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Circ-C*alm4* Regulates Hypoxia-Induced Pulmonary Artery Smooth Muscle Autophagy by Binding Purb

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Abstract

Pulmonary hypertension (PH) is a serious and fatal disease characterized by pulmonary vasoconstriction and pulmonary vascular remodeling. The excessive autophagy of pulmonary artery smooth muscle cells (PASMCs) is one of the important factors of pulmonary vascular remodeling. A number of studies have shown that circular RNA (circRNA) can participate in the onset of PH. Our previous studies have shown that circRNA calmodulin 4 (circ-calm4) is involved in the progression of hypoxic PH. However, the role of circ-calm4 on regulation of hypoxic PH autophagy has not been reported. In this study, we demonstrated for the first time that hypoxia-mediated upregulated circ-calm4 expression has a key regulatory effect on autophagy in hypoxia-induced PASMCs and hypoxic PH mouse models. Knockdown of circ-calm4 both in vivo and in vitro can inhibit the autophagy in PASMCs induced by hypoxia. We also performed bioinformatics predictions and conducted experiments to verify that circ-calm4 bound to the purine-rich binding protein (Purb) to promote its expression in the nucleus, thereby initiating the transcription of autophagy-related protein Beclin1. Interestingly, we found that Beclin1 transcription initiated by Purb was accompanied by a modification of Beclin1 super-enhancer to improve transcription activity and efficiency. Overall, our results confirm that the circ-calm4/Purb/Beclin1 signal axis is involved in the occurrence of hypoxia-induced PASMCs autophagy, and the novel regulatory mechanisms and signals transduction pathways in PASMC autophagy induced by hypoxia.

Introduction

Pulmonary hypertension (PH) is a complex and multi-faceted disease of the cardiovascular system that changes the pressure within the pulmovascular network, thereby causing a reduced cardiac output which can result in heart failure and death[1–4]. The pathogenesis of PH includes vascular remodeling, adventitial fibrosis, and pulmonary vasoconstriction[5, 6]. The vascular remodeling results from excessive proliferation and hypertrophy of smooth muscle cells in the tissue matrix[7], and these changes to the smooth muscle lead to the development of a hypertensive state which alters vascular pressure, and can cause cardiac failure[8]. The exploration of a new mechanism for the pathological process of pulmonary artery smooth muscle under hypoxic conditions is conducive to the early diagnosis and treatment of PH.

Autophagy is a cellular process of engulfing proteins or organelles and into vesicles, which then fuse with lysosomes and the contents are degraded[9, 10]. This process is dynamic, and provides substrates for cell regeneration and repair through the recycling of the raw materials inside cells. Under basic conditions, autophagy is constant and ubiquitous[11]. Autophagy plays an important role in maintaining cell homeostasis during stress; however, excessive stress can lead to excessive autophagy or its inhibition[12]. The dysregulation of autophagy contributes to the development of a variety of cardiovascular diseases, including PH, and regulatory mechanisms of autophagy are extremely complex[13–15]. Our previous studies have confirmed that acetylated cyclophosphamide A is an important mediator of hypoxia-induced autophagy and pulmonary angiogenesis in pulmonary artery endothelial cells (PAECs)[16]. Further, we found that in hypoxic PASMCs, BCAT1 combined with IRE1 on the ER to activate the expression of its downstream pathway, the XBP-1-RIDD axis, to activate

autophagy[17], suggesting that a myriad mechanisms are involved autophagy regulation. It is important to explore new potential mechanisms of hypoxia to regulate PASMC autophagy.

Circular RNA (circRNA) is a type of circular covalent molecule without the 3' and 5' ends[18]. It is more stable than linear transcripts and has tissue specificity and time sequence[19]. CircRNA participates in the pathology of a variety of diseases and functions through multiple mechanisms, including adsorption of miRNAs, binding to proteins, regulation of host gene transcription and splicing, and translation into polypeptides[20–23]. We have previously demonstrated that circ-*calm4* expression is upregulated under hypoxic conditions, and influences the proliferation of PASMCs through the adsorption of *miR-337-3p*[24]. However, whether circ-*calm4* can also regulate autophagy in PASMCs during hypoxia is unclear.

In the current study, we aimed to determine if circ-*calm4* could regulate the hypoxia-induced autophagy of PASMCs and to elucidate the underlying mechanism. Using bioinformatics and laboratory experiments, we confirmed that circ-*calm4* bound to Purb and through modification of a super-enhancer, promoted the transcription of autophagy-related protein Beclin1, and promoted the development of autophagy. Our results reveal a new regulatory mechanism of hypoxia-induced autophagy, and provide evidence of a novel, potential therapeutic strategy for the treatment of PH.

Materials And Methods

The authors declare that all supporting data are available within the article and its online-only Data Supplement. Expanded Methods can be found in the online-only Data Supplement.

Animal and lung tissue preparation

All animal procedures have been approved by the Institutional Animal Care and Use Committee, and comply with the guidelines for the care and use of laboratory animals approved by the Animal Care Institution and Use Committee (approval number by Animal Committee: HMUDQ20200601). All mouse operations are performed under pentobarbital sodium anesthesia to minimize pain. Six-week-old male C57BL/6 mice will be obtained from the Animal Management Center of Harbin Medical University and placed in a controlled environment with a light-dark cycle of 12 hours, temperature of 22-24°C, a relative humidity of 50%, and food and water temperature for a week to avoid stress. As previously described, study animals were randomly assigned to normoxic (21% O2) or hypoxic (9% O2) conditions for 28 days1. After this time, to anesthetize and carry out the detection of corresponding indicators, 1% pentobarbital sodium was injected intraperitoneally at 40 mg/kg. The mice were anesthetized and their lungs were quickly removed for follow-up experiments.

Statistical analysis

According to the Shapiro-Wilk test to detect normal distribution, when the data is normally distributed, single comparisons were performed using paired or unpaired t tests. Statistical analysis was performed with 1- or 2-way ANOVA, followed by Dunnett tests where appropriate. Results with two-tailed p values of

less than 0.05 were considered statistically significant. *P<0.05, **P<0.01, ***P<0.001. All data are expressed as means ± standard errors. Statistical analysis mapping and curve fitting were performed using GraphPad Prism 8 (GraphPad Prism Software, Inc, San Diego, CA). All experiments are greater than or equal to three times.

Results

Knockdown of circ-calm4 reverses increased autophagy caused by hypoxia

To determine whether circ-*calm4* exerted a regulatory effect on hypoxia-induced autophagy, we cultured PASMC under hypoxic conditions for 48 hours and transfected small interfering RNA (siRNA) to perform knockdown of circ-*calm4*. We have determined the siRNA interference efficiency of circ-calm4 in previous experiments[24]. In addition, we have verified that siRNA has no effect on linear transcripts[24]. Subsequently, we detected the expression levels of autophagy-related proteins LC3-I/II, Beclin1, p62, and Atg5 were detected by western blotting, and it was found that knocking down circ-*calm4* reversed the expression of these autophagy-related proteins that was observed under hypoxic conditions (Figure 1A). Next, we performed *in vivo* to verify the regulatory effect of circ-*calm4* on autophagy. We constructed a hypoxic pulmonary hypertension mouse model using adeno-associated virus and by knocking down circ-*calm4* as described above[24]. By using HE staining, right heart catheterization, right ventricular systolic pressure (RVSP), and small animal ultrasound to detect pulmonary arterial velocity time integral (PAVTI). The results showed that knockdown of circ-*calm4* reversed the changes in the RVSP and PAVTI caused by hypoxia (Figure S1A-C). In addition, AAV5 infected mice significantly reversed the upregulation of circ-calm4 caused by hypoxia (Figure S1D). Indicating that the model was successfully constructed. The lung tissues of mice were isolated, and the effects of circ-*calm4* was observed *in vivo* through TEM[25]. The results showed that knocking down circ-*calm4 in vivo* could reverse the increase of autophagosomes observed in hypoxic conditions (Figure 1B).

The expression levels of autophagy-related proteins LC3-I/II, Beclin1, p62, and Atg5 were detected by western blotting. The results showed that knocking down circ-*calm4 in vivo* could reverse the upregulated expression of the above-mentioned autophagy-related proteins under hypoxia (Figure 1C), and we found via immunofluorescence analysis that knocking down circ-*calm4* in vivo could reverse the hypoxia-induced increase in the expression of protein Beclin1 expression (Figure 1D). In addition, we also found that knocking down circ-calm4 can reverse the increase in autophagy induced by the autophagy agonist rapamycin (RAPA) (Figure 1E). The above-mentioned results indicate that circ-*calm4* knockdown *in vivo* and *in vitro* reverses the increase in the level of autophagy induced through hypoxia.

Circ-calm4 can form complex with Purb

To explore the mechanism by which circ-*calm4* might influence hypoxia-induced autophagy, we used bioinformatics software to predict the proteins that might bind to circ-*calm4*[26]. We performed GO analysis for the first nine proteins according to the interaction score[27]. We found that the transcription factor Purb was involved in cell death, proliferation, and DNA replication, which might be related to PH

and autophagy (Figure 2A, S2A). Therefore, we chose Purb for conducting follow-up experiments. We predicted the binding region of circ-*calm4* and Purb through the catRAPID fragments and express interaction matrix[28]. CatRAPID fragments can be used to divide Purb into the peptide and nucleotide sequences into fragments, and then predict their respective interaction tendency[29]. The color in the express heat map indicates the interaction score (from -3 to +3) of each amino acid and nucleotide pair. The sum represents the overall interaction score. The results show that circ-*calm4* 350-700 bp has a strong interaction with 150-300 amino acids of Purb (Figure 2B); catRAPID strength can be used to determine the interaction between a molecule (protein/transcript) and the reference set (transcript/nucleotide-binding proteome) of the model organism[30, 31]. The results further confirmed that circ-*calm4* and Purb had a strong binding ability (Figure 2C S2C). Next, we found that Purb-specific antibodies could enrich circ-*calm4* expression as evidenced by immunoprecipitation results (Figure 2D). Through conduction of the RNA pull-down experiment, we found that the specific biotin-labeled circ-*calm4* pull-down protein detected the enrichment of Purb (Figure 2E). To further determine the binding area of circ-*calm4* and Purb, we divided circ-*calm4* into three segments 290-319, 410-439, and 670-699 based on the results of bioinformatics prediction and labeled them with specific biotin probes, and found that Purb could combine with the first two segments (Figure 2F, S2B). The above-mentioned results indicate that circ-*calm4* can bind to Purb.

Purb expression is upregulated under hypoxic conditions

We investigated the expression of Purb in hypoxic PASMCs. Since Purb expression has not been reported in hypoxic PASMCs, we analyzed the database and found that Purb is located on mouse chromosome 11 and exhibits high expression levels of protein and mRNA in the lung tissue (Figure S3A, S3B) (https://www.genecards.org/cgi-bin/carddisp.pl?gene=PURB). We constructed a hypoxic pulmonary hypertension mouse model as described above[24], and observed the thickness of blood vessels by HE staining. The right heart catheter was used to detect RVSP. The results showed that the hypoxic blood vessel wall thickened and the RVSP increased, indicating that a model of PH was successfully constructed (Figure S3C, S3D). Next, we performed real-time qPCR and western blotting to detect the expression of Purb in the lung tissue of hypoxic mice. The results showed that under hypoxia, the protein levels of Purb were upregulated in PASMCs, which were confirmed by immunohistochemistry and immunofluorescence experiments (Figure 3B, 3C). However, the mRNA level of Purb did not change significantly, indicating that circ-calm4 may function by improving the stability of Purb (Figure 3A).

We then performed western blotting to explore whether circ-*calm4* affected the hypoxia-induced upregulation of Purb. The results showed that knocking down circ-*calm4 in vivo* and *in vitro* inhibited the hypoxia-induced upregulation of Purb (Figure 3D). We conducted FISH and cytoplasmic nuclear isolation experiments to clarify the subcellular localization of Purb in PASMCs. Our results showed that Purb was mainly located in the nucleus and that circ-*calm4* and Purb were co-localized (Figure 3E, 3F). We knocked down cytoplasmic circ-*calm4* by using siRNA, and nucleic circ-*calm4* with antisense oligonucleotide (ASO), qPCR experiment found that ASO has a significant knockdown efficiency of circ-calm4 (Figure S3E). We found that after knocking down circ-*calm4* in the cytoplasm and nucleus, the

expression of Purb in the nucleus was reduced, indicating that circ-*calm4* could affect the nuclear displacement of Purb and promote its expression in the nucleus (Figure S3F). The above-mentioned results indicate that hypoxia upregulates the expression of Purb, and this phenomenon is reversed by knocking down circ-*calm4*.

Purb knockdown inhibits hypoxia-induced autophagy

Subsequently, we constructed an siRNA and overexpression plasmid of Purb to explore the function of Purb in hypoxic PASMCs. Via western blotting experiments, we verified that Si-2, a small interfering RNA of Purb, had good knockdown efficiency, at the same time, the overexpression plasmid has better overexpression efficiency (Figure 4A). The expression of Purb at different periods of hypoxia was also determined, and the results showed that Purb reached a higher expression level after 48 hours of hypoxia, which was consistent with our previously reported expression level of circ-*calm4* (Figure 4B). Knockdown of Purb reversed the upregulated expression of hypoxia-induced autophagy-related proteins (Figure 4C). Additionally, we constructed an overexpression plasmid of Purb and detected the expression of Beclin1 through immunofluorescence experiments. The results showed that overexpression of Purb promoted the expression of Beclin1. Similarly, knocking down Purb reversed the increase in Beclin1 fluorescence signal observed under hypoxic conditions (Figure 4D). We used the mRFP-GFP-LC3 dual fluorescent autophagy indicator to detect the extent of autophagy, and observed that the green light was quenched after hypoxia, indicating that the autophagy had increased, which was reversed after knocking down Purb. On the contrary, overexpression of Purb also significantly inhibited the green light under normoxia, indicating that Purb promotes autophagy under normal conditions (Figure 4E). TEM showed that knocking down Purb reversed the increase of autophagosomes caused by hypoxia (Figure 4F). The above-mentioned results indicate that Purb promotes autophagy, and knocking down Purb can reverse the increase in autophagy induced through hypoxia.

Circ-calm4 regulates autophagy via Purb

We next explored whether circ-*calm4* regulated the occurrence of autophagy by combining Purb with a rescue experiment. We co-transfected circ-*calm4* siRNA and Purb overexpression plasmid in PASMCs under hypoxic conditions for 48 hours; the results of western blotting, transmission electron microscopy, and mRFP-GFP-LC3 dual fluorescent autophagy indicator analysis showed that knocking down circ-*calm4* reversed the increase in autophagy usually caused by hypoxia, and this effect was reversed by the overexpression of Purb (Figure 5A-C). We also co-transfected circ-*calm4* siRNA with Purb overexpression plasmid and added the autophagy agonist rapamycin (RAPA). Western blotting results showed that knocking down circ-*calm4* could also reverse the increase in autophagy caused by RAPA, which was also reversed by the overexpression of Purb (Figure 5D). In addition, our previous studies have shown that circ-calm4 can promote proliferation[24], but whether circ-calm4 can regulate proliferation through Purb's autophagy pathway is unclear. We use Image J software to perform a semi-quantitative analysis of the immunofluorescence of the proliferating cell nuclear antigen Ki67, and through EDU and MTT experiments, it was found that the overexpression of Purb can reverse the ability of knocking down circ-

calm4 to inhibit proliferation under hypoxic conditions, and this phenomenon was reversed after adding the autophagy inhibitor bafilomycin (Baf) (Figure 5E-F). The above-mentioned results indicated that circ-*calm4*, whose expression is upregulated by hypoxia, promotes autophagy by forming a complex with Purb, and can affect cell proliferation.

Purb promotes the transcription of autophagy-related protein Beclin1 by super-enhancer modification in the Beclin1 promoter region

We explored the mechanism underlying circ-*calm4* regulation of autophagy. Since it has been reported that Purb can act as a transcription factor to initiate downstream gene transcription, we considered if Purb could bind to promoter regions of autophagy-related genes and promote their transcription. We first analyzed the binding between Purb and the promoter regions of several key autophagy through bioinformatics analysis, and the results showed that Purb had a strong binding ability to the promoter regions of the Beclin1 (Figure 6A). We first confirmed that our experimental conditions could obtain 200-1000-bp DNA fragments by sonication of DNA (Figure 6B), and then the chromatin immunoprecipitation CHIP results further showed that Purb was enriched in the Beclin1 promoter region (Figure 6C).

Subsequently, we constructed a dual-luciferase reporter gene plasmid for the predicted promoter region and transfected PASMCs. The results showed that the Purb overexpression plasmid significantly promoted the fluorescence intensity in the wild-type promoter than that in the mutant type (Figure 6D). Since a considerable number of current studies have shown that super-enhancers participate in the occurrence and development of several diseases by regulating gene transcription, we explored if super-enhancer modifications existed in the process of Purb-mediated promotion of expression of Beclin1. We first analyzed the presence of super-enhancer modification in the promoter region of Beclin1 through analysis using the USCS database (Figure 6E). Then, through conduction of RNA pull-down and co-immunoprecipitation assays, we found that the super-enhancer marker protein H3K27ac not only bound to circ-*calm4*, but also bound to Purb and the expression of H3K27ac was detected through chromatin immunoprecipitation assays (Figure 6F). The above-mentioned results indicate that Purb may be accompanied by a super-enhancer modification to promote the transcription of autophagy-related protein Beclin1.

Discussion

This study focused on exploring the potential role and mechanism of circ-*calm4* in the occurrence and development of autophagy. First, this study confirmed that the upregulated expression of circ-*calm4* in hypoxic pulmonary artery smooth muscle promoted autophagy in vivo and in vitro; second, it was proved that circ-*calm4* directly bound to Purb to promote its expression in the nucleus and affected autophagy; finally, Purb bound to the promoter region of the autophagy-related protein Beclin1, and with the modification of super-enhancer, promoted its transcription. Overall, our results indicate that the circ-*calm4*-Purb-Beclin1 signal axis can regulate the occurrence of autophagy in hypoxic PASMCs.

Autophagy dysregulation is involved in the occurrence and development of cardiovascular diseases such as PH[15, 31, 32]. PH is a serious and fatal disease. In the early stage of PH, autophagy can promote the proliferation and migration of PASMCs; if autophagy does not cease or becomes dysregulated, it can contribute to the excessive proliferation of PASMCs, which in turn leads to pulmonary vascular remodeling and the development of PH[33]. Blocking autophagy by the autophagy inhibitor chloroquine improves PH[15]. We have reported that autophagy occurs in hypoxia-induced pulmonary hypertension both in PAECs and PASMCs[16, 17]. Our current study supports published results of previous research groups and our previously published results. However, there are also reports that the autophagy protein LC3 plays a protective role in hypoxic PH. The dual effects of the above-mentioned two types of autophagy in the promotion or inhibition of disease progression may play at different junctures of PH, or may participate in different signal pathways. The different regulatory mechanisms resulting from the dual effects of autophagy on hypoxic PH should be further studied.

Many factors have been reported to induce autophagy in cells, such as starvation, hypoxia, lack of growth factors, microbial infection, organelle damage, protein folding errors or aggregation, and DNA damage[34, 35]. The mechanism by which these factors are transmitted signals to regulate progress of autophagy is not clear. A variety of signal pathways, such as mTOR, AMPK, P53, JNK-1, and other signal pathways are involved in the regulation of autophagy[34, 36-38]. Recent studies have shown that circRNA can also participate in the regulation of autophagy[39]. It has been reported that circCDYL promotes breast cancer progression through the *miR-1275-ATG7*/ULK1 autophagy axis[40]. CircCUL2 may function as an inhibitor and modulator of cisplatin sensitivity through *miR-142-3p*/ROCK2-mediated autophagy activation[41]. Owing to the key role of circRNA in autophagy, it is extremely important to discover new circRNAs to regulate hypoxia-induced PASMC autophagy and to clarify the regulatory mechanism. Our current study showed that knocking down circ-*calm4* could reverse the upregulation of autophagy-related proteins that occurred in hypoxic PASMCs. Knocking down circ-*calm4* with an adeno-associated virus *in vivo* reversed the increase of autophagosomes and the expression of autophagy-related proteins in the hypoxic pulmonary hypertension mouse model. We have proved for the first time that circ-*calm4* is a key driving factor for hypoxia-induced autophagy *in vivo* and *in vitro* experiments.

Purb is a DNA and RNA binding protein, involved in the regulation of DNA replication and transcription[42]. It has been reported that Purb acts as a repressor in myoblasts and fibroblasts and plays a role in controlling the transcription of the vascular smooth muscle (VSM) α-actin gene[43]. A recent study showed that Purb can combine with circ-*samd4* to inhibit the expression of MHC[44]. However, whether Purb is involved in the regulation of autophagy is unclear. Through bioinformatics analysis, we have predicted that circ-*calm4* directly binds to Purb. We further identified that the mRNA and protein levels of Purb were upregulated in PASMCs, and regulated the expression autophagy-related proteins. Our data showed that the hypoxia-induced increase in Purb expression was regulated by circ-*calm4*. Notably, by engineering the co-transfection of circ-*calm4* siRNA and Purb overexpression plasmids in PASMCs, we observed that knocking down circ-*calm4* reversed hypoxia-induced autophagy, and was ameliorated by the overexpression of Purb. Our results indicate that circ-*calm4*, which is upregulated during hypoxia, promotes autophagy by forming a complex with Purb.

Beclin1 is a critical molecule in the regulation of autophagy, as it mediates the colocalization of other autophagic proteins to phagocytic vesicles, leading to the formation and maturation of the autophagosome[45]. The expression of Beclin1 increases during autophagy[46]. It has been reported that phosphorylated Unc-51 like autophagy activating kinase 1 (Ulk1) can in turn phosphorylate Autophagy and Beclin-1 Regulator 1 (Ambra1) and bind to Beclin1, which initiates autophagy[47]. Owing to the key role of Beclin1 in autophagy, we analyzed the binding ability and binding region of the transcription factor Purb and Beclin1 promoter region through analyses based on bioinformatics software, the dual-luciferase reporter, and CHIP experiment. Our results identified that Purb could bind to the promoter region of Beclin1 to activate the transcription of Beclin1, thereby promoting the occurrence of autophagy. According to the data, circ-*calm4* regulates the expression of Beclin1 by combining with Purb to promote the occurrence of hypoxic pulmonary artery smooth muscle autophagy.

Super-enhancers are large, dense clusters of 'master' transcription factors involved in cell identity that are found on regions of DNA, identified by the high density of transcriptional factors, activators, cofactors, and enhancer histone modification marks[48]. Several studies have reported that super-enhancers are involved in the occurrence and development of many diseases[49-51], but there is no existing report on their involvement in PH. Our present study showed that the super-enhancer of the autophagy-related protein Beclin1 contributed to PH development. We first analyzed the presence of super-enhancer markers H3K27ac and H3K4me1 in the Beclin1 promoter region through analysis based on bioinformatics software. Then, by performing CHIP experiments using H3K27ac- and Purb-specific antibodies, we found that both the antibodies could pull down the Beclin1 promoter region, and that H3K27ac and Purb could interact with each other. Furthermore, we found that circ-*calm4* also pulled down H3K27ac. Our results indicate that circ-*calm4* and Purb complexes together to initiate Beclin1 transcription through super-enhancer modification.

In conclusion, we have determined for the first time that circ-*calm4* is involved in the regulation of hypoxia-induced autophagy in PASMCs. We have also revealed that circ-*calm4* can promote Purb nuclear expression by combining with Purb, and by super-enhancer modification of Beclin1, circ-*calm4*, and Purb complexes to promote the transcription of Beclin1, leading to the activation of autophagy. Our results provide new clues for the study of the molecular mechanisms and signaling pathways involved in the regulation of hypoxia-induced autophagy of PASMCs, and may provide new potential targets for the diagnosis and treatment of hypoxic pulmonary hypertension.

Perspectives

Our findings demonstrated that knocking down circ-calm4 can inhibit the increase in autophagy induced by hypoxia and the autophagy agonist rapamycin in vivo and in vitro. This change through the targeted binding of the transcription factor Purb, and the super enhancer modification to initiate the transcription of the key autophagy protein Beclin1, and then over-stimulate autophagy, and ultimately lead to pulmonary blood vessel remodeling. Therefore, we further clarified the function and mechanism of the circRNA in PH, thus establishing a potential target for the treatment and diagnosis of PH. Future research

is needed to confirm these findings and provide more insights into the promising applications of this circRNA.

Abbreviations

PH Pulmonary hypertension

PASMCs Pulmonary artery smooth muscle cells

CircRNA Circular RNA

Purb Purine-rich binding protein

AAV5 serotype 5 adenovirus-associated virus

HE Hematoxylin and eosin

PAEC Pulmonary arterial endothelial cell

FBS Fetal bovine serum

SiRNA Small interfering RNA

ASO Antisense oligonucleotide

RIP RNA Binding Protein Immunoprecipitation

FISH Fluorescent in situ hybridization

CHIP Chromatin immunoprecipitation

DAPI 4',6-Diamidino-2-phenylindole

PBS Phosphate-buffered saline

HBSS Hank's Balanced Salt Solution

SMA Smooth muscle actin

Declarations

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Author Contributions

J.Z. and Y.L. performed study concept and design; and performed development of methodology and writing, review and revision of the paper; Y.C., X.Y. and H.S. performed cytology and molecular biology related experiment; X.D.Z., Y.W. and L.Z. conducted all animal-related experiments; X.Y. and X.D.Z. provided partial financial support; D.Z. provided technical, financial and material support as well as the writing, review and revision of the paper. All authors read and approved the final paper.

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Compliance with ethical stsndards

Conflict of interest: No conflicts of interest were disclosed.

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Figures

Figure 1 Knockdown of circ-calm4 reverses increased autophagy caused by hypoxia

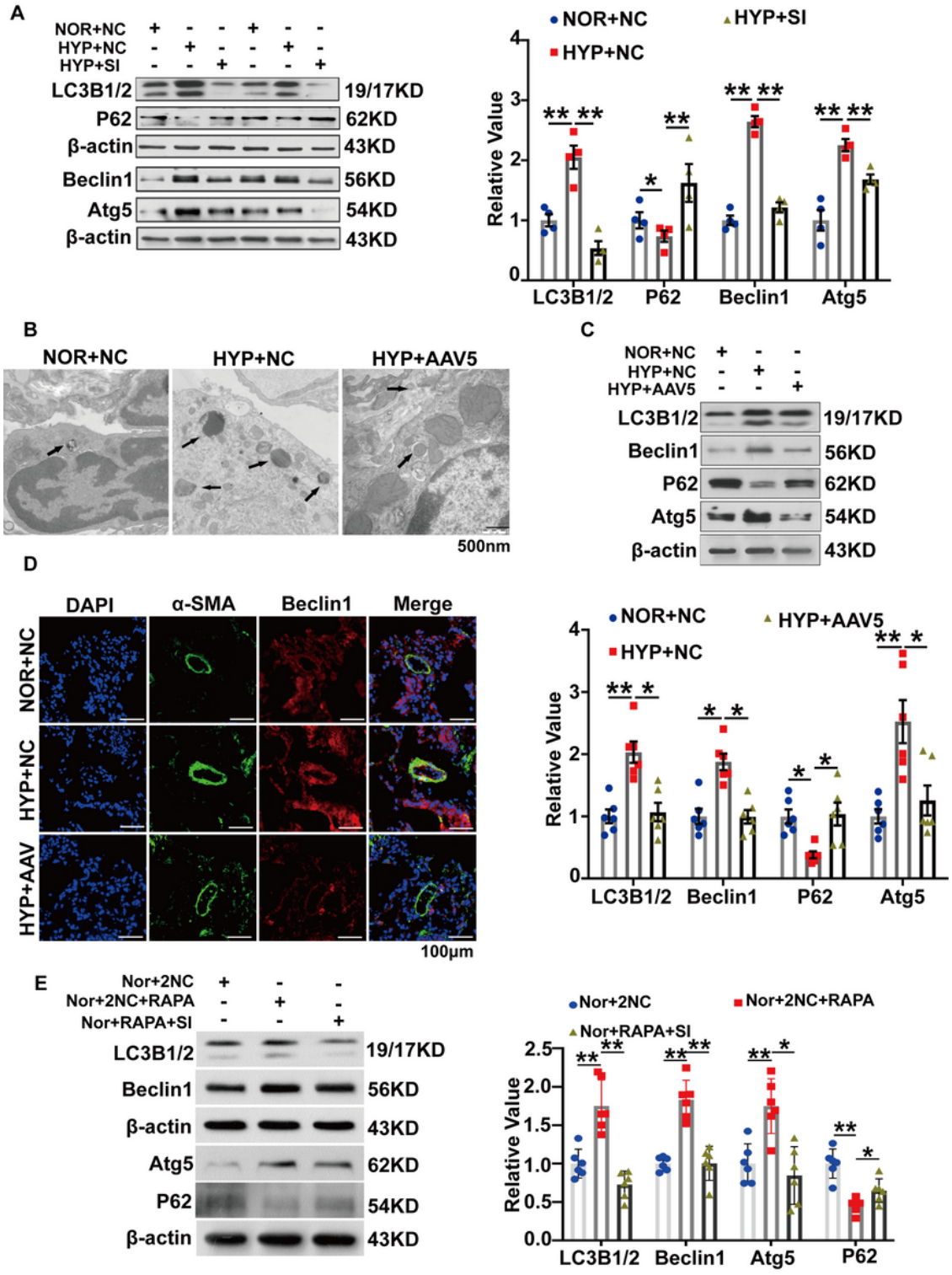

Figure 1

Knockdown of circ-calm4 reverses increased autophagy caused by hypoxia. a Western blotting analysis of the expression of LC3B1/2, P62, Beclin1, and Atg5 in PASMCs (n = 4). b A clear electron micrograph of the pulmonary artery tissue of mice treated with NOR and chronic HYP and AAV5 knockdown circ-calm4. (n = 3). c Extraction of the lung tissue proteins of AAV5 model mice and detection of the expression of autophagy-related proteins LC3B1/2, P62, Beclin1, and Atg5 by western blotting (n = 6). d Immunofluorescence was used to detect Beclin1 in hypoxia and AAV5-treated mouse lung tissues (n = 3). e The expression of autophagy-related proteins LC3B1/2, P62, Beclin1, and Atg5 was detected by western blotting (n = 6). All values are expressed as the mean ± SEM. *P<0.05, **P<0.01, ***P<0.001.

Figure 2 Circ-calm4 can form complex with Purb

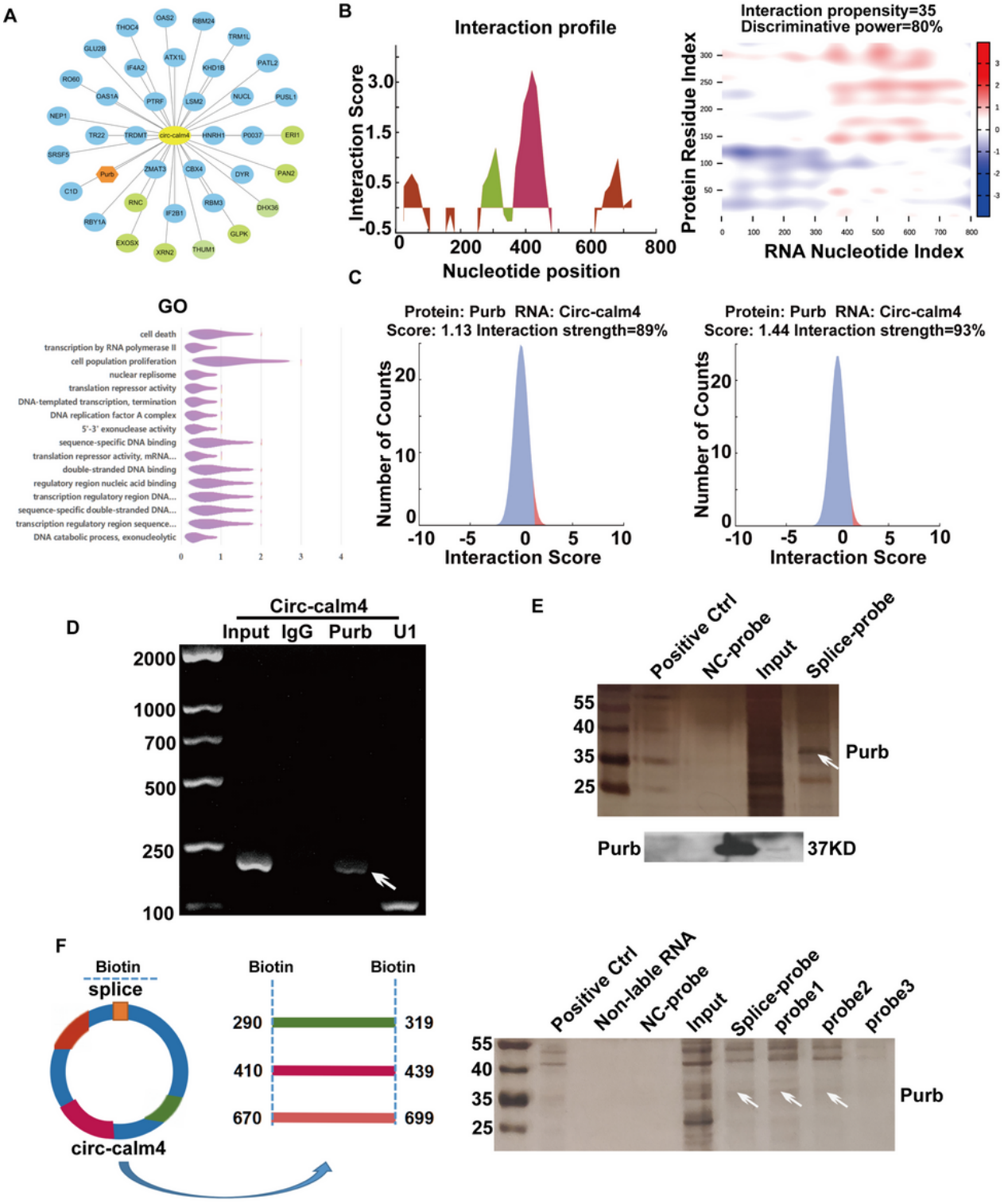

Figure 2

Circ-calm4 can form complex with Purb. a Conduction of functional enrichment analysis (GO) on the screened proteins through DAVID and KOBAS. b, c Analysis was performed for the binding strength of circ-calm4 and Purb through the software catRAPID. d Purb can specifically pull-down circ-calm4 through RNA binding protein immunoprecipitation experiment (n = 3); U1, positive control. e circ-calm4 labeled

with RNA pull-down biotin probe can specifically pull-down Purb (n = 3). f Presence of Purb was assessed by silver staining (n = 3).

Figure 3 Purb expression is upregulated under hypoxic conditions

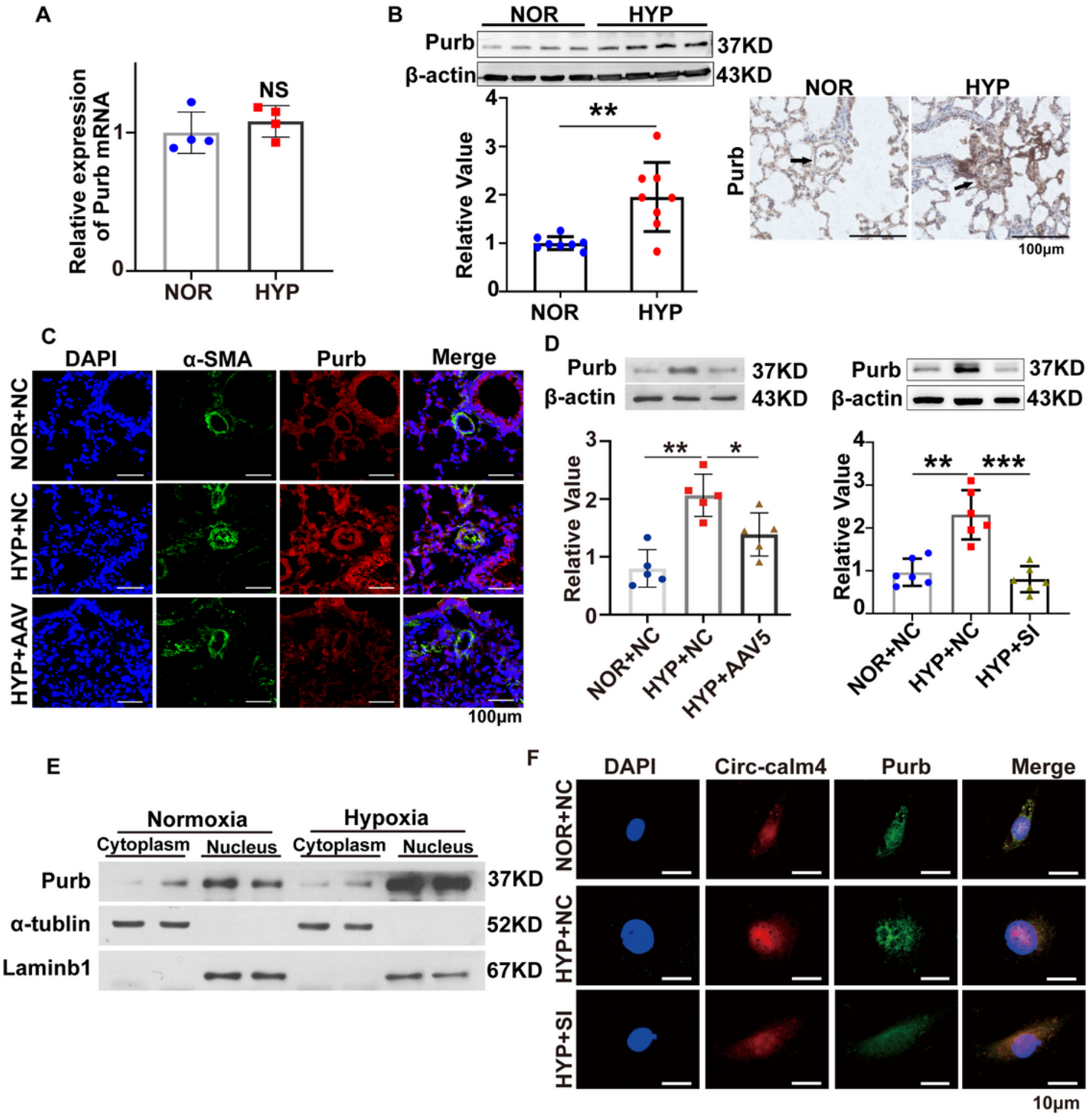

Figure 3

Circ-calm4 can form complex with Purb. a Real-time fluorescence quantitative PCR was conducted to detect the mRNA expression of Purb in the lung tissue of hypoxic mice (n = 4). b Western blotting and immunohistochemistry to detect the expression of Purb protein in the lung tissue of hypoxic mice (n ≥ 3).

c, Immunofluorescence-based detection of Purb expression in hypoxic mice and AAV5 knockdown circ-calm4 model mice. d, The expression of Purb protein in PASMCs, hypoxic mice and AAV5 knockdown circ-calm4 model mice was detected by western blotting (n = 5). e, PASMCs were tested for the distribution of Purb in hypoxic pulmonary artery smooth muscle by separation of the nucleus and cytoplasm (n = 3). f, Fluorescence in situ hybridization experiment (FISH) results showed the co-localization of circ-calm and Purb (n = 3). All values are expressed as the mean ± SEM. *P<0.05, **P<0.01, ***P<0.001.

Figure 4 Purb knockdown inhibits hypoxia-induced autophagy

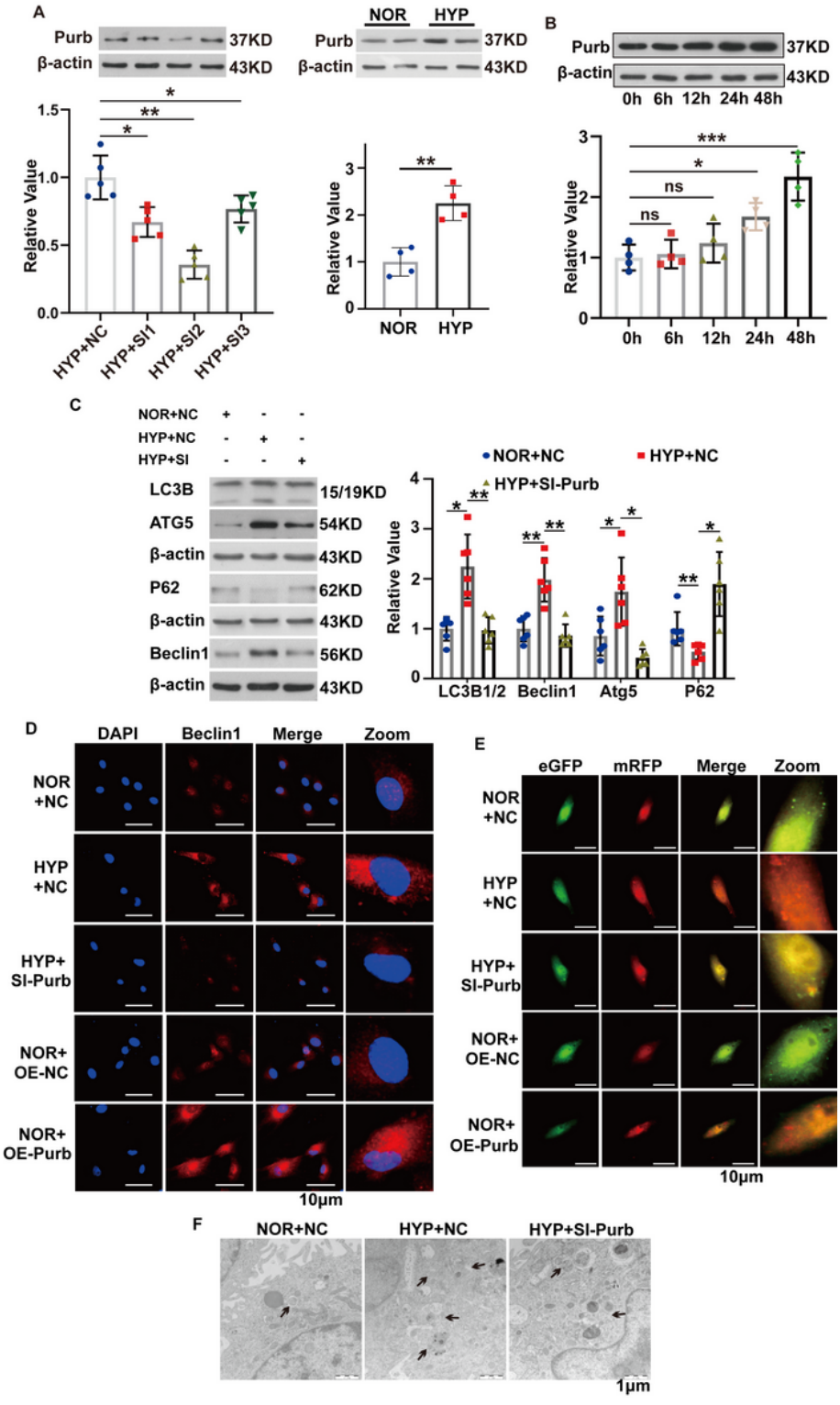

Figure 4

Purb knockdown inhibits hypoxia-induced autophagy. a The interference efficiency (n = 5) and overexpression efficiency (n = 4) were detected by western blotting. b The expression of Purb at different time points by western blotting (n = 4). c PASMCs were tested for the expression of the autophagy-related protein by western blotting (n = 6). d PASMCs were used to detect the expression of autophagy-related protein Beclin1 (n = 3). e PASMCs were photographed using a confocal fluorescence microscope, Scale bar = 10 μm (n = 3). f PASMCs were observed for formation of autophagic vesicles under an electron microscope (n = 3). All values are expressed as the mean ± SEM. *P<0.05, **P<0.01, ***P<0.001.

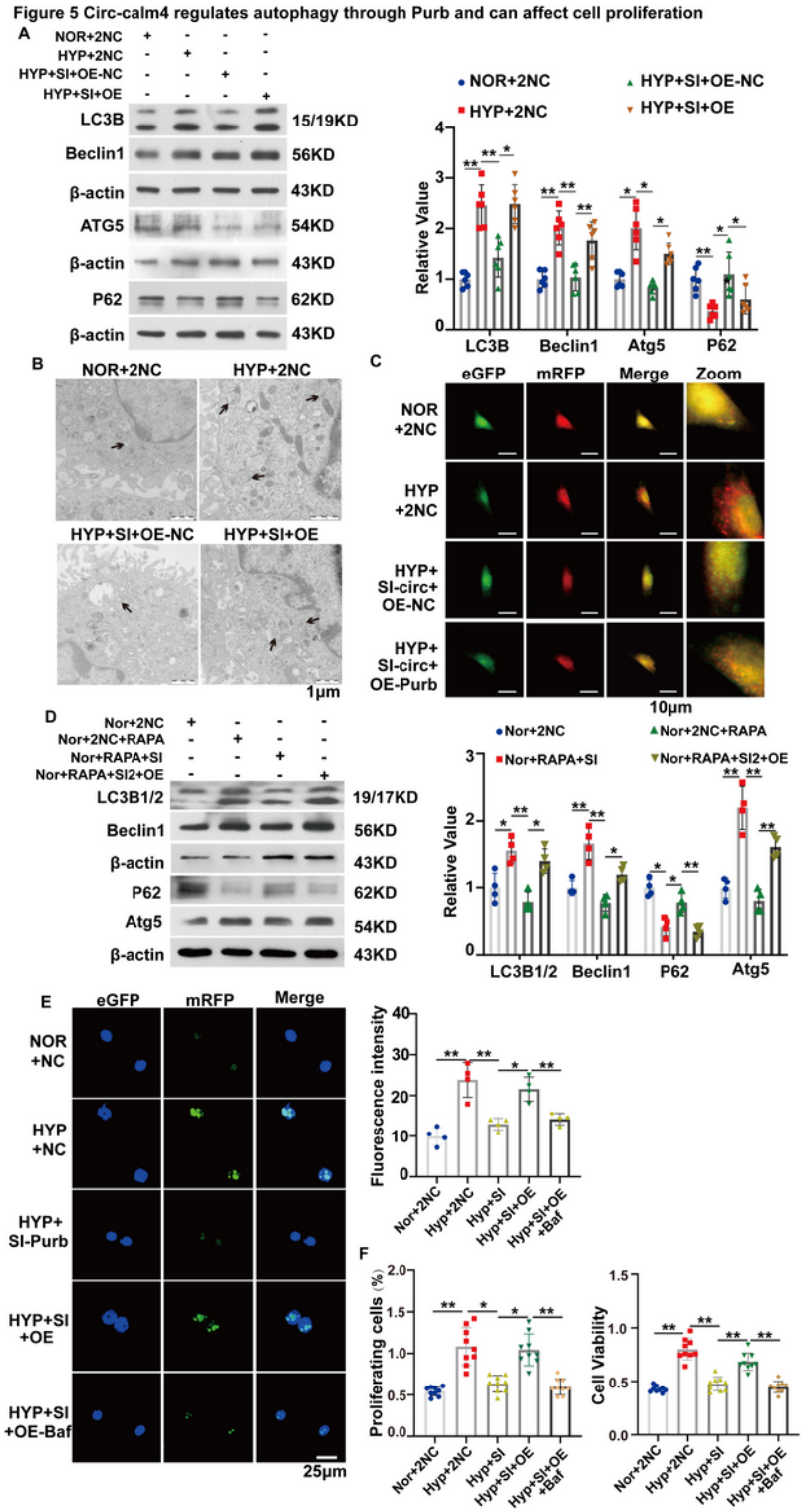

Figure 5

Purb knockdown inhibits hypoxia-induced autophagy. a PASMCs were extracted and tested for the expression of autophagy-related proteins by western blotting (n = 6). b PASMCs were observed for the formation of autophagic vesicles under an electron microscope (n = 3). c PASMCs were photographed using a confocal fluorescence microscope, Scale bar = 10 μm (n = 3). d PASMCs were treated with the autophagy agonist rapamycin (RAPA) (n = 4). e, f Ki67 immunofluorescence semi-quantitative, EDU, MTT

experiments determine the proliferation ability of pulmonary artery smooth muscle cells. All values are expressed as the mean ± SEM. *P<0.05, **P<0.01, ***P<0.001.

Figure 6 Purb promotes the transcription of autophagy-related protein Beclin1 by super-enhancer modification in the Beclin1 promoter region

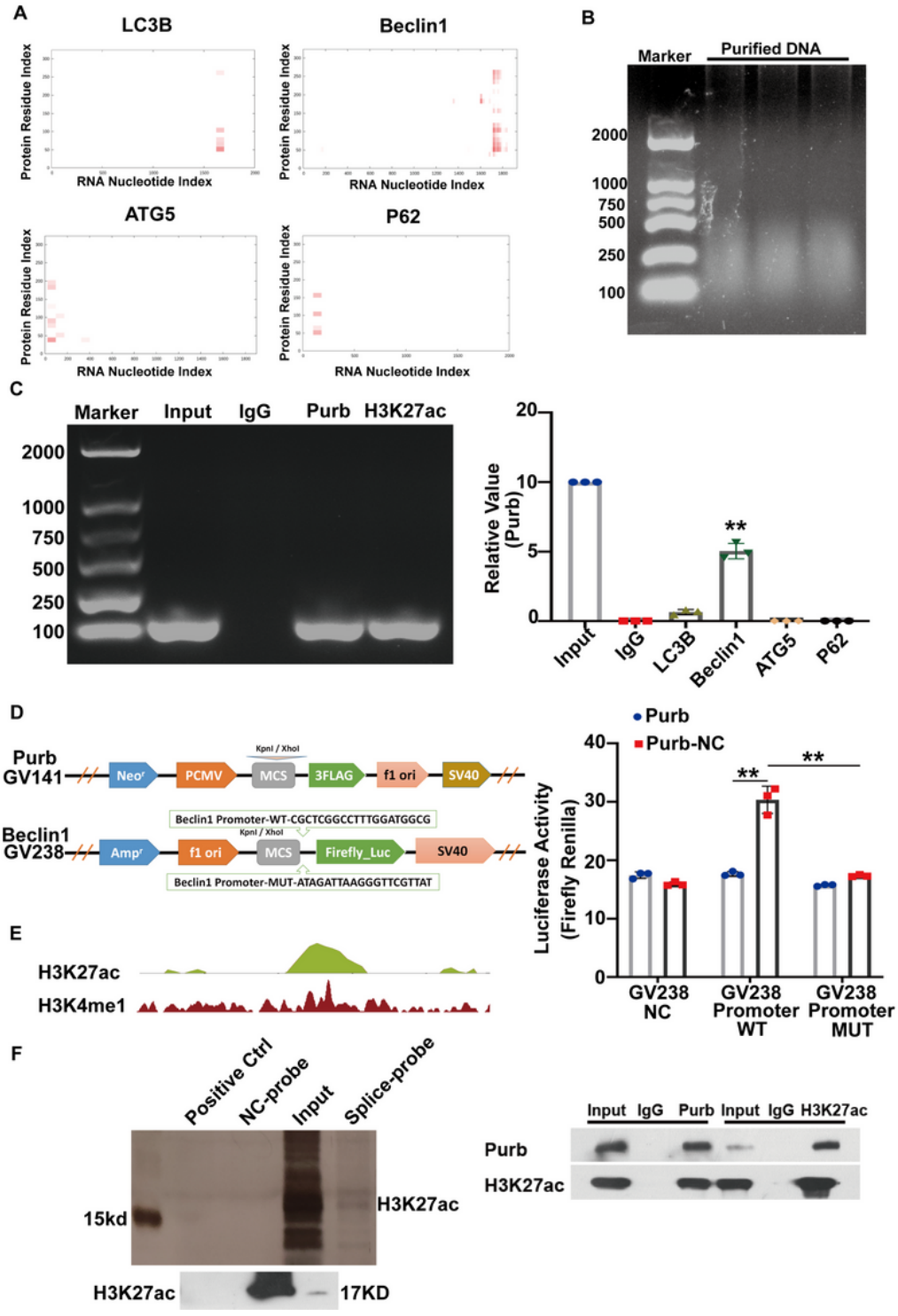

Figure 6

Purb promotes the transcription of autophagy-related protein Beclin1 by super-enhancer modification in the Beclin1 promoter region. a The software catRAPID was used to analyze the binding ability of Purb to the promoter region of autophagy-related proteins. b The ultrasonic conditions were determined by using

an ultrasonic breaker to split the DNA into 200-1000-bp target fragments (n = 3). c Chromatin immunoprecipitation (CHIP) results show that Purb and H3K27ac can bind to the Beclin1 promoter region (n = 3). d Dual-luciferase assays were used to validate the interactions between Purb and Beclin1 promoter (n = 3). e USCS and catRapid software show that the Beclin1 promoter region was modified with super-enhancer markers H3K27ac and H3K4me1. f The RNA pull-down experiment shows that circ-calm4 can pull down H3K27ac (n = 3). Chromatin immunoprecipitation results show that Purb and H3K27ac bind to each other (n = 3). All values are expressed as the mean ± SEM. *P<0.05, **P<0.01, ***P<0.001.

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