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Supplementary Materials for

Interfering MSN-NONO complex–activated CREB signaling serves as a therapeutic strategy for triple-negative breast cancer

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Supplementary Materials

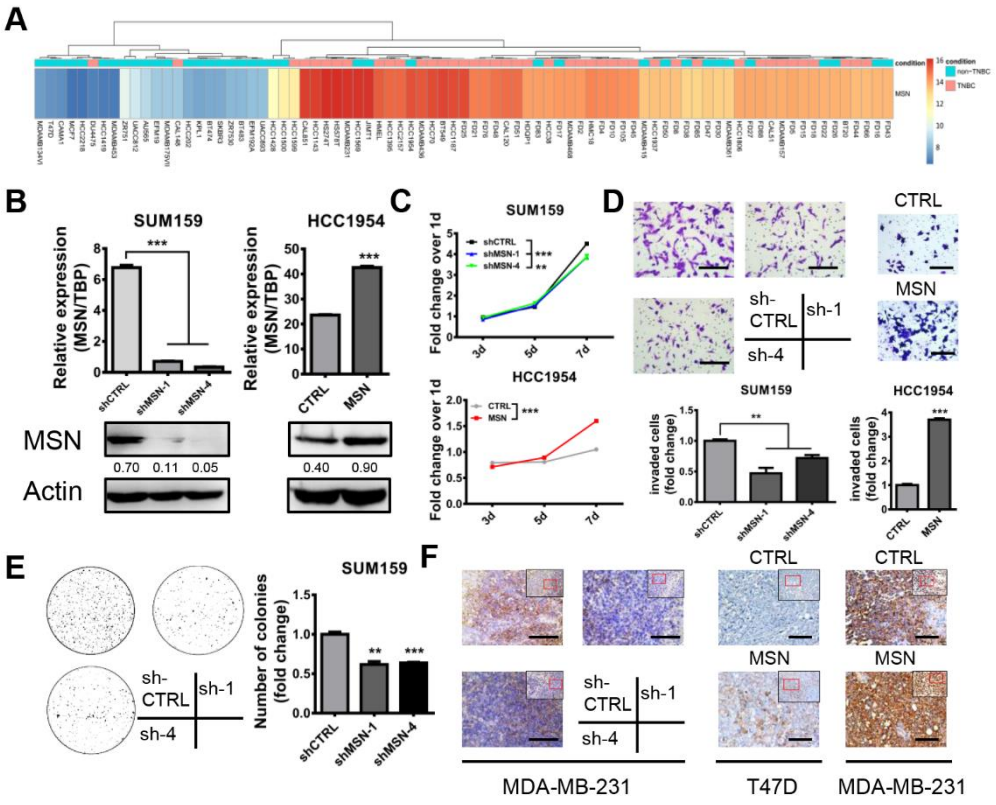


Fig. S1. MSN regulates breast cancer progression. (**A**) We performed RNA-seq on 43 TNBC and 38 non-TNBC cell lines or clinical samples. The heat-map shows the expression of MSN. The relative expression values of MSN used in heat-map are processed as follows: Relative expression value of MSN = $\log_2$ (actual measured value + 1). (**B**) Verification of the MSN knock-down effect in SUM159 and overexpression effect in HCC1954 by qRT-PCR and western blot. (**C**) MTT assay conducted with SUM159 and HCC1954 cells (n=6). (**D**) Invasion assay conducted with SUM159 and HCC1954 cells (n=3). The image (top) and quantitative analysis of the total invasive cells (bottom) are shown. Scale bars: 200μm. (**E**) Soft agar colony formation assay was conducted with SUM159 cells (n=3). The image (left) and quantitative analysis of the total invasive cells (right) are shown. (**F**) The tumor of xenografts in *in vivo* experiments of tumorigenicity in mice was sliced and immunohistochemical staining (IHC) of anti-MSN was performed. Scale bars in the left: 200 μm; Scale bars in the middle and right: 80 μm. **, $P < 0.01$. ***, $P < 0.001$ by unpaired t test of triplicates or test of 2-way ANOVA. Error bars, mean ± SEM.

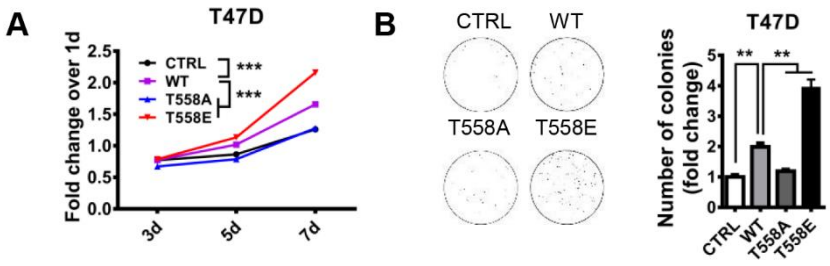


Fig. S2. Effects of MSN in regulating breast cancer are dependent on its T558 phosphorylation. (**A**) Cell proliferation ability was measured by MTT assay (n=6). (**B**) Anchorage-independent growth ability was measured by the soft agar colony formation assay (n=3). The image (left) and quantitative analysis of colonies (right) are shown. **, $P < 0.01$. ***, $P < 0.001$ by unpaired t test of triplicates or test of 2-way ANOVA. Error bars, mean ± SEM.

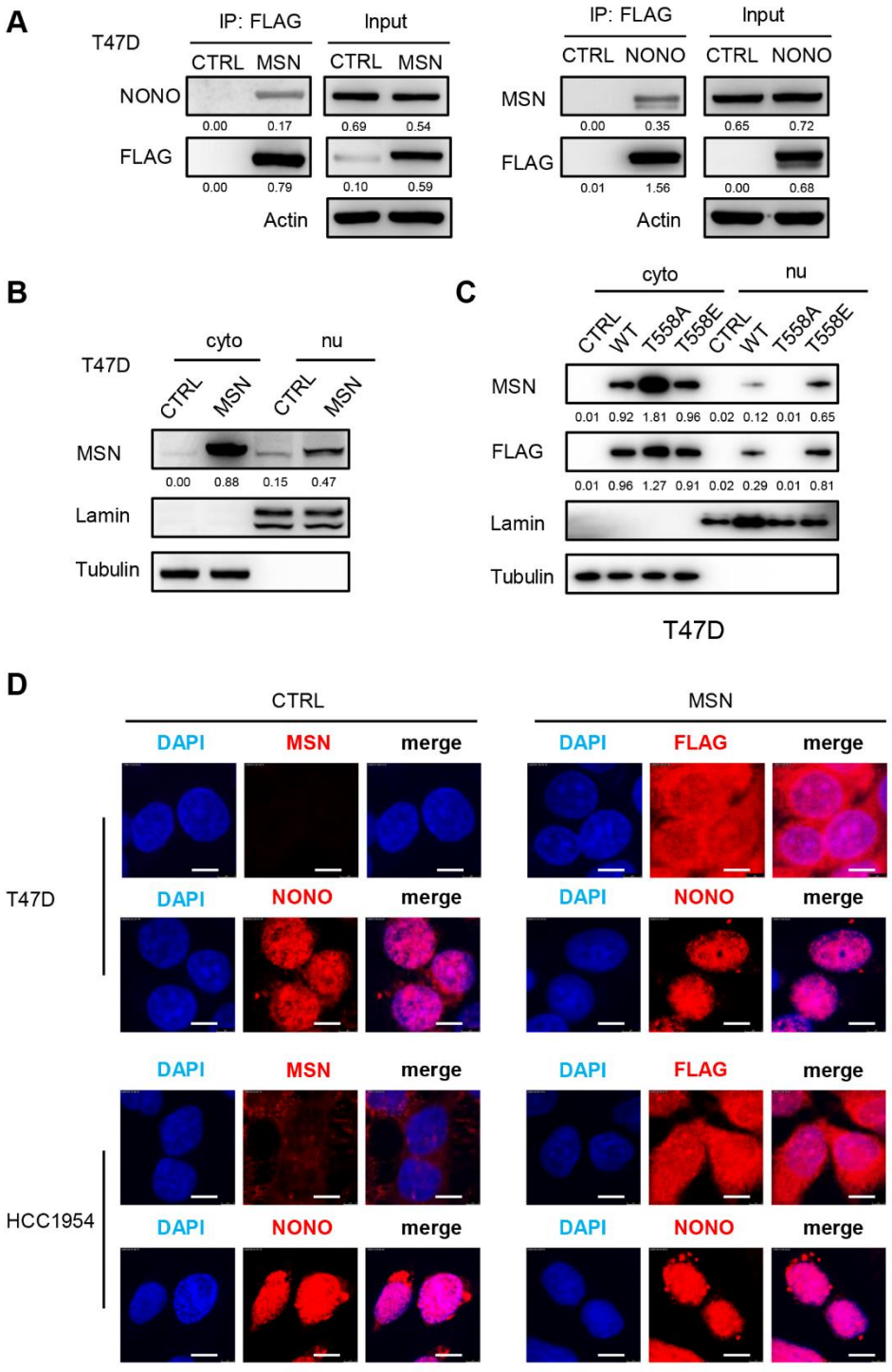


Fig. S3 continued.

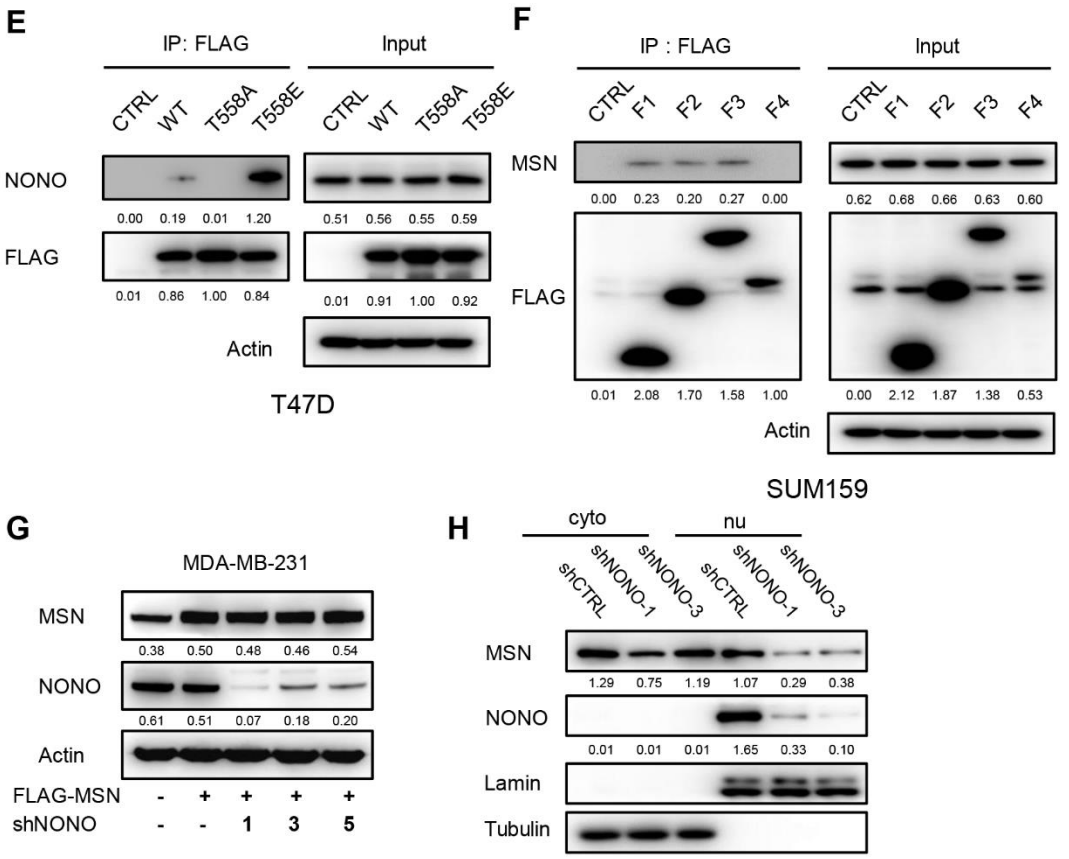


Fig. S3. MSN can interact with NONO and enter the nucleus with the assistance of NONO. (**A**) CTRL or FLAG-MSN-overexpressing T47D cells were immunoprecipitated by anti-FLAG M2 Magnetic Beads and then immunoblotted (left). CTRL or FLAG-NONO-overexpressing MDA-MB-231 cells were immunoprecipitated by anti-FLAG M2 Magnetic Beads and then immunoblotted (right). (**B**) Cytoplasmic (cyto) and nuclear (nu) proteins were separated according to instruction. Western blot was conducted to determine the distribution of MSN in CTRL or FLAG-MSN-overexpressing T47D cells. Tubulin, internal reference for cytoplasmic proteins and lamin for nuclear proteins. (**C**) Western blot was carried out to determine the distribution of wildtype MSN and its mutants in T47D cells. (**D**) Immunofluorescence assay was carried out, in which endogenous MSN was determined with anti-MSN antibody in CTRL cells, exogenous MSN was determined with anti-FLAG antibody in FLAG-MSN overexpressing cells, and endogenous NONO was determined with anti-NONO antibody. Images were captured by confocal laser microscopy. Scale bars: 10 μm. (**E**) CTRL, MSN WT or mutants overexpressing T47D cell lysates were immunoprecipitated with anti-FLAG M2 Magnetic Beads and immunoblotted. (**F**) CTRL or FLAG-tagged different NONO fragments overexpressing SUM159 cells were immunoprecipitated with anti-FLAG M2 Magnetic Beads and immunoblotted. (**G**) Detection of knockdown effect of NONO in MSN-overexpressing MDA-MB-231 cells by western blot. (**H**) Cytoplasmic and nuclear proteins were separated and immunoblotted after NONO knockdown in MDA-MB-231.

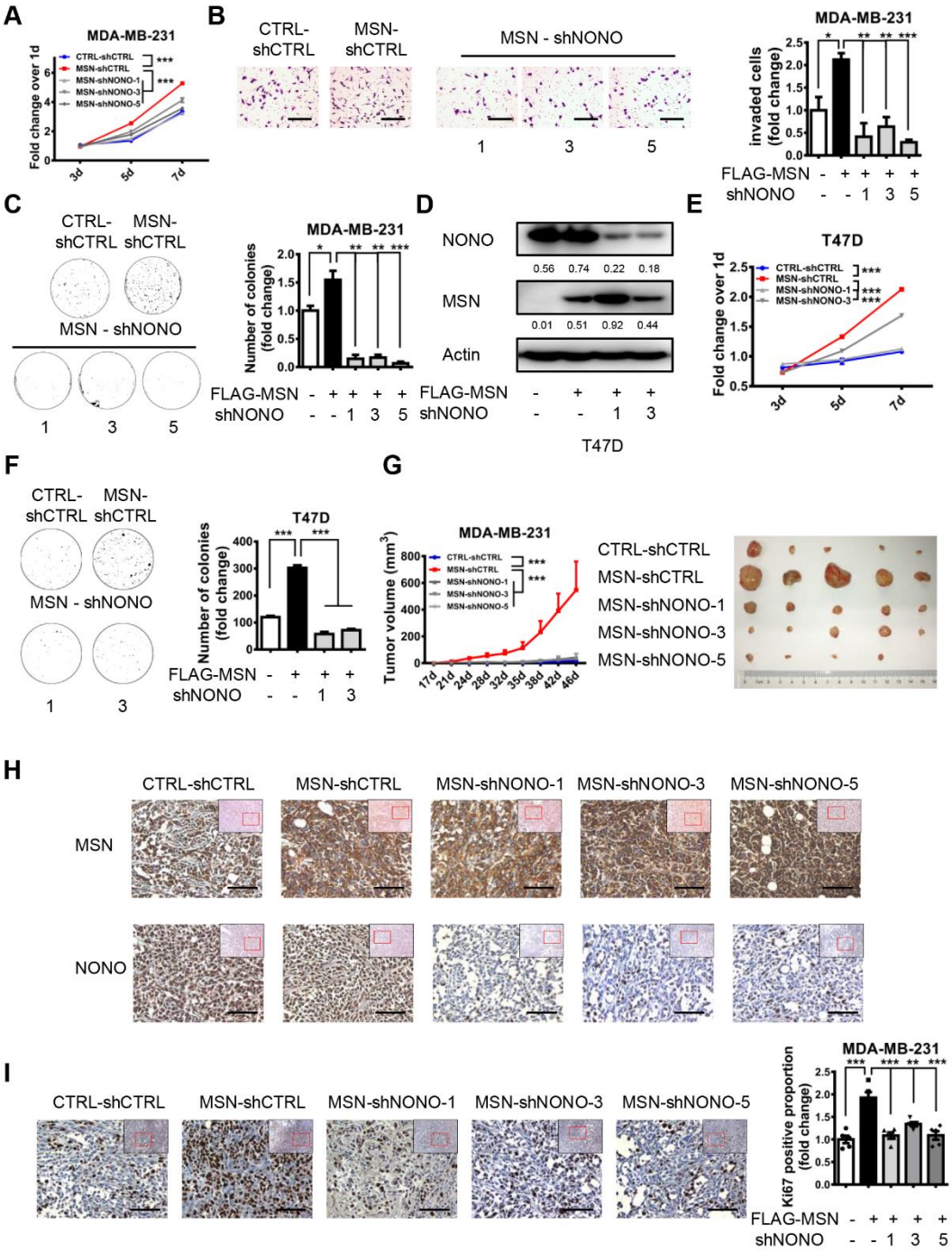

A
MDA-MB-231
Fold change over 1d
CTRL-shCTRL
MSN-shCTRL
MSN-shNONO-1
MSN-shNONO-3
MSN-shNONO-5
3d
5d
7d
B
CTRL-shCTRL
MSN-shCTRL
MSN - shNONO
1
3
5
MDA-MB-231
invaded cells (fold change)
FLAG-MSN - + + + +
shNONO - - 1 3 5
C
CTRL-shCTRL
MSN-shCTRL
MSN - shNONO
1
3
5
MDA-MB-231
Number of colonies (fold change)
FLAG-MSN - + + + +
shNONO - - 1 3 5
D
NONO
0.56 0.74 0.22 0.18
MSN
0.01 0.51 0.92 0.44
Actin
FLAG-MSN - + + +
shNONO - - 1 3
T47D
E
T47D
Fold change over 1d
CTRL-shCTRL
MSN-shCTRL
MSN-shNONO-1
MSN-shNONO-3
3d
5d
7d
F
CTRL-shCTRL
MSN-shCTRL
MSN - shNONO
1
3
T47D
Number of colonies (fold change)
FLAG-MSN - + + +
shNONO - - 1 3
G
MDA-MB-231
Tumor volume (mm3)
CTRL-shCTRL
MSN-shCTRL
MSN-shNONO-1
MSN-shNONO-3
MSN-shNONO-5
17d
21d
24d
28d
32d
35d
38d
42d
46d
H
CTRL-shCTRL
MSN-shCTRL
MSN-shNONO-1
MSN-shNONO-3
MSN-shNONO-5
MSN
NONO
I
CTRL-shCTRL
MSN-shCTRL
MSN-shNONO-1
MSN-shNONO-3
MSN-shNONO-5
MDA-MB-231
Ki67 positive proportion (fold change)
FLAG-MSN - + + + +
shNONO - - 1 3 5

Fig. S4. The interaction between MSN and NONO is critical for MSN function on breast tumor progression. (**A**) Cell proliferation activity was measured by MTT assay (n=6). (**B**) Cell invasion ability was measured by the invasion assay. The image (left) and quantitative analysis of the total invasive cells (right) are shown (n=3) . Scale bars: 200 μm. (**C**) Anchorage-independent growth ability was measured by the soft agar colony formation assay (n=3). The image (left) and quantitative analysis of colonies (right) are shown. (**D**) Detection of NONO knockdown effect in MSN-overexpressing T47D cells by western blot. (**E**) Cell proliferation activity of T47D cells was measured by MTT assay (n=6). (**F**) Anchorage-independent growth ability of T47D cells was measured by the soft agar colony formation assay (n=3). The image (left) and quantitative analysis of colonies (right) are shown. (**G**) MDA-MB-231 cells of 0.5 million were implanted into the 4th mammary fat pads at two flanks of nude mice (n=5). The figure shows the representative results of two independent repetitive experiments. The tumor volume was measured once every three to four days. At the end of experiments, the tumors were taken out and the images are shown on the right. (**H**) The tumors from xenografts in mice were sliced and IHC was performed on MSN or NONO. Scale bars: 80 μm. (**I**) Ki67 staining of tumor tissue was conducted by IHC and positive proportion was shown on the right (n=5). Scale bars: 80 μm. *, $P < 0.05$. **, $P < 0.01$. ***, $P < 0.001$ by unpaired t test of triplicates or test of 2-way ANOVA. Error bars, mean ± SEM.

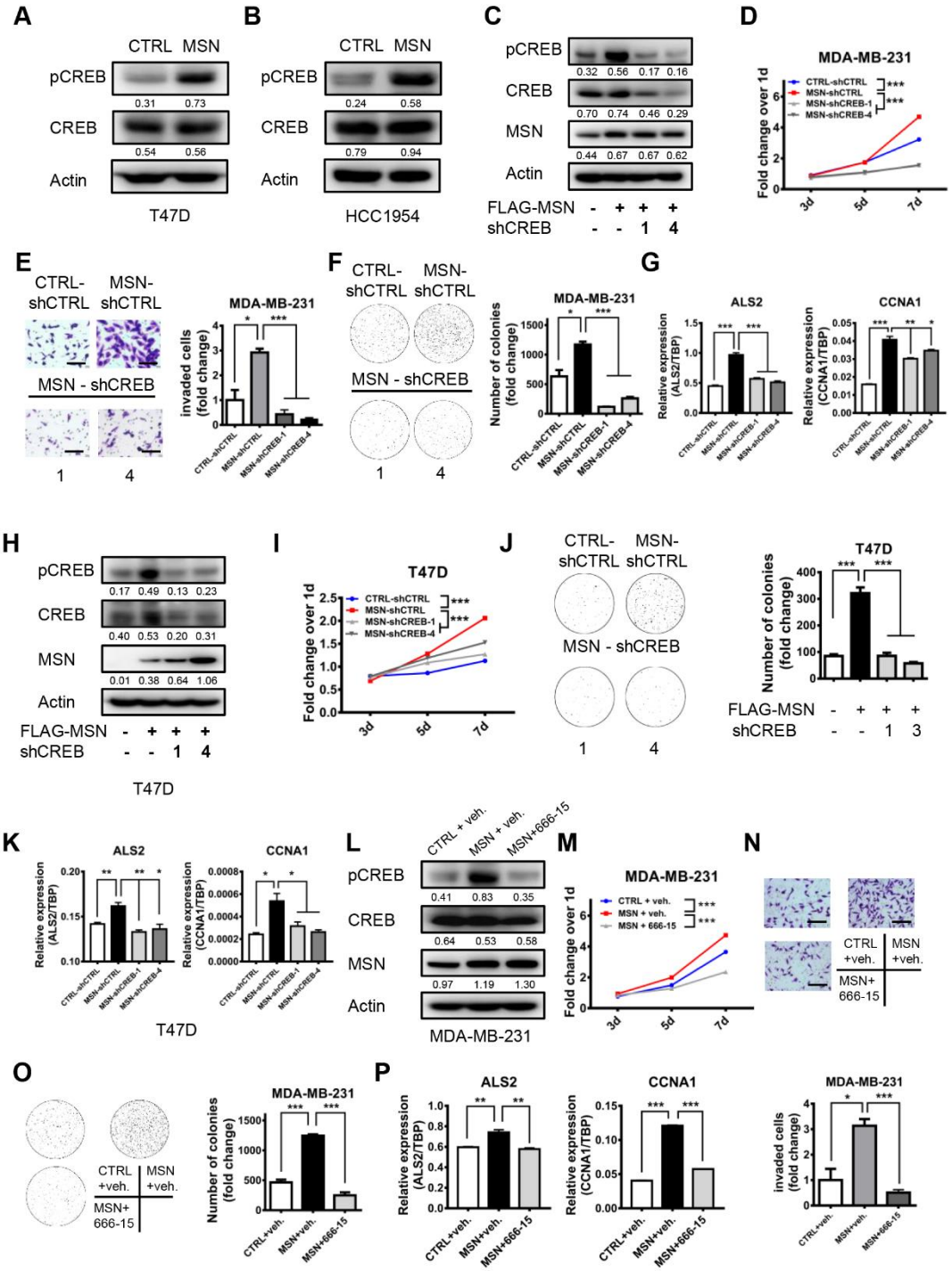

A
CTRL MSN
pCREB
0.31 0.73
CREB
0.54 0.56
Actin
T47D
B
CTRL MSN
pCREB
0.24 0.58
CREB
0.79 0.94
Actin
HCC1954
C
pCREB
0.32 0.56 0.17 0.16
CREB
0.70 0.74 0.46 0.29
MSN
0.44 0.67 0.67 0.62
Actin
FLAG-MSN - + + +
shCREB - - 1 4
D
MDA-MB-231
CTRL-shCTRL
MSN-shCTRL
MSN-shCREB-1
MSN-shCREB-4
Fold change over 1d
3d 5d 7d
E
CTRL-shCTRL MSN-shCTRL
MSN - shCREB
1 4
MDA-MB-231
invaded cells (fold change)
F
CTRL-shCTRL MSN-shCTRL
MSN - shCREB
1 4
G
MDA-MB-231
Number of colonies (fold change)
ALS2
Relative expression (ALS2/TBP)
CCNA1
Relative expression (CCNA1/TBP)
H
pCREB
0.17 0.49 0.13 0.23
CREB
0.40 0.53 0.20 0.31
MSN
0.01 0.38 0.64 1.06
Actin
FLAG-MSN - + + +
shCREB - - 1 4
T47D
I
T47D
Fold change over 1d
J
CTRL-shCTRL MSN-shCTRL
MSN - shCREB
1 4
T47D
Number of colonies (fold change)
FLAG-MSN - + + +
shCREB - - 1 3
K
ALS2
Relative expression (ALS2/TBP)
CCNA1
Relative expression (CCNA1/TBP)
T47D
L
CTRL + veh.
MSN + veh.
MSN+666-15
pCREB
0.41 0.83 0.35
CREB
0.64 0.53 0.58
MSN
0.97 1.19 1.30
Actin
MDA-MB-231
M
MDA-MB-231
CTRL + veh.
MSN + veh.
MSN + 666-15
Fold change over 1d
3d 5d 7d
N
CTRL +veh.
MSN +veh.
MSN+ 666-15
O
CTRL +veh.
MSN +veh.
MSN+ 666-15
MDA-MB-231
Number of colonies (fold change)
P
ALS2
Relative expression (ALS2/TBP)
CCNA1
Relative expression (CCNA1/TBP)
MDA-MB-231
invaded cells (fold change)

Fig. S5. The function of MSN was mediated by phosphorylation of CREB. (**A**) Western blot was performed to detect the phosphorylation level of CREB after overexpressing MSN in T47D cells. (**B**) Western blot was performed to detect the phosphorylation level of CREB after overexpressing MSN in HCC1954 cells. (**C**) Verification of MSN overexpressing and CREB knocking down cell lines of MDA-MB-231 by western blot. (**D**) Cell proliferation activity was measured by MTT assay (n=6). (**E**) Cell invasion activity of each group was measured by invasion assay (n=3). The image (left) and quantitative analysis of the total invasive cells (right) are shown. Scale bars: 200 μm. (**F**) Anchorage-independent growth ability of each group was measured by soft agar colony formation assay (n=3). The image (left) and quantitative analysis of the total colonies (right) are shown. (**G**) qRT-PCR was conducted to detected the expression of CREB downstream genes ALS2 and CCNA1. (**H**) Verification of MSN overexpressing and CREB knock-down cell lines of T47D by western blot. (**I**) Cell proliferation activity of T47D cells was measured by MTT assay (n=6). (**J**) Anchorage-independent growth ability of each group was measured by soft agar colony formation assay (n=3). The image (left) and quantitative analysis of the total colonies (right) are shown. (**K**) qRT-PCR was conducted to detect the expression of CREB downstream genes ALS2 and CCNA1 after knocking-down CREB in T47D MSN-overexpression cell line. (**L**) CTRL or MSN-overexpressing MDA-MB-231 cells were treated with DMSO (veh.) or specific CREB inhibitor (666-15, 73 nM) for 12h and then immunoblotted. (**M**) Cell proliferation activity of CTRL or MSN-overexpressing MDA-MB-231 cells in the presence or absence of CREB inhibitor (666-15, 73 nM) was measured by MTT assay (n=6). (**N**) Cell invasion activity of CTRL or MSN-overexpressing MDA-MB-231 cells in the presence or absence of CREB inhibitor (666-15, 73 nM) was measured by invasion assay (n=3). The image (top) and quantitative analysis of the total invasive cells (bottom) are shown. Scale bars: 200 μm. (**O**) Anchorage-independent growth ability of CTRL or MSN-overexpressing MDA-MB-231 cells in the presence or absence of CREB inhibitor (666-15, 73 nM) was measured by soft agar colony formation assay (n=3). The image (left) and quantitative analysis of the total colonies (right) are shown. (**P**) CTRL or MSN-overexpressing MDA-MB-231 cells were treated with DMSO (veh.) or specific CREB inhibitor (666-15, 73 nM) for 12 h and then qRT-PCR was performed to detect CREB downstream genes ALS2 and CCNA1 expression. *, $P<0.05$. **, $P < 0.01$. ***, $P < 0.001$ by unpaired t test of triplicates or test of 2-way ANOVA. Error bars, mean ± SEM.

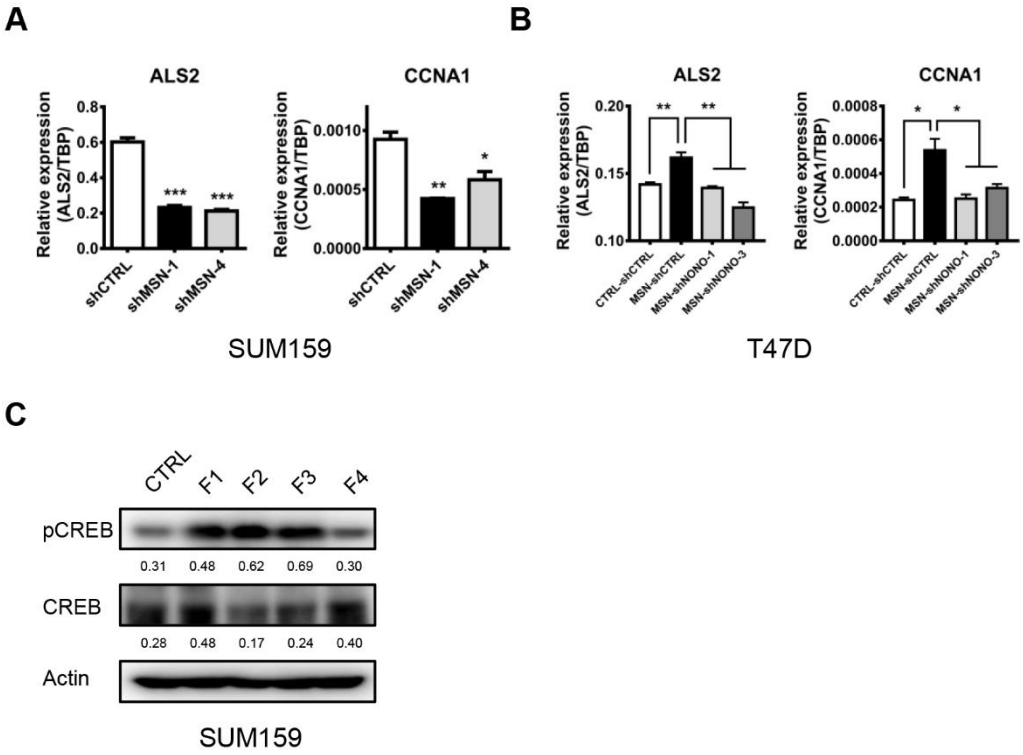


Fig. S6. MSN-NONO interaction enhanced CREB signaling pathway. (**A**) The expression of CREB downstream genes ALS2 and CCNA1 was measured by qRT-PCR in MSN knocking-down SUM159 cells. (**B**) The expression of CREB downstream genes ALS2 and CCNA1 was measured in MSN-overexpressing and NONO-knockdown T47D cells by qRT-PCR. (**C**) Western blot was used to detect CREB phosphorylation level in SUM159 cell line overexpressing NONO in different truncated forms. *, P<0.05. **, P < 0.01. ***, P < 0.001 by unpaired t test of triplicates. Error bars, mean ± SEM.

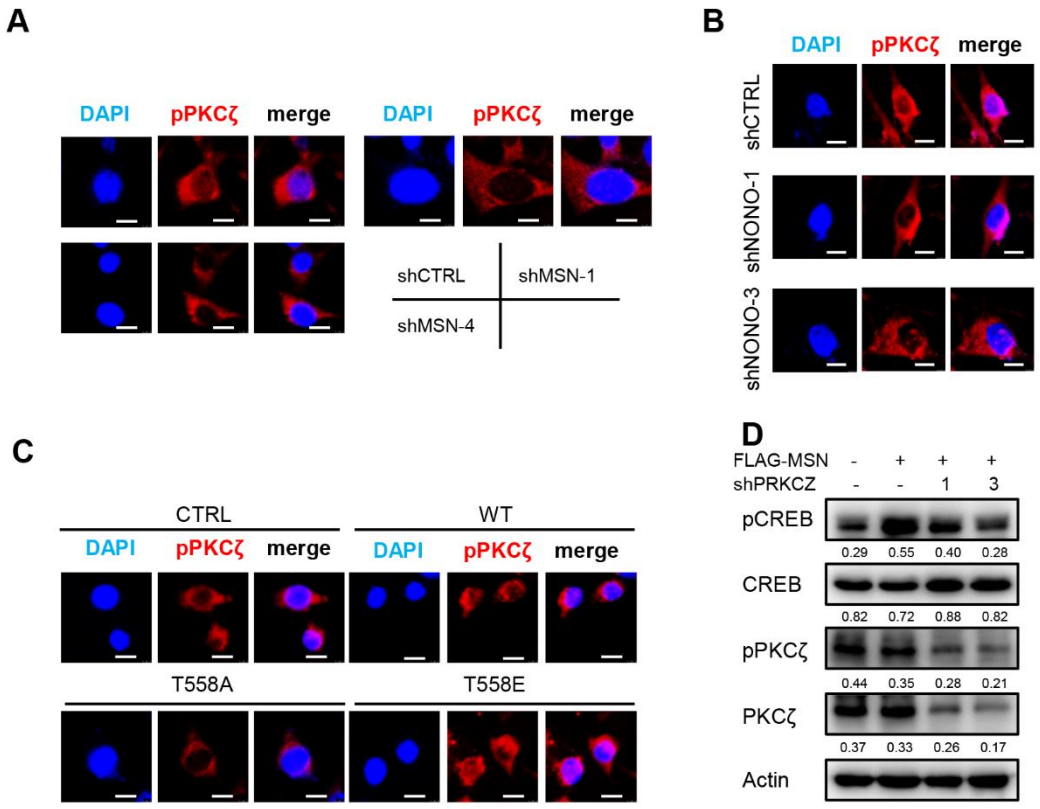


Fig. S7. MSN-NONO interaction promoted CREB phosphorylation by facilitating the nuclear localization of pPKCζ. (**A**) The pPKCζ protein was stained by immunofluorescence after MSN knockdown in MDA-MB-231 cells and images were captured by confocal microscopy. Scale bars: 10 μm. (**B**) The pPKCζ protein was stained by immunofluorescence after NONO knockdown in MDA-MB-231 cells and images were captured by confocal microscopy. Scale bars: 10 μm. (**C**) CTRL or different MSN mutants-overexpressing cells of MDA-MB-231 were performed with immunofluorescence and images were captured by confocal microscopy. Scale bars: 10 μm. (**D**) Phosphorylation level and total proteins of CREB were determined in MSN-overexpressing and PRKCZ-knocking down MDA-MB-231 cells by western blot.

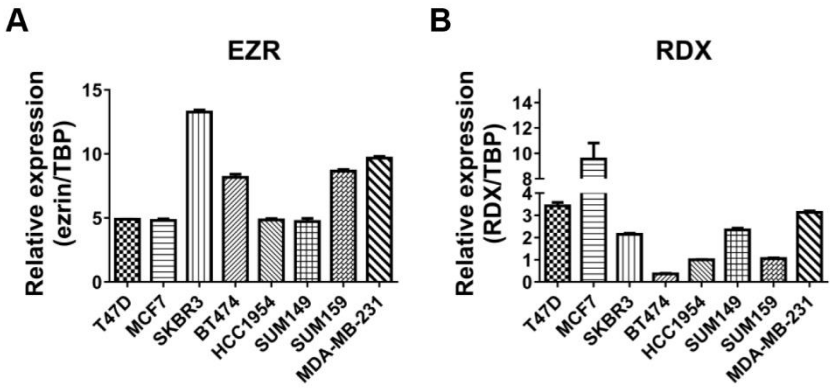


Fig. S8. There was no significant difference in the expression of EZR and RDX in different subtypes of breast cancer cell lines. (**A**) We performed qRT-PCR to measure the expression of ERM family member EZR. (**B**) qRT-PCR was used to measure the expression of ERM family member RDX.

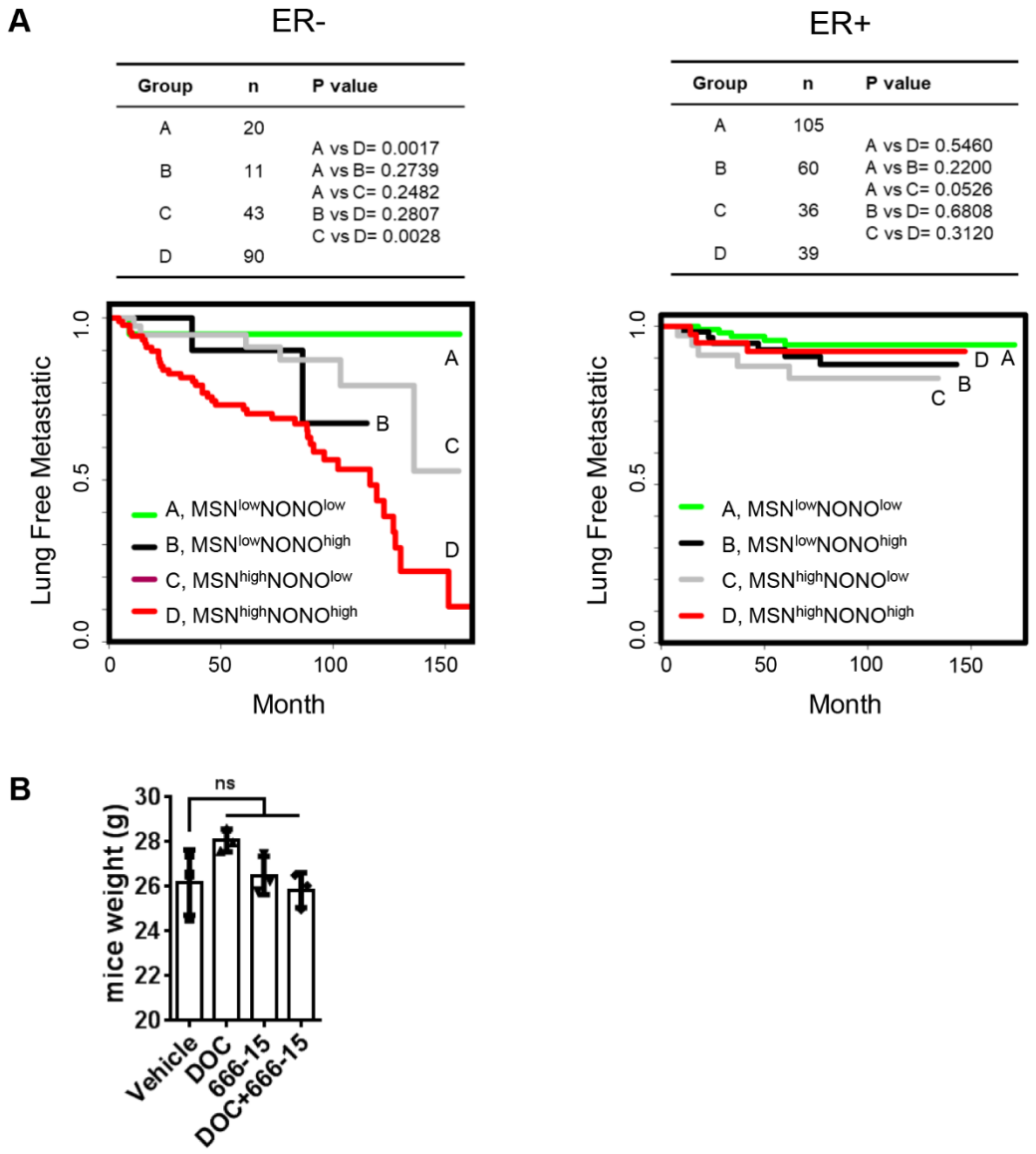
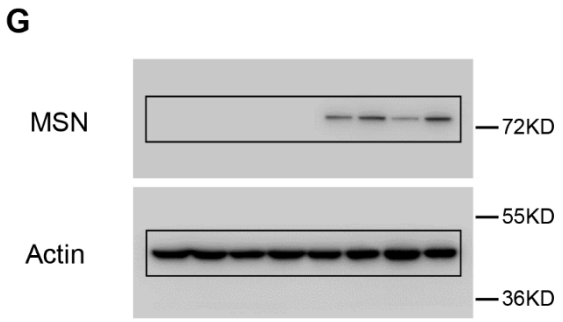
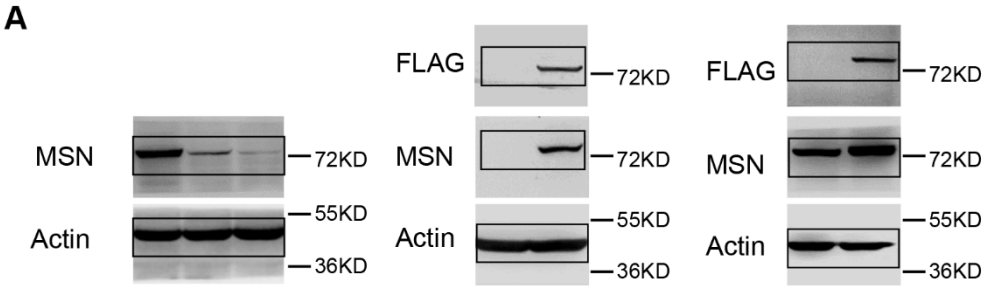


Fig. S9. MSN-NONO complex and downstream CREB signaling pathway could be targeted for TNBC. (**A**) The lung free metastasis survival analysis based different combinations of MSN expression and NONO expression from integrated data sets including GSE5327, GSE2603, GSE2034, which were divided into two groups according to the expression of ER. (**B**) We measured the body weight of mice at the end of the experiment treated with DOC and 666-15 in TNBC PDX model.



Uncropped images for Fig. 1, G.



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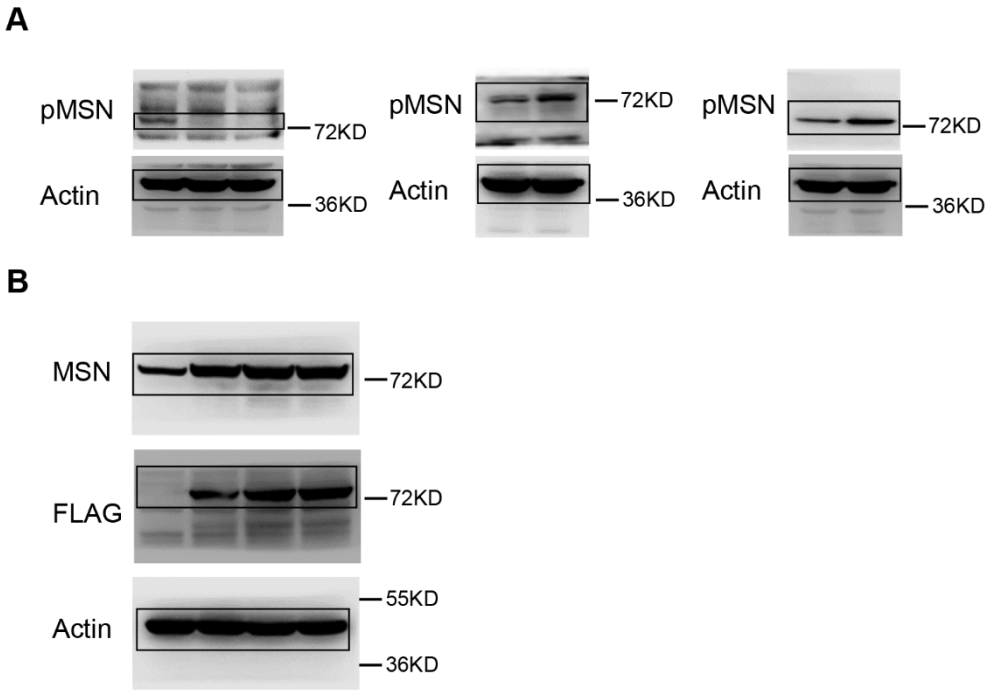

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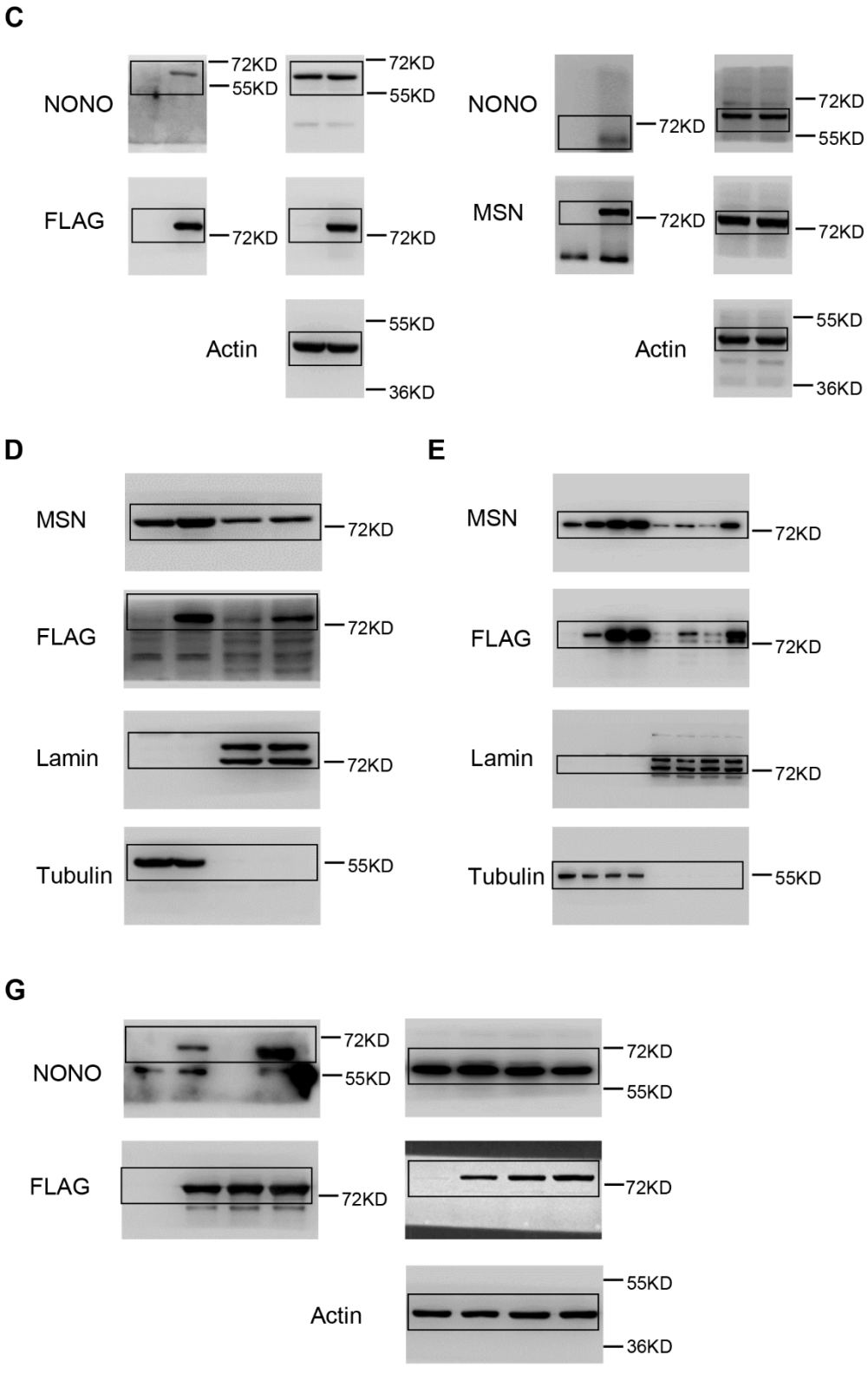
Fig. S10 continued.



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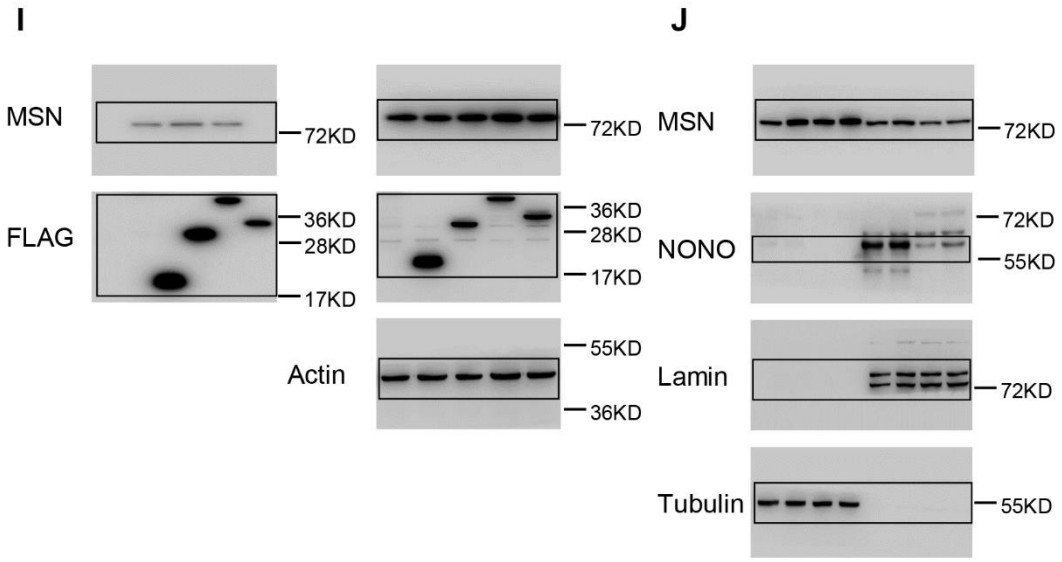

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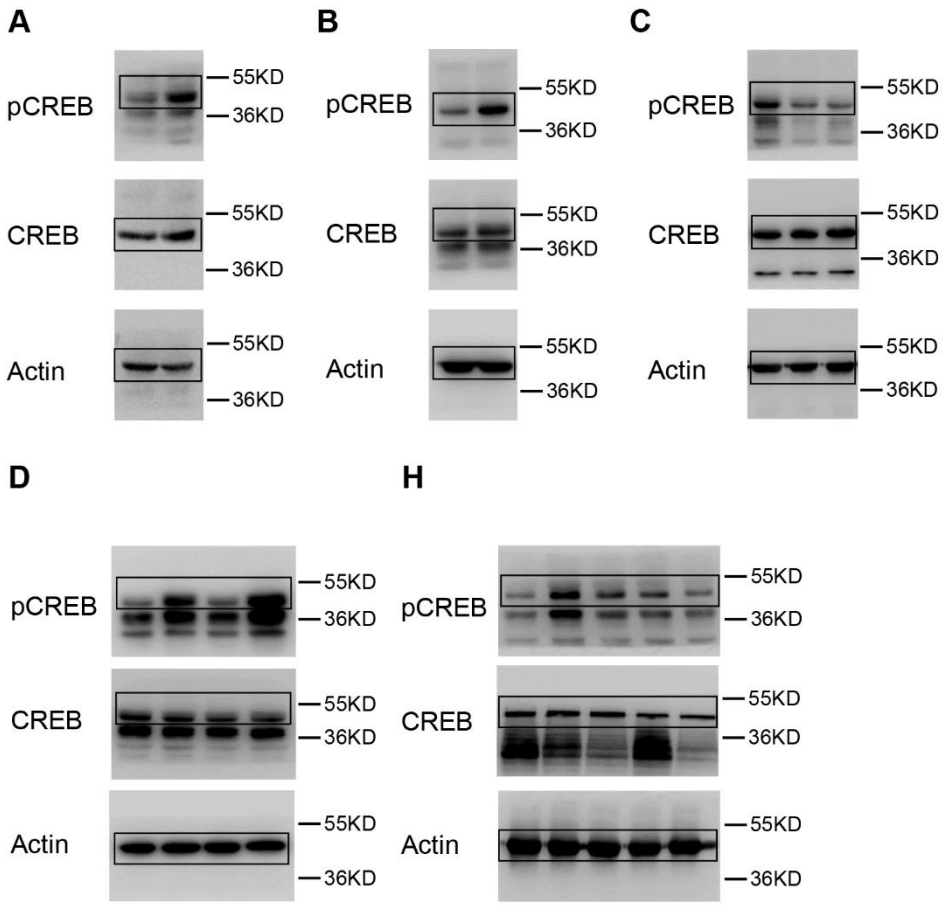

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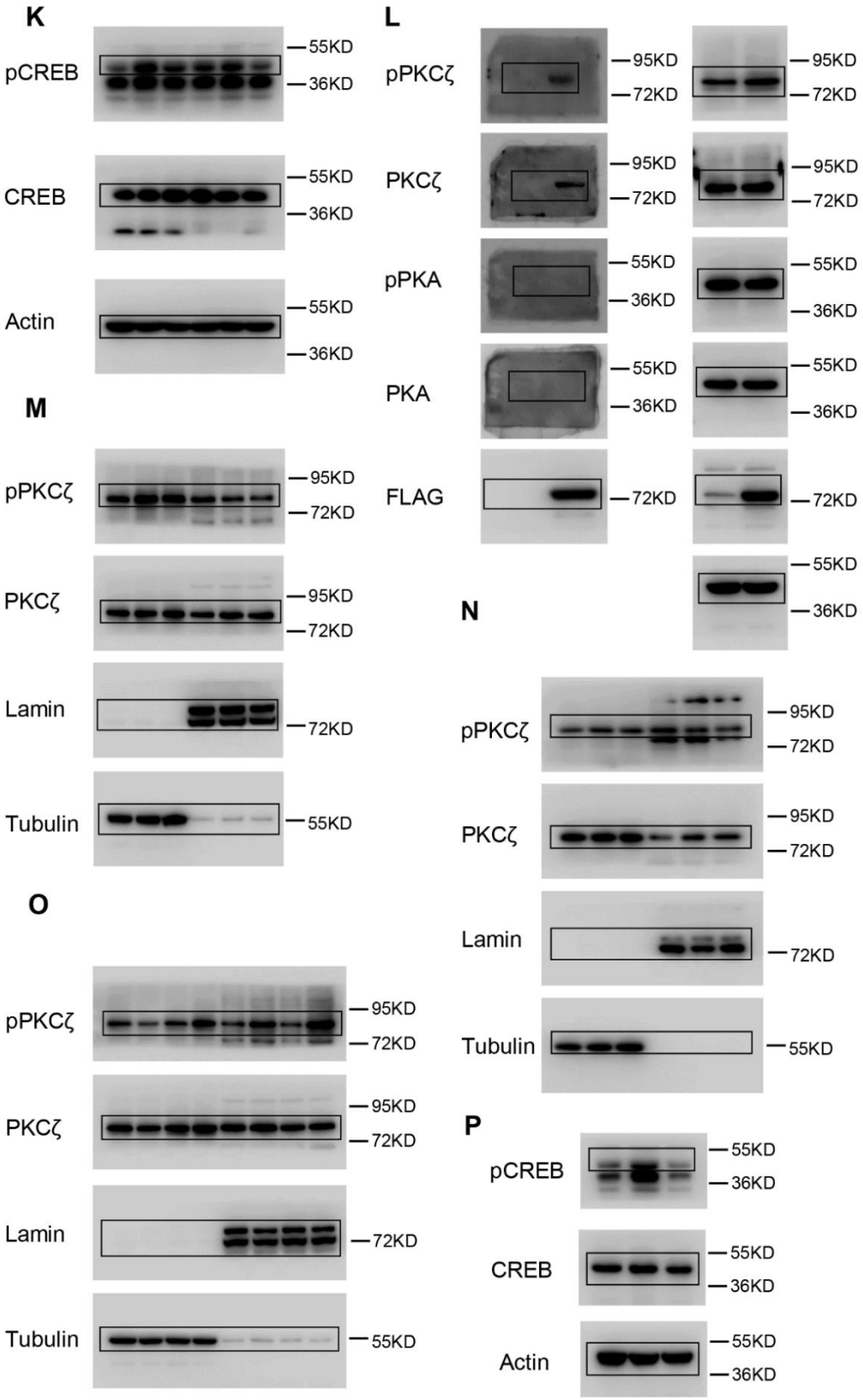

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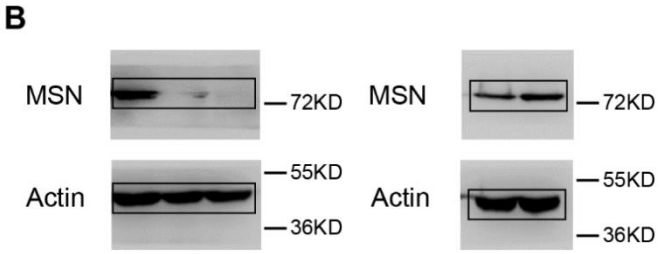

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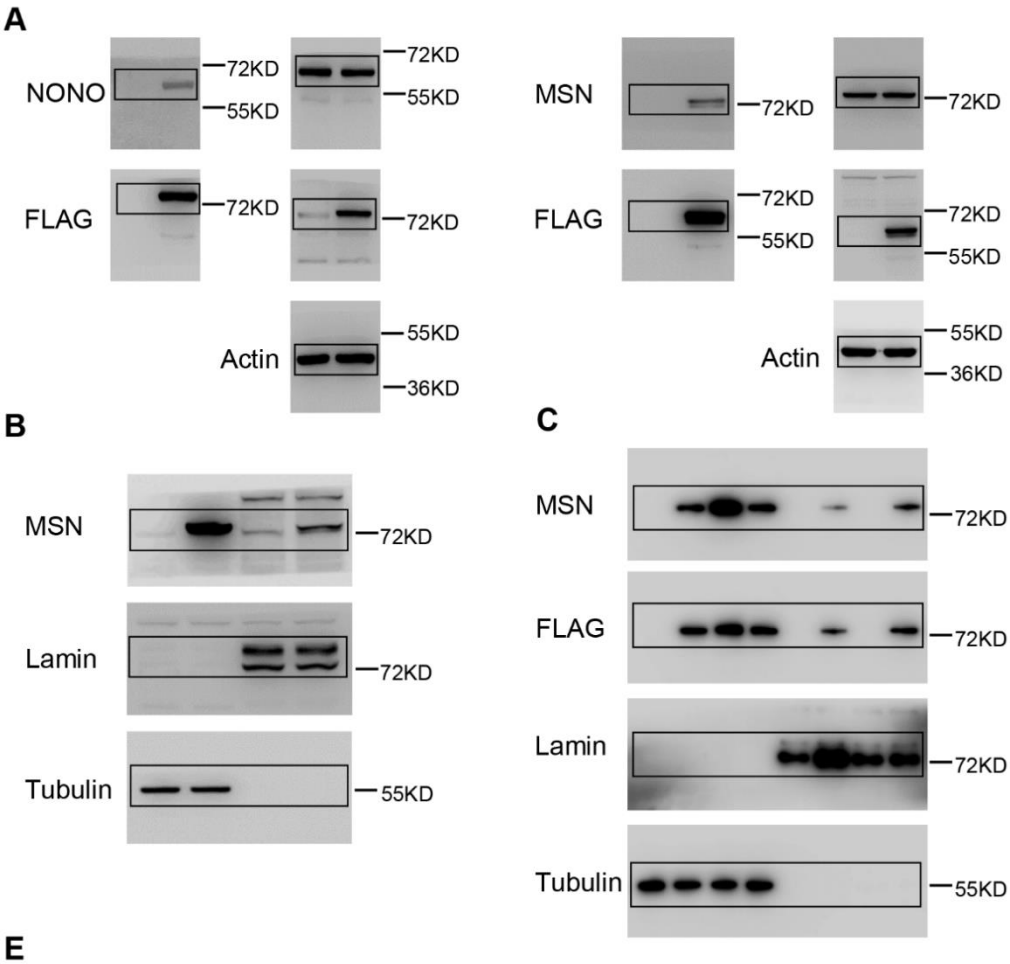

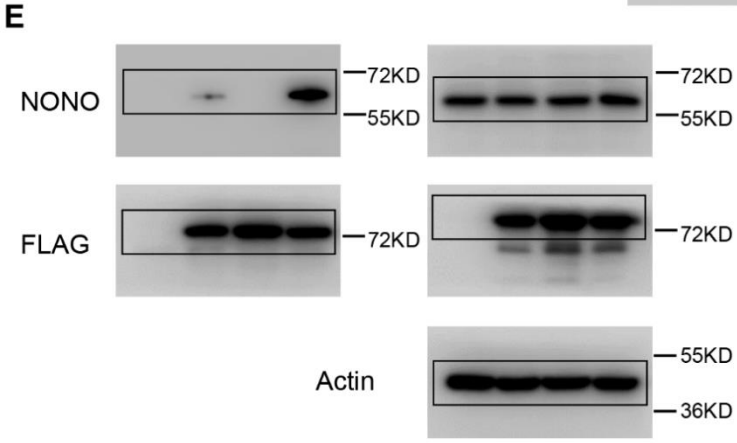

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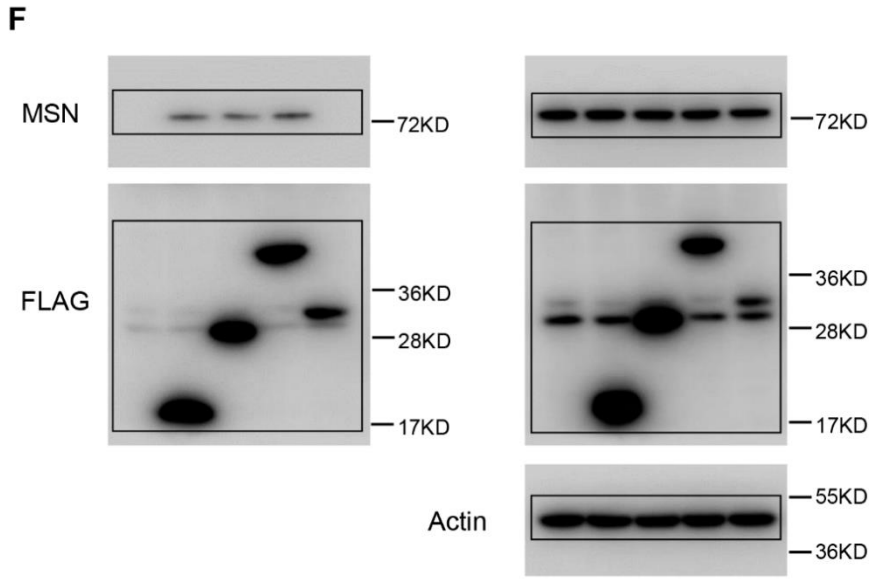

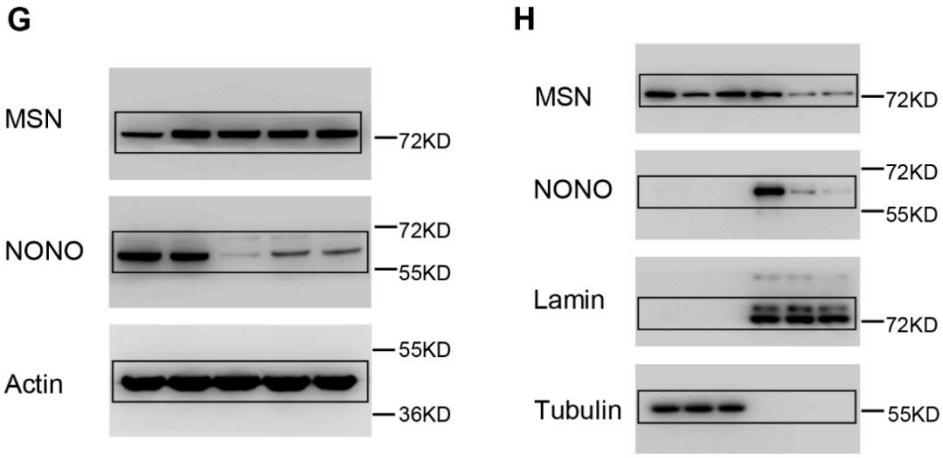

Uncropped images for Fig. S3, A to C and E to H.

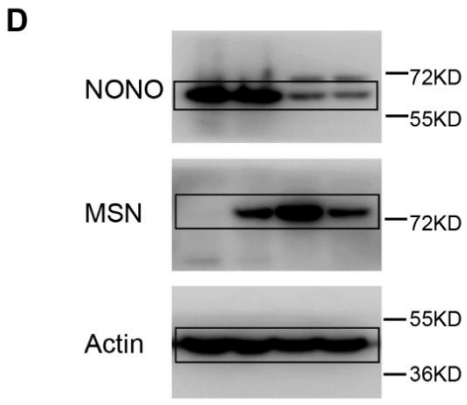

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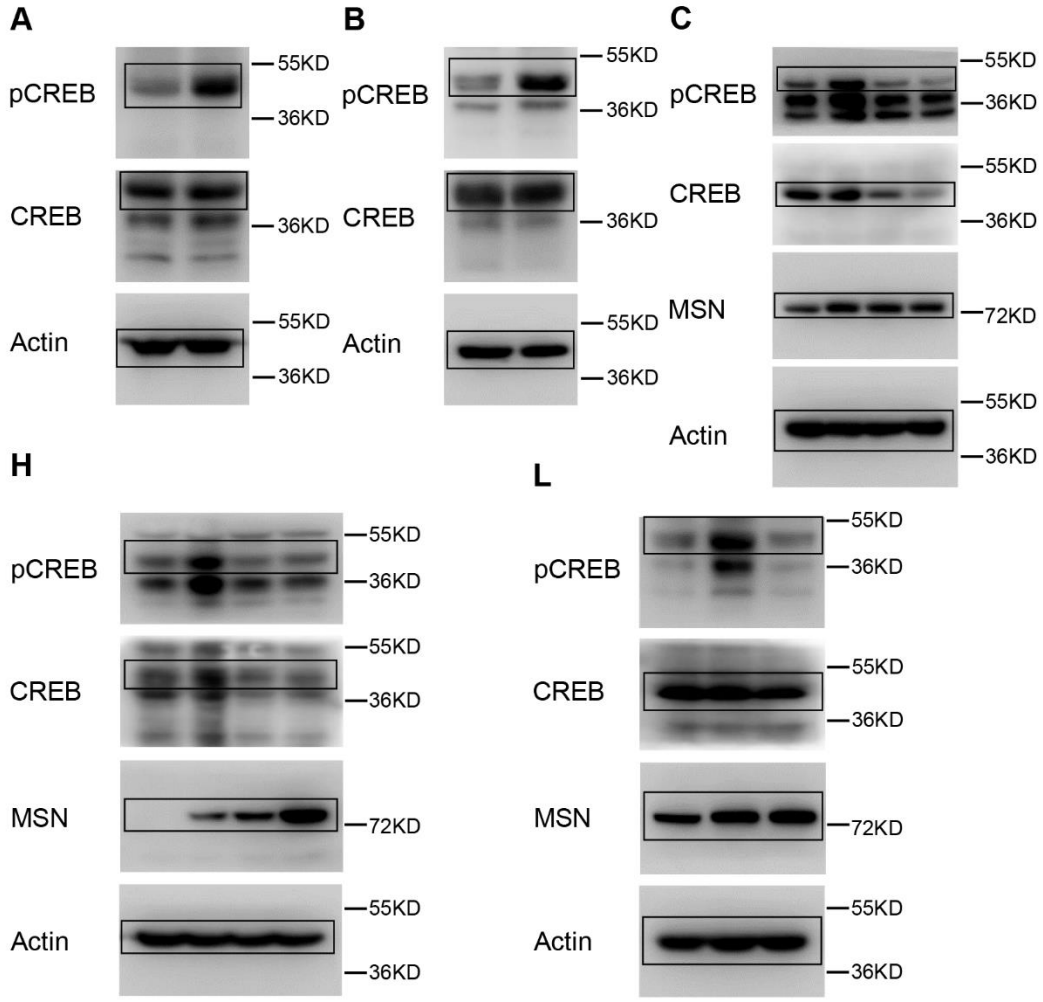
Fig. S10 continued.



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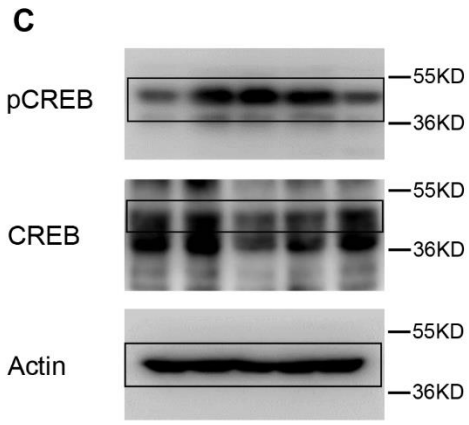

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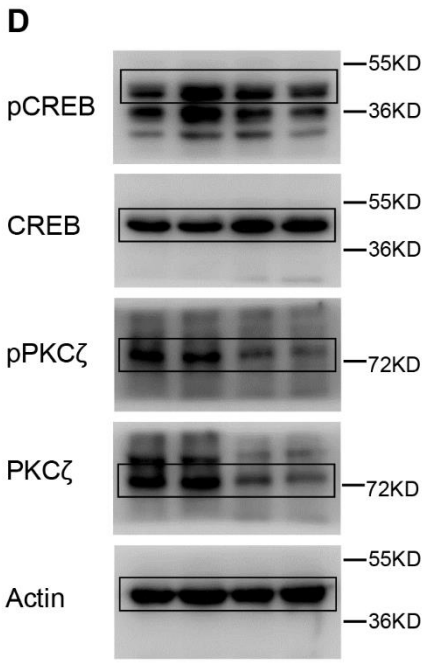

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Fig.S10. Uncropped images from Western blots.