



## ARTICLE

# Downregulation of NUP93 aggravates hypoxia-induced death of cardiomyocytes in vitro through abnormal regulation of gene transcription

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Nuclear pore complex in the nuclear envelope plays an important role in controlling the transportation of RNAs, proteins and other macromolecules between the nucleus and cytoplasm. The relationship between abnormal expression of nucleoporins and cardiovascular diseases is unclear. In this study we investigated how myocardial infarction affected the expression and function of nucleoporins in cardiomyocytes. We separately knocked down 27 nucleoporins in rat primary myocardial cells. Among 27 nucleoporins, knockdown of Nup93, Nup210 and Nup214 markedly increased the expression of *ANP* and *BNP*, two molecular markers of cardiomyocyte function. We showed that Nup93 was significantly downregulated in hypoxic cardiomyocytes. Knockdown of Nup93 aggravated hypoxia-induced injury and cell death of cardiomyocytes, whereas overexpression of Nup93 led to the opposite effects. RNA-seq and bioinformatics analysis revealed that knockdown of Nup93 did not affect the overall transportation of mRNAs from the nucleus to the cytoplasm, but regulated the transcription of a large number of mRNAs in cardiomyocytes, which are mainly involved in oxidative phosphorylation and ribosome subunits. Most of the down-regulated genes by Nup93 knockdown overlapped with the genes whose promoters could be directly bound by Nup93. Among these genes, we demonstrated that Nup93 knockdown significantly down-regulated the expression of YAP1. Overexpression of YAP1 partially rescued the function of Nup93 knockdown and attenuated the effects of hypoxia on cell injury and cardiomyocyte death. We conclude that down-regulation of Nup93, at least partially, contributes to hypoxia-induced injury and cardiomyocyte death through abnormal interaction with the genome to dynamically regulate the transcription of YAP1 and other genes. These results reveal a new mechanism of Nup93 and might provide new therapeutic targets for the treatment of ischemia-induced heart failure.

**Keywords:** myocardial infarction; hypoxia; nuclear pore complex; NUP93; YAP1; transcription

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## INTRODUCTION

Myocardial infarction remains one of the leading causes of mortality and morbidity in developing or developed countries [1]. Considerable advances have been made in our understanding and management of cardiac infarction [2, 3]. However, to date, the cellular and molecular mechanisms underlying myocardial infarction remain largely unclear [4, 5]. In myocardial infarction, cardiomyocyte apoptosis and necrosis are important features that result in heart remodeling and cardiac insufficiency [6]. Several studies have shown that reducing cardiomyocyte apoptosis and necrosis can attenuate cardiac remodeling and improve cardiac function after myocardial infarction [7, 8]. The clarification of cellular and molecular mechanisms of cardiomyocyte apoptosis might help to optimize the prevention and treatment strategies of patients with myocardial infarction and to reduce mortality and morbidity [5].

The nuclear envelope represents a protective membrane barrier that regulates molecular trafficking between the nucleoplasm and cytoplasm [9]. Recent studies have demonstrated the crucial roles of the nuclear envelope in regulating genome architecture [10]. For instance, mutation of lamin A/C promoted aging by abnormally modulating genome structure [11]. In the nuclear envelope, the nuclear pore complex has an important function in controlling the transportation of RNAs, proteins and other macromolecules between the nucleus and cytoplasm [12]. The nuclear pore complex is involved in the regulation of cell proliferation, differentiation, apoptosis, necrosis, and other important cell activities [13, 14]. Therefore, abnormal regulation of the nuclear pore complex might affect cell homeostasis, and thus result in the occurrence and development of diseases.

Nuclear pore complexes are mainly formed by ~30 nucleoporins, which can be divided into two categories, structural scaffold

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nucleoporins, and peripheral component nucleoporins, according to their location and function in the nuclear pore complex (NPC) [15]. The structural scaffold nucleoporins are embedded in the nuclear membrane to form a central channel. The peripheral nucleoporin components generally contain phenylalanine glycine (FG) repeat sequences that form a filter-like structure, which recognizes nuclear localization sequences and nuclear export sequences (NESs) [16]. The reversible interaction of nucleoporins with transporters and substrates controls the entry and exit of macromolecules into or out of the nucleus [17, 18]. Additionally, nucleoporins have been reported to be widely involved in or directly regulate subcellular processes such as DNA replication [19, 20] and mRNA transcription [21].

The linkage between nucleoporins and cardiovascular diseases was first reported in a family with inherited atrial fibrillation (AF) [22]. We reported that Nup35 increased the expression of NHE1 protein by promoting the nucleation of NHE1 mRNA under normal conditions and hypoxic/ischemic conditions, and it regulated the pH balance in cardiomyocytes [23]. However, the relationship between abnormal expression of nucleoporins and cardiovascular diseases is still unclear, and studies about the pathophysiological functions of abnormal regulation of nucleoporins in cardiovascular diseases are relatively weak. In this manuscript, we knocked down 27 nucleoporins [24] to screen for nucleoporins whose abnormal regulation might play an important function in cardiomyocytes. Then, Nup93 was focused on to investigate whether its down-regulation in myocardial infarction accounted for cardiomyocyte apoptosis and necrosis by regulating transcription.

## MATERIALS AND METHODS

### Human heart sample collection

The collection of clinical specimens was approved by the Ethics Committee of the Second Military Medical University. All human heart samples used in this study were from patients who had organ transplants in ChangHai Hospital from 2012 to 2018. ICM samples came from the abandoned hearts of heart transplant acceptors after surgery. Normal heart samples were obtained from donors, whose livers or other organs were transplanted, while the hearts were not suitable for transplantation. All samples were collected with informed consent from the patients or their families. Fresh specimens were obtained during clinical surgery and stored at  $-80^{\circ}\text{C}$  after freezing with liquid nitrogen.

### Rat cardiac ischemia model

All animal procedures were approved by the Committee of Care and Use of Experimental Animals, Second Military Medical University, and followed the Guidelines of the National Institutes of Health (NIH). The cardiac ischemia model was prepared as described previously [8]. In brief, adult male Sprague-Dawley rats (250–280 g) were anesthetized with isoflurane. The standard limb I-conductive electrocardiogram (ECG) was monitored simultaneously. A 6/0 braided silk was used to ligate the left coronary artery pre-descending (LAD), resulting in myocardial ischemia. The rats that underwent the same surgery, except for LAD, were used as surgical controls. The hearts were harvested at 0, 2, 4, 8, and 12 h after surgery and the infarct zone of the hearts was used for further analyzing the expression of NUP93 and other molecules.

### A hypoxia model of rat primary cardiomyocytes

Primary cardiomyocyte culture was performed as described previously [25]. Cardiomyocytes were cultured in Dulbecco's modified Eagle's medium (DMEM, Sigma-Aldrich Corp., St. Louis, MO, USA), containing 10% (*v/v*) fetal bovine serum (FBS, HyClone Laboratories, Logan, UT, USA), in a humid atmosphere of 95% air-5%  $\text{CO}_2$ . After serum deprivation, the cells were exposed to 1%  $\text{O}_2$ -94%  $\text{N}_2$ -5%  $\text{CO}_2$  for 24 h to induce hypoxia. Cardiomyocytes cultured in an aerobic environment were used as controls.

### Transfection of cells with siRNAs

Cardiomyocytes were cultured in serum-free DMEM for 12 h before transfection. Nucleoporin siRNAs were commercially obtained from GenePharma Corp. (SuZhou, China). siRNAs were diluted in RNase-free water to 20 nM and 5  $\mu\text{l}$  siRNA solution/well was transfected into cardiomyocytes with Lipofectamine 2000 following the manufacturer's instructions. The nontargeted siRNA was used as a negative control (NC). If hypoxia was used to induce cell death, 24 h after transfection, cardiomyocytes were exposed to 1%  $\text{O}_2$ -94%  $\text{N}_2$ -5%  $\text{CO}_2$  for 24 h. Forty-eight hours after transfection, the cardiomyocytes were harvested for further analysis.

### Adenovirus establishment and transfection

The adenovirus serotype 5 (Ad5) system was used to overexpress Nup93 and YAP1 in neonatal cardiomyocytes. Briefly, the CDS of rat Nup93 or YAP1 was cloned into pAdtrack vectors (adenoviral shuttle vector). Adtrack-Nup93 or Adtrack-YAP1 was further transferred into BJ5183 bacteria which was pre-transformed with the pAdEasy-1 plasmid (the adenoviral backbone plasmid vector). In BJ5183-AD-1 cells, a recombinant adenovirus was established by a double recombination between pAdEasy-1 and Adtrack vectors. AdEasy-Nup93 or AdEasy-YAP1 was digested with Pac I to expose its inverted terminal repeats (ITRs), and then transfected to 293A cells to construct adenovirus. After amplification and titer determination, 50 Multiplicity of Infection (MOI) was used to transfect cardiomyocytes to overexpress Nup93 and YAP1. If hypoxia was designed for the experiment, 12 h after transfection, cardiomyocytes were exposed to 1%  $\text{O}_2$ -94%  $\text{N}_2$ -5%  $\text{CO}_2$  for 36 h. Forty-eight hours after transfection, the cardiomyocytes were harvested for further analysis.

### Quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR)

Total RNA was extracted with TRIzol (Invitrogen, China, Shanghai). Reverse transcription was performed with M-MLV using oligo-dT as the RT primer. The amounts of target genes were determined by qPCR using SYBR Green methods in a LightCycler-480 machine. The levels of GAPDH were used as endogenous controls with the  $2^{-\Delta\Delta\text{Ct}}$  analysis method.

### Western blot

Samples from tissues or cardiomyocytes were lysed with RIPA buffer (Beyotime, Hangzhou, China) and treated with ultrasound on ice. The protein concentration was determined by a bicinchoninic acid kit (Beyotime, Hangzhou, China). A total of 40  $\mu\text{g}$  protein/well was electrophoresed in a 10% SDS-PAGE gel and transferred to PVDF membrane (Bio-Rad, America). The membranes were blocked with 5% skim milk for 2 h, and then incubated with primary antibodies overnight, and HRP-conjugated secondary antibodies for 2 h. The protein levels were detected by ECL (XinSaiMei, Suzhou, China). The antibodies were anti-Caspase-3 rabbit mAb (A19654, Abclonal, Wuhan, China), anti-cleaved-Caspase-3 rabbit pAb (CST, #9664), anti-NUP93 (A4333, Abclonal, Wuhan, China), anti-BCL2 mouse mAb (CST, #15071), anti-GAPDH rabbit mAb (ABways, Ab0037), and anti-YAP1 (CST, #12395).

### Determination of cell injury and viability

Cell viability was determined by Cell Counting Kit-8 assays (Beyotime, C0037). The structural integrity of myocardial cells after hypoxia was evaluated by measuring the release of lactic acid dehydrogenase (LDH) using an LDH Cytotoxicity Assay Kit (Beyotime, C0016).

### Analysis of cell death

The rate of cell death was determined by an Annexin V-FITC Apoptosis Detection Kit (Beyotime, C1062) according to the manufacturer's instructions. Based on the methods we reported earlier [8], cardiomyocytes were digested with trypsin, washed,

double-stained with AV and PI, and then analyzed with a flow cytometer.

#### RNA isolation from nuclear and cytoplasmic fractions

The nuclear and cytoplasmic fractions from cardiomyocytes were isolated with a PARIS Kit (Life Technologies, AM1921) according to the manufacturer's instructions.

#### mRNA transcriptome sequencing and data analysis

After determination of the quality of the total RNA, mRNA was captured, fragmented and subjected to reverse transcription. After fragment selection and PCR, the libraries were sequenced with an Illumina kit. After determination of the quality of the raw data, the sequences were treated with Cutadapt (version 1.9.1). The clean data were mapped to the rat reference genome with HISAT2 (v2.0.1). Gene expression was evaluated by Htseq (V0.6.1) and normalized to FPKM (fragments per kilo bases per million reads). The relationships among samples were analyzed by the Pearson correlation coefficient and principal component analysis (PCA). The differences in gene expression were analyzed with DESeq (V1.38.0) with the standard parameters (fold change >2 and *q* value (*fdr*, *Padj*) < 0.05). Functional analysis of the genes was performed using the Cytoscape plug-in ClueGO.

#### Analysis of NUP93-interacting proteins

The proteins that interacted with NUP93 were analyzed through the STRING database and BioGRID. Network and functional analyses were performed using the STRING database and Cytoscape ClueGO. The hub genes were analyzed by Cytoscape.

#### Statistics and data analysis

All data are expressed as the mean  $\pm$  standard deviation. Two-tailed unpaired Student's *t* test was used for statistical comparisons between two groups. One-way ANOVA was used to analyze data for more than two groups.

## RESULTS

### Knockdown of nucleoporins resulted in abnormal expression of ANP and BNP in neonatal cardiomyocytes

Normal expression of nucleoporins might play an important role in the maintenance of cardiomyocytes. Here, we knocked down 27 nucleoporins by siRNA transfection into primary cultured myocardial cells. Forty-eight hours after transfection, RNA samples were collected, and the expression of two molecular markers of cardiomyocyte function, ANP and BNP, was detected by quantitative RT-PCR. The expression of ANP was upregulated in Nup210, Nup214, and Nup93 knockdown cardiomyocytes (Fig. 1a). The expression of BNP was increased after knocking down Ndc1, Nup210, Nup214, Nup35, Nup43, and Nup93, and was decreased after knocking down Nup155, Nup160, Nup205, Nup50, Nup54, Nup62, Nup85, Nup88, Nup98, NupL2, and pom121 (Fig. 1b). Both ANP and BNP were dynamically regulated by Nup93, Nup210, and Nup214. The knockdown efficacy of siRNA on Nup93, Nup210, and Nup214 was confirmed by qRT-PCR (Supplementary Fig. 1). These results indicated that abnormal expression of nucleoporins might result in dysfunction of cardiomyocytes and might represent a previously unknown mechanism underlying heart disease. To evaluate the expression of Nup93, Nup210, and Nup214 in human heart diseases, we analyzed the data from the GEO database and found that compared with the control, Nup93 was significantly downregulated (GSE16499), while the expression of Nup210 and Nup214 was not altered in human ischemic hearts (Fig. 1c).

Nup93 was significantly reduced in ischemic human hearts and downregulated in a rat model of LAD-induced cardiac ischemia. We established a rat model of myocardial infarction by ligation of the left front branching of the coronary artery. Heart samples for

analyzing the expression of Nup93 were collected in the acute stage after infarction. The results showed that both the mRNA and protein levels of Nup93 were significantly decreased in the ischemic hearts compared with the sham control hearts (Fig. 1d, e). The expression of Nup93 was then detected using qRT-PCR and Western blotting in heart samples collected by us at ChangHai Hospital from 2012 to 2018. In comparison with the data from normal hearts, the mRNA and protein levels of Nup93 were both significantly decreased in ischemic hearts (Fig. 1f–h). These results indicated that the expression of Nup93 was abnormally regulated in ischemic heart disease, which might have an important role in the cell injury of cardiomyocytes induced by hypoxia.

### Knockdown of Nup93 promoted hypoxia-induced cell injury and cell death of neonatal cardiomyocytes

To investigate the function of Nup93 in hypoxia-induced cardiomyocyte injury, we used siRNAs to knock down Nup93 in cultured primary cardiomyocytes. Using qRT-PCR, we showed that all 4 siRNAs could knock down Nup93 mRNA (Fig. 2a). The protein level of Nup93 was also revealed by Western blots, showing that Nup93 was markedly downregulated by siRNA transfection (Fig. 2b).

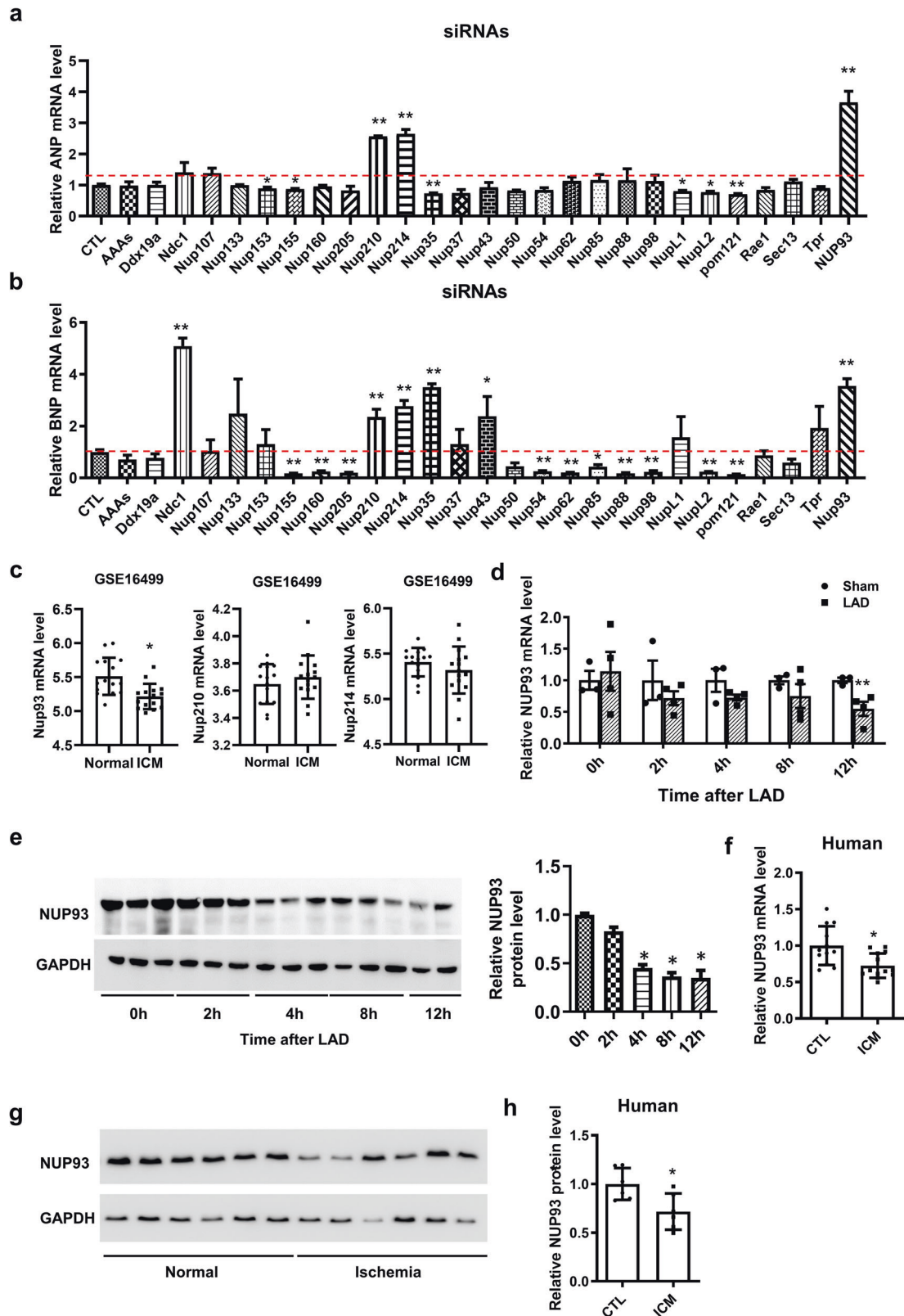
After transfection, we cultured the cells in 1% O<sub>2</sub>-94% N<sub>2</sub>-5% CO<sub>2</sub> for 24 h to induce a hypoxia model, and detected the effects of Nup93 knockdown on hypoxia-induced cell injury and cell death. In normoxic cultured cardiomyocytes, knockdown of Nup93 markedly increased the release of LDH (Fig. 2c). Twenty-four hours after hypoxia, the release of LDH was further increased by knockdown of Nup93 (Fig. 2c). These results indicate that knockdown of Nup93 exacerbated hypoxia-induced damage to the membrane integrity of cardiomyocytes. Additionally, by CCK8 analysis, we showed that knockdown of Nup93 significantly decreased the viability of cardiomyocytes cultured under both normoxic and hypoxic conditions (Fig. 2d). Using Western blotting, we showed that the cleavage of CASP3 was markedly upregulated, and the expression of BCL2 was significantly downregulated by Nup93 knockdown in cardiomyocytes (Fig. 2e). Using flow cytometry, we found that the percentage of viable cells was decreased with Nup93 knockdown in normal cultured cardiomyocytes and in hypoxic cells (Fig. 2f, g). These results indicated that abnormal regulation of Nup93 might act as an endogenous mechanism for ischemia-induced cardiomyocyte injury and cell death.

### Overexpression of Nup93 suppressed hypoxia-induced cardiomyocyte injury and cell death

Further evaluation with data from GSE16499 showed a negative relationship between Nup93 and ANP in human heart samples (Fig. 3a). To determine whether the downregulation of Nup93 induced by ischemia plays an important role in cell injury and cell death, we overexpressed Nup93 in cardiomyocytes with adenovirus at an MOI of 50 (Fig. 3b). Western blot analysis showed that Ad-Nup93 significantly upregulated the expression of Nup93 in cardiomyocytes (Fig. 3b). Twelve hours after transfection, cardiomyocytes were cultured under hypoxic conditions for another 36 h. Through a series of assays, we found that overexpression of Nup93 decreased the level of hypoxia-induced LDH release (Fig. 3c) and suppressed the effects of hypoxia on cell viability (Fig. 3d). Overexpression of Nup93 suppressed the effects of hypoxia on regulating the protein levels of CASP3 and BCL2 (Fig. 3e) and decreased hypoxia-induced apoptosis and necrosis of cardiomyocytes (Fig. 3f, g). Therefore, upregulation of Nup93 might represent a new strategy to suppress hypoxia-induced heart diseases.

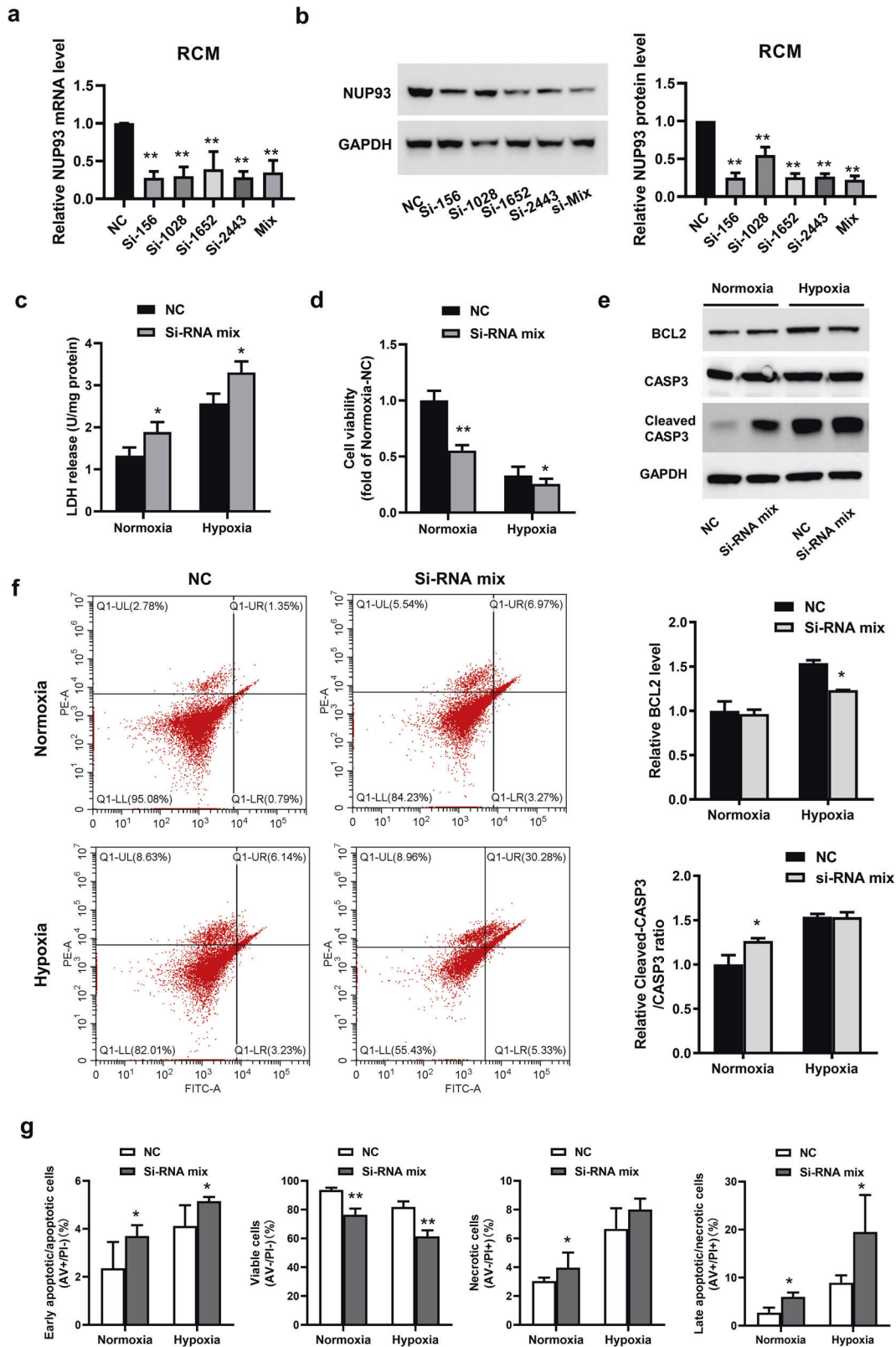
### The function of Nup93 in neonatal cardiomyocytes was involved in chromatin binding-related transcription

Nup93 is a component of the nuclear pore complex, which has an important function in regulating the transportation of cargo.

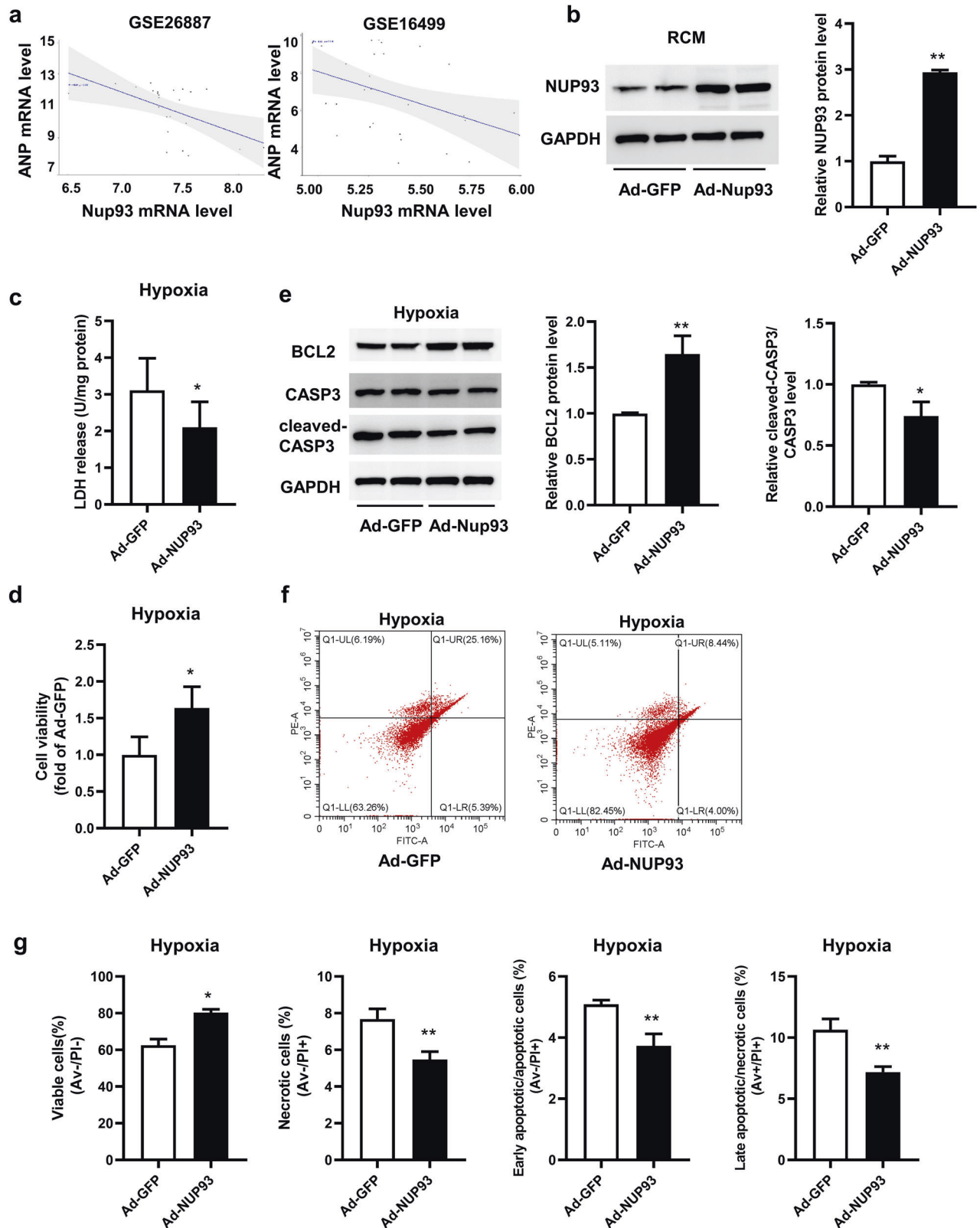


**Fig. 1** Knockdown of nucleoporins resulted in abnormal expression of ANP and BNP in cardiomyocytes. **a, b** The expression of ANP and BNP was upregulated in Nup210, Nup214, and Nup93 knockdown cardiomyocytes ( $n = 3$ ). **c** Nup93 was significantly downregulated in human ischemic hearts in the GSE16499 dataset. **d, e** Both mRNA and protein levels of Nup93 were significantly decreased in the ischemic hearts compared with the sham control hearts from the rat model ( $n = 3$ ). **f–h** In comparison with the data from the normal hearts, the mRNA and protein levels of Nup93 were both significantly decreased in the ischemic hearts ( $n = 6$ ). \* $P < 0.05$ , \*\* $P < 0.01$ .





**Fig. 2** Knockdown of Nup93 promoted hypoxia-induced cardiomyocyte injury and cell death. **a, b** Nup93 was markedly downregulated by siRNA transfection in rat cardiomyocytes (RCMs) ( $n=3$ ). **c** The release of LDH was increased by knockdown of Nup93 ( $n=10$ ). **d** Nup93 significantly decreased the viability of cardiomyocytes ( $n=10$ ). **e** The cleavage of CASP3 was markedly increased, and the expression of BCL2 was significantly downregulated by Nup93 knockdown. **f, g** The percentage of viable cells was decreased with Nup93 knockdown in normal cultured cardiomyocytes, as well as in hypoxic cells ( $n=3$ ). \* $P < 0.05$ , \*\* $P < 0.01$ .



**Fig. 3 Overexpression of Nup93 suppressed hypoxia-induced cardiomyocyte injury and cell death.** **a** A negative relationship between Nup93 and ANP in human heart samples. **b–d** Overexpression of Nup93 in cardiomyocytes with adenovirus decreased the level of hypoxia-induced LDH release and suppressed the effects of hypoxia on cell viability ( $n = 3$ ). **e–g** Overexpression of Nup93 suppressed the effects of hypoxia on regulating the protein levels of BCL2 and cleavage of CASP3 and decreased hypoxia-induced apoptosis and necrosis of cardiomyocytes ( $n = 3$ ). \* $P < 0.05$ , \*\* $P < 0.01$ .

Therefore, we separated the cytoplasmic and nuclear fractions of cardiomyocytes after transfection with siRNA-NC, Nup93 siRNA-156, or the Nup93 siRNA mix. The successful isolation of cytoplasmic and nuclear fractions of cardiomyocytes was verified by qRT-PCR to detect the distribution of U6, 18S, and GAPDH (data not shown). Then, we performed mRNA-seq to illustrate whether the location and expression of mRNAs were affected by Nup93 knockdown in cardiomyocytes. The profiles of mRNAs in different groups were analyzed by PCA and heatmap analysis (Supplementary Fig. 2). The results of PCA and the heatmap showed that total RNA, cytoplasmic RNA, and nuclear RNA were clustered together individually. The correlation coefficients between cytoplasmic RNA and total RNA and between nuclear RNA and total RNA were significantly higher than the correlation coefficients between cytoplasmic RNA and nuclear RNA (Fig. 4a). These results showed that the distribution of mRNAs in the cytoplasm and nucleus was completely different, indicating that the transportation efficiency of distinct mRNAs from the nucleus to the cytoplasm was different. The differentially expressed genes analyzed by the heatmap showed that a collection of genes was markedly modulated by Nup93 knockdown (Fig. 4b). The differences in mRNAs between the siRNA-NC and two siRNA-Nup93 groups in the cytoplasm and nucleus were not as large as those in total samples, indicating that the overall distribution of mRNAs in the nucleus and cytoplasm was not affected by Nup93 knockdown in cardiomyocytes (Figs. 4c, 4d). These results indicated that the effects of Nup93 knockdown on hypoxia-induced cell injury and death might not be due to its function in regulating mRNA transportation from the nucleus to the cytoplasm, but attributed to its participation in transcription.

Knockdown of Nup93 markedly affected the transcription of mRNAs involved in oxidative phosphorylation and ribosome subunits

A volcano plot displays the expression of genes in the siRNA-NC and siRNA-mix-Nup93-transfected cardiomyocytes (Fig. 4c, d), showing that 2163 genes were downregulated and 1138 genes were upregulated by Nup93 knockdown. This result is similar to the differentially expressed genes between the siRNA-NC and siRNA-156-Nup93 groups (Fig. 4d). The Venn diagram showed that 34.9% of these downregulated genes and 35.3% of these upregulated genes overlapped with the results of siRNA-156 transfected cardiomyocytes (Fig. 4e). Furthermore, functional enrichment analysis of these abnormally expressed mRNAs affected by Nup93 knockdown showed that they were enriched in oxidative phosphorylation and ribosome subunits (Fig. 5a). The hub genes were enriched in ribosome and mitochondria located proteins (Fig. 5b). Therefore, we considered that the effects of Nup93 knockdown in cardiomyocytes were, at least partially, mediated by regulating the transcription of mitochondrial or ribosome-related genes.

We further analyzed the abnormally regulated genes in the cytoplasm or nucleus affected by Nup93 knockdown, showing that 217 genes were upregulated and 216 genes were downregulated in nuclear mRNAs, while 468 genes were downregulated and 358 genes were upregulated in cytoplasmic RNAs. The downregulated genes in the nucleus were enriched in neural crest cell migration; upregulated genes in the cytoplasm were enriched in response to ischemia; and downregulated genes in the cytoplasm were enriched in cellular detoxification; and the others were not closely related to hypoxia-induced injury. These aspects might not be as important as the regulation of oxidative phosphorylation and ribosome assembly, indicating that the function of Nup93 knockdown in hypoxia-induced cell injury and death might not be attributed to the abnormal translocation of mRNA but instead be mediated through regulating the transcription of various genes.

Transcriptional regulation by Nup93 occurs through direct interactions between Nup93 and chromosomes

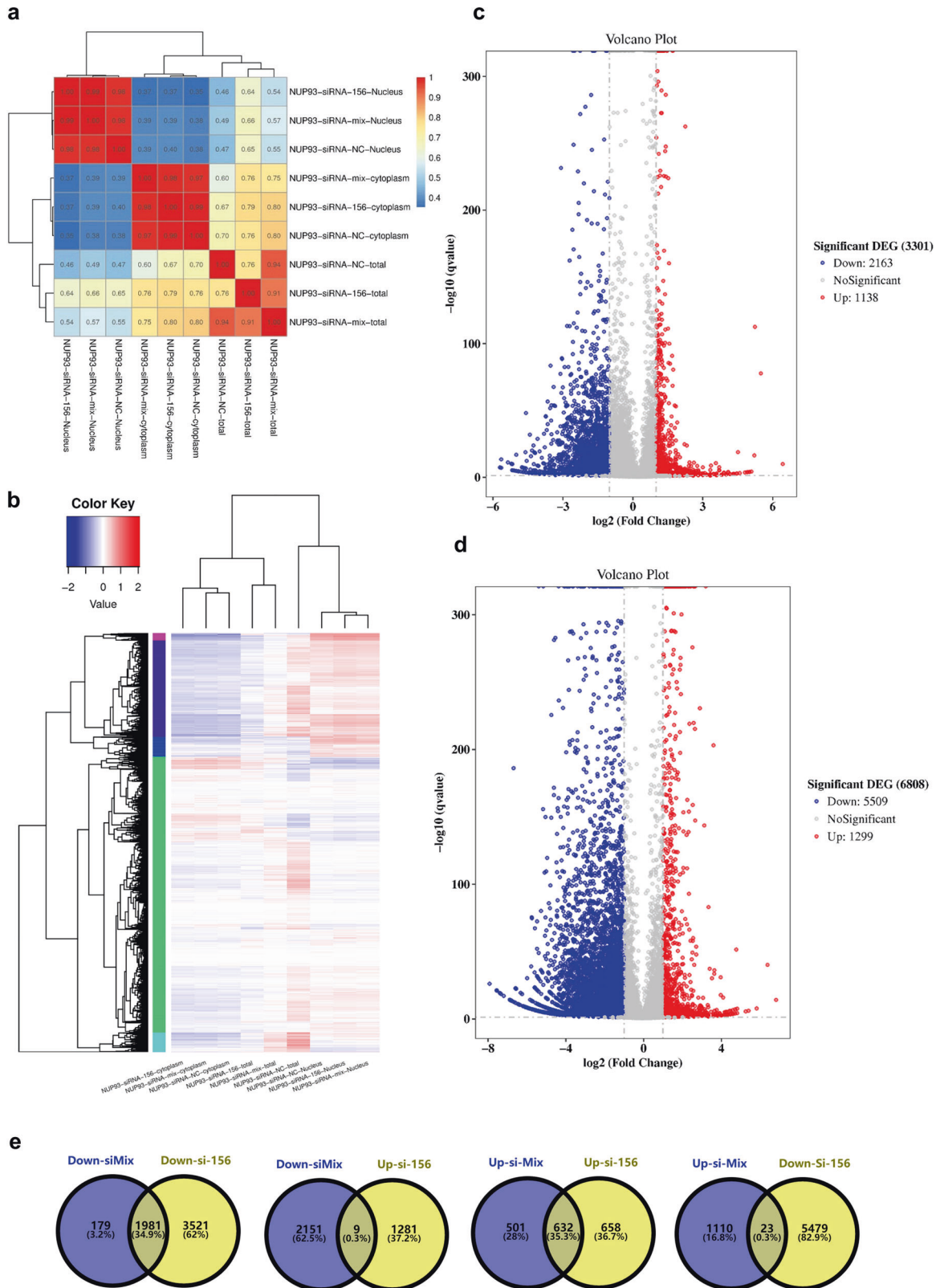
To explore the mechanism through which downregulation of Nup93 increased hypoxic cardiomyocyte injury, we analyzed the proteins that can interact with Nup93 through the STRING database and BioGRID (Fig. 6a). Component analysis showed that Nup93-interacting proteins were enriched in the nuclear pore nuclear basket (Fig. 6b). The functional analysis of these Nup93-interacting proteins showed that they were mainly enriched in RING-like zinc finger domain binding, structural integrity of nuclear pores, histone deacetylase activity, transcription factor binding and coactivator activity, RNA polymerase II proximal promoter, and others (Fig. 6b). The KEGG pathway analysis showed that these Nup93-interacting proteins were enriched in RNA transport, ubiquitin mediated proteolysis, cancers, apoptosis, the HIF-1 signaling pathway, and so on (Fig. 6b). The interactions of these proteins were also analyzed through the STRING database, and the hub genes were selected by Cytoscape (Fig. 6b). These results indicate that the mechanism by which Nup93 protects cardiomyocytes from hypoxia-induced cell injury might involve the regulation of direct interactions with chromosomes.

The genome-wide distribution of Nup93 was determined by Cut-Run and DNA sequencing in previous reports [26]. Through analysis of these data (GSE137691) with our RNA-seq data, we found that after Nup93 knockdown, 546 downregulated genes, and only 38 upregulated genes overlapped with Nup93-interacting genome regions (Fig. 6c). This result indicated that a direct interaction between Nup93 and chromatin might activate the transcription of genes. Among these 546 downregulated genes, we selected YAP1, PDLIM5, and Hif1 $\alpha$  to confirm their downregulation by Nup93 knockdown in cardiomyocytes using qPCR. The results showed that Nup93 knockdown significantly downregulated the expression of YAP1, PDLIM5, and Hif1 $\alpha$  (Fig. 6d). Using ChIP-PCR with an anti-Nup93 antibody, we found that the promoter regions of these three genes were enriched by the Nup93 antibody (Fig. 6e), indicating that transcriptional regulation by Nup93 might occur through, at least partially, a direct interaction between Nup93 and DNA.

Overexpression of YAP1 partially attenuated the effects of Nup93 knockdown and hypoxia on cell injury and death

To investigate whether the downregulation of genes by Nup93 knockdown might contribute to its effects on cardiomyocyte injury, we overexpressed YAP1 $\alpha$  in cardiomyocytes using recombinant adenovirus (Fig. 7a). The effects of Nup93 knockdown on decreasing cell viability were significantly suppressed by YAP1 $\alpha$  overexpression (Fig. 7b–d). Additionally, the increase in ANP and BNP expression induced by Nup93 knockdown was markedly alleviated by YAP1 $\alpha$  overexpression in cardiomyocytes (Fig. 7e). These results indicated that downregulation of YAP1 $\alpha$  induced by Nup93 knockdown might, at least partially, be responsible for the effects of Nup93 knockdown on cell injury and death.

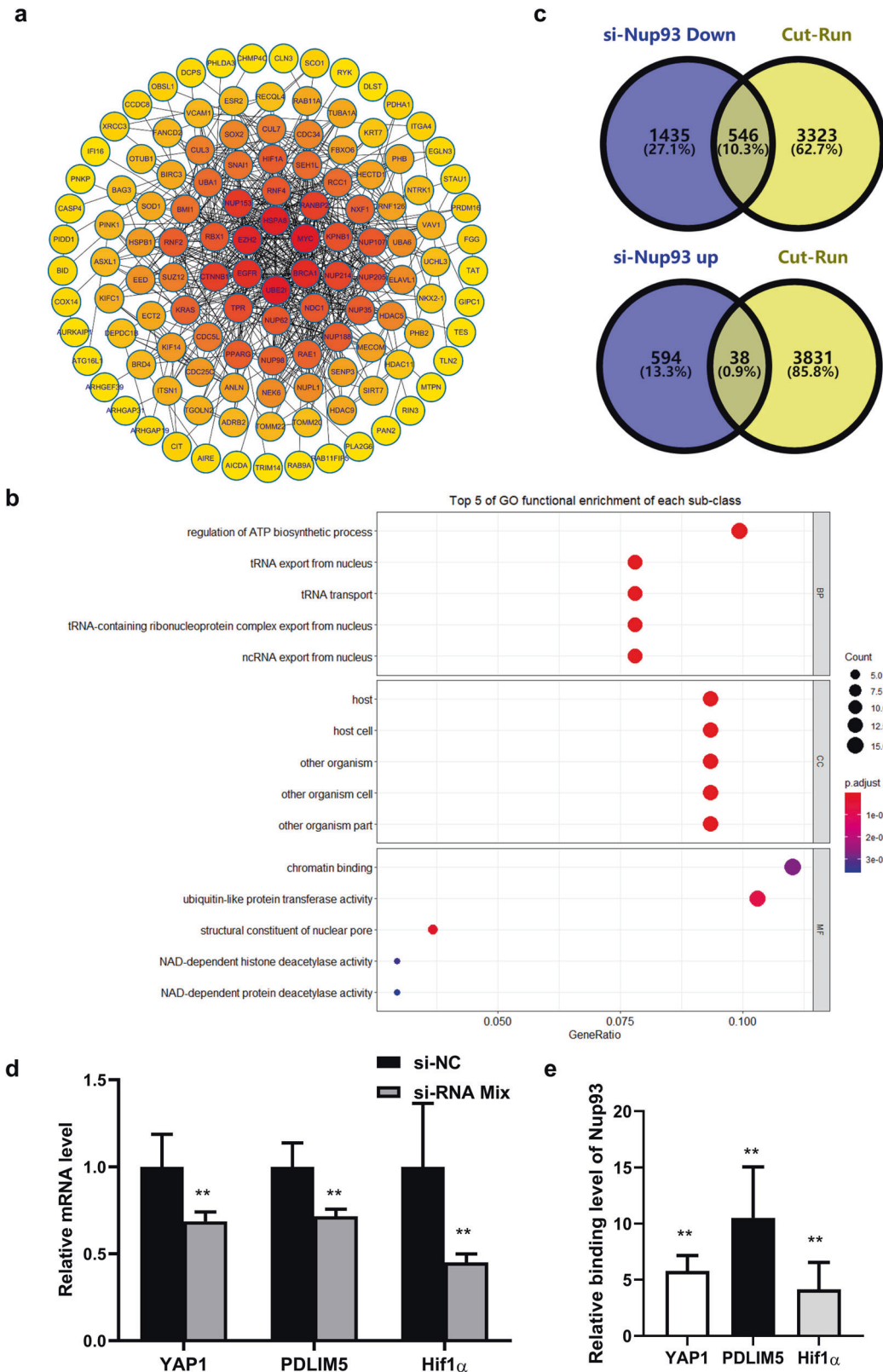
Nup93 was significantly downregulated in ischemic heart diseases (Fig. 1d, e), and knockdown of Nup93 markedly decreased the expression of YAP1 $\alpha$  (Fig. 5d). We propose that downregulation of YAP1 $\alpha$  might contribute to the effects of hypoxia on cell injury and death. Therefore, we analyzed data from GSE16499 and found that YAP1 $\alpha$  was downregulated in ischemic hearts (Fig. 8a). The downregulation of YAP1 was further confirmed by qRT-PCR and Western blot analysis of our collected heart samples (Fig. 8b, c). Therefore, we overexpressed YAP1 $\alpha$  in cardiomyocytes to investigate the effects of YAP1 $\alpha$  on hypoxia-induced cell injury (Fig. 8d). The effects of hypoxia on LDH release (Fig. 8e) and cell viability (Fig. 8f) were suppressed by YAP1 $\alpha$  overexpression. It also showed that over-expression of YAP1 $\alpha$  suppressed the effects of hypoxia on apoptosis and necrosis of cardiomyocytes (Fig. 8g, h). These results indicated that downregulation of YAP1 $\alpha$  induced by Nup93 knockdown might, at least partially, be responsible for the effects of hypoxia on cell injury and death.



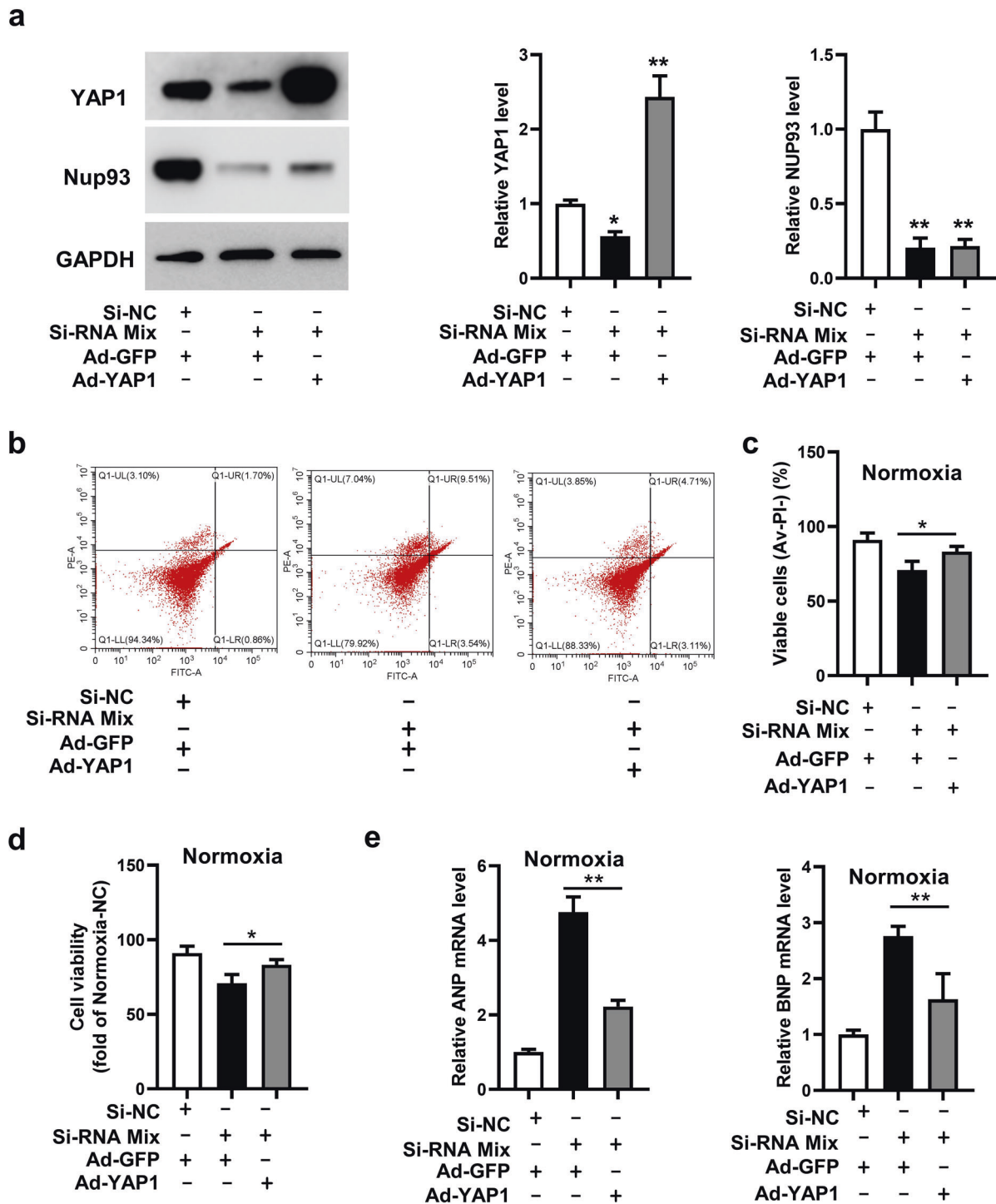
**Fig. 4 The function of Nup93 in cardiomyocytes was involved in chromatin binding related transcription.** **a** The results of the heatmap showed that total RNA, cytoplasmic RNA and nuclear RNA were clustered together individually. **b** The differences in mRNAs between the siRNA-NC and two siRNA-Nup93 groups in the cytoplasm and nucleus were not as large as those in the total samples. **c, d** A volcano plot displays the expression of genes in the siRNA-NC- and siRNA-Nup93-transfected cardiomyocytes. **e** The Venn diagram shows that siRNA-156-Nup93 had similar effects as siRNA-Mix-Nup93.







**Fig. 6 Transcriptional regulation by Nup93 occurs through direct interactions between Nup93 and chromosomes.** **a** The proteins that can interact with Nup93 were analyzed through the STRING database and BioGRID. **b** Functional analysis of Nup93-interacting proteins showed that they were mainly enriched in the regulation of ATP biosynthetic processes and chromatin binding. **c** A direct interaction between Nup93 and chromatin might activate the transcription of genes. **d** Nup93 knockdown significantly downregulated the expression of YAP1, PDLIM5 and Hif1 $\alpha$  ( $n = 3$ ). **e** The promoter regions of YAP1, PDLIM5 and Hif1 $\alpha$  were enriched by the Nup93 antibody ( $n = 3$ ). \* $P < 0.05$ , \*\* $P < 0.01$ .



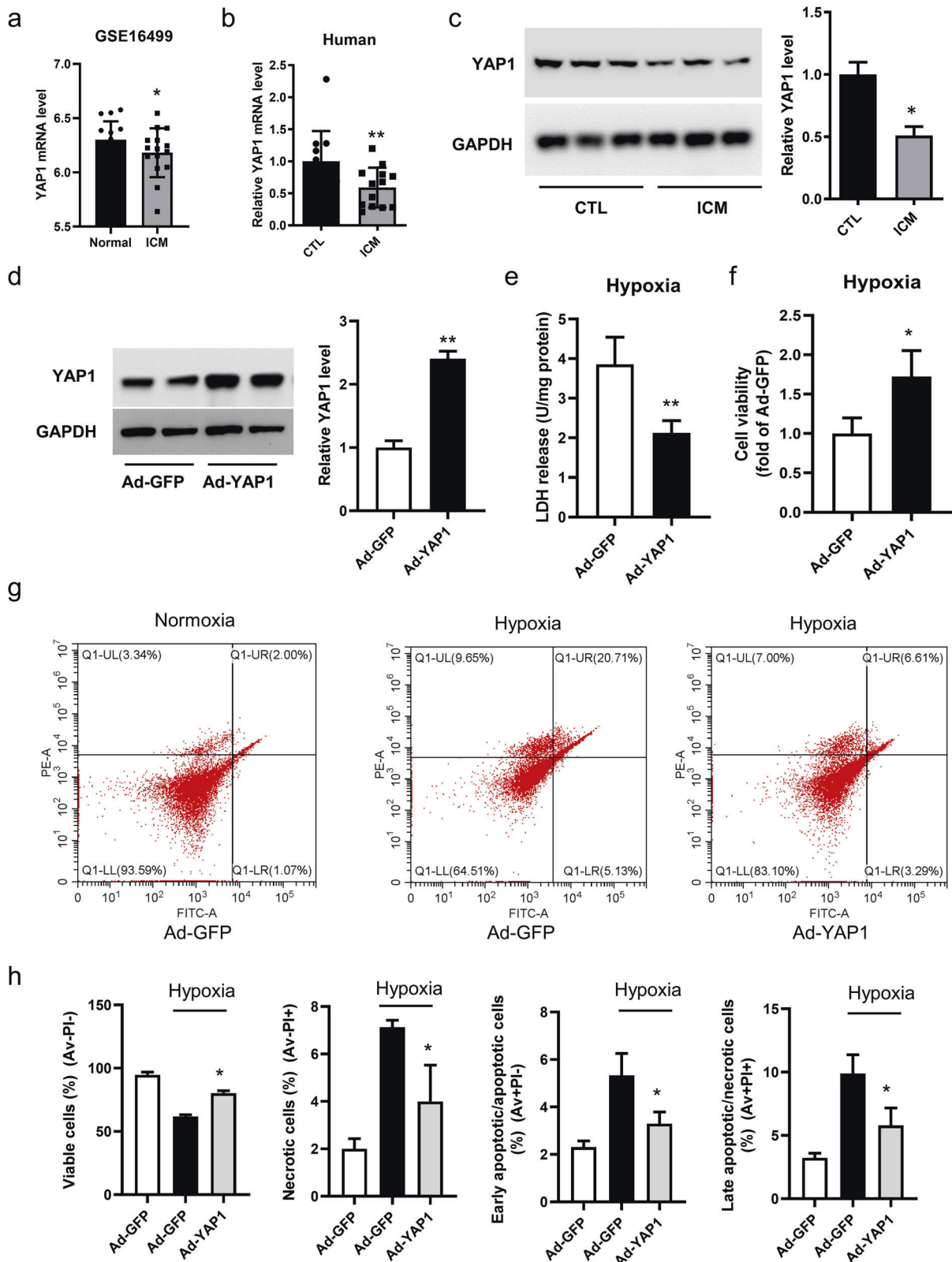
**Fig. 7 Overexpression of YAP1 partially attenuated the effects of Nup93 knockdown and hypoxia on cell injury and death.** **a** Overexpression of YAP1 $\alpha$  in cardiomyocytes using recombinant adenovirus. **b, c** Overexpression of YAP1 $\alpha$  attenuated the effects of Nup93 knockdown on cell death ( $n = 3$ ). **d** The effects of Nup93 knockdown on decreasing cell viability were significantly suppressed by YAP1 $\alpha$  overexpression ( $n = 10$ ). **e** The increase in ANP and BNP expression induced by Nup93 knockdown was markedly alleviated by YAP1 $\alpha$  overexpression ( $n = 3$ ). \* $P < 0.05$ , \*\* $P < 0.01$ .

## DISCUSSION

Nuclear pore complex in the nuclear envelope plays an important role in controlling the transportation of RNAs, proteins and other macromolecules between the nucleus and cytoplasm. As shown in Fig. 9, we showed that the transcription of a collection of genes was regulated by Nup93. These genes regulated by Nup93 are involved in oxidative phosphorylation and ribosome biogenesis. In

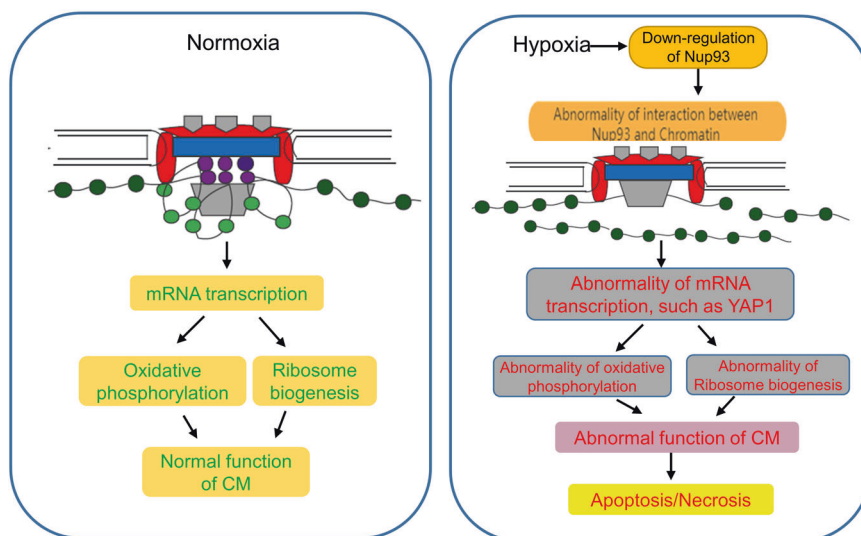
myocardial infarction, hypoxia downregulated the expression of Nup93, which in turn resulted in abnormal interaction between nuclear pore complex and chromatin, leading to aberrant transcription of a set of genes, such as YAP1. Most of these down-regulated genes might, in turn, lead to abnormal function of oxidative phosphorylation and ribosome biogenesis of cardiomyocytes. Therefore, abnormal expression of Nup93 results in





**Fig. 8** Downregulation of YAP1 $\alpha$  might contribute to the effects of hypoxia on cell injury and death. **a** YAP1 $\alpha$  was downregulated in ischemic hearts. **b, c** YAP1 was downregulated in ischemic hearts. **d** Overexpression of YAP1 $\alpha$  in cardiomyocytes. The effects of hypoxia on LDH release (**e**) ( $n = 10$ ), cell viability (**f**) ( $n = 10$ ), and cell death **g, h** ( $n = 3$ ) were suppressed by YAP1 $\alpha$  overexpression. \* $P < 0.05$ , \*\* $P < 0.01$ .





**Fig. 9 Overview of Nup93 on hypoxia-induced cell death.** Nuclear pore complex in the nuclear envelope plays an important role in controlling the transportation of RNAs, proteins, and other macromolecules between the nucleus and cytoplasm. Through interaction with chromatin, nuclear pore complex regulates the transcription of a collection of genes, which are mainly involved in oxidative phosphorylation and ribosome biogenesis. In myocardial infarction, hypoxia decreased the expression of Nup93, leading to abnormal interaction between nuclear pore complex and chromatin, which might result in aberrant transcription of a set of genes, such as YAP1. Therefore, downregulation of Nup93 leads to abnormal function of oxidative phosphorylation and ribosome biogenesis of cardiomyocytes, which might, in turn, results in abnormal function of cardiomyocytes and leads to hypoxia-induced damage to cardiomyocytes.

abnormal function of cardiomyocytes, which in turn leads to hypoxia-induced damage to cardiomyocytes. In this study, we investigated how hypoxia affected the expression and function of nucleoporins in cardiomyocytes and showed for the first time that abnormalities in the nucleoprotein complex may be closely related to the occurrence of myocardial infarction. These results reveal a new mechanism of Nup93 and might provide new therapeutic targets for the treatment of ischemia-induced heart failure.

Nucleoporins have been previously identified to have important functions in the pathophysiological process of cardiovascular diseases. For example, Nup35 regulates the pH homeostasis of cardiomyocytes by regulating the expression of  $\text{Na}^+\text{-H}^+$  exchanger-1. A mutation in Nup155 leads to impairment in both export of Hsp70 mRNA and nuclear import of Hsp70 protein and consequently results in atrial fibrillation and early sudden cardiac death. Abnormality of Nup188 has been identified as a cause of congenital heart disease [27]. Mutations in Nup205 are potentially associated with defects in cardiac left-right patterning [28, 29]. To explore the function of nucleoporins in heart disease, we transfected siRNAs into cardiomyocytes to knock down almost all nucleoporins one by one and measured the expression of ANP and BNP, whose abnormal regulation can reflect heart failure-related phenotypes. Through siRNA screening, we found that several nucleoporins can regulate the expression of ANP and BNP in cardiomyocytes. Here, we focused on the function of nucleoporins in ischemia-related apoptosis of cardiomyocytes. The present study provides multiple lines of evidence for the role of Nup93 in protecting cardiomyocytes from hypoxia-induced cell injury and death. First, clinical data showed that Nup93 expression negatively correlates with the expression of ANP and BNP in human hearts. Second, in both GEO heart samples and our collected heart samples, the mRNA and protein levels of Nup93 in human hearts and rat models were significantly decreased. Third, regardless of the physiological and pathological conditions, knockdown of Nup93 promotes hypoxia-induced cardiomyocyte injury and cell death, whereas overexpression of Nup93 confers powerful protection against the effects of hypoxia on cell viability. These findings suggest that downregulation of Nup93 accelerates

hypoxia-induced injury and cell death. Therefore, upregulation of Nup93 in cardiac ischemia might represent a new mechanism for the treatment of heart disease.

Mammalian Nup93 forms a stable complex with the central transport channel and is responsible for the correct assembly of the nuclear pore complex [2]. Nup93 is a member of the Nup93-205 complex, which includes Nup35, Nup155, Nup188, and Nup205. In this complex, Nup93 interacts with other nucleoporins and plays an important role in regulating the formation of NPC and its transport function. Nup93 was shown to regulate antiviral innate immunity by enhancing TBK1 activity and IRF3 nuclear translocation [30]. Therefore, the downregulation of Nup93 in ischemic heart disease might affect the function of the nuclear pore complex in transporting substances into or out of the nucleus. Interactions between nuclear export factors and nucleoporins with clustered repeats of FG (phenylalanine-glycine) were reported to be required for mRNA export through the nuclear pore complex [3, 4]. We supposed that knockdown of Nup93 might regulate mRNA export indirectly via its interaction with FG nucleoporins, such as Nup54, Nup58, and Nup62 [5]. However, the transport of only a few mRNAs was abnormally regulated in Nup93 knockdown cardiomyocytes. Therefore, the mechanism of Nup93 downregulation in ischemic hearts was not consistent with our primary hypothesis that downregulation of Nup93 might lead to abnormal transportation of mRNAs and proteins. However, it cannot be ruled out that downregulation of Nup93 might lead to abnormal transportation of other molecules. We also attempted to determine whether downregulation of Nup93 would result in abnormal localization of proteins that can interact with Nup93. The localization of more than ten different proteins in both the cytoplasm and nucleoplasm was analyzed. We could not find differences in the localization of these proteins between the control and Nup93 knockdown cardiomyocytes. Therefore, the transport of mRNAs or proteins might not be the main mechanism of Nup93 in cardiac ischemia.

Unexpectedly, we found that a large number of genes were regulated by Nup93 at the transcriptional level. In this manuscript, we showed that knockdown of Nup93 led to significant downregulation of gene transcription, indicating that Nup93 does not

merely regulate molecular trafficking between the cytoplasm and nucleoplasm. These results are consistent with a previous report about the chromatin-binding function of nuclear pore components Nup93 and Nup107. By analyzing Nup93-interacting DNA regions, we found that most of the genes downregulated by Nup93 knockdown could be attributed to abnormal interactions between Nup93 and chromatin. Although our data identify chromatin binding of Nup93 as the driving mechanism for the observed phenotype, we did not perform superresolution microscopy studies to exclude a direct interaction of actin filaments with NPC and, more specifically, with Nup93. In addition to Nup93, other components of nucleoporins might also directly interact with chromatin and act as regulators of transcription to regulate hypoxia-induced cell damage. Therefore, our results revealed for the first time a potential role of nucleoporins in cardiac ischemia beyond their function as regulators of nucleocytoplasmic transport, expanding our knowledge of the functional role of nucleoporins in heart diseases.

Here, we found that knockdown of Nup93 led to a marked decrease in gene transcription. In contrast to previous results, Nup93 targets Polycomb domains and promotes long-range contacts of Polycomb sites. This phenomenon might be attributed to the use of different cell types and different interfering methods. Normal cell functions rely on the accurate spatial and temporal control of transcription through thousands of enhancers. While the precise mechanisms by which Nup93 regulates the genome architecture remain unclear, genomic regions in NPCs appear largely devoid of heterochromatin in human cells. This finding is consistent with our results that Nup93 is an activator of gene transcription. Nup93 was shown to be involved in some tumor diseases by regulating cytoskeletal actin and Rho family proteins [26]. Moreover, Nup153 is required for the accurate expression of target genes in human cells, suggesting that several NPC components might cooperatively regulate gene transcription. Therefore, NPC might act as a platform to regulate genome functions in cardiomyocytes, and the effect of Nup93 on transcriptional regulation might occur through epigenetic modifications. The epigenetics of heart diseases has been intensively evaluated [31]. The interaction between Nup93 and chromatin might alter the chromatin accessibility of transcription factors. We propose that knockdown of Nup93 might affect the acylation of histones to regulate gene transcription. Previous studies have demonstrated that Nup93 can interact with SE regions, which have a high density of H3K27ac marks [32]. It is necessary to investigate the relationship between Nup93 interactions with these marks in the promoter regions of these down-regulated genes. The effects of Nup93 knockdown on the modification of histones, such as active histone marks (H3K27ac, H3K4me1) and repressive histone marks, also remain to be further analyzed in the future.

Our results also suggested that downregulation of Nup93 in ischemia-induced human heart failure and the LAD model might underlie the effects of hypoxia on cardiomyocyte death by regulating gene transcription. Correlation of the RNA-seq and Cut & Run data highlighted that downregulation of Nup93 was directly involved in the transcriptional regulation of a subset of genes. These genes might be responsible for the function of Nup93 in regulating apoptosis. Through GO and KEGG analyses, we found that the chromatin interaction of Nup93 partially modulates the expression of genes associated with oxidative phosphorylation and ribosome subunits. Therefore, the effects of downregulation of Nup93 might account for the abnormal regulation of these effects. Our findings extend previous studies showing that chromatin interactions of Nup93 might underlie the mechanism of hypoxia-induced cardiomyocyte death.

Interestingly, the combination of RNA-seq data with Nup93-chromatin interacting data revealed a subset of genes involved in Nup93-regulated transcription. We do not believe that one or two genes can explain the effects of Nup93 on hypoxia-induced cell death. One gene was tested to determine whether its

downregulation could rescue the effects of Nup93 knockdown on hypoxia-induced cell death. YAP1 (Yes-associated protein 1) is one of the principal factors that modulates organ volume control. YAP1 has been confirmed to play an important role in tissue regeneration, stem cell function, and tumorigenesis by acting as a driver of gene expression. Surprisingly, we found that overexpression of Yes-associated protein 1 (YAP1) decreased the effects of Nup93 knockdown or hypoxia on cell injury and death. The roles of Yap1 in modulating injury after chronic myocardial infarction were investigated before, showing that cardiomyocyte-specific homozygous inactivation of YAP1 in the postnatal heart increased cardiomyocyte apoptosis and fibrosis and led to dilated cardiomyopathy. YAP1 overexpression protected cardiomyocytes against cell death. Thus, downregulation of YAP1 might underlie, at least partially, the effects of Nup93 knockdown on cardiomyocyte apoptosis. In addition to YAP1, other genes influenced by Nup93 knockdown could underlie additional regulatory functions of Nup93. Additional basic and clinical data should be collected to explore the possibility that other Nup93-regulated genes could be therapeutic targets as well as prognostic markers.

#### AVAILABILITY OF DATA AND MATERIALS

The data and materials used in this manuscript are available from the authors, Xian-xian Zhao or Jun-bo Ge, upon request.

#### AUTHOR CONTRIBUTIONS

JBG, XXZ, and XWS designed the study. LP, XWS, JCS, SHL, CYS, ZKW, and SQH conducted experiments. XWS, LP, and JCS wrote and drafted the manuscript. ZFG, XXZ, and JBG analyzed data. All authors read and approved the manuscript.

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#### ADDITIONAL INFORMATION

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**Competing interests:** The authors declare no competing interests.

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