# P66Shc Mediates SUMO2-induced Endothelial Dysfunction

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## Abstract

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. Here, we demonstrate that SUMO2 modifies p66Shc, which impairs endothelial function. In endothelial cells, knockdown of p66Shc blunted SUMO2-induced increases in reactive oxygen species and inflammation. Using multiple approaches, we show that p66Shc is a direct target of SUMO2. 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 (S36), causing it to translocate to the mitochondria, which are key steps regulating the oxidative function of p66Shc. Notably, sumoylation-deficient p66Shc (p66ShcK81R) was resistant to SUMO2-induced p66ShcS36 phosphorylation and mitochondrial translocation. Ingenuity pathway analysis showed that SUMO2ylation of p66ShcK81 regulated multiple pathways in endothelial cells, including Rac1-GTPase. Finally, to examine the physiological significance of this posttranslational modification *in vivo*, we generated p66ShcK81R knockin mice. Unlike wild-type mice, the aortic rings of p66ShcK81R knockin mice were resistant to SUMO2-induced endothelial dysfunction. Collectively, our work uncovers a posttranslational modification of redox protein p66Shc and identifies SUMO2-p66Shc signaling as a regulator of vascular endothelial function.

## Significance Statement

Endothelial cells control vascular function and health. Increased oxidative stress causes vascular endothelial dysfunction and promotes pathologies such as atherosclerosis and hypertension. SUMO2 and p66Shc increase production of reactive oxygen species and cause endothelial dysfunction through unknown mechanisms. We discovered that p66Shc mediates SUMO2-induced ROS production. In the CH2 domain, unique to p66Shc, SUMO2 modifies lysine-81 (K81) a conserved residue essential for SUMO2-induced oxidative activation and mitochondrial translocation of p66Shc and vascular endothelial dysfunction. Thus, the SUMO2-p66Shc axis regulates endothelial function.

## 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 mechanism 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). Yet, the mechanisms by which p66Shc regulates endothelial function, and how this can lead to pathological phenotypes remains unclear.

Here, we examined whether p66Shc mediates SUMO2-induced ED and oxidative stress. 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 and inflammation 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. 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). Increased ROS promotes inflammation (21), therefore, we examined endothelial inflammation when SUMO2 was overexpressed in HUVECs. Indeed, SUMO2 overexpression in HUVECs showed a significant increase in ICAM1 expression (Fig. S1). Further, we asked whether p66Shc mediates SUMO2-induced inflammation. In HUVECs, knockdown of p66Shc abrogated SUMO2-induced increases in ICAM1 levels (Fig. 1 D and E). Together, these findings suggest that p66Shc mediates the effects of SUMO2 on endothelial cells.

**p66Shc is directly modified by SUMO2**. We next asked if 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. S2). 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), 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. 2E). 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 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 identified 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). 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 (22, 23), 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 cells expressing p66ShcK81R were resistant to this increase (Fig. 4E and F). 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). We also verified that SUMO2-dependent regulation of p66ShcS36 phosphorylation is an upstream event by evaluating the effect of SUMO2 on p66Shc binding to prolyl isomerase (PIN1) and noted that SUMO2 does not affect p66Shc-PIN1 association (Fig. S4).

**Mitochondrial p66Shc is not SUMO2ylated.** Since promoting sumoylation increased mitochondrial levels of p66Shc, we examined whether sumoylated p66Shc translocates into mitochondria. To determine this, we generated an antibody using a commercial source (YenZym, CA) to detect sumoylated-p66Shc. This antibody was validated for its specificity in detecting sumoylated p66Shc in HUVECs (Fig. S5). We then assessed sumoylated-p66Shc in mitochondria by overexpressing SUMO2 with p66Shc in HUVECS. Overexpression of SUMO2 with p66Shc showed SUMO2ylated p66Shc in whole cell lysate, but not in the mitochondrial fraction despite the robust increase in mitochondrial levels of p66Shc that were induced by SUMO2 (Fig. 5E). Overall, these findings suggest that even though SUMO2 modification predisposes p66Shc for S36 phosphorylation and mitochondrial translocation, mitochondrial p66Shc is not sumoylated.

**Mice expressing non-sumoylatable p66ShcK81R are protected against SUMO2-induced ED**. To examine whether SUMO2ylation of p66ShcK81 affects endothelial control of vascular tone, we generated a mouse model with endogenous p66Shc mutated to p66ShcK81R (p66ShcK81R knockin mice). Using the CRISPR-Cas9 technique, a mutation was induced that caused arginine to be synthesized in place of lysine at position 81 in p66Shc (Fig. 6 A and B). The p66ShcK81R knockin mice had no alterations in the expression of p66Shc in the aorta (Fig. S6). 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. 6 C 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. 6 G and H). This suggests that the effect of SUMO2 on endothelial function is primarily mediated via p66ShcK81. Collectively, our study shows that p66Shc is required to mediate the effects of SUMO2 on vascular tone.

**SUMO2ylation of p66ShcK81 regulates endothelial cells via diverse molecular pathways**. 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 (Fig. S7). Comparative analysis of protein expression was performed against control (HUVECs infected with an equal number of adenovirsues expressing LacZ). Differential expression analysis revealed distinct protein profiles between p66ShcWT and p66ShcK81R. To gain insights into the biological significance of these changes, we preformed Ingenuity Pathway Analysis (IPA) to identify signaling pathways relevant to cardiovascular disease and cell signaling. Multiple pathways were significantly affected by the presence of SUMO2-p66Shc (Fig. 7). A majority of pathways were downregulated by SUMO2-p66Shc, and a few were upregulated, however these changes were normalized in cells expressing p66ShcK81R. We expanded this analysis to include all pathways in the IPA application. This showed additional

pathways that were downregulated and upregulated in SUMO2-p66ShcWT expressing cells. All of these pathways were normalized in SUMO2-p66ShcK81R expressing cells, except for *cardiac hypertrophy* and *Rho-GTPase signaling*, which were only partially rescued. Interestingly, in endothelial cells expressing p66ShcK81R the *cardiac hypertrophy* pathway was downregulated whereas the *C-type lectin receptors* and *Myelination signaling* pathways were upregulated (Fig. S8). This nuanced analysis provides a comprehensive understanding of the intricate network of pathways affected by SUMO2 modification of p66ShcK81 on p66Shc and highlights the specific impact of the p66ShcK81R mutant on these cellular processes.

## 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 specifically 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. In addition, aortic rings of mice expressing a non-sumoylatable form of (p66ShcK81R KI) were protected against SUMO2-induced ED, 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

p66Shc mediates the endothelial effects of SUMO2, 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, 23). 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 (Fig. 4) suggests the existence of such a crosstalk in regulating the oxidative function of p66Shc. The location of the phosphorylated p66ShcS36 protein band (close to 70kD) suggests that the pool of SUMO2-induced phosphorylated p66ShcS36 is not sumoylated. Therefore, it is likely that K81-SUMOylated and 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 that there are only 3 possible sumoylation motifs present in p66Shc, namely K81, K462, and K582, which can non-specifically be modified by SUMO1/2/3/4. Among these 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. Although other lysines may also get sumoylated, only a single band was noted in immunoblots of *in vitro* SUMO2ylated

p66ShcWT in HUVECs (Figs. 3E and 5E). This indicates that the presence of multiple higher molecular weight protein bands is not due to sumoylation at additional lysines. A better explanation for the presence of multiple higher molecular weight protein bands is that they are binding partners of p66Shc. p66Shc is an adaptor protein; thus it is plausible that sumoylation affects the interaction of p66Shc with other proteins that are also sumoylated. Identifying and characterizing these will be the focus of future studies.

SUMO2-induced increases in mitochondrial levels of p66Shc were dependent on its phosphorylation at S36, as the absence of SUMO2ylation due to expression of p66ShcK81R prevented S36 phosphorylation. SUMO2 did not affect p66Shc-PIN1 binding (Fig. S4), a step that is critical for isomerization and mitochondrial translocation of p66Shc (12). Also, SUMO2ylated p66Shc is not present in mitochondria (Fig. 5E). These findings are in line with that noted with p66ShcS36 phosphorylation (12), which suggest a post-translationally modified form of p66Shc does not translocate to mitochondria; instead it is the downstream modifications that likely facilitate this process. This indicates that facilitating S36 phosphorylation is an upstream mechanism regulating SUMO2-induced mitochondrial translocation of p66Shc.

To understand the mechanism by which SUMO2-p66ShcK81 regulates endothelial cells, we performed global proteomic analysis. Among several downregulated pathways, Rho-GTPase was highly affected by SUMO2-p66Shc (Fig. 7). Shc isoforms differentially participate in Rho-GTPase signaling: p66Shc binds to the RhoA/B/C family of proteins while p52Shc interacts with Ras and Rac (31). Interestingly, HUVECs expressing p66ShcWT showed an upregulation of RhoGDI signaling and downregulation of Rho-GTPase. RhoGDI negatively regulates Rho-GTPase signaling by binding Rho-GTPase and inhibiting its membrane translocation(32). Upregulation of Rho-GDI by p66Shc sumoylation could be another possible pathway by which p66Shc regulates Rho-GTPase signaling. Given the diversity of Rho family GTPases and their complexity, it is difficult to pinpoint the specific pathway being affected by SUMO2-p66Shc; however downregulation of Rho-GTPase was reduced and Rho-GDI signaling was upregulated in cells expressing SUMO2-p66ShcK81R, suggesting the possible involvement of p66ShcK81 in Rho-GTPase signaling. Although not determined here, it would be interesting to determine how SUMO2ylation of p66Shc affects Rho-GTPase signaling.

Additionally, it is noteworthy that cells expressing p66ShcK81R also elicit responses that are not significantly affected by SUMO2-p66Shc, such as activation of C-type lectin receptors and myelination signaling (Fig. S8). Both C-type lectin receptors and myelination signaling are known be active in endothelial cells(33, 34). Although, their relationship with p66ShcK81R will require future studies, preventing p66ShcK81 modification may activate these non-canonical pathways in p66Shc signaling.

Of all the posttranslational modifications on p66Shc, only S36 phosphorylation and K81 acetylation have been examined *in vivo* for their regulatory effects. Despite the robust ex vivo effects of S36 phosphorylation on the oxidative function of p66Shc, mutating S36 to alanine *in vivo* did not prevent p66Shc-dependent inhibition of transient receptor potential (TRPC) channel activity (35). It is worth noting that p66ShcK81R KI prevented diabetes (14) and SUMO2-induced ED(11) in mice. Thus, future studies investigating the effects of posttranslational modifications of p66Shc *in vivo* will help shed light on the pathophysiological significance of these modifications.

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. Prior studies have shown that K81 of p66Shc gets acetylated (14) and that this same lysine can be sumoylated and acetylated (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 it is highly unlikely there is any competition between acetylation and sumoylation for the same target residue. 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 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.

## Materials and Methods

Detailed methods are described in the Supplemental Appendix.

**Animals:** All experimental animal procedures were approved by the University of Iowa Institutional Animal Care and Use Committee and conformed to the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health. The p66ShcK81R knockin mice were generated with the help of the University of Iowa Genome Editing Facility. Wild type mice (C57Bl/6) were procured from the Jackson Laboratory.

**Statistics:** All data met assumptions of the statistical test and all statistical analyses were performed using GraphPad Prism 9 unless specified. Data represent the mean ± SEM of at least three independent assays. Significance of differences between two groups was determined using a two-tailed independent sample Student *t*-test. One-way ANOVA was used for comparing more than two groups and two-way ANOVA was used for comparing two variables simultaneously, with each test followed by multiple comparison analysis. Vascular reactivity data was analyzed by non-linear regression analysis (curve fit) and significance of differences was determined by comparing two curves for the fitting line. Results were considered significant if P values were $\leq$ 0.05.

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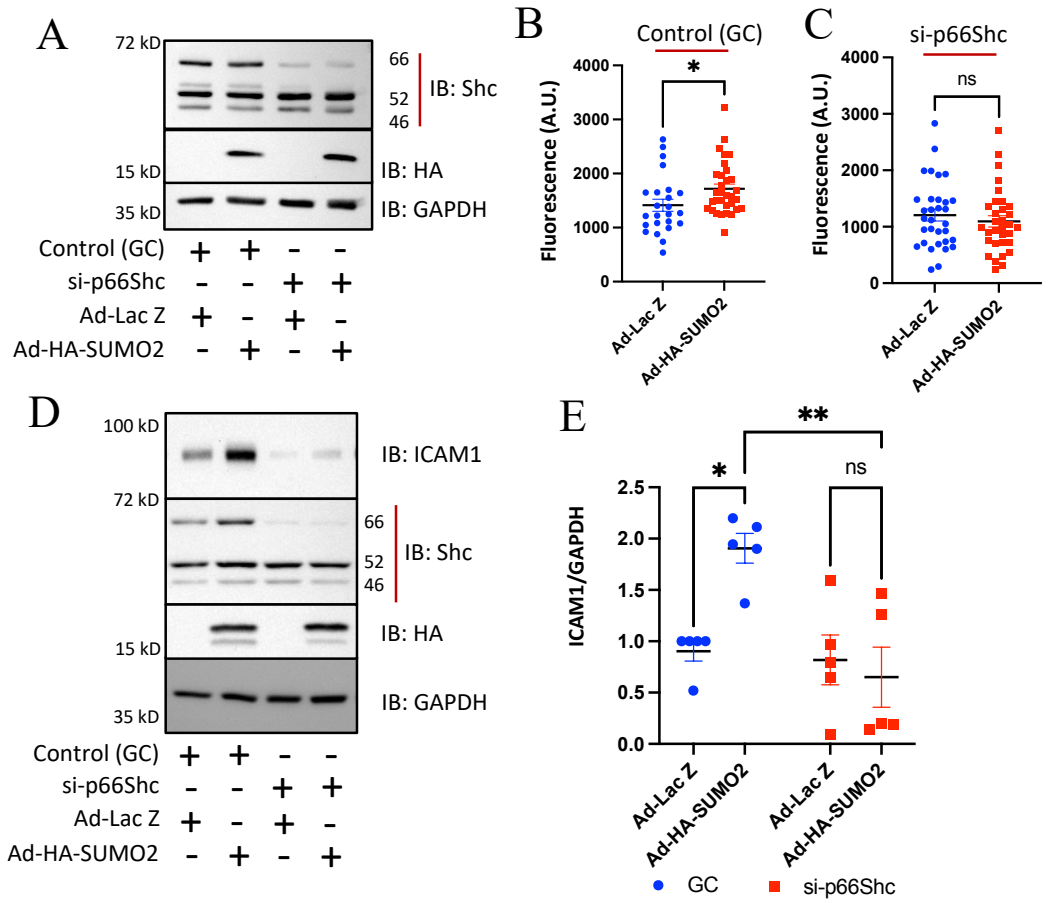


**Figure 1: p66Shc mediates SUMO2-induced ROS production and inflammation in endothelial cells.** (A) Immunoblot for Shc and SUMO2 expression in HUVECs following p66Shc knockdown (si-p66Shc) and SUMO2 overexpression (Ad-HA-SUMO2). (B and C) Quantification of ROS (MitoSOX fluorescence) in HUVECs from A. *P<0.05; n=24-32; student's t-test. (D) Immunoblot for ICAM1 expression following p66Shc knockdown (si-p66Shc) in HUVECs with and without SUMO2 overexpression (Ad-HA-SUMO2). (E) Quantification of ICAM1 from D. *<0.05, **P<0.01; n=3; 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; ICAM1, intercellular adhesion molecule 1.

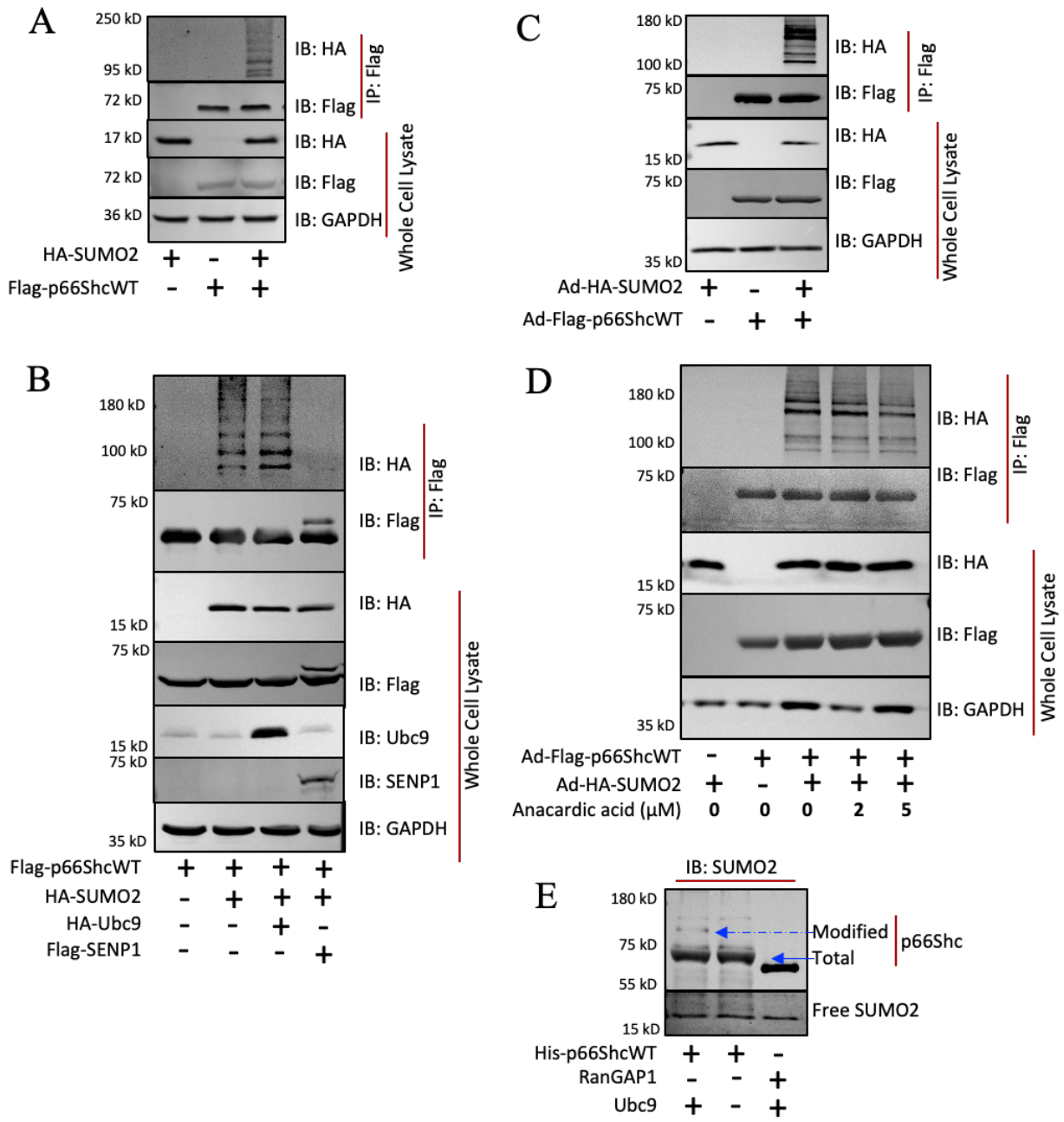


**Figure 2: SUMO2 directly modifies p66Shc.** (A) Immunoblot for sumoylated p66Shc in HEK-293 cells overexpressing SUMO2 (HA-SUMO2) and p66Shc (Flag-p66Shc). (B) Immunoblot for p66Shc sumoylation in HEK-293 cells overexpressing SUMO2 (HA-SUMO2) and p66Shc (Flag-p66Shc) with or without overexpression 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. (E) Immunoblot for sumoylated recombinant p66Shc in the presence of absence of Ubc9. Immunoblots are representative of at least three independent experiments.

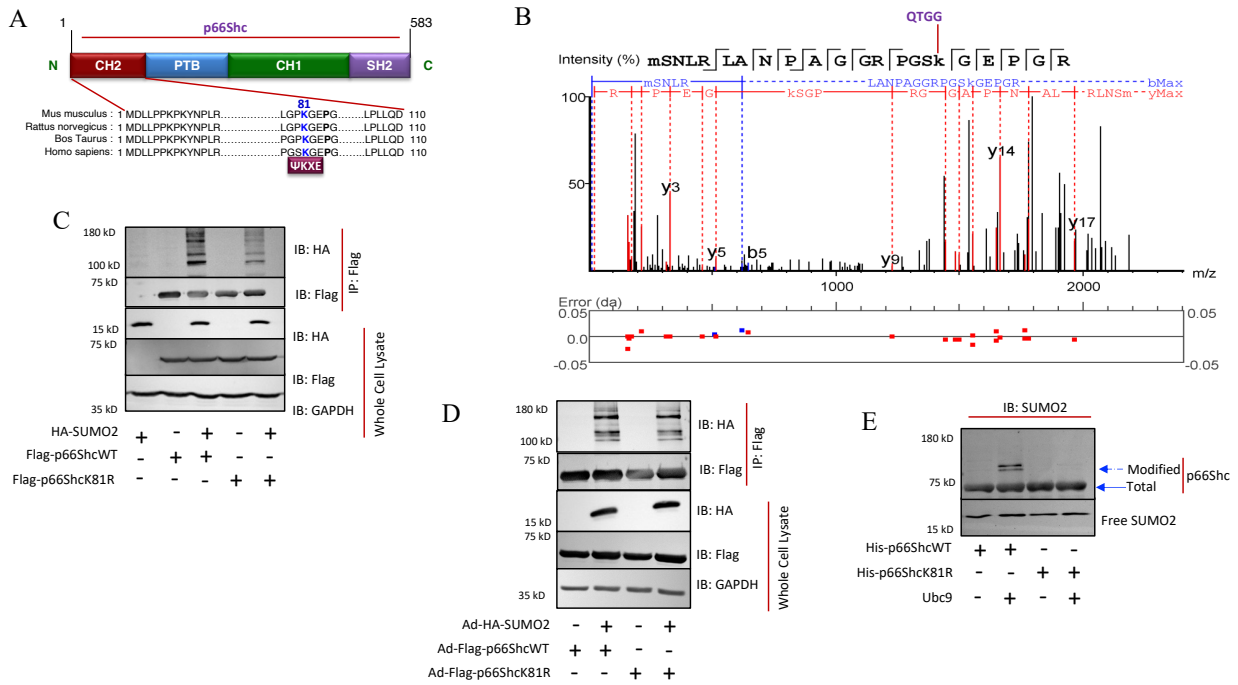


**Figure 3: SUMO2 modifies p66Shc at lysine-81.** (A) Modular structure of p66Shc showing conserved lysine-81 in the CH2 domain. (B) Tandem mass spectrometry of *in vitro SUMO*2ylated recombinant p66Shc. (C) Immunoblot showing sumoylation of p66Shc in HEK-293 cells overexpressing p66ShcWT (Flag-p66ShcWT) 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. Immunoblots are representative of at least three independent experiments. CH2, collagen homology 2; PTB, phosphotyrosine binding; CH1, Collagen homology 1; SH2, Src homology 2.

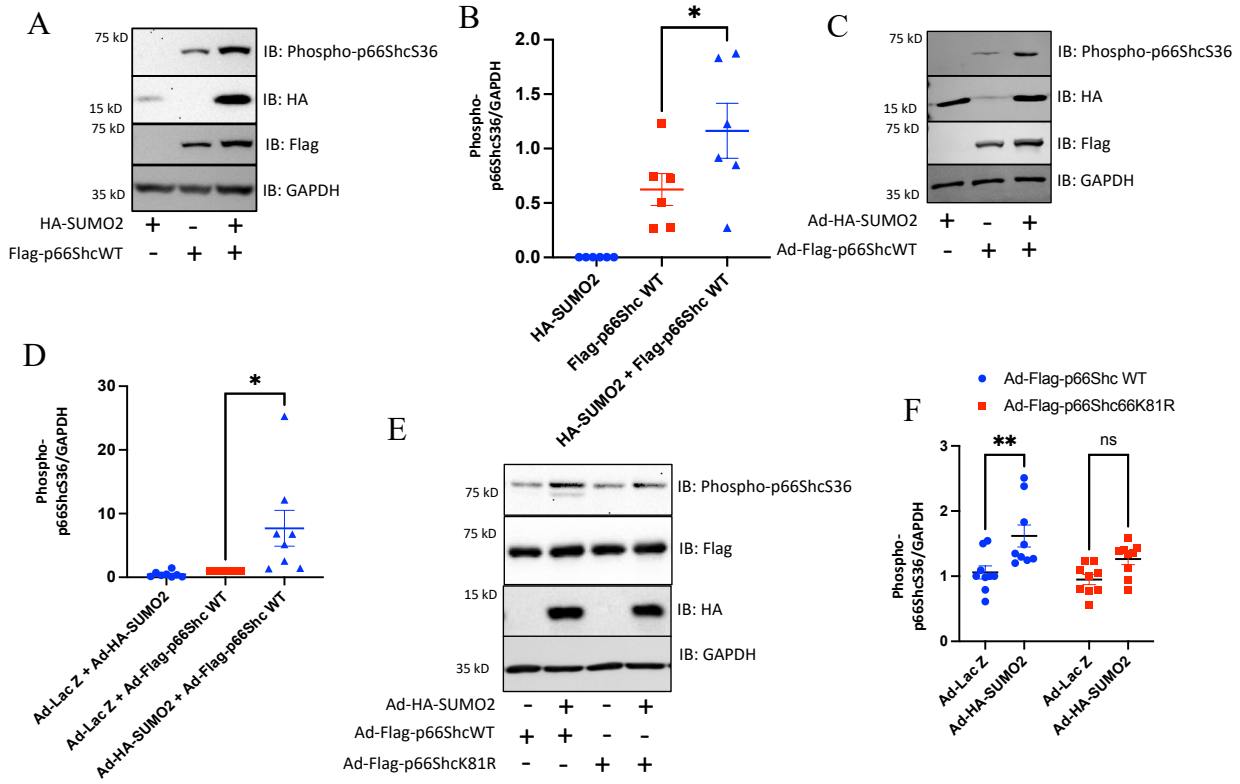


**Figure 4: SUMO2 promotes p66ShcS36 phosphorylation by sumoylation of K81.** (A) Immunoblot for p66ShcS36 phosphorylation in HEK-293 cells overexpressing SUMO2 (HA-SUMO2) and p66ShcWT (Flag-p66ShcWT). (B) Quantification of p66ShcS36 phosphorylation from A. *P<0.05; n=6; one-way ANOVA. (C) Immunoblot 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<0.01; n=6; two-way ANOVA. Immunoblots were quantified with iBright image analysis software. Data represents mean ±SEM. Immunoblots are representative of at least three independent experiments.

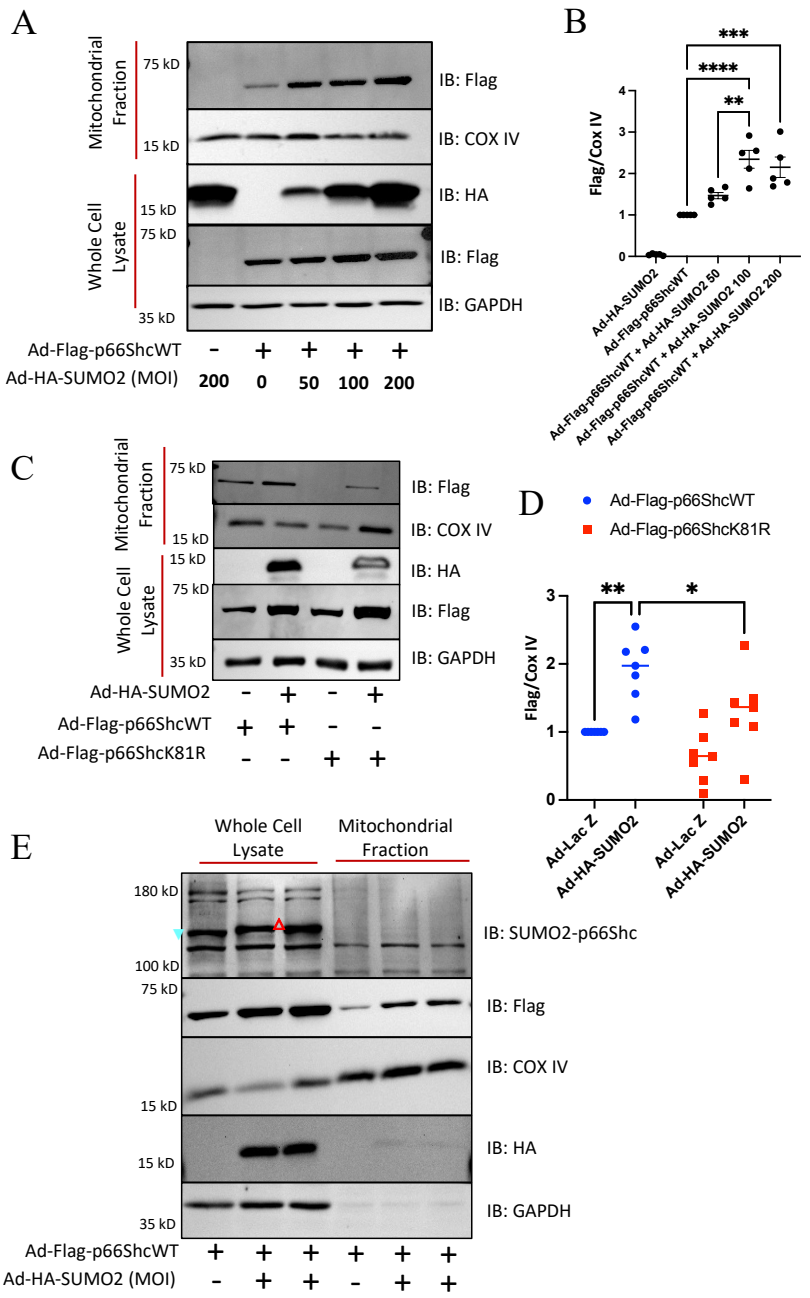


**Figure 5: p66ShcK81 SUMO2ylation of K81 in p66Shc promotes its translocation to the mitochondria.** (A) Immunoblot for p66ShcWT in whole cell lysates or the mitochondrial fraction of HUVECs ectopically expressing SUMO2 (Ad-HA-SUMO2) and p66ShcWT (Ad-Flag-p66ShcWT). (B) Quantification of the relative 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. (E) Immunoblot for SUMO2ylated p66Shc (SUMO2-p66Shc) in whole cell lysates and the mitochondrial fraction of HUVECs ectopically expressing SUMO2 (Ad-HA-SUMO2) and p66ShcWT (Ad-Flag-p66ShcWT). Cox

IV was used as mitochondrial marker. Data represents mean ±SEM. Immunoblots are representative of at least three independent experiments. ▼-Non-specific band, ▲-SUMO2-p66Shc.

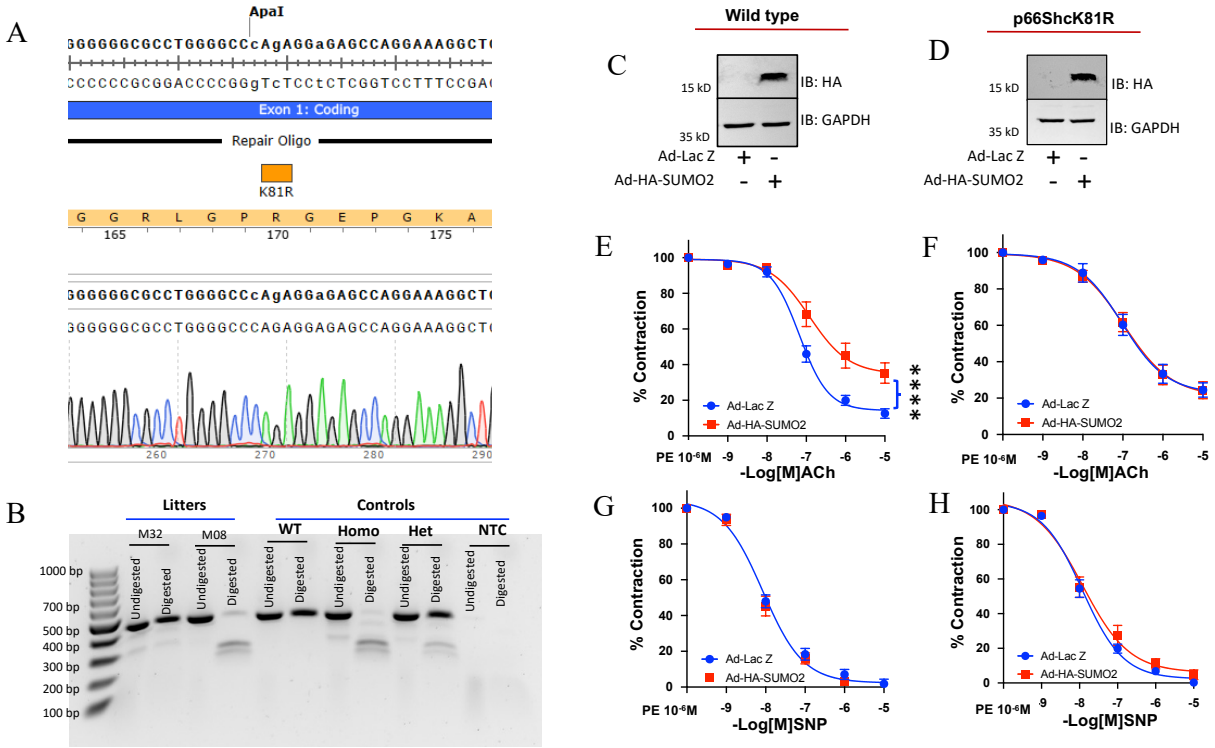


**Figure 6: p66ShcK81R knockin (KI) are resistant to SUMO2-induced endothelial dysfunction.** (A) DNA sequencing data from a p66ShcK81R knockin (KI) mouse showing a point mutation (A>G) resulting in an amino acid change (K→R), and insertion of silent mutation to create a digestion site for ApaI endonuclease 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 ($1.67X10^8$ 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. SUMO2-p66Shc induced changes in endothelial cells are rescued by p66ShcK81R.** Schematic representation of key regulatory pathways impacted by SUMO2-p66Shc in endothelial cells, rescued by p66ShcK81R. Ingenuity Pathway Analysis (IPA) was performed on relative protein expression data from HUVECs expressing SUMO2 with p66ShcWT (top) or p66ShcK81R (bottom) compared to those expressing Ad-Lac Z. The figure illustrates the significant reversal observed in key regulatory pathways between p66ShcWT and p66ShcK81R conditions. Data are derived from a sample size of n=6.