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SUMOylation Fine-Tunes Endothelial HEY1 in the Regulation of Angiogenesis

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Abstract

Background: Angiogenesis, which plays a critical role in embryonic development and tissue repair, is controlled by a set of angiogenic signaling pathways. As a transcription factor belonging to the basic helix-loop-helix family, HEY1 has been identified as a key player in developmental angiogenesis. However, the precise mechanisms underlying HEY1's actions in angiogenesis remain largely unknown. Our previous studies have suggested a potential role for post-translational SUMOylation in the dynamic regulation of vascular development and organization.

Methods: Immunoprecipitation, mass spectrometry and bioinformatics analysis were employed to determine the biochemical characteristics of HEY1 SUMOylation. The promoter-binding capability of HEY1 was determined by chromatin immunoprecipitation, dual luciferase and electrophoretic mobility shift assays. The dimerization pattern of HEY1 was determined by co-immunoprecipitation. The angiogenic capabilities of endothelial cells were assessed by CCK-8, EdU staining, wound healing, transwell and sprouting assays. Embryonic and postnatal vascular

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Author contributions

R. Ren, M. Simons, X. Wei and L. Yu conceived the project; R. Ren, Y. Wang, K. Ma, S. Ding, Y. Jiang , J. Chen, Y. Wang, Y. Kou, X. Wei and X. Fan performed the experiments; R. Ren, X. Wei and Y. Jiang performed the computational and bioinformatics analyses; R. Ren, S. Ding, X. Wei, X. Zhu, L. Qin, C. Qiu, M. Simons and L. Yu analyzed and interpreted the data; and R. Ren, X. Wei and L. Yu wrote the paper and edited the figures.

Disclosures

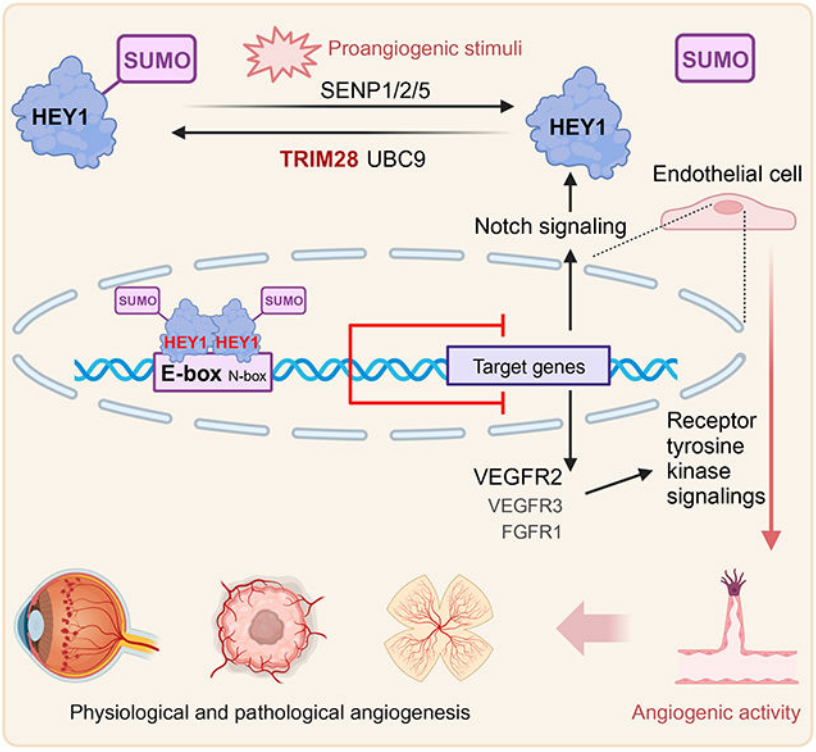
None.

growth in mice tissues, matrigel plug assay, cutaneous wound healing model, oxygen induced retinopathy model and tumor angiogenesis model were employed to investigate the angiogenesis in vivo.

Results: We identified intrinsic endothelial HEY1 SUMOylation at conserved lysines by TRIM28 as the unique E3 ligase. Functionally, SUMOylation facilitated HEY1-mediated suppression of angiogenic receptor tyrosine kinase signaling and angiogenesis in primary human endothelial cells and mice with endothelial cell-specific expression of wild-type HEY1 or a SUMOylation-deficient HEY1 mutant. Mechanistically, SUMOylation facilitates HEY1 homodimer formation, which in turn preserves HEY1's DNA binding capability via recognition of E-box promoter elements. Therefore, SUMOylation maintains HEY1's function as a repressive transcription factor controlling numerous angiogenic genes, including receptor tyrosine kinases and NOTCH pathway components. Pro-angiogenic stimuli induce HEY1 deSUMOylation, leading to heterodimerization of HEY1 with HES1, which results in ineffective DNA binding and loss of HEY1's angiogenesis-suppressive activity.

Conclusions: Our findings demonstrate that reversible HEY1 SUMOylation is a molecular mechanism that coordinates endothelial angiogenic signaling and angiogenesis, both in physiological and pathological milieus, by fine-tuning the transcriptional activity of HEY1. Specifically, SUMOylation facilitates the formation of the HEY1 transcriptional complex and enhances its DNA binding capability in endothelial cells.

Graphical Abstract



Keywords

SUMOylation; HEY1; angiogenesis; transcription

Subject Terms:

Cell Signaling/Signal Transduction; Basic Science Research

INTRODUCTION

Angiogenesis is a stepwise and fine-tuned process that involves the formation of new blood vessels from pre-existing ones[1, 2]. This process is essential during embryonic development and tissue injury repair in adults. However, angiogenesis also occurs in pathological processes, such as tumor growth and retinopathy of prematurity[3-5]. The initiation of angiogenesis is characterized by the migration and proliferation of endothelial cells[6]. Specifically, endothelial tip cells migrate to guide the sprout, while endothelial stalk cells proliferate to elongate the vessel branch[4, 7]. The selection and shuffling of tip-stalk cells is not genetically predetermined but is dynamically orchestrated by both positive and negative pathways. Among these pathways, tyrosine kinase receptor (typically represented by VEGF receptors and FGF receptors) signaling and NOTCH signaling play crucial roles[1].

The hairy/enhancer-of-split related with YRPW motif (HEY) family, along with the hairy and enhancer of split (HES) family, serves as a downstream transducer of NOTCH signaling. Members in this family are highly conserved and versatile to play key roles in cell fate decisions during development[8, 9]. The HEY family is classified as a group of repressive transcription factors (TF) and belongs to the basic helix-loop-helix (bHLH) class. This family is characterized by three conserved domains: the bHLH domain (which contains a basic domain for DNA binding and a helix-loop-helix motif for protein dimerization), the Orange domain, and the C-terminal YRPW motif[10-13]. Previous studies have illuminated the tissue-specific characteristics of HEY within the cardiovascular system[10, 14], specifically highlighting the critical role of HEY1 as a repressor of VEGFR2 in angiogenesis[9, 15, 16]. Nevertheless, the underlying mechanism governing HEY1's function in angiogenesis remains largely elusive.

Small ubiquitin-like modifier (SUMO) is a kind of small peptide that can be covalently conjugated to lysine residues located in SUMO modification motifs of substrate proteins following their translation[17, 18]. Analogous to ubiquitination, SUMOylation requires E1-activating enzymes (SAE1 and SAE2), E2-conjugating enzyme (UBC9) and various SUMO protein E3 ligases[19]. In contrast, SUMO deconjugation, known as deSUMOylation, is under the regulation of the SUMO-specific protease (SENP) family, making SUMOylation a dynamic and reversible process. The impact of SUMOylation and deSUMOylation is extensive, affecting the behavior of target proteins by altering their stability, subcellular localization, protein-DNA interactions, and other biological activities. This dynamic modification has been linked to a variety of physiological and pathophysiological events, with a particular focus on the modulation of nuclear activities such as cell cycle progression,

DNA damage repair, nuclear signaling, and gene transcription[17, 19, 20]. TFs represent a significant subset of SUMOylation substrates. Early studies on SUMOylation demonstrated the transcriptional regulation of several well-known TFs, such as RanGAP1, SP100, and PML, through SUMO modification[21]. Drawing on our previous discovery of the vital role of SUMOylation in the circulatory system, we have identified the regulatory machinery of SUMOylation in vascular pathological remodeling. This machinery functions by targeting a range of TFs from the GATA, NFkB, and HIF families[20, 22-24]. Regarding angiogenesis, we have discovered that SUMOylation is essential for coordinating the activation of VEGFRs and NOTCH receptors in endothelial cells[25, 26]. However, the specific contribution of SUMOylation to the nuclear events of receptor signaling remains unclear.

In our current study, we have identified TRIM28-mediated SUMOylation as a novel posttranslational modification of HEY1 that significantly impacts endothelial cell growth and angiogenesis. The SUMOylation and deSUMOylation of endothelial HEY1 regulate its dimerization and subsequent binding to the promoters of angiogenic genes, including those that encode receptor tyrosine kinases (RTKs) and NOTCH components.

METHODS

Data Availability

The data supporting the conclusions of this study are available from the corresponding author on reasonable requests.

Please refer to Supplemental Methods and the Major Resources Table in Supplemental Material for a detailed description of the Materials and Methods.

The RNA-sequencing data from human umbilical vein endothelial cells (HUVECs) and mouse lung endothelial cells (MLECs) are available in the National Center for Biotechnology Information (NCBI) Gene Expression Omnibus database (accession numbers GSE241150 and GSE241151). The ChIP-sequencing data from HUVECs are available in the NCBI Gene Expression Omnibus database (accession numbers GSE241148). And the mass spectrometry proteomics data from HUVECs are available in the iProX partner repository with the dataset identifier PXD044611.

RESULTS

SUMO modification of HEY1 in endothelial cells.

SUMOylation is a crucial post-translational modification that modulates the function of numerous transcription factors (TFs) and influences the expression of their target genes in a context-dependent manner[27]. To investigate whether HEY1 undergoes SUMOylation, we employed an exogenous system in 293T cells overexpressing Flag-HEY1 and HA-SUMO1 and detected an interaction between these proteins (Figure 1A and 1B). Moreover, endogenous HEY1 SUMOylation was detected in HUVECs (Figure 1C), HMVECs (Figure S1A) and isolated mouse lung endothelial cells (MLECs) (Figure 1D) by immunoprecipitation (IP). The HEY1 SUMOylation in HUVECs could be largely blocked by ML-792, an inhibitor for SUMO-activating enzyme (SAE) (Figure S1B). To gain

insights into the biochemical characteristics of HEY1 SUMOylation, we used bioinformatics analysis to identify putative SUMO-binding sites in HEY1 (Figure S1C). The IP assay showed that mutations in lysine residues at positions 82 (K82R) or 292 (K292R) slightly reduced the level of SUMOylated HEY1, while mutation at position 90 (K90R) markedly reduced it (Figure 1E). These lysine residues are highly conserved among vertebrates, suggesting that they may be critical for HEY1 SUMOylation (Figure 1F). Consistently, a triple mutant of HEY1 at these positions (HEY1-3KR) showed almost no SUMOylation when co-expressed with SUMO1 (Figure 1G).

Unlike the common E1 (SAE1, SAE2) and E2 (Ubc9) enzymes, distinct substrate proteins require different E3 ligases for their SUMOylation. In this regard, TRIM28, previously identified as a SUMO E3 ligase[28], emerged as one of the HEY1-interacting proteins in our immunoprecipitation-mass spectrometry (IP-MS) analysis (Figure S1D). The interaction between HEY1 and TRIM28 was confirmed by IP in 293T cells co-transfected with Flag-HEY1 and Myc-TRIM28 constructs (Figure 1H). Additionally, TRIM28 overexpression in 293T cells significantly enhanced HEY1 SUMOylation in the presence of the E2 enzyme UBC9 (Figure 1I), indicating that TRIM28 plays a role in HEY1 SUMOylation. The interaction between HEY1 and TRIM28 was confirmed in HUVECs (Figure 1J). Consistent with the findings in 293T cells, knockdown of TRIM28 in HUVECs reduced HEY1 SUMOylation (Figure 1K). Altogether, our findings identify TRIM28 as the primary E3 ligase responsible for HEY1 SUMOylation.

We investigated the role of SENPs in HEY1 de-SUMOylation to better understand the removal of SUMO moieties from HEY1. Our immunoprecipitation assay revealed that HEY1 associates with three major functional deSUMOylases in mammalian cells, namely SENP1, SENP2, and SENP5[29] (Figure S1E). Surprisingly, we found that overexpression of SENP1 and SENP2 significantly reduced the level of SUMOylated HEY1, whereas overexpression of SENP5 only slightly decreased it (Figure S1F through S1H). Furthermore, enhanced HEY1 SUMOylation was detected in SENP1-knockdown HUVECs (Figure S1I) and 293T cells overexpressing the SENP1-CA mutant (the catalytically inactive form of SENP1) (Figure S1F).

Moreover, we found that both hypoxia exposure and VEGF-A treatment, the major angiogenic stimuli in sprouting angiogenesis, decreased the level of HEY1 SUMOylation (Figure 1L and 1M). These results indicate that HEY1 is dynamically SUMOylated by TRIM28 E3 ligase and deSUMOylated by SENPs (Figure 1N), and that it may play a crucial role in angiogenic processes.

SUMOylation is vital for HEY1 to repress the angiogenic activity of endothelial cells.

To elucidate the biological function of HEY1 SUMOylation in endothelial cells, we investigated the angiogenic capabilities of primary HUVECs and HMVECs transduced with adenovirus-delivered HEY1-WT or the SUMOylation-deficient mutant HEY1-3KR. During sprouting angiogenesis, endothelial cells migrate and proliferate to form new vessels. Therefore, we first assessed the role of HEY1 SUMOylation in regulating the proliferation and migration ability of endothelial cells. CCK-8 assay and EdU staining assay revealed that overexpression of HEY1-WT disrupted endothelial cell growth, while

overexpression of HEY1-3KR partially rescued this proliferation retardation (Figure 2A and 2B; Figure S2A and S2B). Similarly, results from wound healing and Transwell assay showed that endothelial cells expressing HEY1-WT exhibited significant loss of migration ability, whereas the HEY1-3KR group had even more migrated cells than the control group (Figure 2C and 2D; Figure S2C and S2D). Next, a sprouting assay was employed to assess the effect of HEY1 SUMOylation on the sprouting ability of endothelial cells. We found that compared to the control group, the sprouting length was significantly reduced in endothelial cells transduced with HEY1-WT, while endothelial cells expressing HEY1-3KR exhibited only a slight reduction in the sprouting length (Figure 2E; Figure S2E). Moreover, overexpression of HEY1-WT significantly inhibited tube formation in endothelial cells, whereas the HEY1-3KR group exhibited similar tube formation ability to that observed in the control group (Figure 2F; Figure S2F). Collectively, these findings indicate that SUMOylation endows HEY1 with the ability to suppress endothelial cell angiogenic function.

Notably, previous studies have shown that HEY1 is a major downstream transducer of NOTCH signaling and transcriptionally represses VEGFR2[30]. We aimed to investigate whether SUMOylation is critical for HEY1-mediated transcriptional regulation of angiogenesis in endothelial cells. To address this question, we performed RNA-seq to assess the repressive landscape of HEY1-WT and HEY1-3KR. Interestingly, there were 1841 differentially expressed genes (DEGs) between the HEY1-WT and HEY1-3KR groups (Figure S3A). Gene ontology (GO) analysis of downregulated genes by HEY1-WT and HEY1-3KR, respectively, suggested a potential difference between their target genes (Figure S3B and S3C). HEY1-WT played the canonical role of HEY1 in repressing genes associated with angiogenesis, while SUMOylation-deficient HEY1 (HEY1-3KR) seemed to target genes related to the cell cycle and DNA repair. Additionally, gene set enrichment analysis (GSEA) results suggested that HEY1-WT inhibited angiogenesis, whereas HEY1-3KR facilitated endothelial cell migration (Figure 2G). Specifically, HEY1-WT had a negative feedback effect on NOTCH signaling and suppressed not only VEGFR2 signaling but also most of the angiogenic receptor tyrosine kinase (RTK) signaling (Figure 2G). Focusing on the specific genes that HEY1-WT repressed, as shown in Figure 2H, we found that RTKs such as VEGFR2, VEGFR3, FGFR1, and TIE1 were transcriptionally repressed by HEY1-WT but not by HEY1-3KR. Moreover, HEY1-3KR appeared to lose the feedback effect on NOTCH signaling because it had a poor ability to transcriptionally repress DLL4, JAG2, NOTCH1, and RBPJ. The qPCR and immunoblotting results confirmed the importance of HEY1 SUMOylation in repressing RTKs and NOTCH components in three types of primary human endothelial cells (HUVECs, HMVECs and HAECs) (Figure 2I through 2K; Figure S4A through S4D).

To further determine the role of HEY1 SUMOylation in regulating transcription of the target genes, TRIM28 overexpression was employed to enhance the SUMOylation of HEY1, which aggravated the repression activity of HEY1 on endothelial proliferation, migration and tube formation (Figure S5A through S5C). Correspondingly, overexpressing TRIM28 led to further repressed mRNA and protein levels of angiogenic RTKs (VEGFR2, VEGFR3 and FGFR1) (Figure S5D), while knocking down TRIM28 could partially rescue the repression on angiogenic RTKs induced by HEY1 overexpression (Figure S5E). In contrast,

ML-792, an inhibitor of SUMO-activating enzyme (SAE), attenuated the repressive activity of HEY1 on its target genes including VEGFR2, VEGFR3 and FGFR1 at both mRNA and protein levels (Figure S5F). Moreover, hypoxia, the major angiogenic stimulus that has been shown to induce HEY1 deSUMOylation (Figure 1L), partially rescued the repression of HEY1 on angiogenic RTKs and major members involved in NOTCH pathway including RBPJ and NOTCH1 (Figure S5G).

To further examine the important role of HEY1 SUMOylation in specifying angiogenic signaling in response to angiogenic stimuli, we evaluated signal transduction in classical angiogenic receptor tyrosine kinase (RTK) pathways in endothelial cells. To this end, HMVECs were treated with VEGF-A and bFGF, separately, after overnight serum starvation (Figure 3A and 3B); and serum-starved human dermal lymphatic endothelial cells, HDLECs, were treated with VEGF-C (Figure 3C). We found that HEY1-WT impaired the phosphorylation of VEGFR2, FGFR1, and VEGFR3, as well as the activation of their downstream effectors PLCγ, AKT, and ERK, while overexpression of HEY1-3KR only had slight effects on these pathways. These data indicate that SUMOylation maintains the function of endothelial HEY1 as a transcriptional repressor for angiogenic RTK signaling and completes the effective negative feedback loop of NOTCH signaling.

SUMOylation facilitates the promoter-binding capability of HEY1 to preserve its transcriptional repressive activity.

Given the downregulation of HEY1 SUMOylation upon exposure to proangiogenic stimuli in endothelial cells and the potential repressive activity of HEY1 SUMOylation in angiogenesis, we aimed to determine the mechanisms by which SUMOylation and deSUMOylation regulate HEY1.

The effects of SUMOylation on target proteins are broad-ranging and include alterations in cellular localization, protein stability, protein-protein interactions, and protein-DNA interactions. Therefore, we explored whether SUMOylation might regulate the function of HEY1 via these mechanisms. Immunofluorescence analysis in HUVECs revealed that SUMOylation-deficient HEY1 (HEY1-3KR) exhibited a nuclear distribution pattern similar to that of HEY1-WT (Figure S6A). Regarding HEY1's stability, the half-lives of HEY1-WT and HEY1-3KR were determined in the presence of the protein synthesis inhibitor cycloheximide (CHX). Our immunoblot analysis demonstrated that HEY1-3KR had a shorter half-life than HEY1-WT (Figure S6B). We further found that both HEY1-WT and HEY1-3KR were degraded via the proteasome, as indicated by their rescuing degradation by MG132 treatment (Figure S6C). However, the ineffective repression of target genes by HEY1-3KR was not due to its instability, as blocking its degradation process with MG132 did not restore its repressive effect on target genes (Figure S6D and S6E).

The RNA-seq results suggested that compared to cells expressing HEY1-WT, cells expressing HEY1-3KR could decrease the number of repressive targets and the fold changes in the repression of target genes (Figure S7A and S7B). Furthermore, only 567 common downregulated genes were found between ECs expressing HEY1-WT and ECs expressing HEY1-3KR (Figure S7C). A large proportion of genes downregulated by HEY1-WT was kept unchanged or only slightly downregulated by HEY1-3KR (Figure S7D). These findings

suggest that SUMOylation deficiency might weaken the repressive transcriptional activity of HEY1 on these genes. Additionally, the quantitative IP-MS results in HEY1-WT- and HEY1-3KR-expressing endothelial cells indicated that 55 proteins had reduced binding with HEY1-3KR, with the majority associated with DNA binding (Figure S7E and S7F). These results together suggest that SUMOylation may be critical for the DNA-binding capability of HEY1 to enhance its transcriptional repressive activity.

To confirm our speculation, we conducted ChIP assays in HUVECs. Genome-wide mapping of the HEY1 binding profile by ChIP-seq identified differential binding events between HEY1-WT- and HEY1-3KR-expressing cells, with 85% peaks increased in HEY1-WT-expressing cells but only 15% peaks increased in HEY1-3KR-expressing cells (Figure 4A). Specifically, binding events of HEY1-WT prominently occurred in adjacent transcriptional start sites (TSSs), while HEY1-3KR showed a wider genomic binding region (Figure 4B). In addition, compared to HEY1-WT, binding events of HEY1-3KR showed a decrease in adjacent TSSs (Figure 4C). Interestingly, although both HEY1-WT and HEY1-3KR had binding events in promoter regions, there was an obvious loss of promoter-binding capability in the HEY1-3KR group (Figure 4D). These results revealed that deSUMOylation reduces the DNA-binding capability of HEY1, particularly its promoter-binding capability.

Consistent with our previous findings, many binding events were associated with genes previously repressed by HEY1, such as VEGFR2, FGFR1, and VEGFR3 (Figure 4E). Notably, ChIP-qPCR results revealed that SUMOylation deficiency of HEY1 reduced its binding to FGFR1/VEGFR3 promoter, which may result in a reduction of HEY1 repression on FGFR1/VEGFR3 transcription (Figure 4F). However, the binding to the VEGFR2 promoter was increased with the presence of HEY1-3KR. To investigate the specific regulatory mechanism of HEY1 SUMOylation in HEY1-mediated repression of VEGFR2, we performed a dual luciferase assay by co-expressing HEY1-WT/HEY1-3KR with a firefly luciferase reporter plasmid containing the VEGFR2 promoter (−116 to +268) and a Renilla luciferase control plasmid in 293T cells. Similar to the previous results, SUMOylation significantly enhanced HEY1-mediated transcriptional repression of the VEGFR2 promoter (Figure 4G). We then investigated whether the binding motif of HEY1 on the chromosome was altered by deSUMOylation.

Previous studies have shown that HEY1 binds to E-box motifs (CANNTG) in the promoters of its target genes[11, 31]. Our motif analysis revealed that deSUMOylation of HEY1 decreased its recognition of the E-box motif (the number of targeted E-box sequences was significantly reduced in the HEY1-3KR ChIP-seq samples compared to the HEY1-WT samples) (Figure 4H). However, the promoter motifs bound by HEY1-3KR included the N-box motif (CACNAG), which was not found among the HEY1-WT binding motifs (Figure 4H). Interestingly, there were nearly as many N-box motifs as E-box motifs in the VEGFR2 promoter region, whereas there were fewer N-box motifs in the FGFR1/VEGFR3 promoter region (Figure 4I). The binding preference of HEY1 between E-box and N-box motifs was validated by EMSA, which showed that HEY1-WT bound to more probes containing the E-box sequence than did HEY1-3KR, while HEY1-3KR bound to more probes with the N-box sequence than did HEY1-WT (Figure 4J; Online Figure VII.G). According to the motif identification in the VEGFR2 promoter regions as shown in Figure 4I, we then constructed

a firefly luciferase reporter plasmid containing a 12 x E-box or a 9 x N-box motif to compare HEY1 activity toward these two motifs. The dual luciferase assay demonstrated that compared to HEY1-WT, HEY1-3KR partially lost its transcriptional repression activity toward the 12 x E-box motif (Figure 4K). Neither HEY1-WT nor HEY1-3KR repressed transcription by binding to the 9 x N-box motif (Figure 4K). Furthermore, mutations in the E-box motifs in the VEGFR2 promoter resulted in the loss of transcriptional repression activity of HEY1 (Figure 4L), indicating that the E-box motif is crucial for HEY1 to transcriptionally repress VEGFR2. Accordingly, we conclude that although SUMOylation deficiency increases HEY1 binding to the VEGFR2 promoter via recognition of the N-box motif, this binding is not effective for HEY1 to repress target genes.

SUMOylation deficiency disrupts the dimerization pattern of HEY1.

Previous studies have shown that the choice between forming homodimers or heterodimers with other proteins in the bHLH family can influence HEY1's recognition of different binding motifs[9, 15, 32]. Accordingly, one of the biological functions of SUMOylation is to alter protein-protein interactions. An abnormal dimerization pattern caused by SUMOylation deficiency could explain why HEY1-3KR partially lost its promoter-binding capability. Therefore, we investigated whether SUMOylation has an impact on HEY1 dimerization.

Enhancing HEY1 SUMOylation by co-expressing UBC9 and TRIM28 in 293T cells increased HEY1 homodimer formation (Figure S8A). Additionally, co-immunoprecipitation results in HUVECs and 293T cells co-expressing Flag-HEY1-WT/3KR and HA-HEY1-WT/3KR revealed that it became difficult for HEY1-3KR to form homodimers, and even the dimer formation of HEY1-WT with HEY1-3KR decreased, compared to the formation of HEY1-WT homodimers (Figure 5A; Figure S8B). We then generated a molecular docking model of HEY1-HEY1 homodimer (HEY1 protein represented by the bHLH domain) (Figure S8C). In HEY1-WT homodimer, four hydrogen bonds were formed, and the two HEY1-WT monomers were spatially symmetric. However, in the HEY1-3KR homodimer, the number of hydrogen bonds decreased, and the two HEY1-3KR monomers were not spatially symmetric. These data suggest that SUMOylation might facilitate HEY1 homodimer formation. Furthermore, IP-MS data revealed that HEY2, another transcription factor of the HEY family, was among the proteins with increased binding to HEY1-WT compared to HEY1-3KR (Figure S8D). Consistent with the MS results, immunoblot analysis in HUVECs and 293T cells confirmed that HEY1's SUMOylation deficiency reduced the formation of HEY1-HEY2 heterodimers (Figure 5B; Figure S8E). As HEY1 homodimers primarily recognize E-box promoter elements, the decreased formation of HEY1 homodimer or HEY1-HEY2 heterodimer caused by SUMOylation deficiency could lead to decreased recognition of E-box promoter elements.

Moreover, IP-MS data revealed an increased formation of HEY1-HES1 heterodimers in the HEY1-3KR group (Figure S8D) , which was validated by co-immunoprecipitation in HUVECs and 293T cells co-expressing Flag-HEY1-WT/3KR and HA-HES1 (Figure 5C; Figure S8F). The SENP inhibitor NEM, which restricted HEY1 deSUMOylation, attenuated the interaction between Flag-HEY1 and HA-HES1 (Figure S8G). Interestingly, RNA-seq results showed that HES1 itself was a transcriptional repression target of HEY1 in a manner

dependent on HEY1 SUMOylation (Figure 2H), which was further validated by qPCR and immunoblot analysis in both HUVECs and HMVECs (Figure 5D and 5E; Figure S8H and S8I). Accordingly, co-immunoprecipitation in HUVECs revealed only slight association between HEY1-WT and endogenous HES1, accompanied by inhibited HES1 expression, whereas HEY1-3KR could still form heterodimers with HES1 (Figure 5F). Treatment with NEM further blocked the HEY1-HES1 interaction in HUVECs (Figure S8J). Thus, HEY1 SUMOylation is required for the formation of HEY1 homodimers and HEY1-HEY2 heterodimers, as well as for limiting the HEY1-HES1 interaction and inhibiting HES1 expression.

It has been reported that the association of HEYs with other proteins disrupts their recognition of E-box motifs. Additionally, as another important downstream target of the NOTCH pathway, HES1 binds to N-box motifs in the promoters of target genes[33, 34]. Our molecular docking model further revealed that the DNA-binding sites of HEY1-HES1 heterodimers were entirely different from those of HEY1 homodimers (Figure S8K). Therefore, we focused our investigation on HEY1-HES1 heterodimers to elucidate the mechanism of HEY1's motif recognition using the promoter region of VEGFR2 as an example. The chromatin immunoprecipitation (ChIP) assay revealed that overexpression of HEY1-WT decreased HES1 occupancy on the VEGFR2 promoter, whereas overexpression of HEY1-3KR increased HES1 occupancy (Figure S8L). We then examined whether changes in the HEY1-HES1 interaction affected HEY1's motif recognition. Knocking down HES1 in HUVECs resulted in a decrease of the protein level of HES1 (Figure S8M). And electrophoretic mobility shift assay (EMSA) results revealed that without HES1, HEY1-3KR no longer had an advantage in binding to probes containing N-box sequences compared to HEY1-WT (Figure 5G; Figure S8N), which subsequently led to a decrease in the occupancy of HEY1-3KR on the VEGFR2 promoter (Figure 5H). Since the binding of HEY1-3KR to N-box had no effect on its transcriptional repression activity, knockdown of HES1 certainly had no effect on the transcriptional repression activity of HEY1-3KR towards N-box (Figure S8O).Based on these findings and our earlier discovery of SUMOylation-deficient HEY1 having greater binding to the VEGFR2 promoter, we concluded that SUMOylation prevents HEY1 from recognizing N-box motifs via inhibiting its binding to HES1 (Figure 5I).

SUMOylation facilitates endothelial HEY1 in the repression of developmental angiogenesis.

To investigate the role of endothelial HEY1 SUMOylation in vivo, we used the H11 locus knock-in strategy to generate mice with endothelial cell-specific HEY1-WT/HEY1-3KR knock-in (Figure S9A). These mice were viable and fertile, and Cdh5-Cre^{ERT2} mice were used as controls. The expression of HEY1-WT/3KR was confirmed by qPCR and immunoblot in primary cultured mouse lung endothelial cells (MLECs) and in P7 neonatal retinas (Figure S9B and S9C) and then validated in P7 neonatal retinas using immunostaining (Figure S9D). The specific expression of Flag tagged HEY1-WT and HEY-3KR in endothelial cells was firstly examined in the bone marrows form of HEY1-WT$^{iEC\text{-}KI}$ and HEY1-3KR$^{iEC\text{-}KI}$ mice, in which Flag tagged HEY1-WT or HEY1-3KR were completely co-localized with endothelial cells that marked with CD31, but not expressed in

other cells (Figure S9E). In addition, both HEY1-WT and HEY1-3KR expression is limited in endothelial cells but not other cells, such as vascular smooth muscle cells, as evidenced at mRNA level by real-time qPCR and at protein level by immunoblot assays (Figure S9F through S9H).

During embryonic development, HEY1-WT knock-in did not result in developmental arrest or embryonic lethality, but it did lead to delayed vascular development compared to the control and HEY1-3KR^{KI} groups (Figure S10A and S10B). This highlights the essential role of HEY1 SUMOylation in angiogenesis. Next, we investigated the effect of HEY1 SUMOylation on postnatal angiogenesis. Staining of postnatal day 6 (P6) and day 10 (P10) brains showed a reduction of vascular area in HEY1-WT$^{iEC\text{-}KI}$ pups compared to the control and HEY1-3KR$^{iEC\text{-}KI}$ groups (Figure S10C and S10D). Similarly, reduced angiogenesis was observed in P6 and P10 lungs and hearts in HEY1-WT$^{iEC\text{-}KI}$ pups compared to the control and HEY1-3KR$^{iEC\text{-}KI}$ groups (Figure S10E through S10H). Whole-mount staining of P7 retinas showed that endothelial knock-in of HEY1-WT delayed the expansion of the vascular plexus to the periphery in retinas (Figure 6A). In addition, vessel coverage area, number of branch points, vessel length and number of tip cells were significantly reduced in HEY1-WT$^{iEC\text{-}KI}$ retinas, while knock-in of SUMOylation-deficient HEY1 had only a slight effect on neonatal retinal development (Figure 6B). The number of proliferative ECs also dramatically reduced in HEY1-WT$^{iEC\text{-}KI}$ retinas, but partially recovered in HEY1-3KR$^{iEC\text{-}KI}$ retinas (Figure 6C and 6D). The effect of HEY1 SUMOylation on postnatal retinal angiogenesis was also examined in P4 and P10 pups (Figure S11A through S11D). Overall, the results were similar to those in P7 neonatal retinas, but in P10 retinas, HEY1-3KR expression resulted in further suppressed angiogenesis in deep plexus development. Though HEY1 knock-in resulted in reduced vessel coverage area, number of branch points, vessel length and number of tip cells, which led to delayed vessel development in retinas, HEY1-WT $^{iEC\text{-}KI}$ pups injected with ML-792 showed similar retinal angiogenesis to the control pups, which suggested that delayed angiogenesis by HEY1 knock-in could be rescued by ML-792 (Figure S11E and S11F). Moreover, we constructed Hey1$^{iEC\text{-}KO}$ mice and compared retinal angiogenesis in P7 pups between Hey1$^{iEC\text{-}KO}$ and HEY1-3KR$^{iEC\text{-}KI}$ mice (Figure S11G and S11H). The vascular development in HEY1-3KR$^{iEC\text{-}KI}$ P7 retinas was similar to that in Hey1$^{iEC\text{-}KO}$ P7 retinas. There was no obvious difference in the vessel coverage area, number of branch points, vessel length and number of tip cells between the two groups. Therefore, SUMOylation deficiency might at least result in partial loss-of-function of HEY1 in angiogenesis.

According to our in vitro results in primary endothelial cells (Figure 3), HEY1 SUMOylation impairs angiogenesis by repressing major RTKs and their downstream pathways. Therefore, the phosphorylation of VEGFR2 was examined in P7 retinas. An immunofluorescence signal of phosphorylated VEGFR2 was observed in the angiogenic front, especially in retinal tip cells, in control and HEY1-3KR$^{iEC\text{-}KI}$ mice, but it was obscure and limited to only a few endothelial cells in the vasculature of HEY1-WT$^{iEC\text{-}KI}$ mouse retinas (Figure 6E and 6F). Blocking the phosphorylation of VEGFR2 by SKLB610 (an inhibitor of p-VEGFR2) significantly retarded vascular development in HEY1-3KR$^{iEC\text{-}KI}$ retinas, but had little effect on HEY1-WT$^{iEC\text{-}KI}$ retinas (Figure 6G and 6H). These findings suggest a HEY1 SUMOylation/VEGFR2 axis in regulating angiogenesis. Overall, these data

reveal that SUMOylation facilitates the ability of HEY1 to suppress angiogenesis during both embryonic and postnatal development.

To investigate the role of endothelial HEY1 SUMOylation in VEGF/FGF-mediated angiogenesis and lymphangiogenesis in vivo, we used a subcutaneous Matrigel plug assay. Matrigel plugs containing PBS, VEGF-A, bFGF or VEGF-C were implanted by subcutaneous injection into control (Cdh5-Cre^{ERT2} mice), HEY1-WT$^{iEC\text{-}KI}$ or HEY1-3KR$^{iEC\text{-}KI}$ mice. The degree of angiogenesis/lymphangiogenesis induced by a single growth factor was determined by stereomicroscopy (Online Figure S12A). Vascular or lymphatic vessels were also visualized using immunofluorescence staining with anti-CD31 or anti-LYVE1 antibodies (Figure S12B and S12C), and EC proliferation in Matrigel plugs induced by VEGF-A was accessed by Ki-67 staining (Figure S12D and S12E). In these assays, VEGF-A and bFGF resulted in profound local angiogenesis, and VEGF-C led to an intense local induction of lymphangiogenesis in the implanted Matrigel plugs in control mice. However, the responses to these three growth factors and VEGF-A-induced EC proliferation were significantly reduced in the plugs in HEY1-WT$^{iEC\text{-}KI}$ mice but not in the plugs in HEY1-3KR$^{iEC\text{-}KI}$ mice. Therefore, these in vivo data indicate that SUMOylation maintains the function of endothelial HEY1 as a repressive transcription factor to inhibit angiogenesis and lymphangiogenesis.

SUMOylation of HEY1 in endothelial cells compromises pathological angiogenesis.

The effects of HEY1 SUMOylation on physiological angiogenesis prompted us to evaluate whether HEY1 SUMOylation might be suppressive in pathological angiogenesis. To this end, we firstly employed the mouse model of oxygen-induced retinopathy (OIR) model, which mimics retinopathy of prematurity (ROP) in clinic, represented by pathological intravitreal neovascularization and consequent visual impairment in childhood[5, 35,40]. In this study, mouse pups were exposed to 75% oxygen from P7 to P12 and returned to normoxic conditions until sacrificed for analyses at P17 (Figure 7A), corresponding to a phase of vessel obliteration caused by hyperoxia, followed by the exuberant growth of abnormal vessels as a result of hypoxia. HEY1-WT$^{iEC\text{-}KI}$ retinas showed less surface occupied by neovascular tufts and more avascular area than the control and HEY1-3KR$^{iEC\text{-}KI}$ retinas (Figure 7A and 7B). Moreover, phosphorylation of VEGFR2 and EC proliferation were reduced in HEY1-WT$^{iEC\text{-}KI}$ retinas (Figure 7C and 7D), similar to what was observed in developmental angiogenesis (Figure 6C through 6F). Quantification of preretinal neovascular tufts indicate that HEY1 SUMOylation, or expression of HEY1-WT, is protective against excessive neovascularization in the mouse OIR model (Figure 7E and 7F).

To further determine the effect of HEY1 SUMOylation on pathological angiogenesis, we examined whether HEY1-WT knock-in could affect tumor angiogenesis and consequent tumor growth. Compared with the control and HEY1-3KR$^{iEC\text{-}KI}$ mice, an inhibition of tumor growth was observed in HEY1-WT$^{iEC\text{-}KI}$ mice (Figure 7G), accompanied by a reduction in tumor angiogenesis as evidenced by CD31-labeled microvessels in tumors (Figure 7H). There were no differences between HEY1-WT$^{iEC\text{-}KI}$ and the control/ HEY1-3KR$^{iEC\text{-}KI}$ mice in terms of the amount of NG2 positive pericytes, α-smooth muscle actin

(α-SMA) positive pericyte coverage along tumor vessels, and collagen IV positive basement membrane (BM) coverage (Figure 7H).

Moreover, to investigate the role of endothelial HEY1 SUMOylation in reparative angiogenesis in vivo, cutaneous wound healing model was employed. Compared with mice in the control group and HEY1-3KR$^{iEC\text{-}KI}$ group, HEY1-WT$^{iEC\text{-}KI}$ mice exhibited a significant decrease in wound closure rate and CD31+ capillary density in the wounded tissues (Figure S12F and S12I). Taken together, these results suggested that HEY1 SUMOylation may play a potential role in compromising pathological angiogenesis.

SUMOylation facilitates the transcriptional repressive activity of HEY1 in vivo.

The effect of HEY1 SUMOylation on angiogenesis was further examined in MLECs isolated from Hey1$^{iEC\text{-}KO}$ HEY1-WT$^{iEC\text{-}KI}$, HEY1-3KR$^{iEC\text{-}KI}$ mice (with Hey1$^{iEC\text{-}KO}$ mice used as control). Similar to the results in primary endothelial cells including HUVECs and HMVECs (Figure 2; Figure S2), knock-in of HEY1-WT repressed the proliferation, migration, sprouting and tube formation capability of MLECs, while knock-in of HEY1-3KR had much milder repression on MLEC proliferation and sprouting, but had no effect on the migration and tube formation capability of MLECs (Figure S13A through S13D). RNA-seq suggested that there could be more than 2,000 differently expressed genes (DEGs) between HEY1-WT$^{iEC\text{-}KI}$ and HEY1-3KR$^{iEC\text{-}KI}$ MLECs, with 13579 genes upregulated and 15565 genes down regulated by HEY1-WT$^{iEC\text{-}KI}$ (Figure S13E). In addition, GO analysis of genes identified as downregulated by HEY1-WT knock-in revealed a correlation to angiogenesis and Notch signaling pathway, which was consistent with the RNA-seq data in HUVECs (Figure S13F). The DEGs between the two groups were further showed by heatmap in Figure S13G. A mass of genes was down-regulated in HEY1-WT$^{iEC\text{-}KI}$ MLECs compared with HEY1-3KR$^{iEC\text{-}KI}$ MLECs. Moreover, consistently, angiogenic RTKs and major Notch components were downregulated in HEY1-WT$^{iEC\text{-}KI}$ MLECs compared to HEY1-3KR$^{iEC\text{-}KI}$ MLECs (Figure S13H). The downregulation of angiogenic RTKs and major Notch components induced by HEY1-WT knock-in was further validated by qPCR and immune blot (Figure 8A). The binding preference of HEY1 between E-box and N-box motifs was examined in MLECs isolated from HEY1-WT$^{iEC\text{-}KI}$ and HEY1-3KR$^{iEC\text{-}KI}$ mice, and the EMSA results further validated the binding preference of HEY1-WT for E-box and the preference of HEY1-3KR for N-box (Figure 8B). These results together suggested that SUMOylation could facilitate the transcriptional repressive activity of HEY1 in MLECs and further validated the action mechanism of HEY1 SUMOylation in vivo.

DISCUSSION

HEY1, a repressive TF of the bHLH family, is well known for its role in NOTCH signaling. However, the action mechanism of HEY1 in angiogenesis is still unclear. In the present study, we identified that HEY1 coordinated both positive (VEGF and FGF pathways) and negative (NOTCH pathway) signaling pathways involved in endothelial cell growth, thus functionally regulating angiogenesis in a SUMOylation/deSUMOylation dependent manner. Mechanistically, SUMOylation-maintained HEY1 homodimer changes to a HEY1-

HES1 heterodimer due to deSUMOylation induced by proangiogenic stimuli. SUMOylation deficiency alters the promoter element recognition and DNA binding capability of HEY1, weakening HEY1's transcriptional repression activity toward its target genes, including RTKs and NOTCH pathway components (Figure 8C).

SUMOylation is regarded as a regulator of gene expression because hundreds of TFs are targets of posttranslational SUMO modification. In this study, we identified HEY1 as a SUMO-modified TF and found the responsiveness of HEY1 SUMOylation to angiogenic stimuli, indicating the physiological role of HEY1 SUMOylation in adaptation to different angiogenic contexts. Specifically, the dynamic SUMO modification of HEY1 is accomplished by the SUMO E3 ligase TRIM28 and SENP deSUMOylases. While the role of SENPs in deSUMOylation is clear, the E3 ligases participating in SUMOylation are diverse. The tripartite motif (TRIM) family is a group of ubiquitin E3 ligases, but among these proteins, tripartite motif-containing protein 28 (TRIM28) has also been identified as a SUMO E3 ligase for proteins such as CD9 and PCNA [36, 37]. We found that TRIM28 associates with HEY1 to drive HEY1 SUMOylation. Interestingly, co-expression of TRIM28 and UBC9 dramatically promoted HEY1 SUMOylation, while overexpression of TRIM28 alone only slightly elevated HEY1 SUMOylation, indicating that TRIM28 facilitates HEY1 SUMOylation by interacting with UBC9.

HEY1 is a classical transcriptional repressor involved in cell fate decision and differentiation. Previous research has shown that HEY1 undergoes ubiquitination and phosphorylation to play roles in Kaposi's sarcoma-associated herpesvirus reactivation and tumor cell growth, respectively[38, 39]. In this study, we discovered that SUMOylation is a novel posttranslational modification of HEY1. Our investigation revealed that HEY1 is regularly modified in a SUMO conjugation-preferred manner during its cellular function. Specifically, SUMOylation mainly affects transcriptional complex formation and consequent DNA binding capability. We found that SUMOylation ensures the formation of HEY1 homodimers, which are necessary for HEY1 to recognize the correct sequence of the E-box promoter element and act as a repressive TF by mediating dimerization through the interaction surface in the helix-loop-helix (HLH) domain. This facilitates the DNA binding capability of HEY1 by directing the adjacent basic domain. DeSUMOylation alters the binding of HEY1, promoting heterodimerization with HES1 and leading to recognition of N-box promoter elements in target DNA, resulting in ineffective DNA binding and loss of repression of angiogenic genes. This mechanism may also apply to other TFs in the bHLH family and the basic domain superclass due to the common DNA binding pattern shared by these family members. It is also important to examine the crosstalk between SUMOylation and ubiquitination/phosphorylation of HEY1 in future studies since certain modification sites are close to each other.

HEY1 is known to play an important role in developmental angiogenesis by regulating VEGFR2 expression in endothelial cells, as previously recognized [32, 40, 41]. However, our RNA sequencing and protein assays revealed that the target genes of endothelial HEY1 are not limited to VEGFR2, but rather include major endothelial receptor tyrosine kinases (RTKs) and NOTCH signaling components. Intriguingly, we found that HEY1 requires SUMOylation for transcriptional repression of these RTKs and their

downstream signal transduction, which is essential for inhibition of angiogenesis and lymphangiogenesis. Moreover, our study demonstrated that HEY1 SUMOylation undergoes quick downregulation upon proangiogenic stimuli of hypoxia exposure and VEGF-A treatment, which are known to activate VEGFR signaling in endothelial cells. Importantly, the protein level of HEY1 remained unchanged. We also observed a decrease in mRNA and protein levels of members involved in NOTCH signaling upon HEY1-WT overexpression, indicating a negative feedback loop in NOTCH signaling based on SUMOylated HEY1. Together with the fact that deSUMOylation removes the block on RTK signaling, HEY1 may act as a key node that regulates the crosstalk between NOTCH and VEGFR signaling pathways. Reversible HEY1 SUMOylation may therefore represent an essential regulatory mechanism that orchestrates angiogenic signaling for endothelial cell growth during angiogenesis.

In our previous study, we revealed that SENP1-regulated SUMOylation played a positive role in framing angiogenesis by targeting on NOTCH signaling in endothelial cells, in which NOTCH1 was the SUMOylation substrate in the membrane receptor complex. Upon hypoxia, SUMOylation facilitates NOTCH1 cleavage, N1ICD stability and subsequent transcriptional complex formation, while it suggested that SUMOylation might also be involved in regulating other NOTCH pathway components in angiogenesis[25]. According to those clues, the current study further identified HEY1 as the major SUMOylation target in nuclei of endothelial cells. Interestingly, SUMOylation of HEY1 modulates a mutual interaction between NOTCH receptor complex and transcription complex, and in turn coordinates the quiescence of RTK signaling and endothelial angiogenesis, while deSUMOylation removes the blockage in the presence of SENP1 as an intrinsic rheostat of these processes. Therefore, dynamic SUMOylation/deSUMOylation can systematically reconcile NOTCH signaling in different spatio-temporal scenarios in endothelial cells, which may broaden our understanding of the fine tuning of molecular networking during angiogenesis.

Beside the function in physiological angiogenesis, SUMOylation of HEY1 is also indispensable in angiogenesis in pathological states, restricting angiogenesis in tumor growth, oxygen-induced retinopathy (OIR) and wound healing. This specifies the role of endothelial SUMOylation in pathogenesis of tumor progression and metastasis, and aberrant retinal neovascularization-related ocular diseases that account for a major cause of blindness worldwide[42, 43]. Similar to the classic regulatory mechanism in tumor angiogenesis, hypoxia-VEGF/VEGFR axis drives the aberrant retinal neovascularization, represented by the intravitreal neovascularization in retinopathy of prematurity and OIR. Müller cells and astrocytes in the retina were shown to overproduce VEGFA and an increase of VEGFR2 level was observed in retinal endothelium during these disease progressions in both clinical specimens and animal models[44-49]. Intriguingly, knock-down of VEGFR2 in endothelial cells significantly reduced IVNV but partially allowing physiological vascular growth of the peripheral retina[50]. Considering the specific role of HEY1 SUMOylation in limiting pathological angiogenesis by targeting VEGFR, it could be a potential therapeutic approach for inhibiting excessive angiogenesis in ocular diseases, tumor growth and other related diseases.

In summary, our results establish reversible SUMOylation as a molecular mechanism that coordinates endothelial angiogenic signaling and angiogenesis by modulating the transcriptional regulatory activity of HEY1. SUMOylation/deSUMOylation fine-tunes the formation of the HEY1 transcriptional complex and its DNA binding capability in response to angiogenic stimuli in endothelial cells. These findings expand our understanding of the orchestration of transcriptional regulation in different physiological or pathological states. Moving forward, we plan to investigate the role of SUMOylation and related post-translational modifications (PTMs) in bHLH TF-regulated nonvascular biological events, specifically tumor cell growth, where HEY1 functions as an oncogene rather than a repressive transcription factor.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Nonstandard Abbreviations and Acronyms

bHLH	basic helix-loop-helix
ChIP-seq	chromatin immunoprecipitation sequencing
EdU	5-ethynyl-2-deoxyuridine
HDLEC	human dermal lymphatic endothelial cell
HES1	hairy and enhancer of split 1
HEY1	hairy/enhancer-of-split related with YRPW motif 1
HMVEC	human microvascular endothelial cell
HUVEC	human umbilical vein endothelial cell
IP-MS	immunoprecipitation-mass spectrometry
IVNV	intravitreal neovascularization
MLEC	mouse lung endothelial cell
OIR	oxygen-induced retinopathy

RNA-seq	RNA sequencing
ROP	Retinopathy of prematurity
RTK	receptor tyrosine kinase
SENP	sentrin-specific protease
SUMO	small ubiquitin-like modifier
TRIM28	tripartite motif containing 28
VSMC	vascular smooth muscle cell

References

1. Blanco R, Gerhardt H. Vegf and notch in tip and stalk cell selection. Cold Spring Harb Perspect Med. 2013;3:a006569 [PubMed: 23085847]
2. Chen W, Xia P, Wang H, Tu J, Liang X, Zhang X, et al. The endothelial tip-stalk cell selection and shuffling during angiogenesis. J Cell Commun Signal. 2019;13:291–301 [PubMed: 30903604]
3. Aspalter IM, Gordon E, Dubrac A, Ragab A, Narloch J, Vizan P, et al. Alk1 and alk5 inhibition by nrp1 controls vascular sprouting downstream of notch. Nat Commun. 2015;6:7264 [PubMed: 26081042]
4. Welch-Reardon KM, Ehsan SM, Wang K, Wu N, Newman AC, Romero-Lopez M, et al. Angiogenic sprouting is regulated by endothelial cell expression of slug. J Cell Sci. 2014;127:2017–2028 [PubMed: 24554431]
5. Ramshekar A, Hartnett ME. Vascular endothelial growth factor signaling in models of oxygen-induced retinopathy: Insights into mechanisms of pathology in retinopathy of prematurity. Front Pediatr. 2021;9:796143 [PubMed: 34956992]
6. Chang GH, Lay AJ, Ting KK, Zhao Y, Coleman PR, Powter EE, et al. Arhgap18: An endogenous inhibitor of angiogenesis, limiting tip formation and stabilizing junctions. Small GTPases. 2014;5:1–15
7. Yokota Y, Nakajima H, Wakayama Y, Muto A, Kawakami K, Fukuhara S, et al. Endothelial ca 2+ oscillations reflect vegfr signaling-regulated angiogenic capacity in vivo. Elife. 2015;4
8. Taylor KL, Henderson AM, Hughes CC. Notch activation during endothelial cell network formation in vitro targets the basic hlh transcription factor hesr-1 and downregulates vegfr-2/kdr expression. Microvasc Res. 2002;64:372–383 [PubMed: 12453432]
9. Noguchi YT, Nakamura M, Hino N, Nogami J, Tsuji S, Sato T, et al. Cell-autonomous and redundant roles of hey1 and heyl in muscle stem cells: Heyl requires hes1 to bind diverse DNA sites. Development. 2019;146
10. Fischer A Hey genes in cardiovascular development. Trends in Cardiovascular Medicine. 2003;13:221–226 [PubMed: 12922017]
11. Heisig J, Weber D, Englberger E, Winkler A, Kneitz S, Sung WK, et al. Target gene analysis by microarrays and chromatin immunoprecipitation identifies hey proteins as highly redundant bhlh repressors. PLoS Genet. 2012;8:e1002728 [PubMed: 22615585]
12. Buas MF, Kabak S, Kadesch T. The notch effector hey1 associates with myogenic target genes to repress myogenesis. J Biol Chem. 2010;285:1249–1258 [PubMed: 19917614]
13. Villaronga MA, Lavery DN, Bevan CL, Llanos S, Belandia B. Hey1 leu94met gene polymorphism dramatically modifies its biological functions. Oncogene. 2010;29:411–420 [PubMed: 19802006]
14. Woltje K, Jabs M, Fischer A. Serum induces transcription of hey1 and hey2 genes by alk1 but not notch signaling in endothelial cells. PLoS One. 2015;10:e0120547 [PubMed: 25799559]
15. Fischer A, Gessler M. Delta-notch--and then? Protein interactions and proposed modes of repression by hes and hey bhlh factors. Nucleic Acids Res. 2007;35:4583–4596 [PubMed: 17586813]

16. Maier MM, Gessler M. Comparative analysis of the human and mouse hey1 promoter: Hey genes are new notch target genes. Biochem Biophys Res Commun. 2000;275:652–660 [PubMed: 10964718]
17. Sriramachandran AM, Dohmen RJ. Sumo-targeted ubiquitin ligases. Biochim Biophys Acta. 2014;1843:75–85 [PubMed: 24018209]
18. Varejao N, Lascorz J, Li Y, Reverter D. Molecular mechanisms in sumo conjugation. Biochem Soc Trans. 2020;48:123–135 [PubMed: 31872228]
19. Hay RT. Sumo-specific proteases: A twist in the tail. Trends Cell Biol. 2007;17:370–376 [PubMed: 17768054]
20. Qiu C, Wang Y, Zhao H, Qin L, Shi Y, Zhu X, et al. The critical role of senp1-mediated gata2 desumoylation in promoting endothelial activation in graft arteriosclerosis. Nat Commun. 2017;8:15426 [PubMed: 28569748]
21. Hay RT. Sumo: A history of modification. Mol Cell. 2005;18:1–12 [PubMed: 15808504]
22. Yu L, Ji W, Zhang H, Renda MJ, He Y, Lin S, et al. Senp1-mediated gata1 desumoylation is critical for definitive erythropoiesis. J Exp Med. 2010;207:1183–1195 [PubMed: 20457756]
23. Shao L, Zhou HJ, Zhang H, Qin L, Hwa J, Yun Z, et al. Senp1-mediated nemo desumoylation in adipocytes limits inflammatory responses and type-1 diabetes progression. Nat Commun. 2015;6:8917 [PubMed: 26596471]
24. Bae SH, Jeong JW, Park JA, Kim SH, Bae MK, Choi SJ, et al. Sumoylation increases hif-1alpha stability and its transcriptional activity. Biochem Biophys Res Commun. 2004;324:394–400 [PubMed: 15465032]
25. Zhu X, Ding S, Qiu C, Shi Y, Song L, Wang Y, et al. Sumoylation negatively regulates angiogenesis by targeting endothelial notch signaling. Circ Res. 2017;121:636–649 [PubMed: 28760777]
26. Zhou HJ, Xu Z, Wang Z, Zhang H, Zhuang ZW, Simons M, et al. Sumoylation of vegfr2 regulates its intracellular trafficking and pathological angiogenesis. Nat Commun. 2018;9:3303 [PubMed: 30120232]
27. Rosonina E A conserved role for transcription factor sumoylation in binding-site selection. Curr Genet. 2019;65:1307–1312 [PubMed: 31093693]
28. Neo SH, Itahana Y, Alagu J, Kitagawa M, Guo AK, Lee SH, et al. Trim28 is an e3 ligase for arf-mediated npm1/b23 sumoylation that represses centrosome amplification. Molecular and Cellular Biology. 2015;35:2851–2863 [PubMed: 26055329]
29. Geiss-Friedlander R, Melchior F. Concepts in sumoylation: A decade on. Nat Rev Mol Cell Biol. 2007;8:947–956 [PubMed: 18000527]
30. Jin Y, Kaluza D, Jakobsson L. Vegf, notch and tgfbeta/bmps in regulation of sprouting angiogenesis and vascular patterning. Biochem Soc Trans. 2014;42:1576–1583 [PubMed: 25399573]
31. Holderfield MT, Henderson Anderson AM, Kokubo H, Chin MT, Johnson RL, Hughes CC. Hesr1/chf2 suppresses vegfr2 transcription independent of binding to e-boxes. Biochem Biophys Res Commun. 2006;346:637–648 [PubMed: 16782059]
32. Weber D, Wiese C, Gessler M. Hey bhlh transcription factors. Curr Top Dev Biol. 2014;110:285–315 [PubMed: 25248480]
33. Liu ZH, Dai XM, Du B. Hes1: A key role in stemness, metastasis and multidrug resistance. Cancer Biol Ther. 2015;16:353–359 [PubMed: 25781910]
34. Rani A, Greenlaw R, Smith RA, Galustian C. Hes1 in immunity and cancer. Cytokine Growth Factor Rev. 2016;30:113–117 [PubMed: 27066918]
35. Xu M, Jiang Y, Su L, Chen X, Shao X, Ea V, et al. Novel regulators of retina neovascularization: A proteomics approach. J Proteome Res. 2022;21:101–117 [PubMed: 34919406]
36. Ma X, Yang T, Luo Y, Wu L, Jiang Y, Song Z, et al. Trim28 promotes hiv-1 latency by sumoylating cdk9 and inhibiting p-tefb. Elife. 2019;8
37. Li M, Xu X, Chang CW, Liu Y. Trim28 functions as the sumo e3 ligase for pcna in prevention of transcription induced DNA breaks. Proc Natl Acad Sci U S A. 2020;117:23588–23596 [PubMed: 32900933]

38. Gould F, Harrison SM, Hewitt EW, Whitehouse A. Kaposi's sarcoma-associated herpesvirus rta promotes degradation of the hey1 repressor protein through the ubiquitin proteasome pathway. J Virol. 2009;83:6727–6738 [PubMed: 19369342]
39. Lopez-Mateo I, Arruabarrena-Aristorena A, Artaza-Irigaray C, Lopez JA, Calvo E, Belandia B. Hey1 functions are regulated by its phosphorylation at ser-68. Biosci Rep. 2016;36
40. Fischer A, Schumacher N, Maier M, Sendtner M, Gessler M. The notch target genes hey1 and hey2 are required for embryonic vascular development. Genes Dev. 2004;18:901–911 [PubMed: 15107403]
41. Watanabe Y, Seya D, Ihara D, Ishii S, Uemoto T, Kubo A, et al. Importance of endothelial hey1 expression for thoracic great vessel development and its distal enhancer for notch-dependent endothelial transcription. J Biol Chem. 2020;295:17632–17645 [PubMed: 33454003]
42. Geisen P, Peterson LJ, Martiniuk D, Uppal A, Saito Y, Hartnett ME. Neutralizing antibody to vegf reduces intravitreous neovascularization and may not interfere with ongoing intraretinal vascularization in a rat model of retinopathy of prematurity. Mol Vis. 2008;14:345–357 [PubMed: 18334951]
43. Budd SJ, Thompson H, Hartnett ME. Association of retinal vascular endothelial growth factor with avascular retina in a rat model of retinopathy of prematurity. Arch Ophthalmol. 2010;128:1014–1021 [PubMed: 20697002]
44. Bai YY, Ma JX, Guo JJ, Wang JJ, Zhu M, Chen Y, et al. Muller cell-derived vegf is a significant contributor to retinal neovascularization. J Pathol. 2009;219:446–454 [PubMed: 19768732]
45. Robbins SG, Rajaratnam VS, Penn JS. Evidence for upregulation and redistribution of vascular endothelial growth factor (vegf) receptors flt-1 and flk-1 in the oxygen-injured rat retina. Growth Factors. 1998;16:1-+ [PubMed: 9777366]
46. Gonzalez CR, Diaz CM, Garreton CR. Anti-vascular endothelial growth factor (vegf) drugs compared to laser photocoagulation for treatment of type 1 retinopathy of prematurity. Medwave. 2022;22:e8507 [PubMed: 35100244]
47. McCloskey M, Wang HB, Jiang YC, Smith GW, Strange J, Hartnett ME. Anti-vegf antibody leads to later atypical intravitreous neovascularization and activation of angiogenic pathways in a rat model of retinopathy of prematurity. Invest Ophth Vis Sci. 2013;54:2020–2026
48. McColm J, Geisen P, Hartnett M. Vegf isoforms and their expression after a single episode of hypoxia or repeated fluctuations between hyperoxia and hypoxia: Relevance to clinical rop. Molecular Vision. 2004;10:512–520 [PubMed: 15303088]
49. Sankar MJ, Sankar J, Chandra P. Anti-vascular endothelial growth factor (vegf) drugs for treatment of retinopathy of prematurity. Cochrane Database Syst Rev. 2018;1:CD009734 [PubMed: 29308602]
50. Simmons AB, Bretz CA, Wang HB, Kunz E, Hajj K, Kennedy C, et al. Gene therapy knockdown of vegfr2 in retinal endothelial cells to treat retinopathy (vol 21, pg 751, 2018). Angiogenesis. 2018;21:765–765 [PubMed: 29943214]
51. Li RQ, Li YR, Kristiansen K, Wang J. Soap: Short oligonucleotide alignment program. Bioinformatics. 2008;24:713–714 [PubMed: 18227114]
52. Kim D, Landmead B, Salzberg SL. Hisat: A fast spliced aligner with low memory requirements. Nat Methods. 2015;12:357–U121 [PubMed: 25751142]
53. Langmead B, Trapnell C, Pop M, Salzberg SL. Ultrafast and memory-efficient alignment of short DNA sequences to the human genome. Genome Biol. 2009;10
54. Pitulescu ME, Adams RH. Regulation of signaling interactions and receptor endocytosis in growing blood vessels. Cell Adh Migr. 2014;8:366–377 [PubMed: 25482636]
55. Tovchigrechko A, Vakser IA. Gramm-x public web server for protein-protein docking. Nucleic Acids Res. 2006;34:W310–314 [PubMed: 16845016]
56. Zhu X, Qiu C, Wang Y, Jiang Y, Chen Y, Fan L, et al. Fgfr1 sumoylation coordinates endothelial angiogenic signaling in angiogenesis. Proc Natl Acad Sci U S A. 2022;119:e2202631119 [PubMed: 35733256]
57. Pitulescu ME, Schmidt I, Benedito R, Adams RH. Inducible gene targeting in the neonatal vasculature and analysis of retinal angiogenesis in mice. Nat Protoc. 2010;5:1518–1534 [PubMed: 20725067]

Novelty and Significance

What Is Known?

- As a major member of the basic helix-loop-helix family, HEY1 is a repressive transcription factor downstream of Notch signaling.
- HEY1 plays an important role in development and cell fate decision.
- The mechanisms of action of HEY1 in angiogenesis remains largely elusive.

What New Information Does This Article Contribute?

- Reversible HEY1 SUMOylation is an intrinsic molecular mechanism active in coordinating endothelial angiogenic signalings, both receptor tyrosine kinases pathway and NOTCH pathway.
- SUMOylation/deSUMOylation fine-tunes the formation of the HEY1 transcriptional complex and its DNA binding capability in response to angiogenic stimuli in endothelial cells.
- The SUMOylation-dependent regulatory mechanism may be applicable to other transcription factors in the basic helix-loop-helix family and even the basic domain transcription factor superfamily because of the common DNA binding pattern shared by these family members.

Angiogenesis is critical for embryonic development, tissue homeostasis and also pathological tissue growth. The neovascularization is a stepwise process dynamically orchestrated by both positive and negative signalings in endothelial cells. HEY1 is a classical transcriptional repressor involved in development and cell differentiation. However, the underlying mechanisms governing HEY1's function in angiogenesis remain unclear. The present study identifies TRIM28-mediated SUMOylation as a novel posttranslational modification of HEY1. In a SUMOylation/deSUMOylation dependent manner, HEY1 coordinates both positive (VEGF and FGF pathways) and negative (NOTCH pathway) signaling pathways involved in endothelial cell growth, thus functionally regulating both physiological and pathological angiogenesis. SUMOylation-maintained HEY1 homodimer formation tends to be altered to HEY1-HES1 heterodimer due to HEY1 deSUMOylation upon proangiogenic stimuli. This deSUMOylation alters the promoter element recognition and DNA binding capability of HEY1 and consequently abolishes HEY1's transcriptional repression activity toward its target genes, including those encoding receptor tyrosine kinases (VEGFR2, FGFR1 and VEGFR3) and NOTCH pathway components. These findings reveal the precise function of HEY1 in controlling neovascularization and indicate the underappreciated role of HEY1 beyond the downstream transducer of the NOTCH signaling. This may help to deep understand the basic helix-loop-helix family-regulated vascular and developmental events and provides a novel therapeutic strategy for angiogenic diseases.

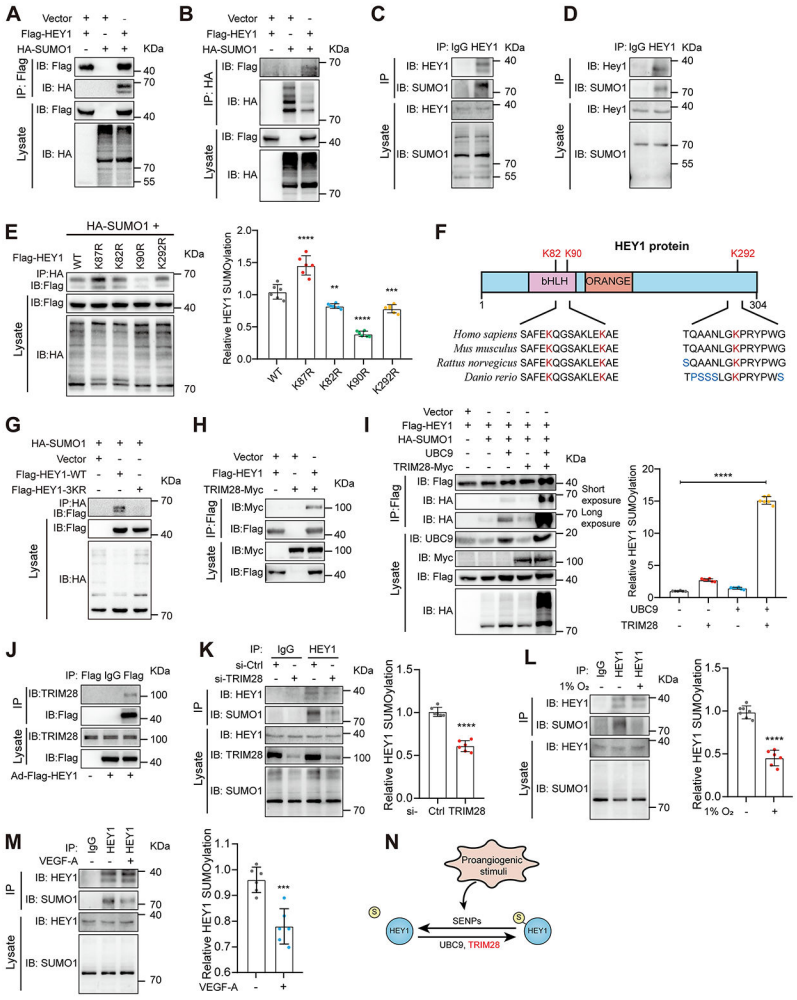

Figure 1. HEY1 can be SUMOylated in endothelial cells.

A and **B**, SUMO modification of HEY1 in 293T cells transfected with Flag-HEY1 and HA-SUMO1 plasmids. **C** and **D** Endogenous HEY1 interacts with SUMO1 in HUVECs (**C**) and MLECs (**D**). **E**, Identification of HEY1 SUMOylation sites. Relative HEY1 SUMOylation was normalized to that of input Flag-HEY1 and the WT group. **F**, Pattern diagram indicating the distribution of the consensus SUMOylation sites in HEY1. **G**, Detection of the interaction between SUMO1 and HEY1-WT/3KR in 293T cells. **H**, Detection of the exogenous HEY1-TRIM28 interaction in 293T cells. **I**, SUMOylation of HEY1 upon overexpression of UBC9 and TRIM28 in 293T cells. Relative HEY1 SUMOylation was normalized to that of input Flag-HEY1 and the "vector + Flag-HEY1" group. **J**, Detection of the HEY1-TRIM28 interaction in HUVECs. **K**, SUMOylation of HEY1 upon TRIM28 knockdown in HUVECs. Relative HEY1 SUMOylation was normalized to that of input HEY1 and the si-Ctrl group. **L**, SUMOylation of HEY1 under hypoxic conditions. HUVECs were treated under normoxic or hypoxic conditions for 48 h. Relative HEY1 SUMOylation was normalized to that of input HEY1 and the normoxia group. **M**, SUMOylation of HEY1 upon VEGF-A treatment. HUVECs were starved in basic EBM-2 medium for 12 h before treatment with VEGF-A (10 ng/mL) for 15 min. Relative HEY1 SUMOylation

was normalized to that of input HEY1 and the no-VEGF-A group. **N**, Schematic model for the dynamic SUMOylation and de-SUMOylation of HEY1. **P < 0.01, ***P < 0.001 and ****P < 0.0001 (means±SDs; n=6) analyzed by one-way ANOVA with Dunnett's multiple comparisons test (**E**), two-way ANOVA with Sidak's multiple comparisons test (**I**) or Student's t test (**K-M**). The exact P values are presented in the "Supplemental Statistical Table".

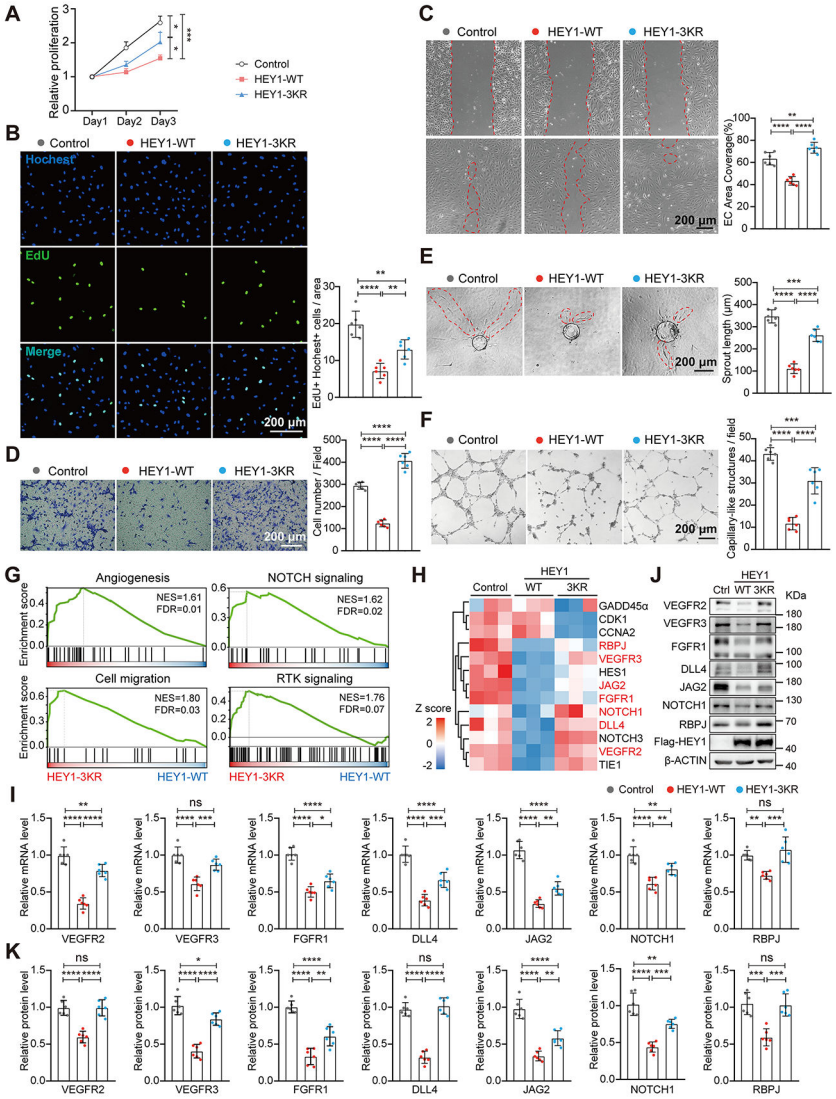

Figure 2. SUMOylation facilitates HEY1-mediated repression of angiogenic activity in HUVECs.

The angiogenic capability of HUVECs transduced with Ad-LacZ (as control), Ad-HEY1-WT or Ad-HEY1-3KR vectors was evaluated. **A** and **B**, Cell proliferation measured by the Cell Counting Kit-8 (**A**) and the EdU assay (**B**). Relative cell proliferation of each group was normalized to that of Day1. **C** and **D**, Cell migration measured by the wound healing (**C**) and the transwell assay (**D**). **E** and **F**, Angiogenic function measured by the sprouting (**E**) and the tube formation assay (**F**). Scale bar, 200 μm. **G**, Gene set enrichment analysis (GSEA) was performed to identify differentially affected functionally related gene sets in HUVECs transduced with Ad-HEY1-WT or Ad-HEY1-3KR vectors. **H**, RNA-seq heat map of representative genes differentially expressed in HUVECs transduced with Ad-LacZ (as control), Ad-HEY1-WT or Ad-HEY1-3KR vectors. **I-K**, Validation of downregulation of selected genes upon expression of HEY1-WT in HUVECs by quantitative PCR (**I**) and immunoblotting (**J** and **K**). The mRNA/protein level was normalized to that of β-ACTIN and the control group. *P < 0.05, **P < 0.01, ***P < 0.001 and ****P < 0.0001 (means±SDs; n=6) analyzed by one-way ANOVA with Tukey's multiple comparisons test

(**B-F, I** and **K**) or two-way ANOVA with Tukey's multiple comparisons test (**A**) (ns, not significant). Benjamini-Hochberg adjustment was employed for multiple testing correction in **I** and **K**, in which adjusted P (false discovery rate corrected Q) values are shown in the “Supplemental Statistical Table”. The exact P values are presented in the “Supplemental Statistical Table”.

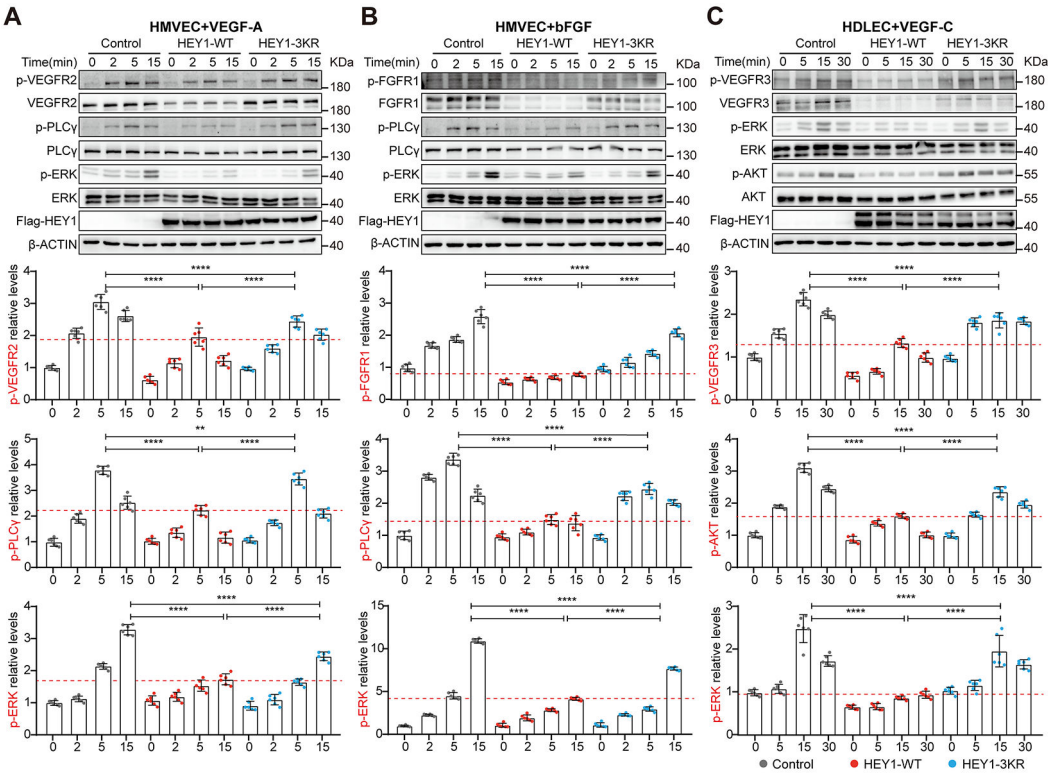

Figure 3. SUMOylation is required for HEY1-mediated inhibition of receptor tyrosine kinase signaling cascades in endothelial cells.

A and **B**, Immunoblots showing VEGFA-VEGFR2 (A) and bFGF-FGFR1 (B) signaling in HMVECs with HEY1-WT or HEY1-3KR expression by adenoviral delivery. **C** Immunoblots showing VEGFC-VEGFR3 signaling in HDLECs with HEY1-WT or HEY1-3KR expression by adenoviral delivery. The protein level was normalized to that of β-ACTIN and the "0 min" control group. ***P < 0.001 and ****P < 0.0001 (means±SDs; n=6) analyzed by two-way ANOVA with Tukey's multiple comparisons test (ns, not significant). The exact P values are presented in the "Supplemental Statistical Table".

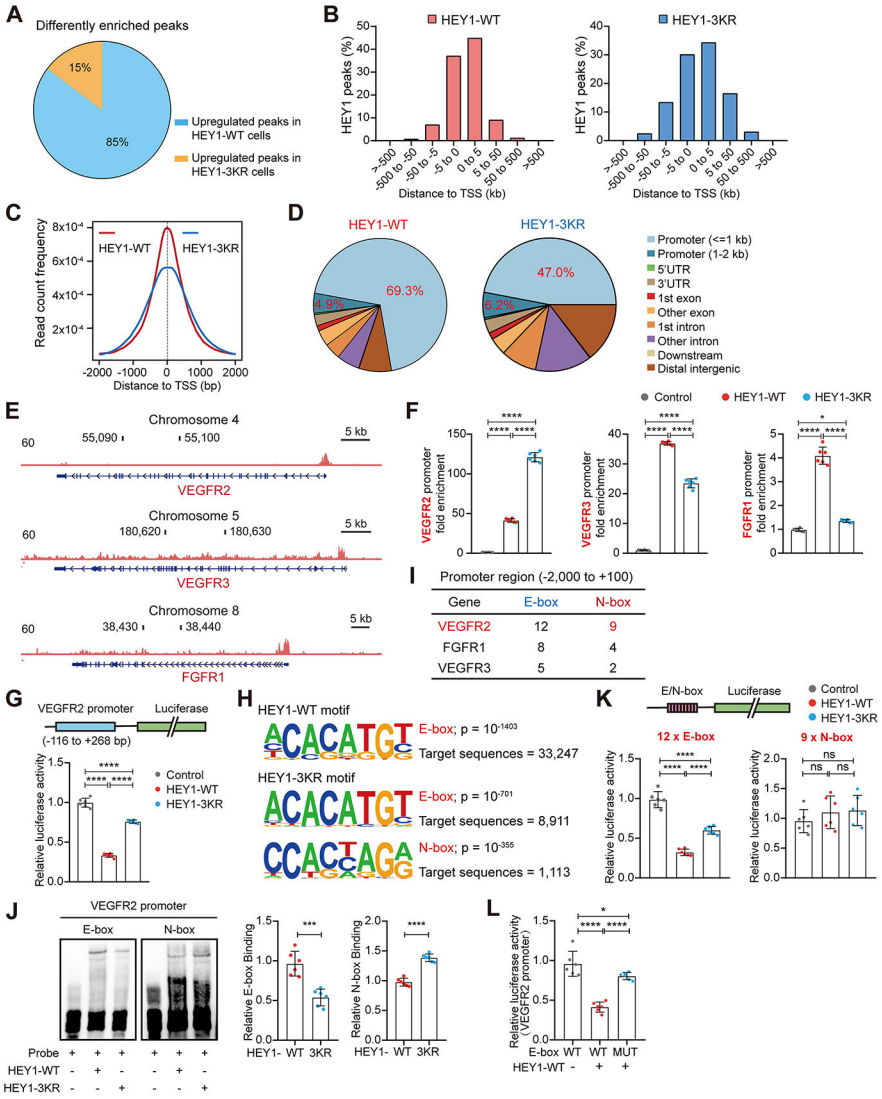

Figure 4. SUMOylation of HEY1 promotes its promoter-binding capability.

A, Differently enriched peaks between HEY1-WT and HEY1-3KR from ChIP-seq data analysis. **B**, Location of HEY1-WT and HEY1-3KR binding events in relation to closest annotated TSS. **C**, Enrichment profile of HEY1-WT and HEY1-3KR in relation to closest annotated TSS. **D**, Location of HEY1-WT and HEY1-3KR binding events respective to various genomic features. **E**, IGV snapshots showing HEY1-WT ChIP-seq signals at binding sites associated with the VEGFR2, VEGFR3 and FGFR1 promoters in HUVECs. **F**, ChIP-qPCR results in HUVECs showing the recruitment of HEY1-WT/3KR to the promoters of VEGFR2, VEGFR3 and FGFR1 in HUVECs. The relative promoter enrichment was normalized to that of the input and of the control group. **G**, Dual luciferase assay showing HEY1-WT/3KR-mediated repression of the VEGFR2 promoter. The relative luciferase activity was normalized to that of Renilla and the control group. **H**, HEY1-WT/3KR binding motifs identified by HOMER is enriched under ChIP-seq peaks. **I**, Numbers of E-box/N-box motifs identified in the VEGFR2, FGFR1 or VEGFR3 promoter regions. **J**, EMSA showing HEY1-WT/3KR binding to E-box and N-box motifs in HUVECs. The

relative E/N-box binding was normalized to that of the HEY1-WT group. **K**, Dual luciferase assay indicating the repressive activity of HEY1-WT/3KR toward E-box or N-box motifs. The relative luciferase activity was normalized to that of Renilla and the control group. **L**, Dual luciferase assay indicating the repressive activity of HEY1-WT toward wild type E-box or E-box mutant. The relative luciferase activity was normalized to that of Renilla and the WT E-box group. (For ChIP-seq, ChIP-qPCR and EMSA assay, HUVECs were employed; and for Dual luciferase assay, 293T cells was employed.) *P < 0.05, ***P < 0.001 and ****P < 0.0001 (means±SDs; n=6) analyzed by one-way ANOVA with Tukey's multiple comparisons test (**F, G, K** and **L**) or Student’s t test (**J**) (ns, not significant). The exact P values are presented in the “Supplemental Statistical Table”.

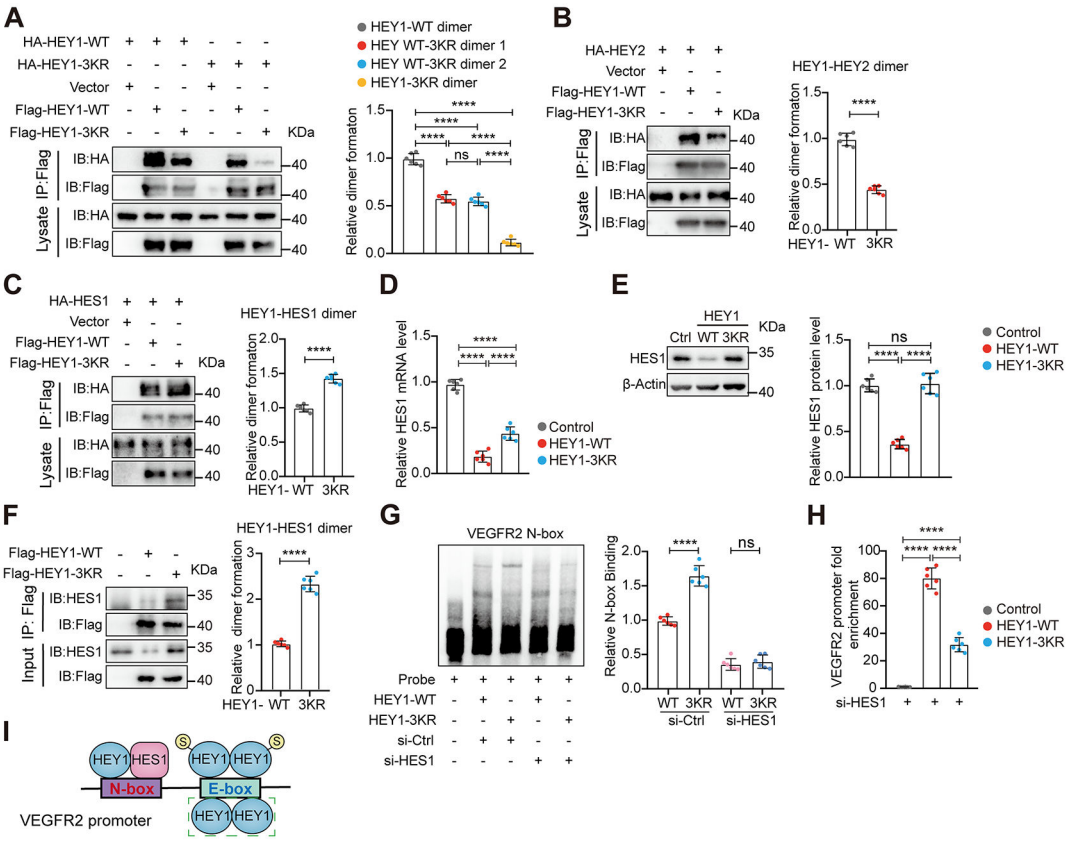

Figure 5. SUMOylation affects the formation of HEY1 dimer, thus altering its preference for the promoter-binding motif.

A, Detection of HEY1-WT/3KR homodimer formation in HUVECs. The relative dimer formation was normalized to that of the HEY1-WT dimer group. **B**, Detection of HEY1-WT/3KR-HEY2 heterodimer formation in HUVECs. The relative dimer formation was normalized to that of the HEY1-WT group. **C**, Detection of HEY1-WT/3KR-HES1 heterodimer formation in HUVECs. The relative dimer formation was normalized to that of the HEY1-WT group. **D** and **E**, mRNA and protein levels of HES1 in Ad-HEY1-WT/3KR-transduced HUVECs. The mRNA/protein level was normalized to that of β-ACTIN and the control group. **F**, Detection of the interaction between HEY1-WT/3KR and endogenous HES1 in HUVECs. The relative dimer formation was normalized to that of the HEY1-WT group. **G**, Interaction between HEY1-WT/3KR and N-box motifs decreased upon HES1 knockdown in HUVECs. The relative N-box binding was normalized to that of the si-Ctrl-HEY1-WT group. **H**, Interaction between HEY1 and N-box motifs decreased upon HES1 knockdown in HUVECs. The relative promoter enrichment was normalized to that of the input and of the control group. **I**, Schematic showing the preference of HEY1 for E-box or N-box motif. ****P < 0.0001 (means±SDs; n=6) analyzed by one-way ANOVA with Tukey's multiple comparisons test (**A, D, E** and **H**), two-way ANOVA with Sidak's multiple comparisons (**G**) or Student's t test (**B, C** and **F**) (ns, not significant). The exact P values are presented in the "Supplemental Statistical Table".

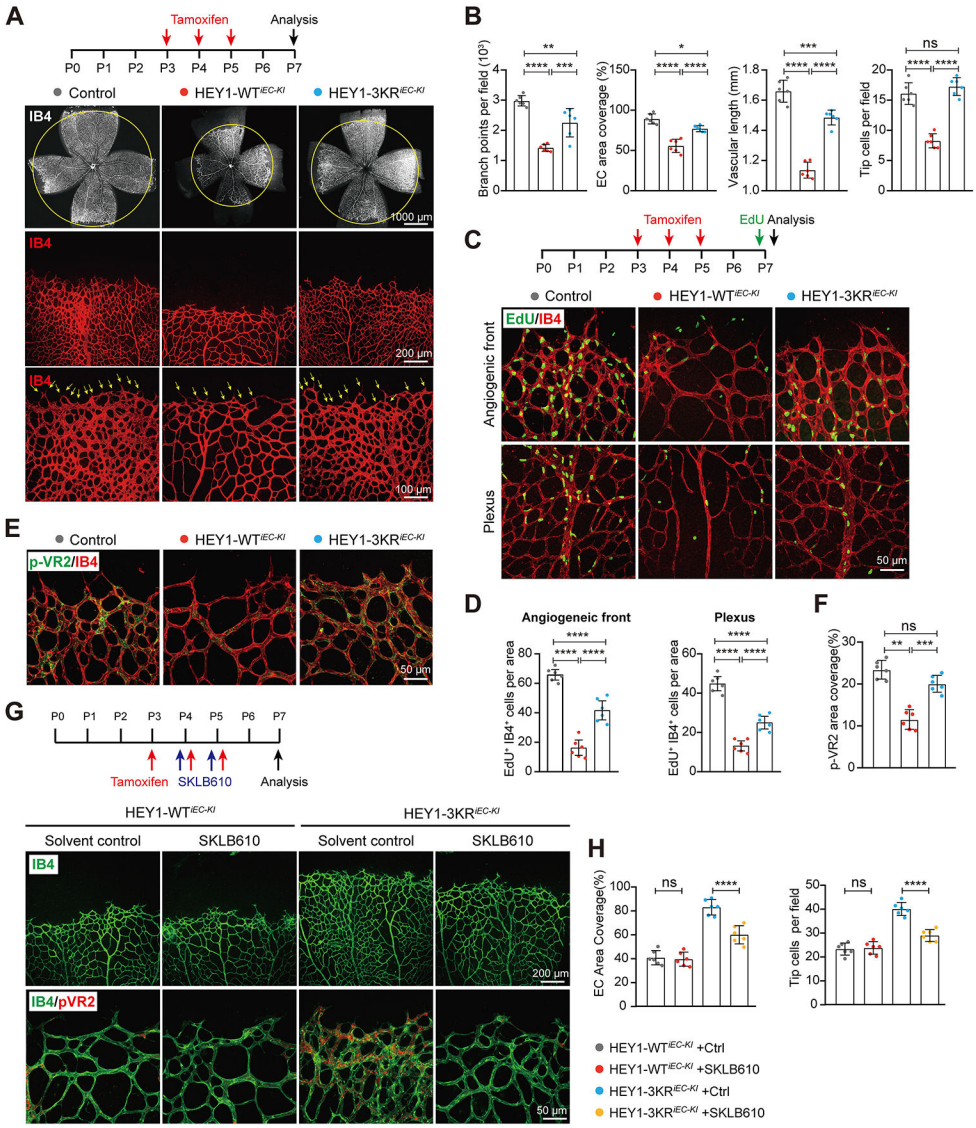

Figure 6. SUMOylation facilitates HEY1 in the repression on of retinal angiogenesis.

A and **B**, Retinal angiogenesis in HEY1-WT/3KR$^{iEC\text{-}KI}$ neonatal mice. Representative images and statistical analysis of vascular outgrowth on P7 in control, HEY1-WT$^{iEC\text{-}KI}$ and HEY1-3KR$^{iEC\text{-}KI}$ retinas stained with IB4 are shown. Scale bar: the upper panel, 1000 μm; the middle panel, 200 μm; and the lower panel, 100 μm. **C** and **D**, Proliferation of endothelial cells in P7 HEY1-WT/3KR EC-KI retinas. Representative images and statistical analysis of EdU+ IB4+ cells on P7 in control, HEY1-WT$^{iEC\text{-}KI}$ and HEY1-3KR$^{iEC\text{-}KI}$ retinas are shown. Scale bar, 50 μm. **E** and **F**, Phosphorylated VEGFR2 in P7 HEY1-WT/3KR EC-KI retinas. Representative images and statistical analysis of p-VEGFR2 area on P7 in control, HEY1-WT$^{iEC\text{-}KI}$ and HEY1-3KR$^{iEC\text{-}KI}$ retinas are shown. Scale bar, 50 μm. **G** and **H**, Retinal angiogenesis was repressed by SKLB610 (an inhibitor of p-VEGFR2) in HEY1-3KR but not HEY1-WT EC-KI neonatal mice. Representative images and statistical analysis of vascular outgrowth on P7 in HEY1-WT$^{iEC\text{-}KI}$ and HEY1-3KR$^{iEC\text{-}KI}$ retinas stained with IB4 are shown. Scale bar: the upper panel, 200 μm; and the lower

panel, 50 μm. *P < 0.05, **P < 0.01, ***P < 0.001 and ****P < 0.0001 (means±SDs; n=6) analyzed by one-way ANOVA with Tukey's multiple comparisons test (**B, D** and **F**) or two-way ANOVA with Sidak's multiple comparisons (**H**) (ns, not significant). P <0.0125 was considered statistically significant for multiple testing correction in **B** and P <0.025 was considered statistically significant for multiple testing correction in **D** and **H** based on the Bonferroni adjustment. The exact P values are presented in the “Supplemental Statistical Table”.

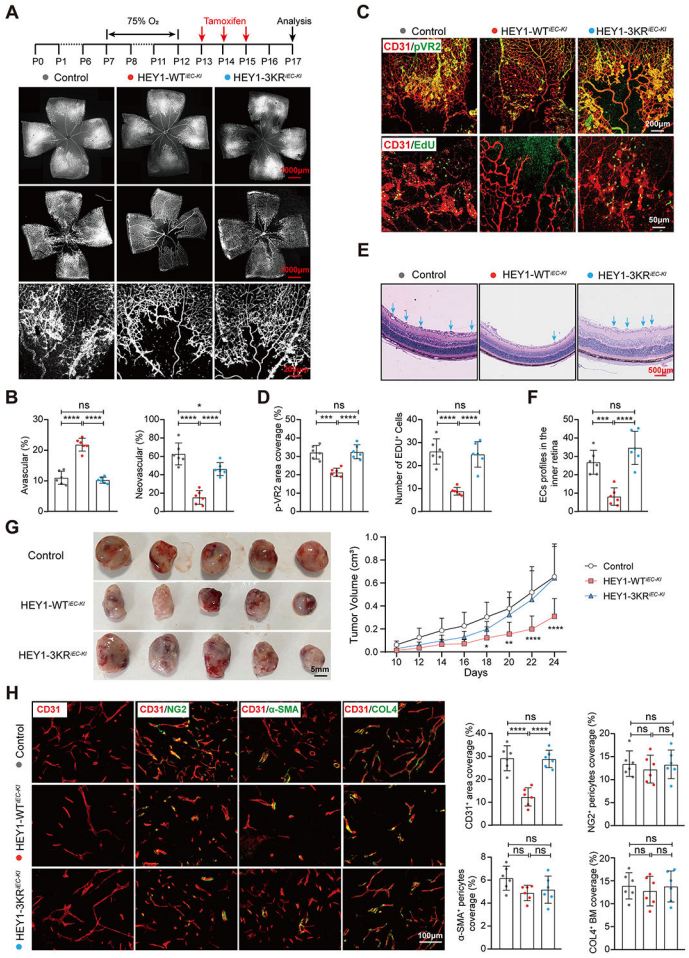

Figure 7. SUMOylation of HEY1 in endothelial cells effectively compromises pathological angiogenesis.

A and **B**, Endothelial expression of HEY1-WT but not the HEY1-3KR mutant protects against aberrant neovascularization in the oxygen-induced retinopathy (OIR) model. Representative images and statistical analysis of vascular area on P17 in control, HEY1-WT$^{iEC\text{-}KI}$ and HEY1-3KR$^{iEC\text{-}KI}$ retinas stained with IB4 are shown. Scale bar: the upper and the middle panels, 1000 μm; the lower panel, 200 μm. **C** and **D**, Proliferation of endothelial cells and phosphorylation of VEGFR2 in P17 HEY1-WT/3KR retinas in the OIR model. Representative images and statistical analysis of p-VEGFR2+ CD31+ and EdU+ CD31+ cells area respectively, on P17 in control, HEY1-WT$^{iEC\text{-}KI}$ and HEY1-3KR$^{iEC\text{-}KI}$ retinas are shown. Scale bar: the upper panel, 200 μm; the lower panel, 50 μm. **E** and **F**, Endothelial expression of HEY1-WT but not the HEY1-3KR mutant is able to protect against preretinal neovascular tufts development in the OIR model. Representative images of H&E-stained sagittal sections of eyes from P17 mice and statistical analysis of preretinal neovascular tufts are shown. Scale bar, 500 μm. **G**, Representative images and statistical analysis of explant LLC tumors from control and HEY1-WT/3KR$^{iEC\text{-}KI}$ mice. Scale bar, 5 mm. **H**, Representative images and statistical analysis of immunofluorescence staining with CD31, NG2, α-SMA, Collagen IV (Col4) antibody in explant LLC tumors from control and HEY1-WT/3KR$^{iEC\text{-}KI}$ mice. Scale bar: 100 μm. *P < 0.05, ***P < 0.001 and ****P

< 0.0001 (means±SDs; n=6 for **B, D, F** and **H**; n=8 for **G**) analyzed by one-way ANOVA with Tukey's multiple comparisons test (**B, D, F** and **H)** or two-way ANOVA with Tukey's multiple comparisons test (**G**) (ns, not significant). P <0.025 was considered statistically significant for multiple testing correction in **B** based on the Bonferroni adjustment. The exact P values are presented in “Supplemental Statistical Table”.

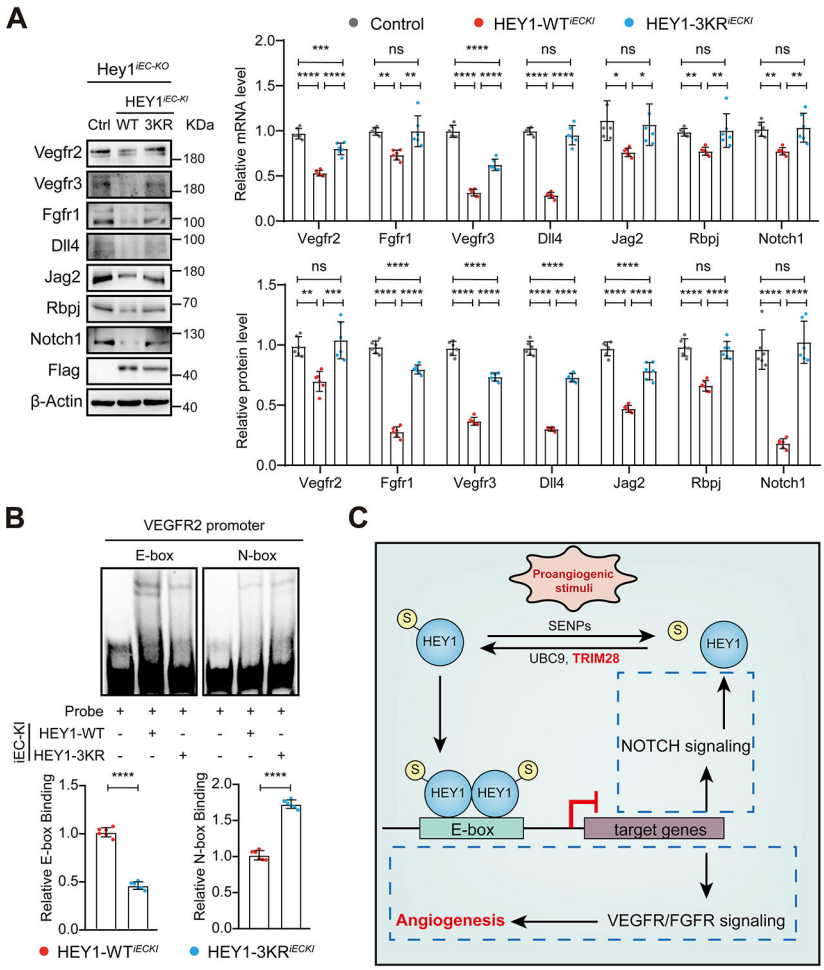

Figure 8. The critical role of HEY1 SUMOylation in the regulation of angiogenesis.

A, Validation of downregulation of selected genes upon expression of HEY1-WT but not HEY1-3KR in Hey1$^{iEC\text{-}KO}$ MLECs by quantitative PCR and immunoblotting. The mRNA/protein level was normalized to that of β-ACTIN and the control group. **B**, EMSA showing HEY1-WT/3KR binding to E-box and N-box motifs in MLECs. The relative E/N-box binding was normalized to that of the HEY1-WT$^{iEC\text{-}KI}$ group. **C,** Schematic diagram illustrating the critical role of HEY1 SUMOylation in the regulation of angiogenesis. *P < 0.05, **P < 0.01, ***P < 0.001 and ****P < 0.0001 (means±SDs; n=6) analyzed by one-way ANOVA with Tukey's multiple comparisons test (A) Student's t test (**B**) (ns, not significant). The exact P values are presented in "Supplemental Statistical Table".