SUPPLEMENTAL INFORMATION

1. Experimental Procedures:

Molecular Biology

The p68-GFP plasmid was generated by cloning p68 RT-PCR product using RNA from normal human skeletal muscle into the SacI and KpnI sites of pGFP-C1. The sequences of the p68 primers are: forward 5'-AGGAGCTCCCATGTCGGGTTATTCGAGTGA-3' and reverse: 5'-TTGGTACCTTATTGGGAATATCCTGTTGGC-3'.

The AAV8-GFP and AAV8-p68-GFP adeno-associated viral vectors were produced at SignaGen Laboratories. P68-GFP was cloned into an AAV8 *cis* vector under control of a CMV promoter. The titer of AAV8-GFP and AAV8-p68-GFP was 1.7 - 6.4 x 10^{12} GC/ml.

Cell culture

Generation and analysis of the tet-regulated CHO and HeLa cell lines expressing CUG_{914} and $CCUG_{100}$ RNAs have been described (16). Tet-regulated CHO and HeLa cells expressing CUG_{914} and $CCUG_{100}$ RNA were grown in DMEM containing 10% tet-free FBS (Clontech Laboratories) supplemented with geneticin and hygromycin B to 80% density. Dox was added to CHO cells at a concentration (0.5 μg/ml) and to HeLa cells at a concentration of 1 μg/ml. Protein extracts were collected at various time points after Dox addition and subjected to analysis.

DM1 myoblasts were generated from biopsied muscle from DM1 patients with 60, 300 and 500 CTG repeats. DM1 fibroblast line (GM05163) was purchased from the Coriell Institute. DM2 fibroblasts were established from skin biopsies of patients with DM2 with approximately 2,000 CCTG repeats. Normal myoblasts and fibroblasts were generated from patients with normal muscle histology. Human myoblasts were maintained in an F10 medium (Gibco) containing 1% Sodium Bicarbonate (Gibco), 15% fetal bovine serum (Hyclone), 5% defined supplemental calf serum (Hyclone), 1% penicillin/streptomycin (Gibco) and 1% L-glutamine (Gibco). Human fibroblasts were grown in DMEM medium (Gibco), supplemented with 10% FBS (HyClone) and 1% penicillin/streptomycin (Gibco).

Western blotting

Human muscle biopsies and mouse muscle samples were homogenized in RIPA buffer. Western blot analysis was performed as described (10) using mouse monoclonal antibodies to CUGBP1 (3B1), p68 (A5) and α-Tubulin (TU02) from Santa Cruz Biotechnologies. Mouse monoclonal antibodies to β-actin were from Sigma-Aldrich.

Histological analyses

To assess the number of fibers with central nuclei, muscle sections from the age- and gender-matched WT and HSA^{LR} muscles, treated with PBS or p68, were stained with H/E. The fibers with central nuclei were counted in 200-400 fibers from the matched WT and PBS- or p68-treated HSA^{LR} muscles (3-4 mice per group) using MetaMorph (Molecular Devices) software. The data describing the number of fibers with central

nuclei were presented as the percentage of the total fibers. A change in the relative number of centralized nuclei was calculated as follows: 100 x (central nuclei (HSA control) – central nuclei (HSA-p68)) / (central nuclei (HSA controls) – central nuclei (wild type)). Muscle fiber diameters were examined in 800-1,000 fibers in each mouse group by measuring the mean minimal Feret's diameter as described in Supplemental Reference 1, using NIS Elements AR Imaging Software V. 4.20.

Fluorescence *in Situ* Hybridization and Quantitative analysis

DM1 myoblasts and DM2 fibroblasts were transfected with 2-6 μg of p68-GFP or GFP per 500,000 cells. Transfections were performed with the AMAXA nucleofector system with an average efficiency 75%. Seventeen hours after transfection cells were fixed with 3.7% formaldehyde in PBS and pre-hybridized in 40% formamide and 2X SSC for 10 minutes at 37°C, followed by hybridization for 1 hour at 37°C. The hybridization mix consisted of 40% formamide, 4 × SSC, 1 mg/ml tRNA, salmon sperm DNA (200 μg/ml), 0.2% BSA, 2 mmol vanadyl guanoside, and 0.5 μg/ml CAG_{15} or $CAGG_{12}$ labeled with Alexa555. The images were analyzed on the Nikon microscope using the same conditions of brightness and exposure time. The percentage of CUG/CCUG aggregates was calculated in 100 cells using the same conditions for time of exposure and brightness. The experiment was repeated 3 times for a total of 300 cells analyzed. The number of CUG foci without transfection with p68 was set as 100%. To determine the number of nuclear and cytoplasmic CUG foci, randomly selected cells (100 cells for each experiment) were analyzed by FISH and foci were counted. The analysis was repeated three times for a total of 300 cells counted to determine the average value.

Frozen mouse muscle sections were subjected to pre-hybridization in the solution of 40% formamide and 2 x SSC at 37°C for 2 hours. Sections were hybridized in the mix of 40% formamide, 10% dextran sulfate and 2 x SSC at 37°C overnight. The hybridization solution contained 30ng/ml CAG_{15} probe labeled with Alexa555. After hybridization, sections were washed three times in 2 x SSC, covered with mounting media containing DAPI and examined on the Nikon microscope under the same conditions of time exposure and brightness. The average number of CUG foci was counted in 300-400 randomly selected fibers using three mice per group including WT mice and HSA^{LR} mice treated with GFP-AAV8 or p68-GFP-AAV8. The number of CUG foci in the GFP-treated muscle was set as 100%.

Electron Transmission Microscopy (ETM)

Fresh whole gastrocnemius from WT mice and from HSA^{LR} mice injected with PBS or AAV8-GFP-p68 were sequentially fixed in 4% formaldehyde in PBS at 4°C overnight and in 3% glutaraldehyde, containing 0.4% of tannic acid for 24 hours. Small pieces of the gastrocnemius muscles from mice of different groups were cut at the similar areas, used for the injections, with surgical scissors under dissecting microscope. EM analysis was performed in the Pathology Research Core of Cincinnati Children's Hospital. Samples were post-fixed in 1% osmium tetroxide for 1 hour at 4°C and counterstained with 1% uranyl acetate for 2 hours and infused with epoxy resin. Because gastrocnemius is a multipennate muscle special care has been taken to assure that the areas with similar fiber orientation were used for the EM analysis. Prior to preparation of slides for EM analysis, muscle sections stained with 1% Toluidine Blue O were analyzed by light

microscopy at magnification 20 x to compare the fiber orientation in the muscle sections from different mouse groups. Once the orientation of fibers was verified, ultrathin sections (50-70 nm) were contrasted with uranyl acetate and Sato lead stain and analyzed using Hitachi Transmission Electron Microscope, model H-7650. Myofibrillar organization was compared in the similar muscle areas with similar fiber orientation in the matched WT and HSA^{LR} muscle injected with PBS or with AAV8-p68-GFP at 5,000 x (39-77 images per mouse). The same sections were also examined at 20,000 x; 30,000 x and 50,000 x with total analysis of 300-600 myofibrils per slide.

Semi-quantitative RT-PCR

Total RNA was extracted from the gastroc of the matched WT and HSA^{LR} mice un-treated and treated with p68-AAV8 using Trizol. The integrity of RNA was examined by gel electrophoresis. Reverse transcription was performed using 1 μg of total RNA with SuperScript III. The semi-quantitative conditions of PCR were established using series of dilutions of 1 μl of reverse transcription mix with the internal standard primers for GAPDH. The sequence of the forward primer for GAPDH is 5' - AACTTTGGCATTGTGGAAGGGCTC - 3'. The sequence of the reverse primer is 5' - TGGAAGAGTGGGAGTTGCTGTTGA - 3'. The RT-PCR assay with these primers produced a GAPDH-specific 380 bp product. The splicing of BIN1 was examined with the BIN1 specific primers for the isoform lacking exon 11 under semi-quantitative conditions established for GAPDH. The sequence of the BIN1 forward primer is 5'-TCAATGATGTCCTGGTCAGC -3'. The sequence of the BIN1 reverse primer is 5'-GCTCATGGTTCACTCTGATC-3'. The RT-PCR with these primers produced two

BIN1-specific products: the 207 bp (with the inclusion of exon 11) and 162 bp (with the exclusion of exon 11). The RT-PCR products were separated on 4.5% agarose gel and the intensities of the bands were quantified by the scanning densitometry using GAPDH as control. The amounts of the BIN1 mRNA isoform lacking exon 11 were determined as percentage of the total BIN1 mRNA. The amounts of total BIN1 mRNA was set as 100%. The experiment was repeated four times.

Statistical Analysis

Data are presented as mean +/- SEM. Statistical analysis was performed using two-way ANOVA and two-tailed Student's *t* test. A P value <0.05 was considered statistically significant.

References for the Supplementary Materials

1. Briguet A et al. (2004) Histological parameters for the quantitative assessment of muscular dystrophy in the mdx-mouse. *Neuromuscular Disorders* 14 (10), 675-682.

2. SUPPLEMENTAL FIGURE LEGENDS

Figure S1. Ectopic expression of p68 causes complete dissociation of RNA foci in DM1 myoblasts from a patient with 60 CTG repeats. (A) FISH assay with CAG probe detects CUG foci in DM1 myoblasts. Nuclei were stained with DAPI. **(B)** Fluorescent analysis detecting p68-GFP. Transfected cells (green) are shown by arrows.

The variability of the intensity of the green signal likely reflects different number of molecules of p68 plasmid transfected in cells. **(C)** UV cross-link analysis of p68, immunoprecipitated from normal human fibroblasts, with ^{32}P-RNA, containing 123 CUG repeats. As controls, IgG without antibodies to p68 (IgGs) and purified recombinant CUGBP1 were used. **(D)** Cytoplasmic and nuclear protein extracts from un-transfected and transfected with p68-GFP HeLa cells were analyzed by UV-cross link with the ^{32}P-labeled RNA containing 123 CUG repeats.

Figure S2. Quantification of CCUG foci in DM2 fibroblasts transfected with p68 shown in the Figure 2G. Foci were counted in un-transfected cells and in cells transfected with 2 μg of plasmid coding for p68-GFP. One hundred cells were analyzed in each set; and the average data of three repeats were presented. * $P<0.023$.

Figure S3. Ectopic expression of p68 disintegrates CCUG foci in a tet-regulated HeLa cell model of DM2. (A) Detection of CCUG foci by FISH assay in HeLa-$CCUG_{100}$ cells, transfected with GFP (control) and with p68-GFP after induction of $CCUG_{100}$ RNA by Dox. Arrows point out nuclear and cytoplasmic foci in $CCUG_{100}$ HeLa cells transfected with empty vector. In HeLa-$CCUG_{100}$ cells, transfected with p68-GFP, CCUG signal is diffused (shown by arrows). **(B)** Quantification of results from A. The number of CCUG foci was counted in un-transfected, transfected with GFP or p68-GFP cells, expressing $CCUG_{100}$. 150-200 randomly selected cells were analyzed in each group. The average data from three repeats are presented. * $P<0.05$.

Figure S4. Quantification of the mutant *DMPK* mRNA, mutant CUG and CCUG repeats and normal *ZNF9* mRNA in DM1/2 fibroblasts and in CUG/CCUG stable clones described in the Figure 3. Intensities of the mutant *DMPK* mRNA in DM1 fibroblasts **(A)**, mutant CUG repeats in CHO stable cells **(B),** mutant CCUG repeats in DM2 fibroblasts **(C),** normal *ZNF9* mRNA in DM2 fibroblasts **(D)** and mutant CCUG repeats in HeLa stable clones **(E)** were determined by scanning densitometry and adjusted to the *GAPDH* signal used as a control for RNA loading. Data are presented as mean +/- SEM. * $P < 0.05$.

Figure S5. Recombinant p68, injected into *gastrocnemius* of *HSA* mice, binds to the mutant CUG repeats in vitro. (A) Structure of AAV8-p68-GFP recombinant vector. **(B)** Fluorescent analysis of HSA^{LR} skeletal muscle (gastroc) injected with PBS or GFP-p68 confirms expression of p68-GFP. All images were taken at the same exposure. **(C)** UV cross link analysis of the recombinant p68, injected into *gastroc* of HSA^{LR} mice, with CUG RNA. Cytoplasmic and nuclear protein extracts prepared from gastroc of WT mice and HSA^{LR} mice injected with PBS or p68-GFP were examined by UV cross link assay with RNA containing 123 CUG repeats. Analysis of muscle extracts from two HSA^{LR} mice injected with p68-GFP is shown.

Figure S6. FISH analysis of frozen muscle sections from gastroc of matched WT mice and HSA^{LR} mice treated with AAV8-GFP (A) or AAV-p68-GFP (B). CUG foci were detected with CAG-Alexa555 at magnification 60x and 100x. Nuclei were stained with DAPI. CUG foci are shown by arrows. Note a reduction of the CUG signal in the

p68-treated muscle. **(C)** P68-treated HSA^{LR} muscle has reduced number of CUG foci. The numbers of CUG foci were counted in 300-400 fibers in HSA^{LR} muscle treated with GFP or GFP-p68 as described in the Methods. Y shows the average percentage of CUG foci per view at magnification 60x. The number of CUG foci in the HSA^{LR} muscle treated with GFP was set as 100%. ***P=0.000406.

Figure S7. Improvement of muscle histopathology in HSA^{LR} mice treated with p68. **(A)** Comparison of the average myofiber size in gastroc isolated from matched WT and HSA^{LR} mice treated with PBS or p68-GFP. One hundred fibers were measured in similar regions of muscle sections from each mouse using 4-5 mice per group. **(B)** P68 corrects the mean minimal Feret's diameter in muscle of HSA^{LR} mice. The minimal Feret's diameter is significantly increased in the PBS-treated muscle from HSA^{LR} mice relatively WT muscle (P=2.3375E-131); whereas p68 treatment normalizes the minimal Feret's diameter in HSA^{LR} mice [P=2.1067E-138 (PBS treated versus p68-treated HSA^{LR} mice)]. **(C) Increase of myofiber number in gastroc of HSA^{LR} mice treated with p68-GFP.** Total fiber number was counted in five 20 x fields in similar regions of muscle sections using 4-5 mice per group. **(D) The number of central nuclei is reduced in HSA^{LR} mice treated with p68.** Comparison of average percentage of myofibers with centralized nuclei in WT muscle and in muscle from HSA^{LR} mice treated with PBS or p68. P values are 0.0214 (WT versus PBS-treated HSA^{LR} muscle) and 0.02391 (PBS-treated muscle versus p68-treated muscle from HSA^{LR} mice). The change of the number of myofibers with central nuclei in the PBS-treated muscle versus the p68-treated muscles is 90.7%.

Figure S8. Improvement of myofibrillar structure of *HSA^{LR}* muscle treated with p68. Electron transmission microscopy of muscle from the matched WT mice and *HSA^{LR}* mice treated with PBS (control) or with AAV-p68-GFP. The images were taken at magnification 30,000 x **(A)** and 5,000 x **(B).** The sarcomeres are shown by arrows. Note the presence of thin myofibrils and reduced myofibril density in *HSA^{LR}* muscle treated with PBS relatively WT muscle and the improvement of myofibrillar organization in *HSA^{LR}* muscle treated with p68.

Figure S9. The splicing of BIN1 is improved in *HSA^{LR}* mice treated with p68. **(A)** Representative semi-quantitative RT-PCR analysis of the BIN1 spliced isoforms with exclusion and inclusion of exon 11 is shown. The 162 and 207 bp products correspond to the BIN1 isoform lacking exon 11 (shown by red arrow) and including exon 11 (shown by black arrow). **(B)** Quantitative analysis of the BIN1 isoform lacking exon 11 is shown. The levels of BIN1 were adjusted to GAPDH and the percentage of the BIN1 isoform lacking exon 11 was calculated. The total amount of BIN1 RNA was set as 100%. Data are presented as mean +/- SEM.

Figure S10. GSK3 inhibitor, TDZD-8, corrects p68 levels in *HSA^{LR}* mice. P68 levels were evaluated by Western blot analysis in gastroc from the matched WT and un-treated and treated with TDZD-8 *HSA^{LR}* mice. The membrane was re-probed with Abs to calreticulin as the control.

SUPPLEMENTAL FIGURES

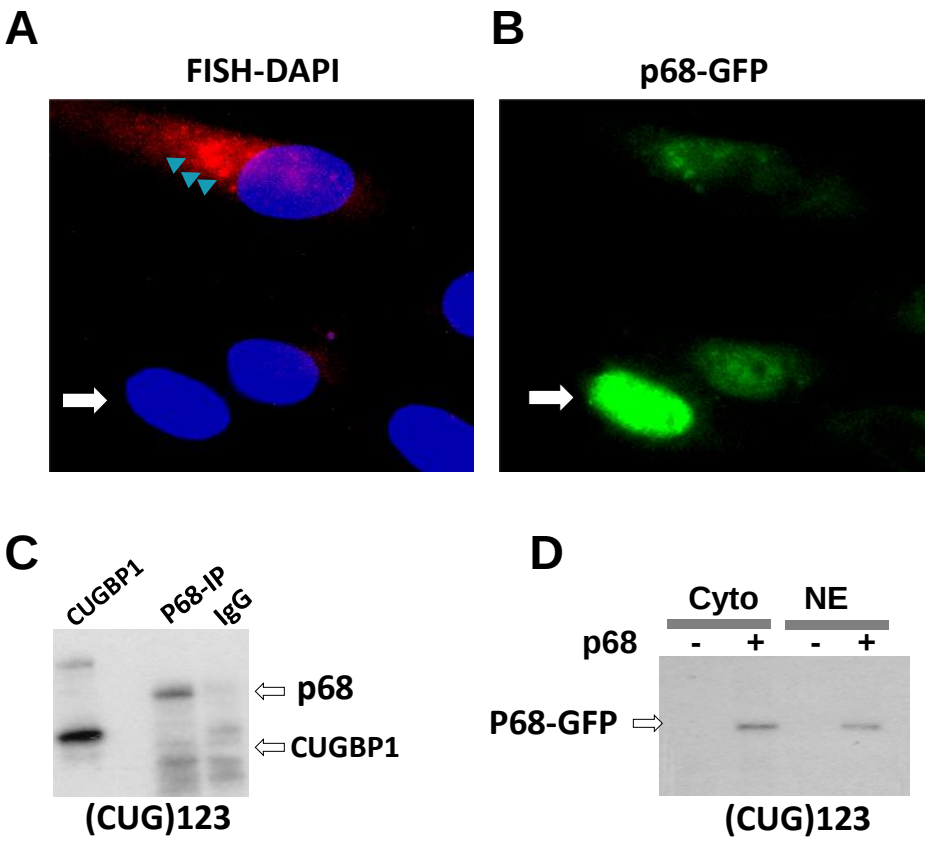

A
FISH-DAPI
B
p68-GFP
C
CUGBP1
P68-IP
IgG
p68
CUGBP1
(CUG)123
D
Cyto
NE
p68
- + - +
P68-GFP
(CUG)123

Figure S1

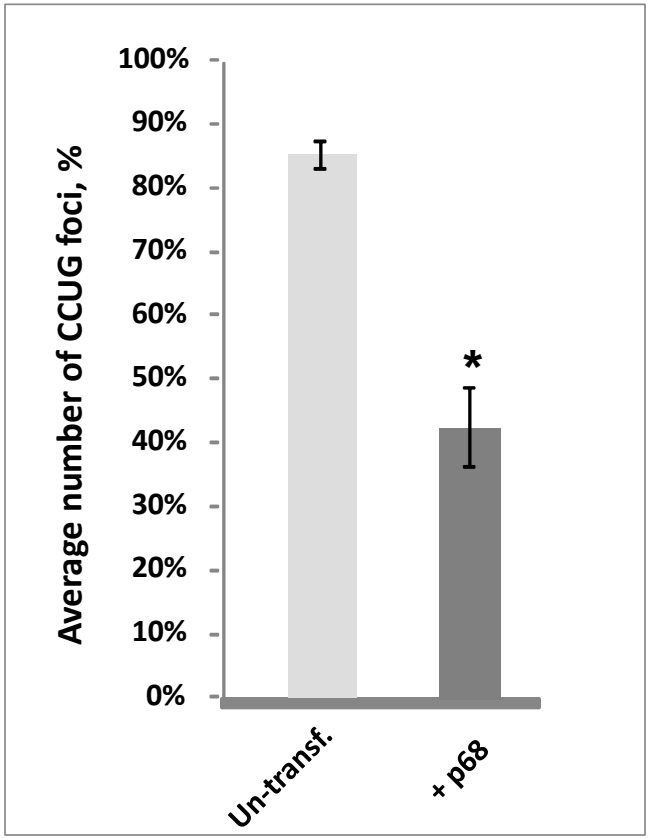

Average number of CCUG foci, %
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
*
Un-transf.
+ p68

Figure S2

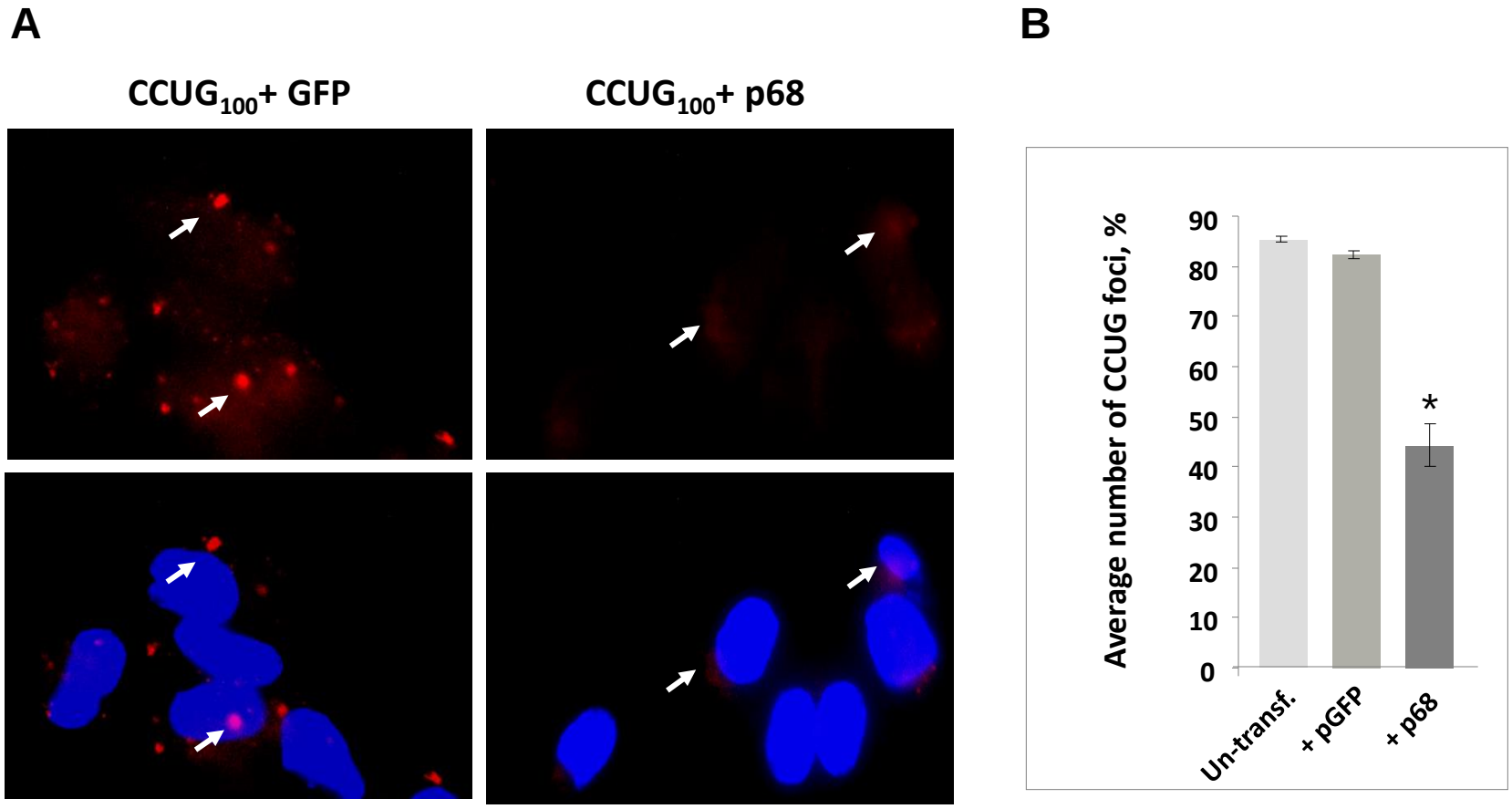

A
CCUG100+ GFP
CCUG100+ p68
B
Average number of CCUG foci, %
90
80
70
60
50
40
30
20
10
0
*
Un-transf.
+ pGFP
+ p68

Figure S3

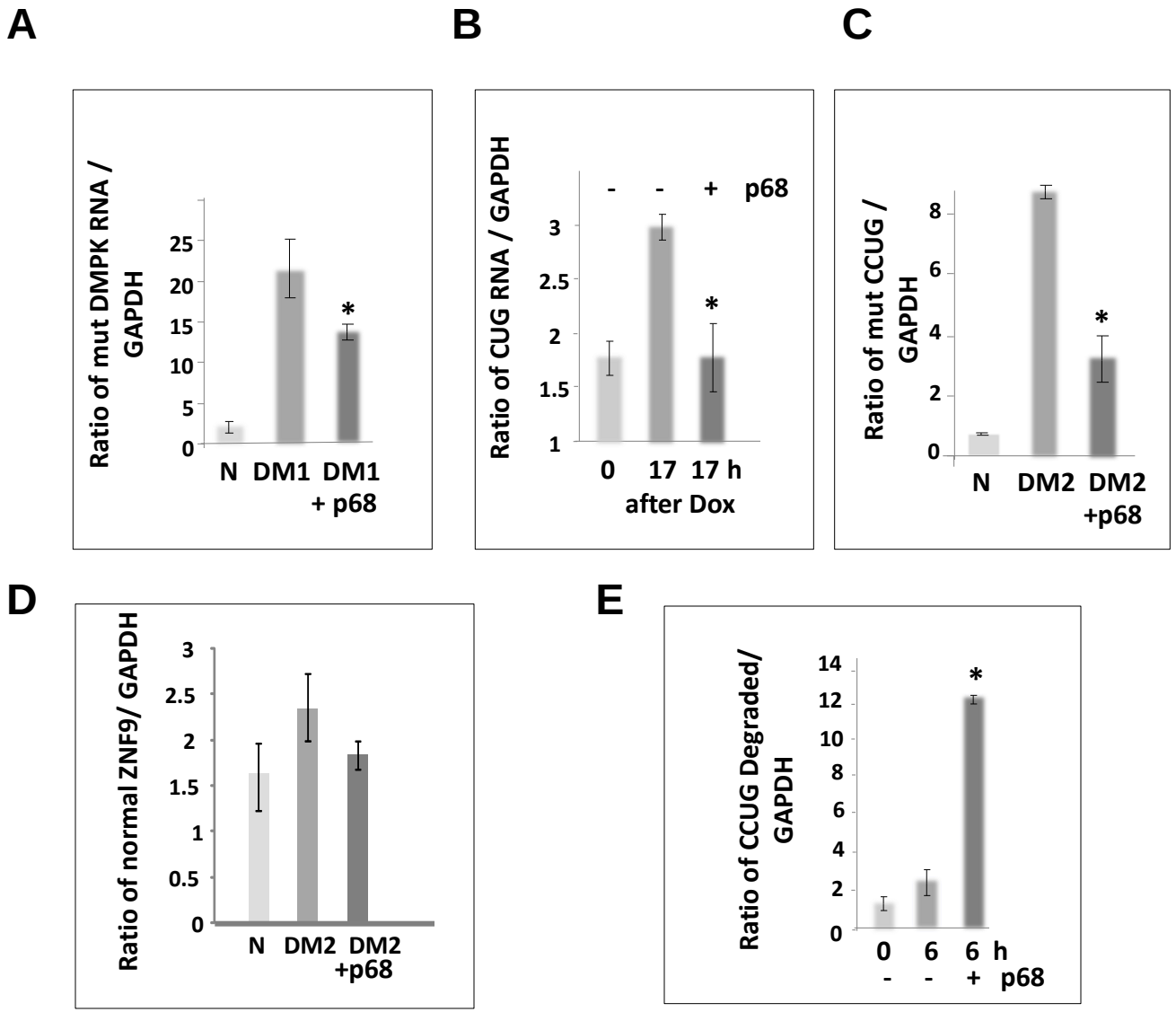
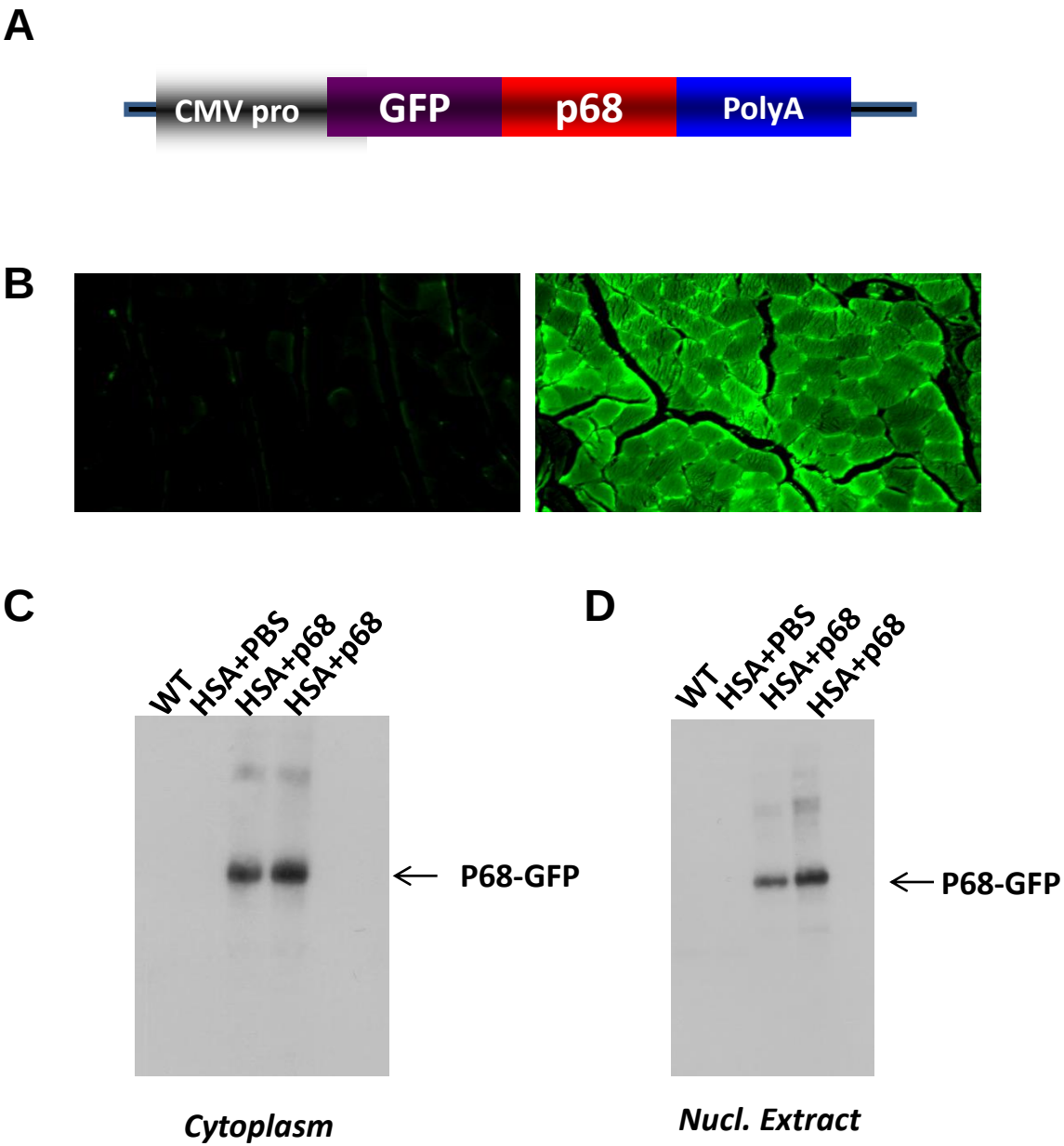

A
Ratio of mut DMPK RNA / GAPDH
25
20
15
10
5
0
*
N
DM1
DM1 + p68
B
Ratio of CUG RNA / GAPDH
- - + p68
3
2.5
2
1.5
1
*
0
17
17 h
after Dox
C
Ratio of mut CCUG / GAPDH
8
6
4
2
0
*
N
DM2
DM2 +p68
D
Ratio of normal ZNF9/ GAPDH
3
2.5
2
1.5
1
0.5
0
N
DM2
DM2 +p68
E
Ratio of CCUG Degraded/ GAPDH
14
12
10
8
6
4
2
0
*
0
6
6 h
- - + p68

Figure S4


A
CMV pro
GFP
p68
PolyA
B
C
WT
HSA+PBS
HSA+p68
HSA+p68
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Cytoplasm
D
WT
HSA+PBS
HSA+p68
HSA+p68
P68-GFP
Nucl. Extract

Figure S5

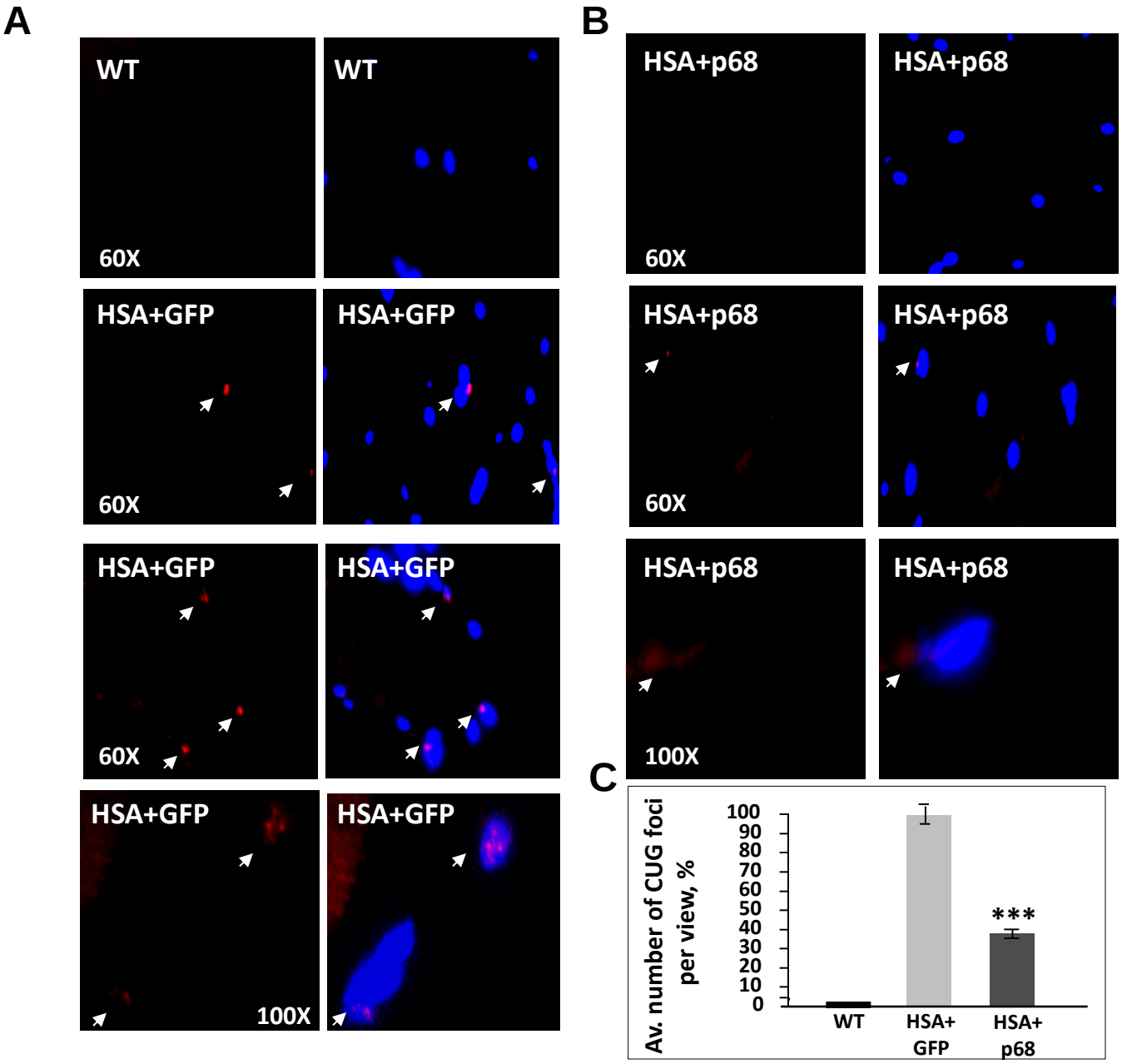
A
WT
WT
60X
HSA+GFP
HSA+GFP
60X
HSA+GFP
HSA+GFP
60X
HSA+GFP
HSA+GFP
100X
B
HSA+p68
HSA+p68
60X
HSA+p68
HSA+p68
60X
HSA+p68
HSA+p68
100X
C
Av. number of CUG foci per view, %
100
90
80
70
60
50
40
30
20
10
0
***
WT
HSA+ GFP
HSA+ p68

Figure S6

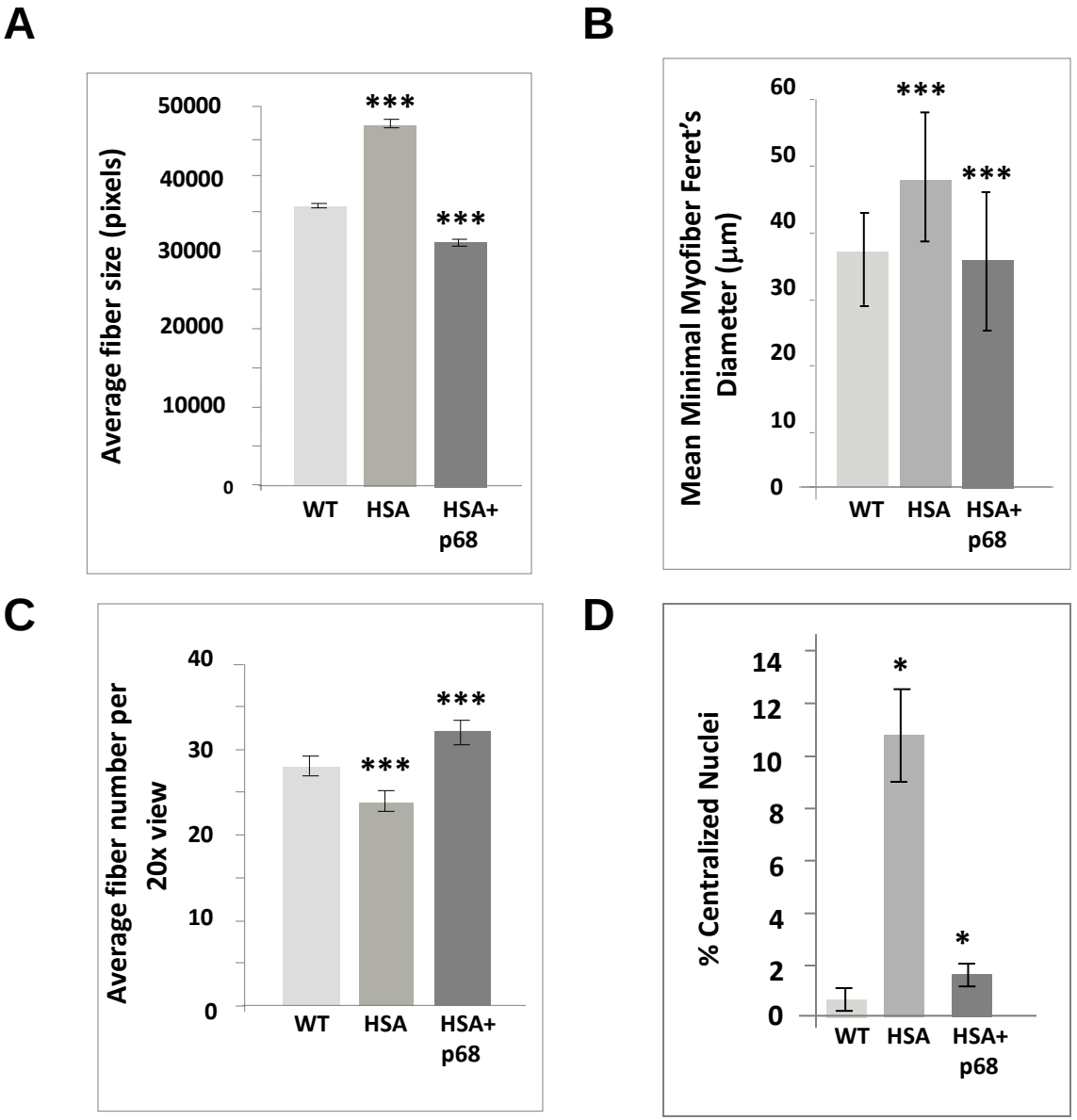
A
Average fiber size (pixels)
50000
40000
30000
20000
10000
0
***
***
WT
HSA
HSA+ p68
B
Mean Minimal Myofiber Feret's Diameter (μm)
60
50
40
30
20
10
0
***
***
WT
HSA
HSA+ p68
C
Average fiber number per 20x view
40
30
20
10
0
***
***
WT
HSA
HSA+ p68
D
% Centralized Nuclei
14
12
10
8
6
4
2
0
*
*
WT
HSA
HSA+ p68

Figure S7

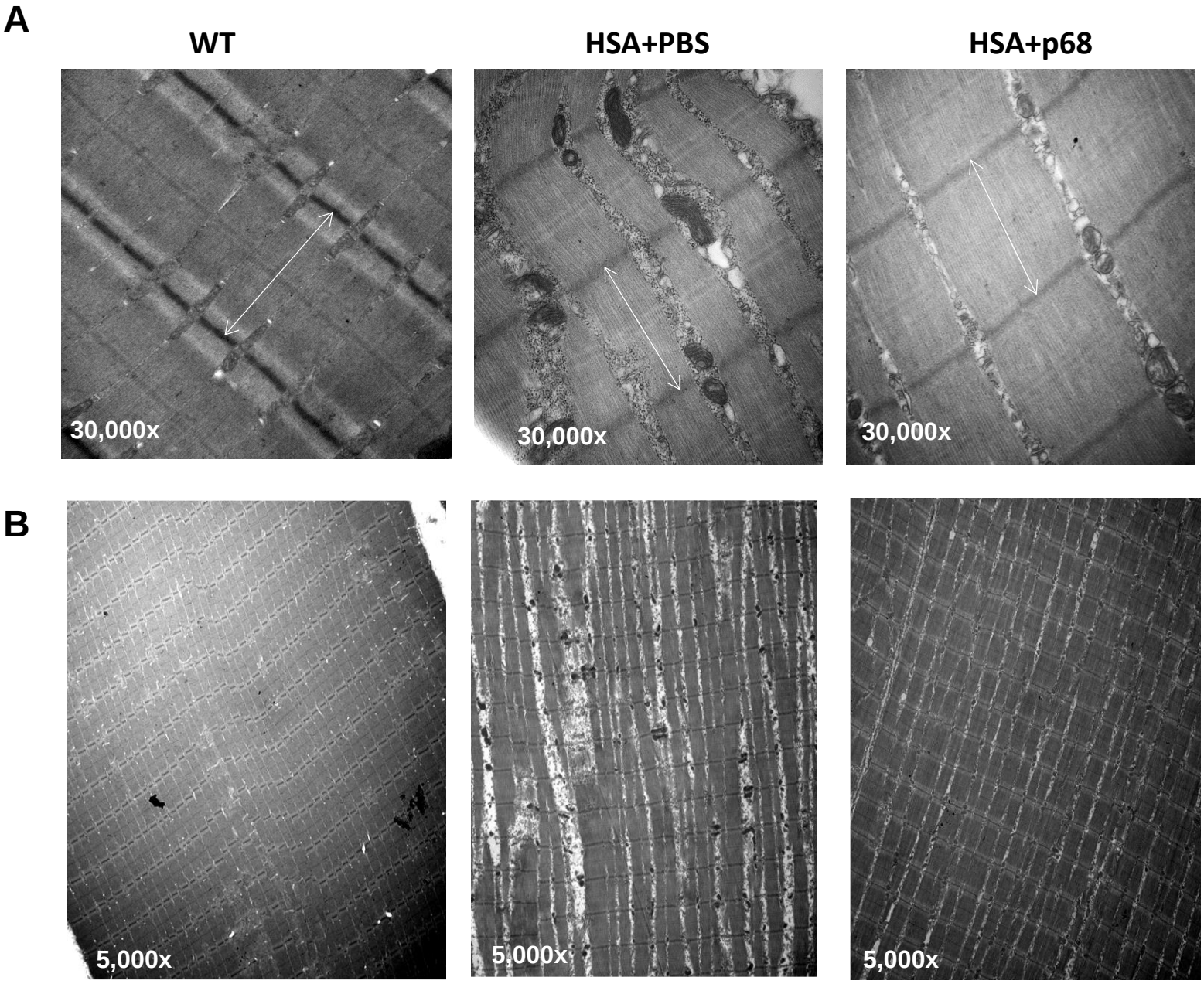

Figure S8

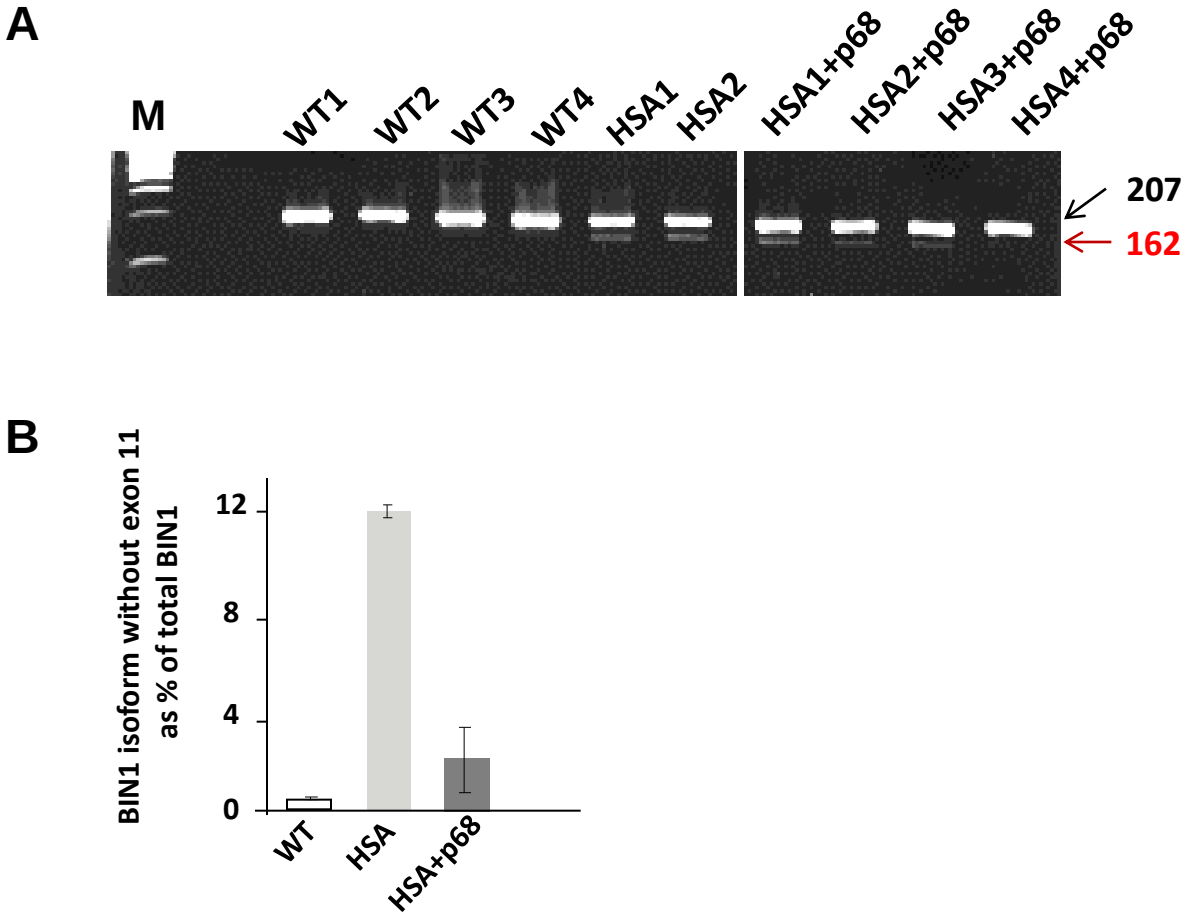
A
M
WT1
WT2
WT3
WT4
HSA1
HSA2
HSA1+p68
HSA2+p68
HSA3+p68
HSA4+p68
207
162
B
BIN1 isoform without exon 11
as % of total BIN1
12
8
4
0
WT
HSA
HSA+p68

Figure S9

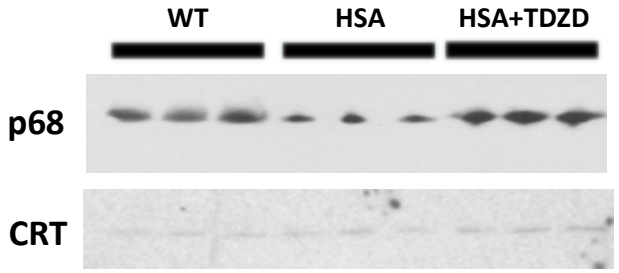

Figure S10