 and 282-286: These sentences can be rewritten for more clarity.*

We have modified these sentence for clarity.

> *(j) Line 512 should read, "Two-way ANOVA, ***P<0.001."*

Thank you, we have corrected the mistake.

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