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Mechanisms Regulating Muscle Protein Synthesis in Chronic Kidney Disease

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ABSTRACT

Background CKD induces loss of muscle proteins partly by suppressing muscle protein synthesis. Muscles of mice with CKD have increased expression of nucleolar protein 66 (NO66), as do muscle biopsy specimens from patients with CKD or those undergoing hemodialysis. Inflammation stimulates NO66 expression and changes in NF-κB mediate the response.

Methods Subtotal nephrectomy created a mouse model of CKD with BUN >80 mg/dl. Crossing NO66$^{flox/flox}$ with MCK-Cre mice bred muscle-specific NO66 (MCK-NO66) knockout mice. Experiments assessed the effect of removing NO66.

Results Muscle-specific NO66 knockout in mice blocks CKD-induced loss of muscle mass and improves protein synthesis. NO66 suppression of ribosomal biogenesis *via* demethylase activity is the mechanism behind these responses. In muscle cells, expression of NO66, but not of demethylase-dead mutant NO66, decreased H3K4me3 and H3K36me3 and suppressed pre-rRNA expression. Knocking out NO66 increased the enrichment of H3K4me3 and H3K36me3 on ribosomal DNA. In primary muscle cells and in muscles of mice without NO66, ribosomal RNA, pre-rRNA, and protein synthesis all increased.

Conclusions CKD suppresses muscle protein synthesis *via* epigenetic mechanisms that NO66 mediates. Blocking NO66 could suggest strategies that counter CKD-induced abnormal muscle protein catabolism.

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The loss of muscle proteins stimulated by CKD results from an imbalance between mechanisms that stimulate muscle protein degradation and/or those that impair rates of protein synthesis. Identifying mechanisms that cause loss of muscle mass is necessary because these losses of muscle mass contribute substantially to the morbidity and mortality associated with CKD.[1] We and others have discovered that complications of CKD including metabolic acidosis,[2,3] insulin resistance,[3] and inflammation,[5,6] as well as glucocorticoids and [2,4] angiotensin II, stimulate muscle proteolysis by activating caspase-3 and the ubiquitin-proteasome system (UPS).[7,8] In contrast, mechanisms that suppress the rates of muscle protein synthesis are unclear.

How could muscle protein metabolism be improved in patients with CKD? Currently, these

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patients are treated by restricting their dietary protein to reverse the progressive decline in kidney function.[9] However, the problem is complicated because an inadequate intake of nutrients could jeopardize the maintenance of protein stores. In 1981, the Food and Agriculture Organization/World Health Organization/United Nations University declared a recommended daily allowance (RDA) of dietary protein of 0.8 g protein/kg per day. In studies of elderly subjects, however, Wolfe and colleagues[10] measured the adequacy of protein intake and concluded that an RDA of 0.8 g protein/kg per day would be insufficient to raise the level of protein synthesis and prevent the losses in protein storage. They recommended that the RDA be almost doubled, with the goal of increasing protein synthesis in muscle. In contrast, Bhasin *et al.*[11] tested the adequacy of the RDA by measuring protein turnover in elderly patients eating 0.8 versus 1.3 g protein/kg per day; they concluded a protein intake >0.8 g protein/kg per day would not increase lean body mass, muscle performance, or physical functions. We studied the turnover of muscle proteins in a subtotal nephrectomy model of CKD in rats and found the rate of muscle protein synthesis was 34% lower in rats with CKD versus pair-fed, control rats; the defect in protein synthesis was not corrected by treating metabolic acidosis.[12] Identifying the mechanisms involved in impaired muscle protein synthesis is required because correcting such defects could uncover strategies to improve the complications of CKD.

What mechanisms could improve muscle protein metabolism? Nucleolar protein 66 (NO66) is a JmjC domain–containing protein.[13] Reportedly, NO66 exhibits histone demethylase activity that is involved in the methylation of H3K4 and H3K36.[14–19] NO66 stimulates the osteoblast transcription factor osterix that leads to the inhibition of the expression of osterix target genes[14,17] and, during embryonic stem cell differentiation, NO66 is recruited to the polycomb repressive complex 2. These responses lead to loss of H3K36me3, increased levels of H3K27me3, and result in silencing of certain activated genes.[16] These findings are relevant because NO66 actually mediates the repression of genes. Moreover, mice with mesenchymal deletion of NO66 exhibit an increase in bone formation.[20] In contrast, we have found that mesenchymal overexpression of NO66 in mice inhibits skeletal growth and bone formation, demonstrating the presence of an important *in vivo* organogenesis role for NO66.[21] Our experimental results demonstrated that the expression of NO66 is increased in muscles of mice with CKD and expression of NO66 inhibits muscle protein synthesis by suppressing ribosomal DNA (rDNA) transcription *via* a demethylase mechanism. Our results have uncovered a new strategy that influences muscle protein mass in CKD and potentially other catabolic conditions.

Significance Statement

The morbidity and mortality of CKD arise from acceleration of muscle protein degradation and suppression of muscle protein synthesis. Responses such as caspase-3 mediation of apoptosis and activation of the ubiquitin-proteasome system drive CKD-induced proteolysis. However, CKD-induced mechanisms that impair protein synthesis in muscle are less well studied. This investigation reports that CKD-stimulated, chromatin-modifying, nucleolar protein 66 (NO66) suppresses both ribosomal DNA transcription and muscle protein synthesis *via* a demethylase mechanism. Notably, muscle-specific knockout of NO66 in mice improved muscle protein metabolism despite the presence of CKD. Additionally, NO66 is present in muscle biopsy specimens of patients with CKD or those on hemodialysis. These findings might lead to clinical strategies that counter CKD-induced muscle protein catabolism.

METHODS

Reagents

The antibodies used are listed in Supplemental Table 1. For cell culture, DMEM and FBS were purchased from Cellgro Mediatech (Manassas, VA). The *NO66* expression plasmids were obtained from Dharmacon (GE Healthcare Life Sciences, Lafayette, CO). The mutant *NO66 AKA* plasmid was a gift from Dr. K. M. Sinha *et al.*[14] L-(^{14}C[U]) phenylalanine was purchased from PerkinElmer (Santa Clara, CA).

Animal Studies

Transgenic mice with a loxP-flanked ("floxed") *NO66* (NO66$^{flox/flox}$) were a gift from Dr. De Crombrugghe (University of Texas MD Anderson Cancer Center, Houston, TX).[20] MCK-Cre transgenic mice (the Cre recombinase gene is driven by the creatine kinase promoter in muscles) were purchased from the Jackson Laboratory (Bar Harbor, ME).[22] Mice with muscle-specific NO66 knockout (KO) (MCK-NO66) were created by crossing MCK-Cre mice with NO66$^{flox/flox}$ mice.

To create CKD in NO66$^{flox/flox}$ and in MCK-NO66 mice, anesthetized male and female mice (10–12 weeks old) underwent subtotal nephrectomy in two stages as described.[23] In the first stage, 60%–70% of the left kidney was removed and mice were fed 6% protein diet (Harlan Teklab, Indianapolis, IN) to minimize mortality from uremia. Seven days later, the right kidney was removed and mice were continued on the 6% protein diet. Two weeks later, mice were fed a 40% protein diet (Harlan Teklab) to induce advanced CKD.[24] Sham-operated, control mice underwent surgery without damaging the kidneys and were fed the same diets. Mice of the same sex were housed in cages with a 12-hour light/dark cycle. NO66$^{flox/flox}$ mice with CKD and MCK-NO66 mice with CKD were paired based on their BUN and the amount of food eaten daily.

Isolation and Culture of Mouse Primary Myoblasts

We created global deletion of NO66 mice (NO66$^{-/-}$) by crossing transgenic, Sox2-Cre mice with NO66$^{flox/flox}$ mice. The muscle from these mice was used for RNA-sequencing (RNA-seq) analysis and they were also used to isolate primary mouse myoblasts as described.[25] For isolation and culture of mouse primary myoblasts, neonatal NO66$^{flox/flox}$ and global NO66 KO (NO66$^{-/-}$) mice were euthanized by decapitation and muscles were removed, placed in PBS, and minced with razor blades. The resulting slurry was transferred into a sterile

tube with 2 ml of a collagenase/dispase/calcium chloride solution per gram of tissue and then incubated at 37°C for 20 minutes before the slurry was gently triturated with a plastic pipette to break up clumps. The slurry was filtered through 100-μm nylon mesh and then centrifuged for 5 minutes at 350 $\times$ *g*. The pellet was resuspended in 4 ml of Ham F-10 medium (including 20% FCS, 2.5 ng/ml basic fibroblast growth factor, and 100 U/ml penicillin/streptomycin) and then plated in a 35-mm matrigel-coated cell culture dish. Cells were incubated at 37°C in a 5% carbon dioxide incubator. Subsequently, cells were removed from the dish using trypsin and preplated for 15 minutes on a matrigel-coated dish before moving the remaining cells into a new matrigel-coated dish. This procedure was repeated until no additional fibroblasts were attached to the plates during 15 minutes of observation. Myoblasts were cultured in F-10/DMEM-based primary myoblast growth medium and, subsequently, myoblasts were differentiated into myotubes by replacing the medium with 2% horse serum (HS).[23] These cells were transfected with plasmids using Invitrogen (Carlsbad, CA) Neon Transfection System (the transfection rate with this method can be >90%).[26]

Measurements of Myofiber Areas

Myofiber sizes were measured in cryo-cross sections of muscles that had been immunostained with anti-laminin. Briefly, cross sections of muscles were fixed in 4% paraformaldehyde and permeabilized by incubating in 0.3% Triton X-100 in PBS followed by blocking treatment with protein block (DAKO, Carpinteria, CA) for 1 hour at room temperature. Sections were incubated with anti-laminin diluted with Antibody Dilute (DAKO) overnight at 4°C, followed by incubation with the secondary antibody, which was conjugated to Alexa 568 (Invitrogen). The cross section of muscles were visualized using a Nikon 80i microscope and images were acquired using a DS-cooled camera with myofiber sizes measured in images using NIS-Elements Br 3.0 software (Melville, NY).

Chromatin Immunoprecipitation Assay

Chromatin immunoprecipitation (CHIP) assays were performed using a Millipore Kit as described.[26] Briefly, DNA-protein complexes from cultured muscle cells were crosslinked in 1% formaldehyde (Sigma-Aldrich) for 10 minutes before cells were washed three times with ice-cold PBS containing a protease inhibitor (Sigma-Aldrich). The cells were lysed, vortexed, and sonicated for 10 seconds at power setting 4 (VibraCell Sonicator). This procedure was repeated four times to obtain DNA fragments ranging between 300 and 800 bp. After centrifugation, the protein-DNA lysate was diluted tenfold in CHIP buffer and precleared for 1 hour at 4°C using salmon sperm DNA and protein A/G agarose beads. Each 100 μl of protein-DNA lysate was used as an input control. Cellular lysates of protein-DNA were immunoprecipitated overnight at 4°C with antibodies against H3K4me3, H3K36me3, NO66, P65, or H3. Lysates were incubated with IgG overnight at 4°C and used as negative control. This was followed by incubation with protein A/G agarose beads at 4°C for 1 hour. As described in the kit, immune complexes were washed and the immunoprecipitated DNA was reverse crosslinked at 65°C for 4 hours in the presence of 0.2 M sodium chloride. The DNA was then purified using phenol/chloroform/isoamyl alcohol and subjected to PCR amplification. The primer sequences are presented in Supplemental Table 2.[27] The quantitative PCR was normalized using percentage of input.[28]

Measurements of Rates of Protein Synthesis and Degradation in Skeletal Muscles

Extensor digitorum longus (EDL) or soleus muscles were maintained at resting length during incubation in Krebs–Henseleit bicarbonate buffer with 10 mM glucose as described.[23,29] The incorporation of L-(^{14}C[U]) phenylalanine into muscle protein and the release of tyrosine from muscle were measured as rates of muscle protein synthesis and degradation, respectively.[24,30]

The Rates of Protein Synthesis in Myotubes

Mouse C2C12 myoblasts (ATCC, Manassas, VA) were cultured in DMEM (Cellgro Mediatech) supplemented with 10% FBS (Invitrogen) plus 100 U/ml of penicillin/streptomycin. At a 80%–90% confluence of C2C12 cells or primary muscle cells, the culture medium was switched to DMEM with 2% HS for myotube formation. Myotubes were treated with a cytokine mixture including IL-6 (100 ng/ml), TNFα (10 ng/ml), and IFN-γ (200 U/ml) for 24 or 48 hours. Treated myotubes were incubated in DMEM containing 2% HS and 0.6 mM phenylalanine for 2 hours before they were placed in the same media containing 0.5 μCi L-(^{14}C[U]) phenylalanine for 4 hours (we included 0.6 mM nonradioactive phenylalanine to the cell culture medium to equilibrate the extracellular and intracellular phenylalanine pools as we and Gulve and Dice reported[30,31]). Cells were then washed three times with cold PBS and fixed with 10% TCA before they were collected by centrifugation (13,000 rpm for 15 minutes). Pellets were washed three times with ethanol/ether (1:1) before being solubilized overnight in 1 ml of 0.3 M sodium hydroxide. The supernatant was analyzed by Liquid Scintillation. The protein synthesis rate was calculated as nmol L-phenylalanine/mg protein per hour.

Ribosomal RNA Analysis

Ribosomal RNA (rRNA) comprises approximately 80% of the total cellular RNA. Thus, total RNA was used to evaluate rRNA. Total RNA was extracted from soleus or EDL muscles using an RNA extraction kit (Qiagen). The total RNA obtained from 125 μg of muscle tissues was electrophoresed on a 1% agarose gel with ethidium bromide and viewed under ultraviolet light. Using captured electrophoresis images, densitometry of 18S rRNA and 28S rRNA were determined using the National Institutes of Health ImageJ software.

Calculation of Muscle Protein Synthesis Capacity

Changes in muscle protein synthesis are dependent on the efficiency of mRNA translation and/or the abundance of ribosomes in muscles. The abundance of ribosomes in muscle (calculated as the ratio of RNA to muscle) define the protein synthesis capacity.[32] RNA concentration was measured using a NanoDrop Spectrometer at 260 nm, whereas the total RNA content per gram EDL or soleus muscle (RNA in μg/muscle in g) was calculated as the muscle protein synthesis capacity.

RT-PCR

RNA was extracted from cells or muscles using an RNA extraction kit; cDNA was prepared using an iScript cDNA Synthesis Kit (Bio-Rad). Duplicate PCR reactions were performed using SYBR green (Bio-Rad) on a Bio-Rad CFX96 real-time thermal cycle.[6,33] The relative gene expression was calculated from cycle threshold values using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as the internal control (Ct; relative expression $=2^{[\text{sample Ct} - \text{GAPDH Ct}]}$). Primer sequences used for RT-PCR are detailed in Supplemental Table 2.

RNA Sequence Assay

Total RNA was extracted from the soleus muscles of $NO66^{flox/flox}$ and global NO66 KO mice ($NO66^{-/-}$) using RNeasy Mini Kit (Qiagen). RNA samples were sequenced using the standard Illumina protocol to create raw sequence files (.fastq files) at LC Sciences (Houston, TX). The ontology analysis and KEGG pathways were evaluated by bioinformatics at LC Sciences. RNA-seq data have been deposited in the ArrayExpress database at European Molecular Biology Laboratory European Bioinformatics Institute (EMBL-EBI; www.ebi.ac.uk/arrayexpress) under accession number E-MTAB-7285.

Studies in Patients with CKD

For studies of muscle metabolism in patients with CKD, we used procedures that were approved by the Ethical Committee at the Department of Internal Medicine, University of Genoa, in accordance with the Declaration of Helsinki regarding the ethics of human research. Samples of abdominal muscles were obtained from patients with CKD during the placement of peritoneal dialysis catheters. The biopsy samples were obtained before patients began dialysis treatments and they were frozen at −80°C and stored until analyzed. Muscle biopsy specimens of control subjects were obtained from otherwise normal adults who were undergoing abdominal hernia surgery. Patient information is provided in Table 1 and Supplemental Table 3.

Muscle biopsy specimens from patients on hemodialysis at Vanderbilt University Medical Center (VUMC) were obtained as described.[34] The studies were approved by VUMC patient review board(s); written, informed consent was obtained from participants before their inclusion in the study. Patient characteristics included age ≥18 years and regular maintenance hemodialysis treatments for >3 months prescribed as an adequate dialysis dose (single-pool Kt/V >1.2) of a thrice-weekly dialysis program using biocompatible hemodialysis membranes. The information regarding the patients on hemodialysis is in Table 2 and Supplemental Table 4.

Statistical Analyses

Results are expressed as means±SEM. GraphPad Prism 8 was used for data analysis and graphing of figures. Significance testing was performed using *t* test when results from two groups were compared or two-way ANOVA when data from three or more groups were evaluated. Statistical significance was set at $P<0.05$. A minimum of three replicates was performed for each experimental condition.

RESULTS

CKD Induces NO66 Expression in Skeletal Muscles of Mice

It has been reported that NO66 suppresses both stem cell functions and the differentiation of bone growth.[16,21] Our experiments were aimed at determining whether NO66 expression in skeletal muscles (Supplemental Figure 1) also influences the metabolism of skeletal muscle proteins. As shown in Figure 1, A and B, our mouse CKD model was characterized by higher BUN values and decreased body weights. CKD was also associated with decreased weights of skeletal muscles including soleus muscles with a higher proportion of red fibers (Figure 1C), EDL muscles with a higher proportion of white fibers (Figure 1D), and the mixed-fiber gastrocnemius and tibialis anterior muscles (Supplemental Figure 2, A and B). Consistent with these estimates of decreased muscle mass in mice with CKD, we found that myofibers in soleus muscles were smaller compared with sham-operated, control mice (Figure 1E). In these mice, we also found that CKD increased

Table 1. The characteristics of patients with CKD

Characteristic	Age (yr)	Sex	Height (cm)	Serum Creatinine (mg/dl)	eGFR (ml/min per 1.73 m^2)	BUN (mg/dl)	Diabetes
Healthy controls	67.2±2.4	*n*=20 M, *n*=4 F	170.9±0.8	0.9±0.03	76.7±2.9	21.9±1.2	No
Patients with CKD	65.7±1.41	*n*=23 M, *n*=4 F	171.6±1.2	7.2±0.27	10.4±0.4	112.4±5.6	No
P value (CKD versus control)	0.63		0.66	2.28×10^{-15}	1.57×10^{-11}	5.82×10^{-11}	

Data are average±SEM. M, male; F, female.

Table 2. The characteristics of patients on dialysis with CKD or healthy controls

Characteristic	Sex	Age (yr)	Weight (kg)	BUN (mg/dl)	Creatinine (mg/dl)	Albumin (g/dl)	Total Protein (g/dl)	Diabetes
Healthy controls	n=6 M, n=2 F	48.3±4.1	86.1±9.0	12.6±1.1	0.94±0.1	4±0.03	6.7±0.1	No
Patients on dialysis with CKD	n=6 M, n=2 F	47.3±4.6	90.3±7.0	40.2±4.2	9.59±1.0	3.9±0.1	6.9±0.2	No
P value		0.44	0.36	0.000066	0.000016	0.28	0.17	

Data are average±SEM. M, male; F, female.

both mRNA and protein expression of NO66 (Figure 1, F and G). Similar results were discovered in EDL muscles of CKD mice.

High Levels of NO66 are Present in Muscles of Patients with CKD

To begin to assess the influence of NO66 on muscle protein metabolism, we examined muscle biopsy specimens from nondiabetic patients with CKD who were not being treated with hemodialysis (Supplemental Table 3). These patients with CKD exhibited significant increases in BUN and serum creatinine and low eGFR values (Table 1). In muscles of these patients with CKD, we found significant increases in the mRNA expression of inflammatory cytokines (*i.e.*, IL-6 and TNFα) and atrophy genes (Atrogin-1 and MuRF-1) (Figure 2, A–D). There were also significant increases in NO66 mRNA and protein in muscles of patients with CKD ($P<0.05$, Figure 2, E and F). In addition, we examined NO66 protein from patients on hemodialysis and discovered increases in NO66 versus control (Figure 2G, Supplemental Table 4, Table 2). We conclude that patients with CKD as well as those being treated by hemodialysis express NO66 in their skeletal muscles.

NF-κB Signaling Regulates NO66 Expression in Muscle

Next, we investigated mechanisms that could upregulate the expression of NO66 in muscles of mice as well as patients with CKD. Because CKD is associated with the expression of inflammatory cytokines, we initially hypothesized that CKD stimulates the expression of inflammatory cytokines and these in turn increase the expression of NO66 *via* an NF-κB signaling pathway. Our conclusion was based on two factors: firstly, CKD is associated with inflammation[23,29] and, secondly, the NO66 promoter contains two potential NF-κB binding sites (-607, 5′-GGAGAAGTCCC-3′, -597; $+332$, 5′–GGGCTTATCCT-3′, $+344$) that were identified using the Transfac program. To mimic CKD conditions previously, we combined IL-6, TNFα, and IFN-γ and found this cytokine mixture stimulates NF-κB activity in muscles, resulting in increased expression of proteasome subunits.[35] The combination of cytokines was also shown to upregulate the expression of SIRP-α in muscles, which causes dysregulation of intracellular insulin signaling in mice with CKD.[36] Using the same concentration of cytokines,[35,36] we treated C2C12 myotubes with the cytokine mixture and determined that it increased the expressions of both the NO66 mRNA (Figure 3A) and protein (Figure 3B) while suppressing the expression of pre-rRNA (Figure 3A). Notably, the cytokine mixture decreased the rate of protein synthesis in myotubes (Figure 3C). To examine whether the inflammatory cytokines stimulate NO66 *via* an NF-κB pathway, we treated C2C12 myotubes with the NF-κB inhibitor QNZ and found that QNZ suppressed the expression of both NO66 mRNA and its protein in response to treatment with the cytokine mixture (Figure 3, D and E). Results obtained with CHIP assays also confirmed that NF-κB mediates the expression of NO66. Specifically, we treated C2C12 myotubes with the cytokine mixture for 24 hours. Cell lysates were immunoprecipitated with an anti-P65 antibody or IgG (used as negative control). DNA from the immunocomplex was subjected to PCR analysis using primers covering two potential NF-κB sites (see Supplemental Table 2 for primer sequences). The "percentage to input" was used to calculate the enrichment of P65 on the promoter of the NO66 gene. Our results indicate the cytokine mixture increases P65 binding to the promoter of NO66 (Figure 3F).

MCK-NO66 Mice Are Resistant to CKD-Induced Muscle Protein Loss

To test for a pathophysiologic role of NO66 in muscle, we created MCK-NO66 mice by crossing $NO66^{flox/flox}$ mice with MCK-Cre transgenic mice. In MCK-NO66 mice versus results from $NO66^{flox/flox}$ mice, NO66 protein is substantially decreased in skeletal muscles but not in other tissues, including spleen and kidneys. There is also a low level of NO66 in heart tissue of MCK-NO66 mice versus that in $NO66^{flox/flox}$ mice, but these differences did not reach a significant level (Figure 4A). We then created the CKD model in mice and found, in $NO66^{flox/flox}$ mice with CKD, NO66 protein and mRNA were increased in muscles. However, NO66 protein and mRNA were abolished in skeletal muscles of MCK-NO66 mice even in the presence of CKD (Figure 4, B and C).

CKD caused a significant loss of body and muscle weight (*e.g.*, soleus and EDL muscles) in $NO66^{flox/flox}$ mice (Figure 4, D and E). In contrast, the examination of MCK-NO66 mice revealed no significant differences in body and muscle weight between mice with CKD and the sham, control mice. Notably, there were significant increases in body and muscle weight of MCK-NO66 mice with CKD versus $NO66^{flox/flox}$ mice with CKD (Figure 4, D and E, Supplemental Figure 3, A and B). To confirm the difference in muscle mass, we measured myofiber areas in soleus muscles. The results indicate the average myofiber size in MCK-NO66 mice with CKD were larger than in $NO66^{flox/flox}$ mice with CKD (Figure 4F). Similar results were found in mixed-fiber tibialis anterior muscles (Supplemental Figure 3C).

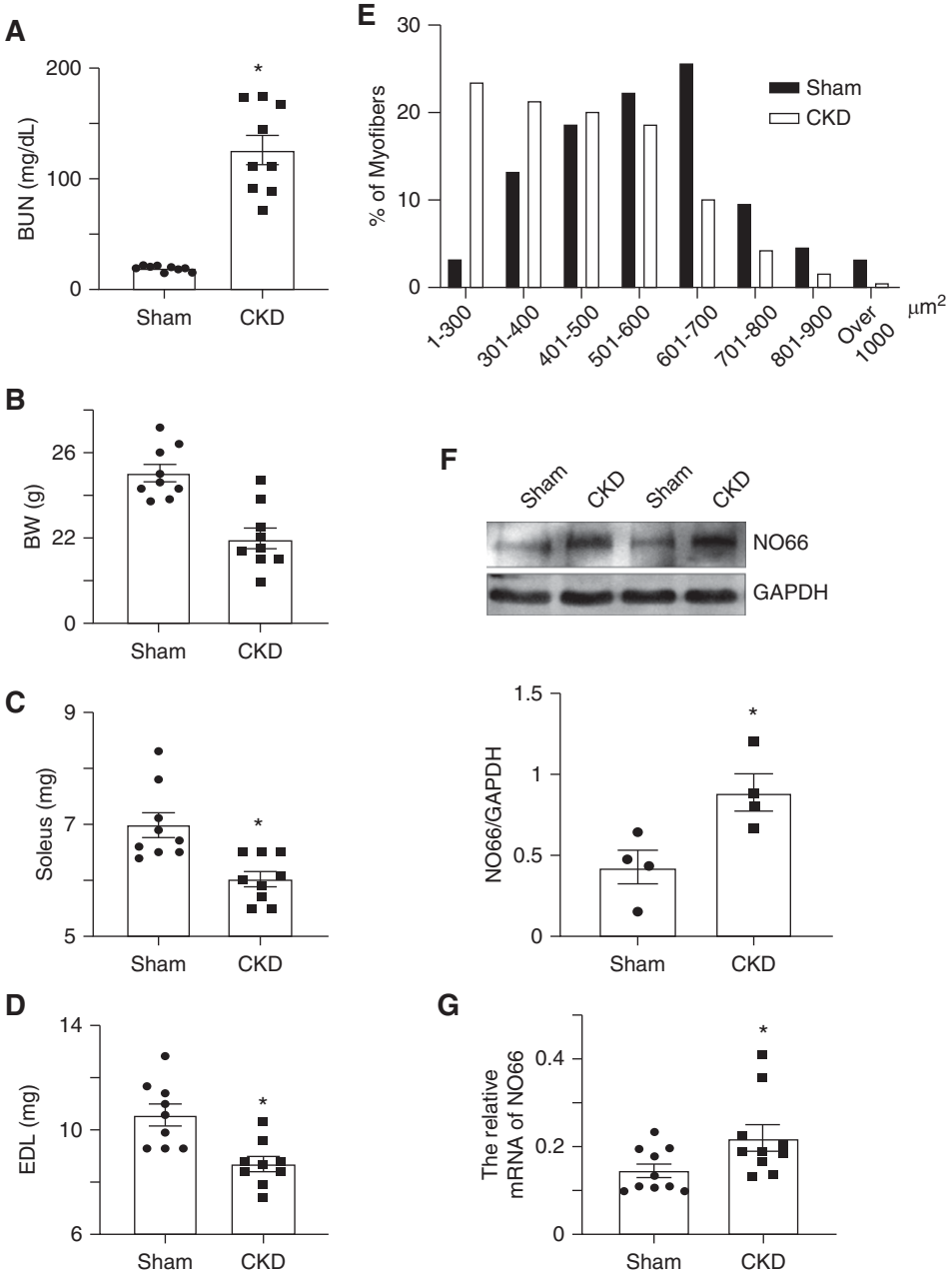

Figure 1. CKD induces NO66 expression in mouse skeletal muscles. (A–D) Compared with values of sham-operated, control mice, CKD mice have (A) higher BUN levels, (B) lower body weight (BW), plus (C) reduced weight of soleus and (D) EDL muscle (n=9 mice in each group. (E) The distribution of myofiber areas in soleus muscles of CKD mice are shifted to the left (*i.e.*, smaller size; n=246 in CKD and n=220 in control myofibers). (F) Western blotting shows higher levels of NO66 protein in soleus muscles of CKD mice, quantification is shown in the lower panel (n=4 mice in each group). (G) Increased NO66 mRNA was identified in soleus muscles of CKD mice (n=10 mice in each group). *P<0.05 versus sham control.

In soleus muscles of NO66$^{flox/flox}$ mice with CKD, the rate of protein synthesis was significantly decreased (P<0.05, Figure 4G) and the rate of protein degradation was increased (P<0.05, Figure 4H). Similar results were found in EDL muscles. In muscles of MCK-NO66 mice with CKD, the rate of protein synthesis was not significantly different from those measured in muscles of sham-operated control MCK-NO66 mice. CKD still stimulated the rate of protein degradation in muscle of MCK-NO66 mice (Figure 4, G and H). However, the rate of protein synthesis in muscles of MCK-NO66 mice with CKD were substantially greater compared with that in NO66-$^{flox/flox}$ mice with CKD (Figure 4G). Although CKD stimulated protein degradation in MCK-NO66 mice (Figure 4H), the muscle mass in MCK-NO66 mice was still greater than that

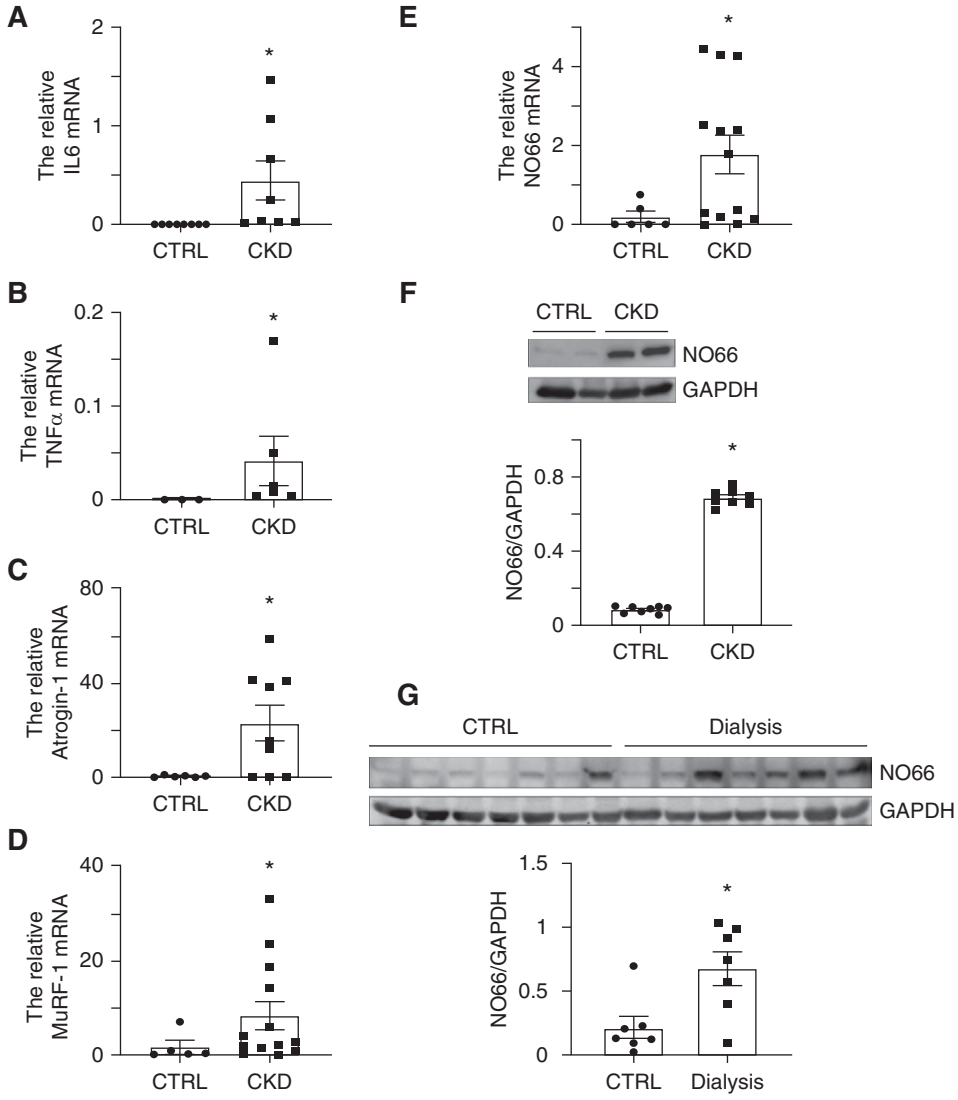

Figure 2. High levels of NO66 are present in muscles of patients with CKD. (A–E) In muscles of patients with CKD, the mRNA of (A) IL-6, (B) TNFα, (C) Atrogin-1, (D) MuRF-1, and (E) NO66 was significantly higher compared with values in control subjects ($n \geq 5$ in each group). (F) Western blotting detected high levels of NO66 protein in muscle lysates from patients with CKD. Quantification is in the lower panels ($n=8$ in each group). (G) CKD is associated with increased NO66 protein in muscles of patients on hemodialysis ($n=7$ in each group). *$P<0.05$ versus control subjects.

in muscles of $NO66^{flox/flox}$ mice with CKD (Figure 4E). These results suggest that stimulation of protein synthesis is responsible for the increase in muscle mass in MCK-NO66 mice.

To explore mechanisms by which NO66 could regulate muscle mass in mice with CKD, we assessed changes in myostatin because its expression regulates muscle mass.[23] We found the expression of myostatin mRNA was not significantly different in muscles lacking NO66 versus control (Supplemental Figure 4A). In addition, we transfected C2C12 cells with plasmids that express NO66 and found no significant change in the level of myostatin proteins compared with results obtained from cells transfected with cDNA3 (Supplemental Figure 4B). Thus, NO66 does not cause muscle wasting by increasing myostatin expression.

Loss of NO66 Stimulates Ribosomal Biogenesis in Mouse Muscles

To identify genes that contribute to muscle mass in muscles lacking NO66, we performed a transcriptome analysis (RNA-seq analysis) using soleus muscles from 2-month-old $NO66^{flox/flox}$ and global NO66 KO ($NO66^{-/-}$) mice. The RNA-seq analysis identified 1955 (5.19%) upregulated genes and 1306 (3.47%) downregulated genes when the results were evaluated on a threshold of more than twofold changes of the 37,622 genes analyzed in the soleus muscles of $NO66^{-/-}$ mice versus results from soleus muscles of control, $NO66^{flox/flox}$ mice (ArrayExpress database at EMBL-EBI, number E-MTAB-7285). A Gene Ontology analysis revealed the largest proportion of up-regulated genes was involved in protein-metabolic processes

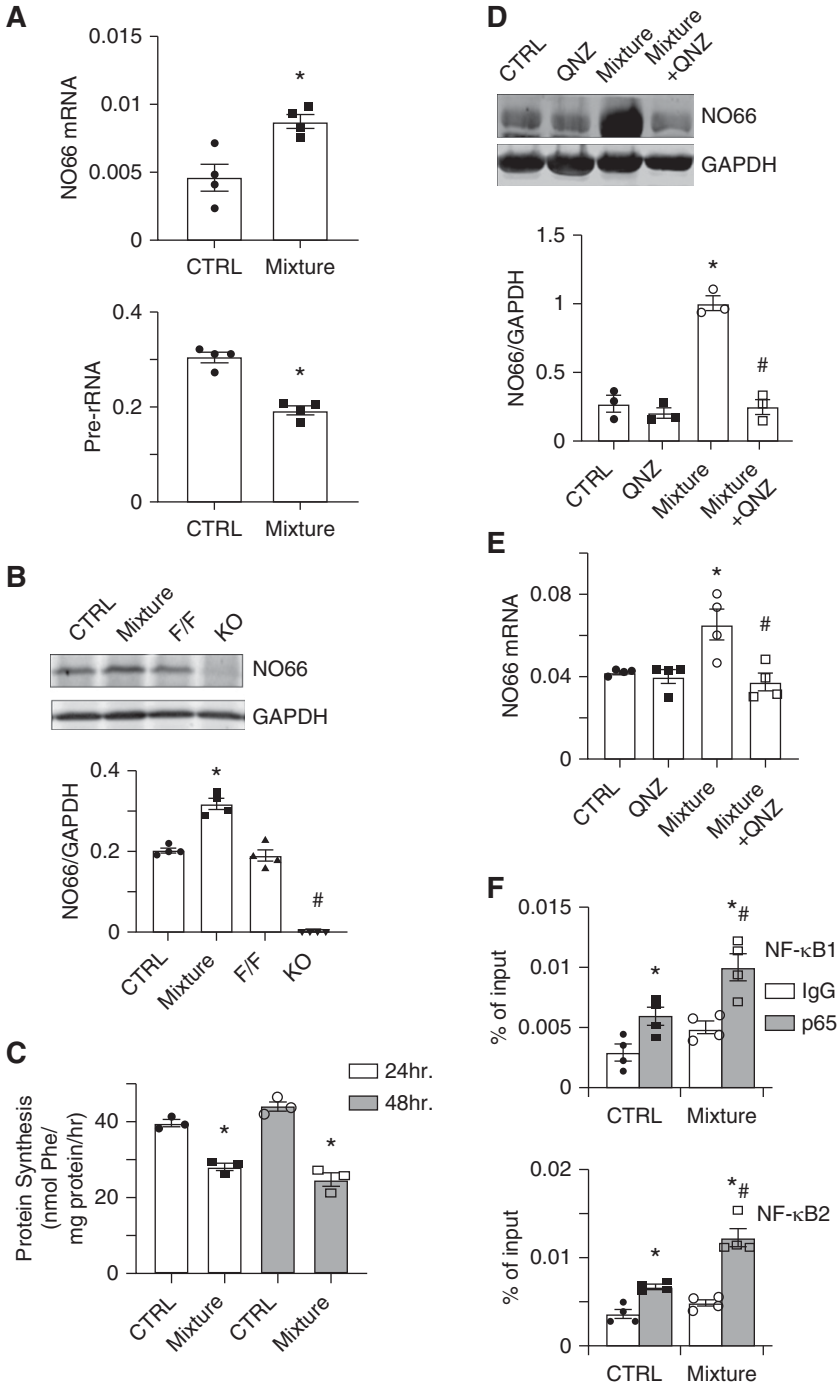

Figure 3. NF-κB signaling regulates NO66 expression in muscle. (A) C2C12 myotubes treated with cytokine mixture (including IL-6, TNFα, and INFγ) for 8 hours stimulated NO66 mRNA and suppressed pre-rRNA (n=4 repeat). *P<0.05 versus control. (B) Cytokine mixture is associated with increased NO66 protein in C2C12 myotubes after 32-hour incubation. Lysates of NO66$^{flox/flox}$ or NO66$^{-/-}$ myotubes were used as controls. Quantification relative to GAPDH is shown in the lower panel (n=4 repeat). *P<0.05 versus control. (C) The cytokine mixture suppressed the rate of protein synthesis in C2C12 myotubes after 24- or 48-hour treatment (n=3 repeat). *P<0.05 versus control. (D and E) C2C12 myotubes were treated with the NF-κB inhibitor QNZ for 30 minutes before adding cytokine mixture for 24 hours. The cytokine mixture stimulated (D) NO66 protein and (E) mRNA was suppressed by QNZ. *P<0.05 versus control; #P<0.05 versus mixture. (F) The CHIP assay shows the enrichment of P65 at the NO66 after cytokine treatment for 24 hours (n=4). *P<0.05 versus IgG control; #P<0.05 versus P65 control.

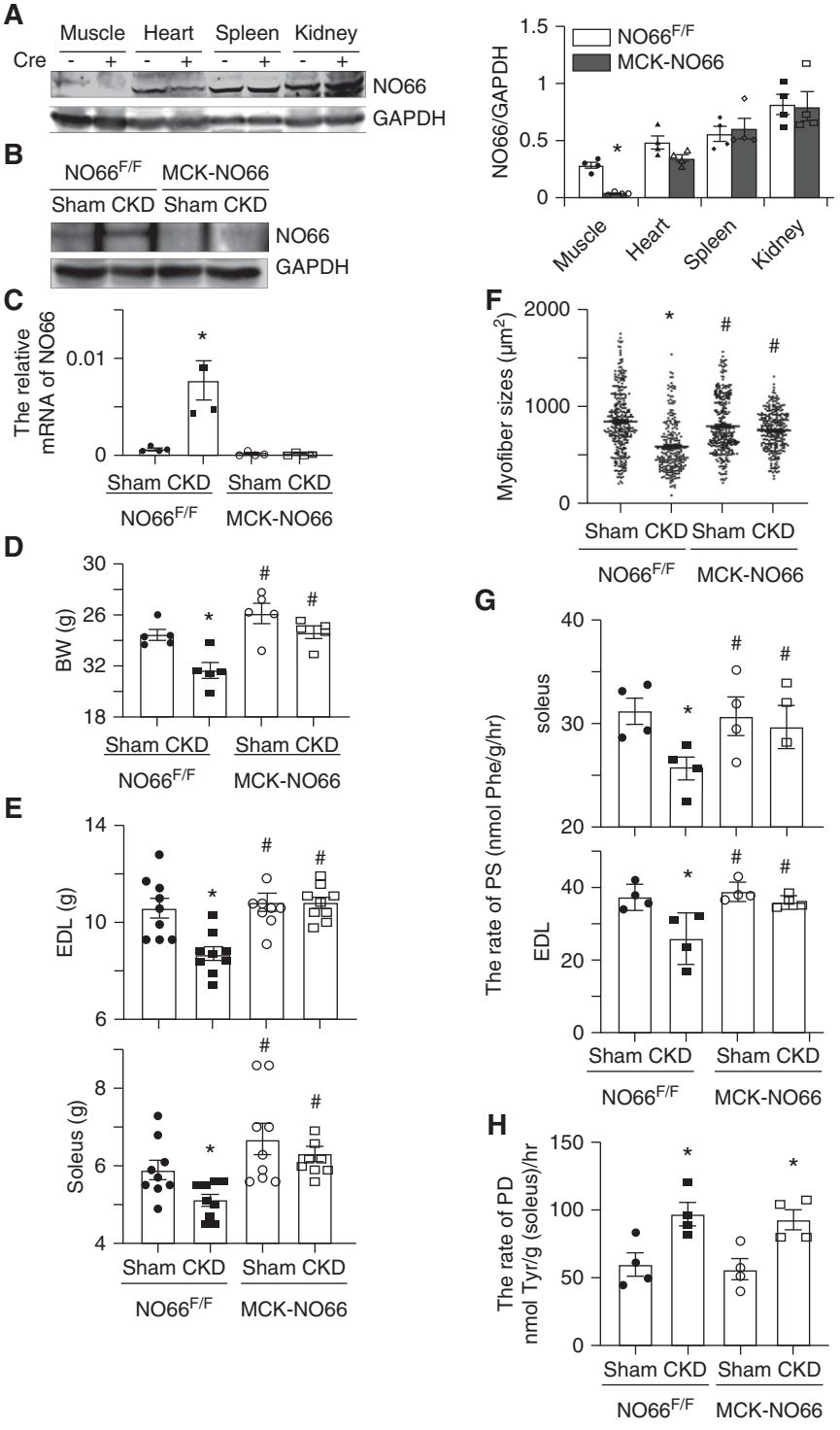

Figure 4. MCK-NO66 mice are resistant to CKD-induced muscle wasting. (A) NO66 protein was evaluated in different tissues of MCK-NO66 mice versus that in NO66$^{flox/flox}$ mice (left panel). The densitometry shown in the right panel indicates the significant decrease of NO66 in muscles of MCK-NO66 mice (right panel). (B and C) In muscles of NO66$^{flox/flox}$ mice, NO66 was measurable and CKD raised its level of (B) protein and (C) mRNA. These results were abolished in muscles of MCK-NO66 mice despite the presence of CKD. (D and E) CKD decreased (D) body and (E) muscle weight in NO66 $^{flox/flox}$ mice, but not that in MCK-NO66 mice. (F) The average myofiber area in soleus muscles of MCK-NO66 mice with CKD was significantly larger than that of NO66$^{flox/flox}$ with CKD (dots represent myofibers). (G) In the presence of CKD, higher rates of protein synthesis were present in soleus and EDL muscles of MCK-NO66 mice versus results in NO66$^{flox/flox}$ mice. (H) CKD increases the rates of protein degradation in soleus muscles of NO66$^{flox/flox}$ as well as soleus muscle of MCK-NO66 mice ($n \geq 4$ in each group). *$P<0.05$ versus sham- NO66$^{flox/flox}$ mice; $^{\#}P<0.05$ versus CKD-NO66$^{flox/flox}$ mice. CTRL, control; F/F, flox/flox; Phe, phenylalanine.

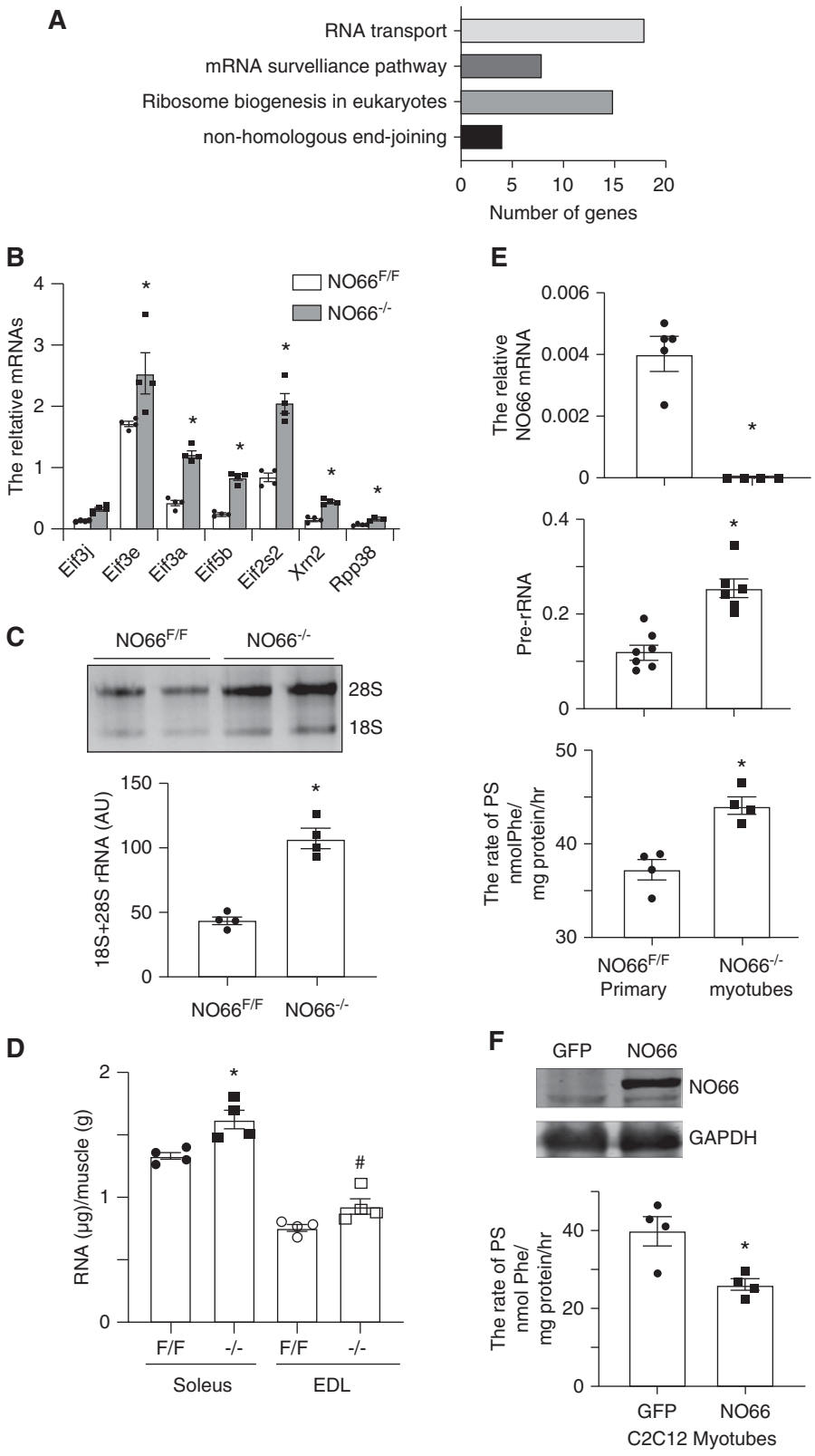

Figure 5. Loss of NO66 stimulates ribosomal biogenesis in mouse muscles. (A) KEGG pathway analysis using the gene differential expression identified by RNA-seq reveals that there is increased ribosomal biogenesis signaling in the response to NO66$^{-/-}$ from soleus muscles. (B) In muscles lacking NO66, the mRNA of eukaryotic translation-initiation factors is increased (n=4). *P<0.05 versus soleus-NO66$^{flox/flox}$. (C) rRNA from soleus muscles of NO66$^{-/-}$ mice is higher than that of NO66$^{flox/flox}$ mice (n=4 mice in each group).

(Supplemental Figure 5). Interestingly, a KEGG pathway analysis showed a significant proportion of the increased genes in muscles of NO66$^{-/-}$ mice were involved in ribosomal biogenesis signaling (Figure 5A). There are also 18 genes (Eif3j2, Xpo1, Eif3a, Fmr1, Fxr1, Pnn, Ranbp2, Thoc1, Eif5b, Rpp38, Nxt2, Upf2, Magohb, Eif2s2, Ncbp2, Upf3b, Trnt1, and Eif3j1) overexpressed in muscles of NO66$^{-/-}$ mice that are categorized as components of RNA transport (Figure 5A). These genes are mainly involved in mRNA nuclear export for gene expression processes, including control of transcription, splicing, 3′-end formation, and even translation. Notably, in muscles lacking NO66, a pairwise analysis of the differential expression of genes uncovered significant increases in genes controlling eukaryotic translation-initiation factors (Eif3j2, Eif3j1, Eif3e, Eif3a, Eif5b, and Eif2s2), exo-ribonucleases (Xrn1 and Xrn2), and ribonuclease P protein subunits of p38 (Rpp38), *etc.* (Figure 5B). Thus, the influence of NO66 on the regulation of ribosomal biogenesis could be a mechanism that changes the rate of muscle protein synthesis.

To evaluate this possibility, we measured rRNA in muscles *via* evaluation of total RNA. RNA isolated from 125 μg of soleus muscle of NO66$^{-/-}$ mice was subjected to electrophoresis on 1% agarose gel with ethidium bromide and we found a significant increase in 18S, 28S, and total rRNA when compared with muscles of control NO66$^{flox/flox}$ mice (Figure 5C) (similar results were present in EDL muscles). Notably, the agarose gel image included a large ribosomal subunit (28S RNA) plus a small ribosomal subunit (18S RNA). These RNAs were present in a 2:1 ratio, although 28S and 18S rRNAs are produced by cleavage of the same single RNA transcript. Presumably, rRNAs detected on agarose gels depend on the number of nucleotides present in each molecule. For example, in humans, 28S rRNA has approximately 5070 nucleotides, and 18S has 1869 nucleotides, which gives a 28S/18S ratio of approximately 2.7.

Because the abundance of ribosomes influences the rate of protein synthesis, we evaluated the translational capacity that is based on the number of ribosomes per unit of muscle.[32] In this case, we found a higher level of rRNA per gram of muscle in both the soleus and EDL muscles from NO66$^{-/-}$ mice when compared with results from NO66$^{flox/flox}$ mice (Figure 5D). Based on this association, we infer that the absence of NO66 in muscle participates in stimulating the capacity of muscle protein synthesis.

To evaluate the influence of NO66 on protein synthesis, we measured the rate of protein synthesis in primary myotubes that lack NO66. In these cells, there was a significant ($P<0.05$) increase in ribosomal pre-rRNA and rates of protein synthesis versus that in primary muscle cells isolated from NO66$^{flox/flox}$ mice (Figure 5E). We also found overexpression of NO66 in C2C12 myotubes results in suppression of protein synthesis (Figure 5F). We conclude that NO66 inhibits ribosomal biogenesis, thereby decreasing protein synthesis in muscle cells.

NO66 Regulates rDNA Transcription *via* a Demethylase Mechanism

To explore mechanisms by which NO66 could suppress protein synthesis, we tested whether NO66 regulates ribosomal biogenesis *via* demethylase activity. This possibility was raised because NO66 exhibits demethylase activity.[14,16,37] For this experiment, we isolated primary cells from muscles of NO66$^{-/-}$ and NO66$^{flox/flox}$ mice. Cultures of primary NO66$^{-/-}$ myoblasts were transfected with plasmids that express NO66 or "demethylase-dead" NO66 (NO66AKA)[14] using the Invitrogen Neon Transfection System. Cells transfected with green fluorescent protein were used as a negative control. RT-PCR and Western blotting analyses confirmed the mRNA and protein levels, respectively, of NO66 in these cells (Figure 6, A and B). In cells expressing NO66 (but not the mutant, NO66AKA) there were significant decreases in the trimethylated levels of H3K4 and H3K36 (Figure 6A). In addition, NO66 but not NO66AKA significantly ($P<0.05$) suppressed pre-rRNA (Figure 6B, lower panel). Expression of NO66 (but not of NO66AKA) significantly decreased the cellular translational capacity (*i.e.*, the amount of RNA in mg/protein in g; Figure 6C). The results suggest mutation of the demethylase activity of NO66 will abolish the suppression of NO66 on rDNA transcription. In short, NO66 can influence gene expression in a demethylase-dependent mechanism.

Next, we performed CHIP assays to examine whether histone trimethylation of H4K4 or H3K36 on rDNA differs in primary muscle cells from NO66$^{flox/flox}$ or NO66$^{-/-}$ mice using the primers listed in Figure 6D and Supplemental Table 2. Results of these experiments indicate that, in cells lacking NO66, there is enrichment of H3K4me3 and H3K36me3 in rDNA (Figure 6, E and F). These results demonstrate NO66 expression in muscle cells causes demethylation of H3K4me3 and H3K36me3 in rDNA, resulting in suppression of rDNA transcription.

DISCUSSION

For decades, CKD has been associated with increased protein degradation and repressed synthesis of muscle proteins.[38] We and others have found that CKD induced muscle protein

*$P<0.05$ versus soleus-NO66$^{flox/flox}$. (D) Protein synthesis capacity in both soleus and EDL muscles of NO66$^{-/-}$ mice is higher than that in muscles of NO66$^{flox/flox}$ mice ($n=4$ mice in each group). *$P<0.05$ versus soleus-NO66$^{flox/flox}$; #$P<0.05$ versus EDL-NO66$^{flox/flox}$. (E) Primary muscle cells from NO66$^{-/-}$ mice exhibit increases in the expression of pre-rRNA plus increases in rates of protein synthesis ($n\geq4$ repeat). *$P<0.05$ versus primary cell of NO66$^{flox/flox}$. (F) NO66 overexpression in C2C12 myotubes suppressed the rate of protein synthesis ($n=4$ repeat). *$P<0.05$ versus green fluorescent protein (GFP)–C2C12. F/F, flox/flox.

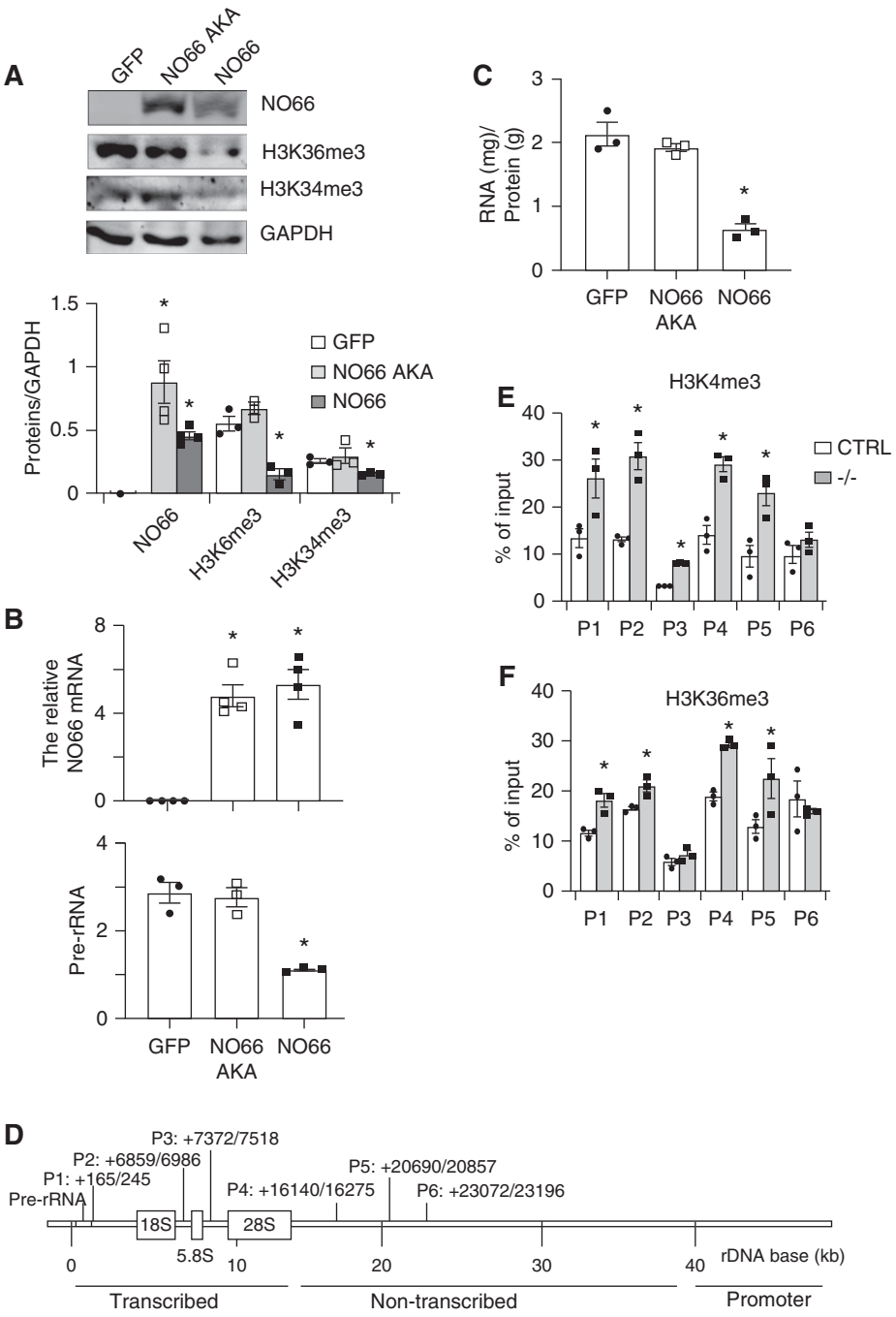

Figure 6. NO66 regulates rDNA transcription *via* a demethylase mechanism. (A–C) Primary cultures of NO66$^{-/-}$ cells were transfected with plasmids expressing wild-type NO66, mutant NO66AKA, or green fluorescent protein (GFP; used as control) and the protein levels of (A) trimethylated H3K4 and H3K36, (B) the pre-rRNA, and (C) protein synthesis capacity were evaluated (n=3 repeats). *P<0.05 versus GFP. (D) The primer information for a CHIP assay in rDNA of mice (see also Supplemental Table 2 for primer sequence). (E and F) CHIP assay shows the enrichment of (E) H3K4me3 and (F) H3K36me3 in rDNA from cells lacking NO66 (n=3 repeats). *P<0.05 versus NO66$^{flox/flox}$. CTRL, control.

degradation *via* a mechanism of activation of proteolytic responses, caspase-3, and the UPS. However, CKD-induced mechanisms that impair protein synthesis in muscle have not been identified. Fujii *et al.*[39] have demonstrated that protein synthesis in muscle is suppressed in uremic rats and found this metabolic defect is not improved by raising dietary protein. The influence of dietary protein is emphasized because muscle protein losses in other conditions (*e.g.*, aging) exhibit impaired protein synthesis that is not corrected by increasing dietary protein.[11] Specifically, feeding high levels of protein to

patients with kidney disease not only raises their production of uremic toxins but this treatment also increases phosphate intake, resulting in accelerated complications of CKD.[40] In contrast to the disappointing metabolic responses achieved by raising dietary protein in catabolic conditions, protein metabolism can develop beneficial responses to exercise or the administration of growth hormone, including increasing the level of muscle protein synthesis.[38,41] Unfortunately, exercise is not widely prescribed for patients with CKD.[42] Our experiments identify a novel pathway that can increase muscle protein synthesis in models of CKD without relying on raising nutrient intake or increasing exercise. We found that deletion of NO66 in skeletal muscle prevents the suppression of protein synthesis and loss of muscle mass. NO66 elimination does not affect the rate of muscle protein degradation in mice with CKD. In rats with CKD, however, we have shown that metabolic acidosis activates the degradation of muscle proteins; this response is reversed when acidosis is corrected. In contrast, however, treatment of acidosis did not improve the rate of protein synthesis.[12,24] We conclude that the control of muscle protein degradation occurs independently of the activation of protein synthesis. In addition, we speculate that strategies directed at improving muscle protein synthesis and decreasing protein degradation will greatly improve muscle mass in catabolic conditions.

All cells of the body have essentially the same DNA, but different organs and tissues serve vastly different functions. These functions are determined by epigenetic responses. Epigenetic modification encompasses three forms: (*1*) DNA methylation; (*2*) post-translational modifications of nucleosomal histones including their acetylation, methylation, phosphorylation, ubiquitinylation, and sumoylation; or (*3*) higher-order chromatin structures. Specifically, histone modifications are associated with gene activities, gene silencing, or insulation between active and inactive gene regions. Environmental and metabolic responses can cause histone modifications that account for approximately 80% of the risks for developing human or animal diseases. For example, widespread acceptance of the Western diet is a leading cause of type 2 diabetes, whereas smoking is a leading cause of several cancers and autoimmune and respiratory diseases.[43] In contrast, exercise is able to mediate changes in the skeletal-muscle epigenome.[44] Our findings represent another disorder that is related to histone modification: CKD has elements of an inflammatory disease and it induces the expression and function of NO66 *via* activation of NF-κB. Specifically, an increase in NO66 can result in demethylation of H3K4me3 and H3K36me3 in rDNA (Figure 7). These responses led to a decrease in the expression of pre-rRNA plus suppression of the rate of protein synthesis in muscle. Our findings are consistent with the report that a decrease in H3K4 methylation can be associated with a decrease in the expression of pre-rRNA, which leads to decreased activity of mouse embryonic stem cells.[27,45]

Our findings are consistent with the conclusion that ribosomal biogenesis is a central mechanism regulating muscle

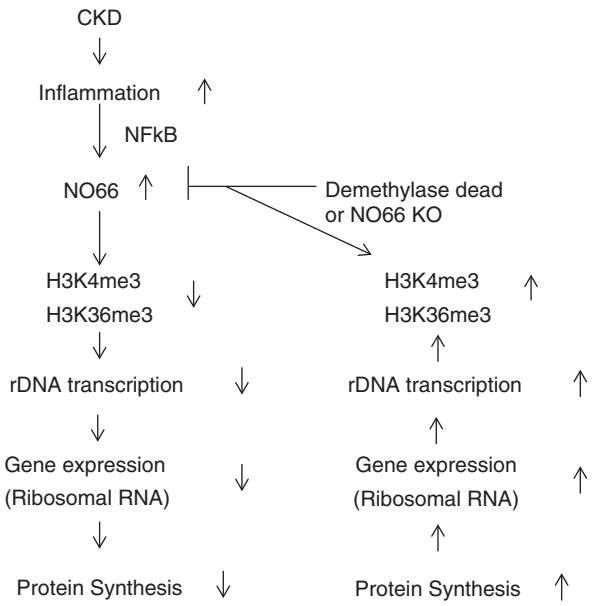

Figure 7. A model illustrating a mechanism that CKD suppresses muscle protein synthesis. NO66 suppresses rDNA transcription *via* a demethylase mechanism, leading to impairment of the rate of protein synthesis.

protein synthesis, thereby contributing to changes in muscle mass.[46] Ribosomal biogenesis is in turn regulated by signaling pathways including mTORC1 or Wnt/β-catenin/c-myc that can be stimulated by anabolic or catabolic conditions.[47,48] The importance of ribosomal biogenesis in regulating cell growth has mainly been demonstrated in yeast or tumor cells, whereas only limited reports have been identified in skeletal or cardiac muscles.[49,50] Few studies have examined the regulation of ribosome biogenesis in skeletal muscles,[51] but no reports have focused on the regulation of ribosomal biogenesis in catabolic conditions. Our results are the first to document that ribosomal biogenesis is regulated by NO66 in muscles of mice and patients with CKD. In addition to the regulation of ribosomal biogenesis, our data suggest that NO66 may affect mechanisms besides rRNA transcription. This is suggested because we found increases in components of RNA transport in muscles lacking NO66. These genes are involved in gene expression processes including transcription, splicing, 3′-end formation, and even translation that could influence responses of muscle protein synthesis.

In summary, we have uncovered a CKD-mediated process that silences rRNA genes. Our discoveries are significant because they have uncovered an epigenetic mechanism that impairs muscle protein synthesis contributing to CKD-induced protein energy wasting. Specifically, our results are independent of other CKD-stimulated mechanisms, namely the increase in muscle protein degradation after activation of UPS and caspase-3.[7,8] Understanding how this pathway is regulated could uncover new strategies for preventing the loss of muscle in CKD and other catabolic diseases.

DISCLOSURES

All authors have nothing to disclose.

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Q. Chen, Z. Chen, Y. Wang, and L. Zhang performed animal and cell experiments; J. Gamboa, G. Garibotto, and T. Ikizler participated in interpreting metabolic changes resulting from renal insufficiency in mice with CKD as well as patients with kidney disease; W. Mitch and L. Zhang designed and coordinated the experiments and wrote the manuscript.

SUPPLEMENTAL MATERIAL

This article contains the following supplemental material online at http://jasn.asnjournals.org/lookup/suppl/doi:10.1681/ASN.2019121277/-/DCSupplemental.

Supplemental Figure 1. NO66 is expressed in muscle nuclei.

Supplemental Figure 2. CKD decreases mixed-fiber muscle weights.

Supplemental Figure 3. MCK-NO66 mice are resistant to CKD-induced muscle wasting.

Supplemental Figure 4. NO66 and myostatin independently regulate muscle mass.

Supplemental Figure 5. The Gene Ontology analysis.

Supplemental Table 1. Antibody lists.

Supplemental Table 2. The primer list for RT-PCR.

Supplemental Table 3. Information about CKD patients.

Supplemental Table 4. Information about hemodialysis patients.

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