SUPPLEMENTAL DATA:

Supplemental Tables:

Supplemental Table I. Antibodies, source, and applications.

Target	Source	Catalog Number	Application	Working Concentration
Phospho-NF-κB (Ser536, p65 subunit)	Cell Signaling Technology	3033	WB	1:1000
NF-κB (p65 subunit)	Cell Signaling Technology	4764	WB	1:1000
NF-κB (p65 subunit)	Cell Signaling Technology	6956	IF-C	1:800
Nck1	Cell Signaling Technology	2319	WB	1:1000
Nckb	abcam	ab109239	WB	0.45 μg/ml
Nck1/2	Millipore	06-288	WB	0.1 μg/ml
VCAM-1	abcam	ab134047	WB/IHC-IF	0.44 μg/ml
GAPDH	Cell Signaling Technology	2118	WB	1: 5000
von Willibrand Factor	abcam	ab11713	IHC-IF	0.3 μg/ml
ICAM-1 (YN1/1.7.4)	abcam	ab119871	IHC-IF	0.25 μg/ml
ICAM-1	Santa Cruz	sc-1511	WB	0.5 μg/ml
Mac2	Accurate Chemical	CL8942AP	IHC-IF	0.1 μg/mL
Smooth Muscle Actin	Sigma Aldrich	C6198	IHC-IF	0.25 μg/ml
Phospho-eNOS (Ser1177)	Cell Signaling Technology	9571	WB	1:500
Anti-eNOS/NOS Type III	BD Transduction Lab	610297	WB	0.25μg/ml
Phospho-Akt (Ser473)	Cell Signaling Technology	4060	WB	1:1000
AKT1/2 (N-19)	Santa Cruz	sc-1619	WB	0.2 μg/ml
ERK1	Santa Cruz	Sc-94	WB	0.1 μg/mL
Phospho-p44/42 MAPK (ERK1/2)	Cell Signaling Technology	4377	WB	1:1000
IRAK-1 (D51G7)	Cell Signaling Technology	4504	WB/IP	1:1000 WB 1:100 IP
Anti-IRAK-1 (phospho T209)	abcam	Ab218130	WB/IHC-IF	1:1000 WB 1:100 IHC-IF
Human Nck1	R&D Systems	MAB7008	IHC-IF	1:100

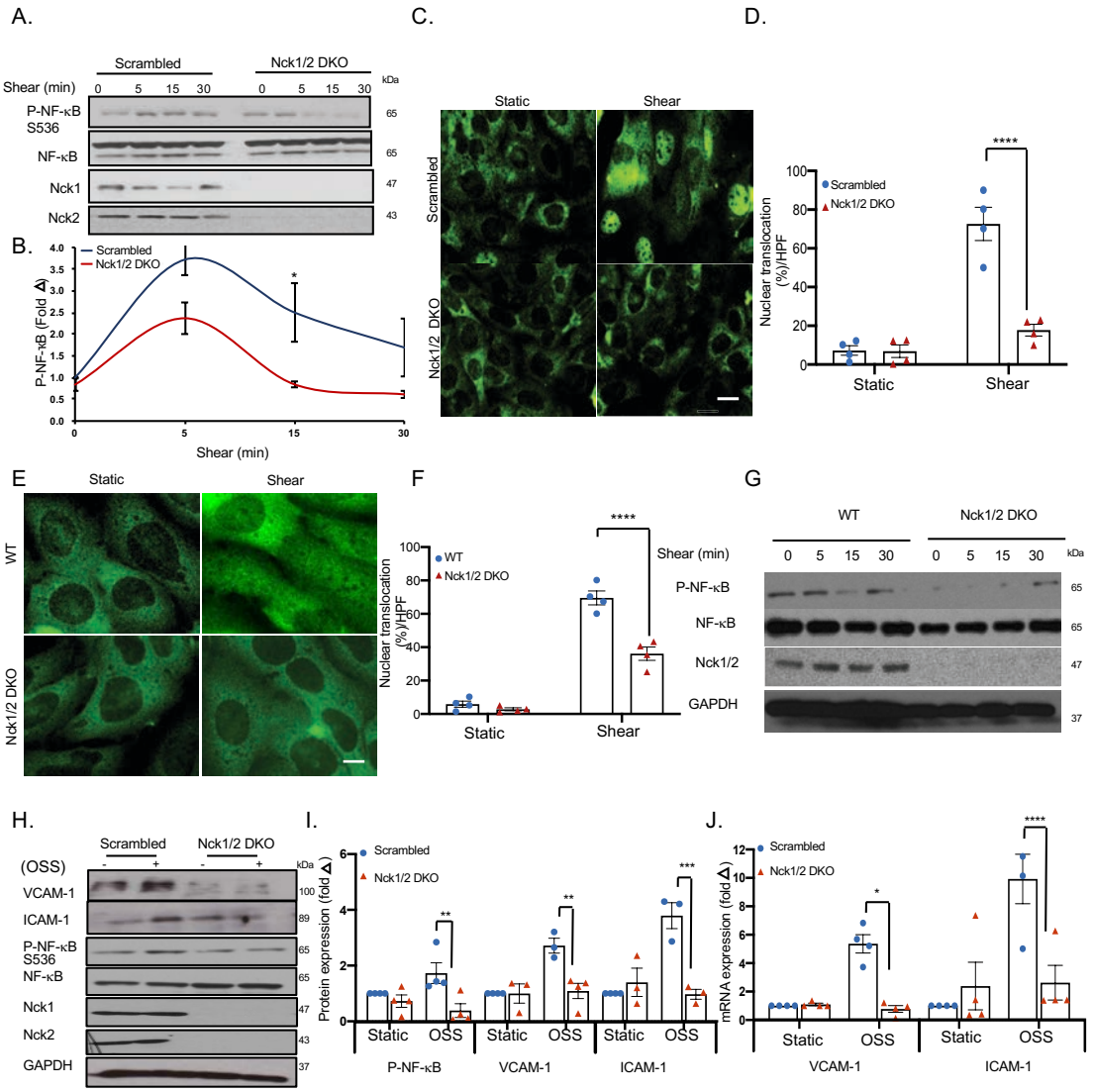
Supplemental Table II. Primers used for qRT-PCR.

Gene	Species	Forward	Reverse
β2-microglobulin	Mouse	TTCTGGTGCTTGTCTCACTGA	CAGTATGTTCGGCTTCCCATTC
Rpl13a	Mouse	GGGCAGGTTCTGGTATTGGAT	GGCTCGGAAATGGTAGGGG
β2-microglobulin	Human	AGCATTCGGGCCGAGATGTCT	CTGCTGGATGACGTGAGTAAACCT
Rpl13a	Human	GCCATCGTGGCTAAACAGGTA	GTTGGTGTTCATCCGCTTGC
VCAM-1	Mouse	TCAAAGAAAGGGAGACTG	GCTGGAGAACTTCATTATC
VCAM-1	Human	ATGAGGGGACCACATCTACG	CACCTGGATTCCTTTTTCCA
ICAM-1	Human	TGTCCCCCTCAAAAGTCATC	TAGGCAACGGGGTCTCTATG
ICAM-1	Mouse	CTGGCTGTCACAGAACAGGA	AAAGTAGGTGGGGAGGTGCT
KLF2	Mouse	AGAATGCACCTGAGCCTGCTAG	AATTTCCCCGAAAGCCTGC
SMA	Mouse	GGACGTACAACTGGTATTGTGC	CGGCAGTAGTCACGAAGGAAT
Nck2	Mouse	GTCATAGCCAAGTGGGACTACA	GCACGTAGCCTGTCCTGTT
PECAM-1	Mouse	GGAGTCAGAACCCATCAGGA	CAGCTGGTCCCCTTCTATGA

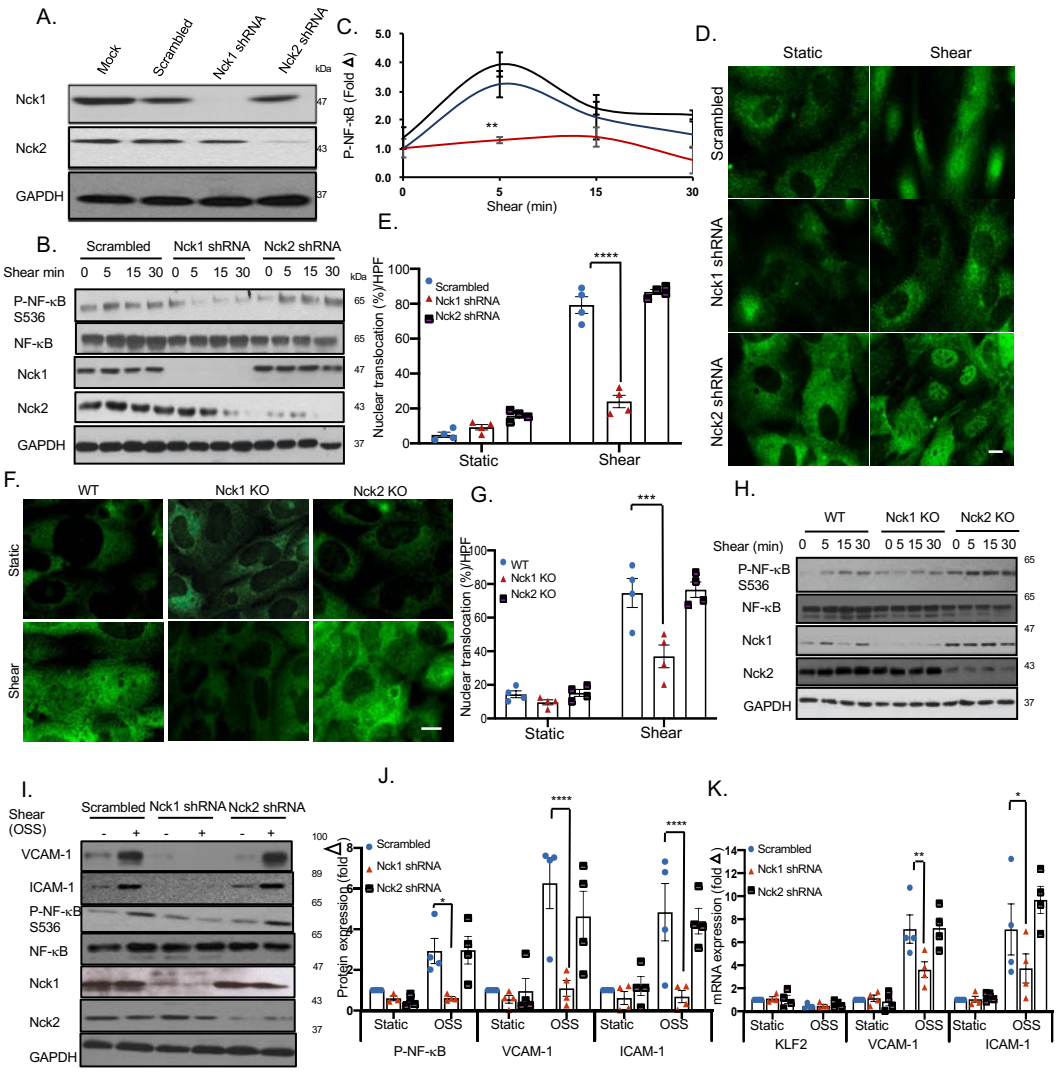
Supplemental Table III: Genotyping Primers:

Gene	Sequence
Nck1	GCATGTAGACAATTACACTTC AGC ACC
	ATTCATGGAATTTCGAACTCGCCACC
	CTGATTGAAGCAGAAGCCTGCGATG
	TATTGGCTTCATCCACCACATACAGG
APOE	GCCGCCCCGACTGCATCT
	TGTGACTTGGGAGCTCTGCAGC
	GCCTAGCCGAGGGAGAGCCG
VeCad Cre	ACT GGG ATC TTC GAA CTC TTT GGA C
	GAT GTT GGG GCA CTG CTC ATT CAC C
	CCA TCT GCC ACC AGC CAG
	TCG CCA TCT TCC AGC AGG
Nck2	GGA TAC CAC CAT TGG CAT TAG TAG
	GTG CTC ATT TGA CAA GTG ACA C

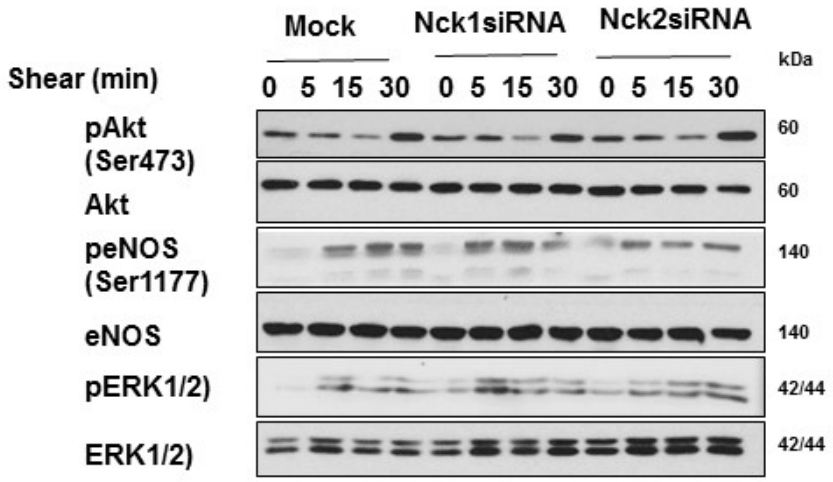
Supplemental Figures:



Supplemental Figure 1. A/B) Nck1/2 was deleted from HAECs using CRISPR/Cas9 editing, and shear stress-induced NF-κB activation was assessed by **(A/B)** Western blotting for p65 phosphorylation and **(C/D)** staining for p65 nuclear translocation**. E-G) Mouse aortic endothelial cells (MAECs) from iEC-Nck1/2 DKO and wild type (WT) were isolated and subjected to shear stress.** NF-κB activation was assessed by analysis of p65 nuclear translocation and **(E/F)** p65 Ser536 phosphorylation **(G)**, showing significant reduction in Nck1/2 DKO MAECs compared to Wild-type cells (WT). Data are mean ± SEM, analyzed by unpaired student *t* test, n=4, ***p<0.001. Scale bar=50μm. **H/I)** Nck1/2 was deleted from HAECs using CRISPR/Cas9 and OSS-induced proinflammatory gene expression and signaling was assessed by Western blotting. **J)** HAECs were treated as in **(H)** & **(I)**, and mRNA expression was assessed by qRT-PCR. Data are mean ± SEM, n=3-4, analyzed by 2-Way ANOVA followed by Bonferroni's post-test, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.



Supplemental Figure 2. A) Lentiviral Nck1 and Nck2 knocking down by shRNA. **B-E)** HAECs expressing either Nck1 shRNA or Nck2 shRNA were subjected to acute shear stress for the indicated times, and NF-κB activation was assessed by measuring **(B/C)** NF-κB phosphorylation and **(D/E)** nuclear translocation. Scale bars=50μm. Data are mean ± SEM, n=4, analyzed by 2-Way ANOVA followed by Bonferroni's post-test, **p<0.01, ****P<0.0001. **F-H) Mouse aortic endothelial cells (MAECs) from Nck1 KO, iEC-Nck2 KO, or Wild type (WT) were isolated and subjected to shear stress. F/G)** NF-κB activation was assessed by analysis of p65 nuclear translocation and **(H)** p65 Ser536 phosphorylation, showing significant reduction in Nck1 KO MAECs compared to Wild-type cells (WT). Data are mean ± SEM, analyzed by 1-Way ANOVA, and Tukey's post-test, n=4, ***p<0.001. Scale bar=50μm. **I/J)** HAECs were transfected with either Nck1 or Nck2 shRNA, and oscillatory shear stress (OSS, 18h) induced proinflammatory gene expression (ICAM-1/VCAM-1) and signaling (P-NF-κB Ser536) were assessed by Western blotting. **K)** mRNA expression as assessed by qRT-PCR. Data are from n=4, analyzed by 2-Way ANOVA and Bonferroni's post-test, *p<0.05, **p<0.01, ****p<0.0001.



Supplemental Figure 3. Endothelial cells subjected to acute shear stress for the indicated times, and activation of Akt, eNOS, and ERK1/2 assessed by Western blotting. Representative blots are from n=4.

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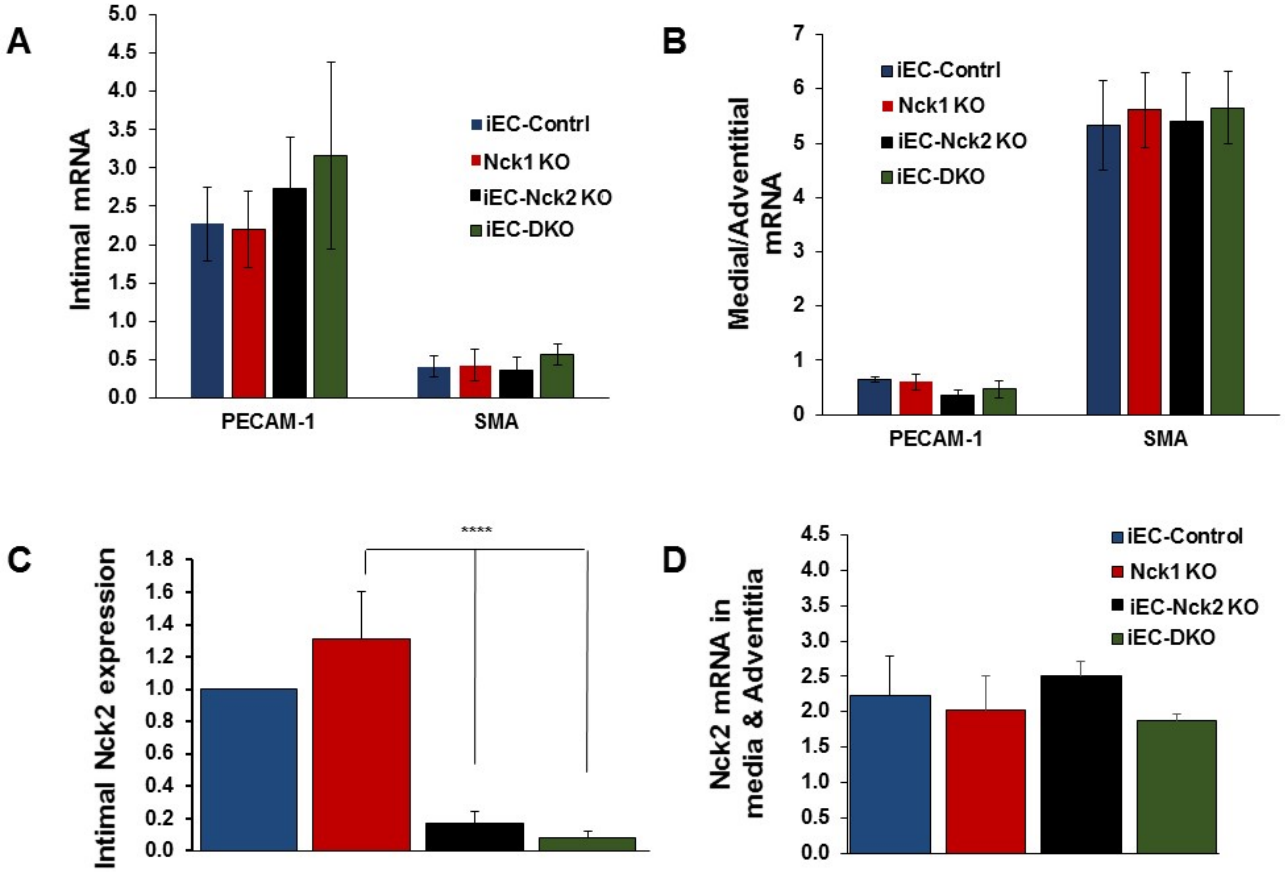
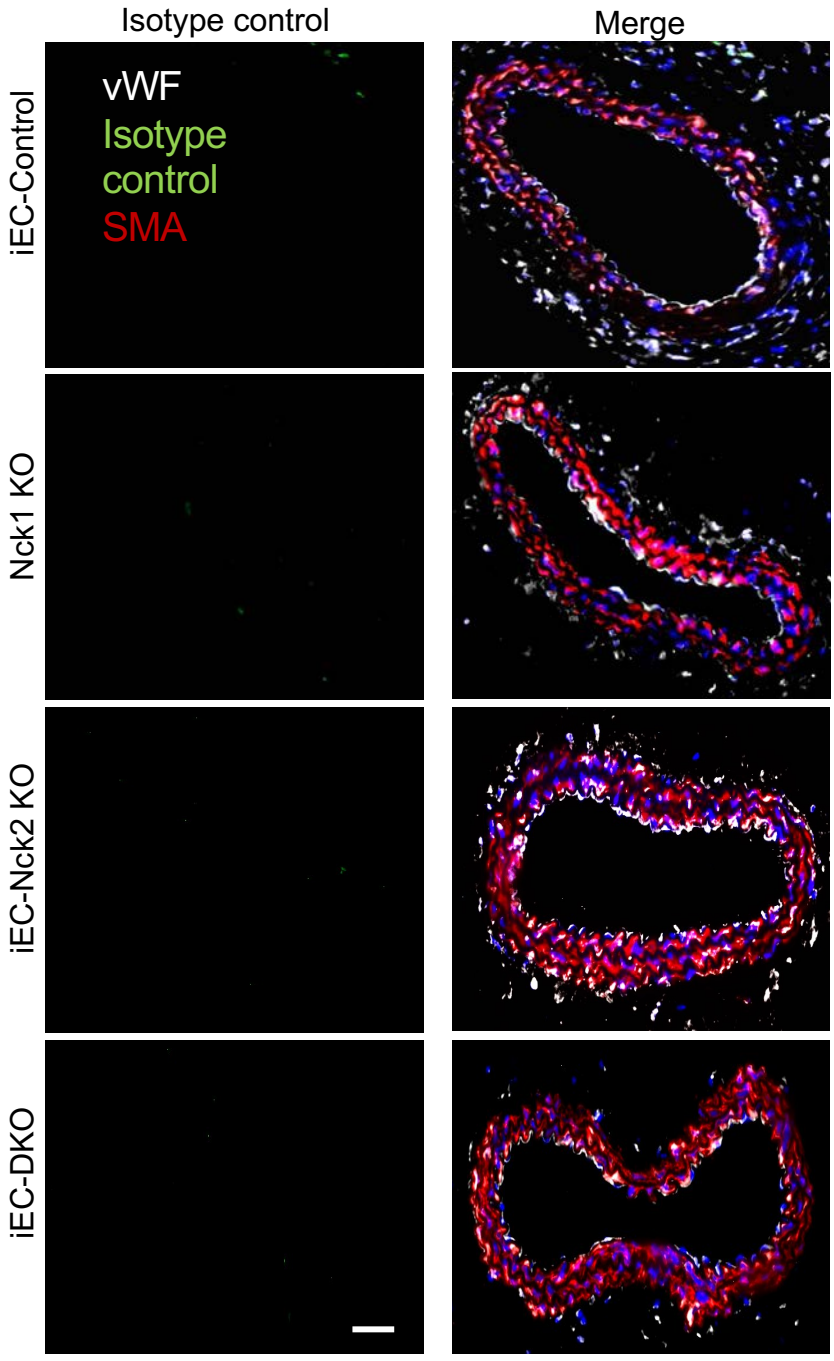
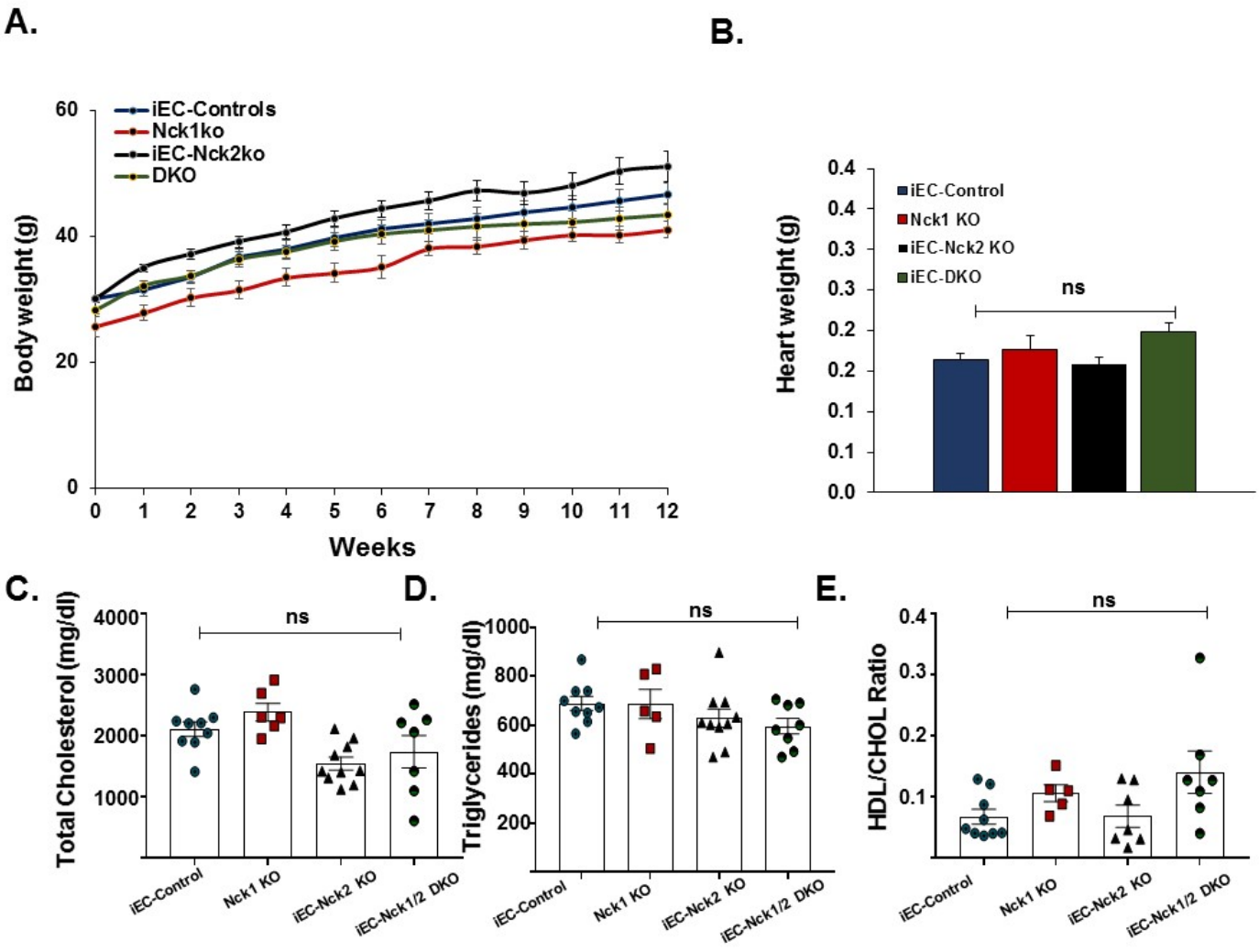


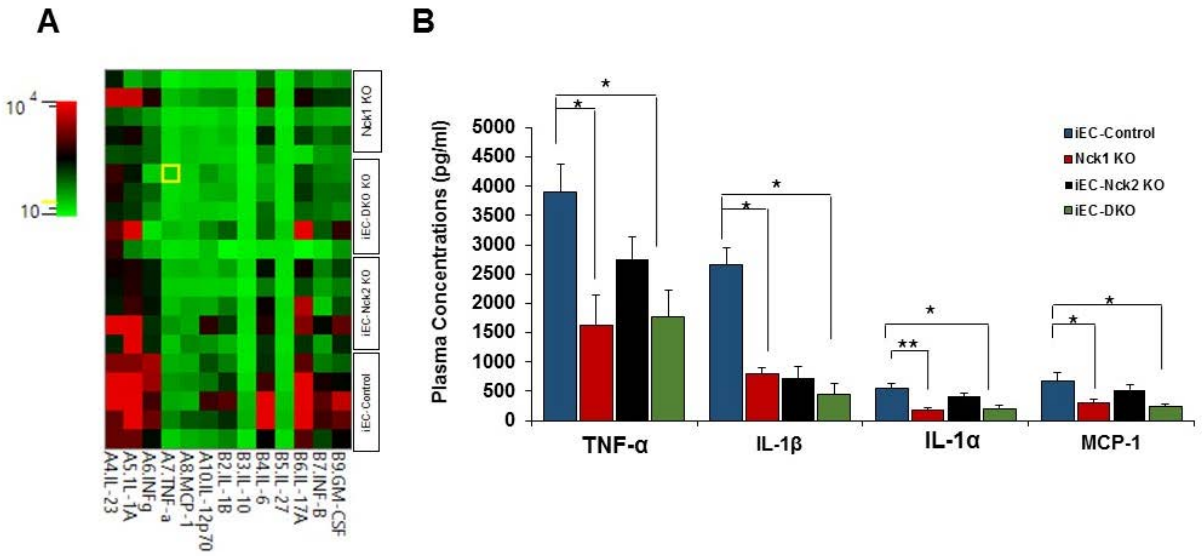
Supplemental Figure 4. A/B) Intimal mRNA and Medial/Adventitial mRNA were obtained 2 days post-ligation surgery from right and ligated left carotid arteries. mRNA levels were assessed for the endothelial marker gene platelet endothelial cell adhesion molecule-1 (PECAM-1) and the smooth muscle marker gene α-smooth muscle actin (SMA). **C/D)** Nck2 mRNA expression after tamoxifen injection shows selective Nck2 depletion from the intima and not the media/adventitia. Graphs are mean ± SEM, n=7-10/group. Data analyzed by 1-Way ANOVA and Tukey's post-test, ****p<0.0001.



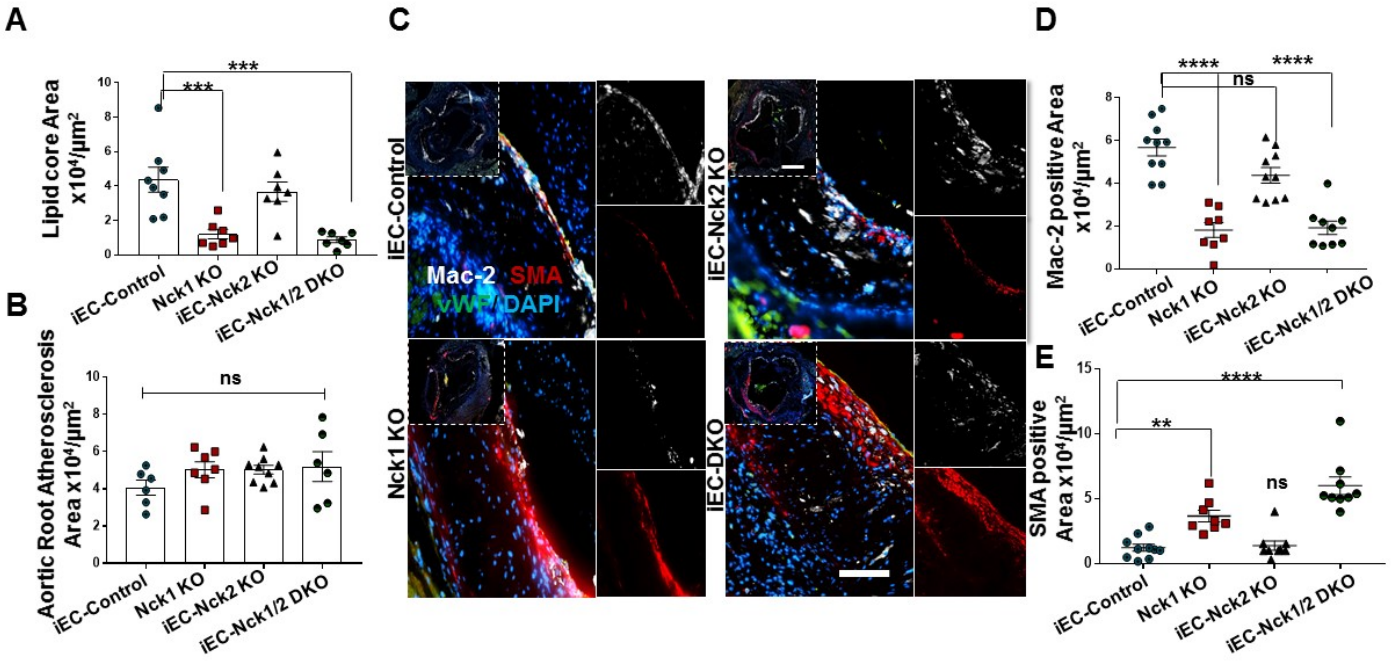
Supplemental Figure 5. Isotype control staining for mac-2 in the ligated carotid arteries among experimental groups. Smooth muscle cells (α-smooth muscle actin (SMA), red), and endothelium (vWF, white). Scale bars=200μm



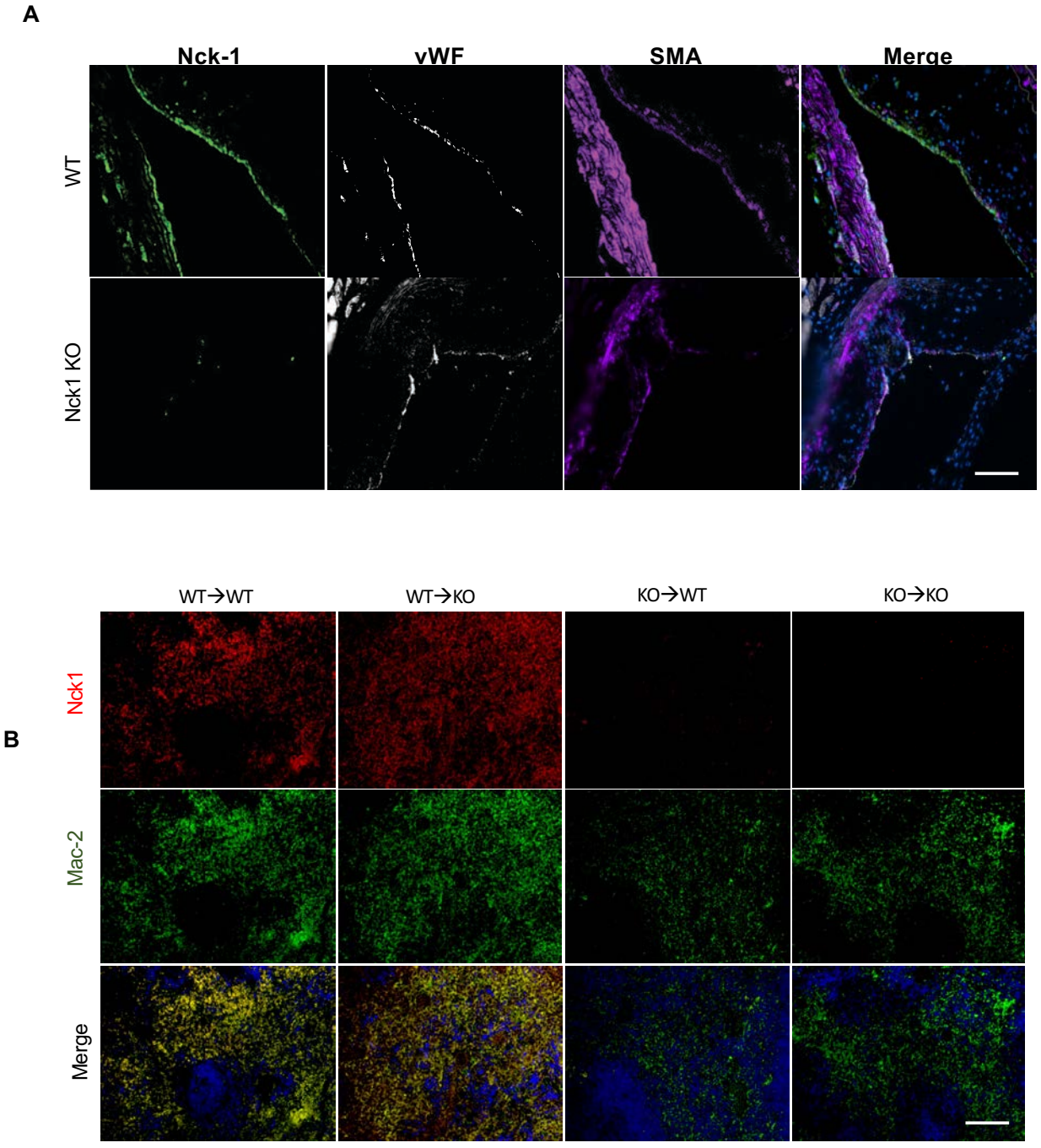
Supplemental Figure 6. A) Body weight changes in response to HFD feeding in iEC-Controls, Nck1 KO, iEC-Nck2 KO, iEC-DKO mice. Body weight in grams were recorded weekly for 12 weeks. **B)** Heart weight among experimental groups after 12 weeks of HFD feeding. **C)** Total plasma cholesterol (mg/dl) **(D)** Triglycerides and **(E)** HDL/total Cholesterol ratio were measured. Data are mean ± SEM, n=5-10/group. Data analyzed by 1-Way ANOVA and Tukey's post-test, ns: not-significant.



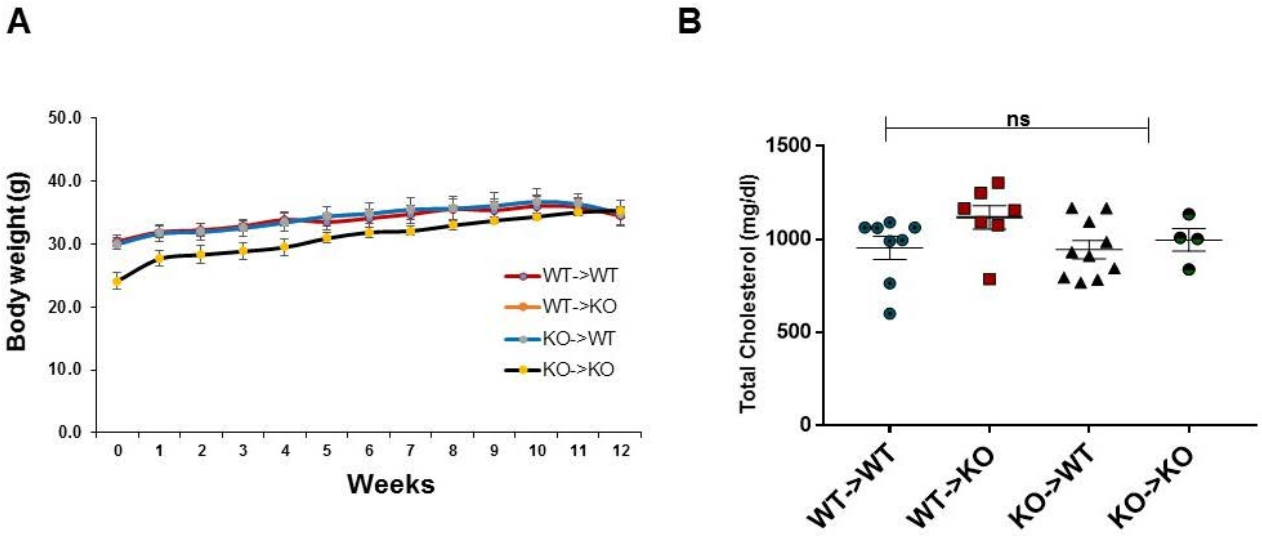
Supplemental Figure 7. Nck1 KO mice show attenuated plasma pro-inflammatory cytokine/chemokine profiles. A) Heat map and **(B)** a graphical representation of plasma cytokine concentrations (pg/ml), analyzed using LEGENDplex™. Data are mean ± SEM, n=6-10/group. Data analyzed by 2-Way ANOVA and Tukey's post-test, *p<0.05, **p<0.01.



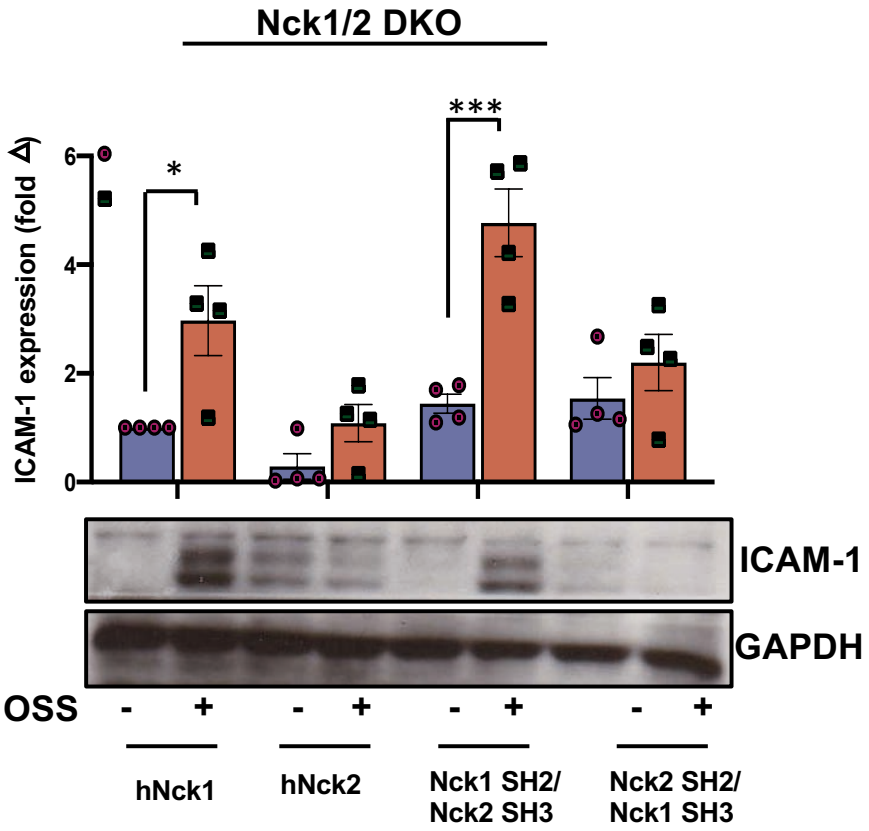
Supplemental Figure 8. A) Lipid core area quantification in carotid atherosclerosis. **B)** Aortic Root atherosclerosis and **(C-E)** analysis of aortic root plaque cellular content following staining for macrophages (Mac-2; white), smooth muscle cells (α-smooth muscle actin; SMA, red) and endothelium (vWF; green). Analysis was performed using NIS Elements software and data are mean ± SEM, n=6-10/group. Data analyzed by 1-Way ANOVA and Tukey's post-test, **p<0.01, ***p<0.001, ****p<0.0001. Scale bars=50-200µm, ns=not significant.



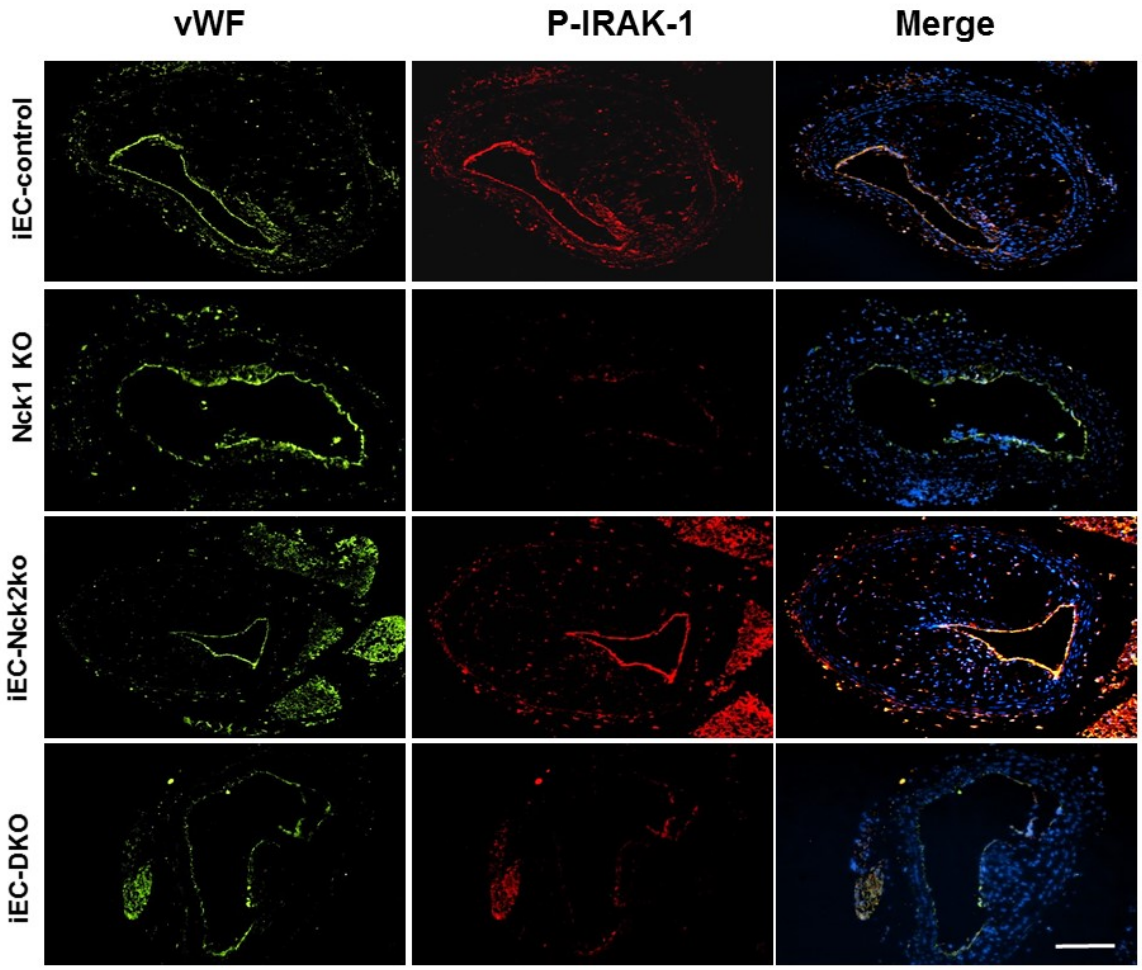
Supplemental Figure 9. A) Testing staining specificity for Nck1 antibody (R7D Systems) in sections of mouse aortic atherosclerosis from wild type (WT) or Nck1 KO mice stained for Nck1, vWF, SMA. **B)** Splenic sections from bone marrow chimeric mice stained for Nck1 (red) and mac-2 (green). From n=5/ group. Scale bars=100μm.



Supplemental Figure 10. Bone marrow chimera were produced as described in methods. **A)** Average body weight measurement assessed weekly for 12 weeks during the period of HFD feeding. **B)** Plasma cholesterol levels were assessed using an enzymatic assay. Data are mean ± SEM, 2-Way ANOVA and Bonferroni's post-test was used, ns=not significant.



Supplemental Figure 11. ICAM-1 protein expression in Nck1/2 DKO HAECs after rescuing the expression of Nck1, Nck2, the Nck1 SH2/Nck2 SH3 chimera, or the Nck2 SH2/Nck1 SH3 chimera. Data are mean ± SEM, n=4 independent experiment. Data are analyzed by 2-Way ANOVA and Bonferroni's post-test, *p<0.05, ***p<0.001.



Supplemental Figure 12. Immunostained images from brachiocephalic arteries (BCA) after HFD feeding in iEC-Controls, Nck1 KO, iEC-Nck2 KO, iEC-DKO mice. Representative staining for endothelium (vWF; green), p-IRAK-1 (red). Analysis was performed using NIS Elements software and images are from n=4/group. Scale bar=100μm.

Figure 1.

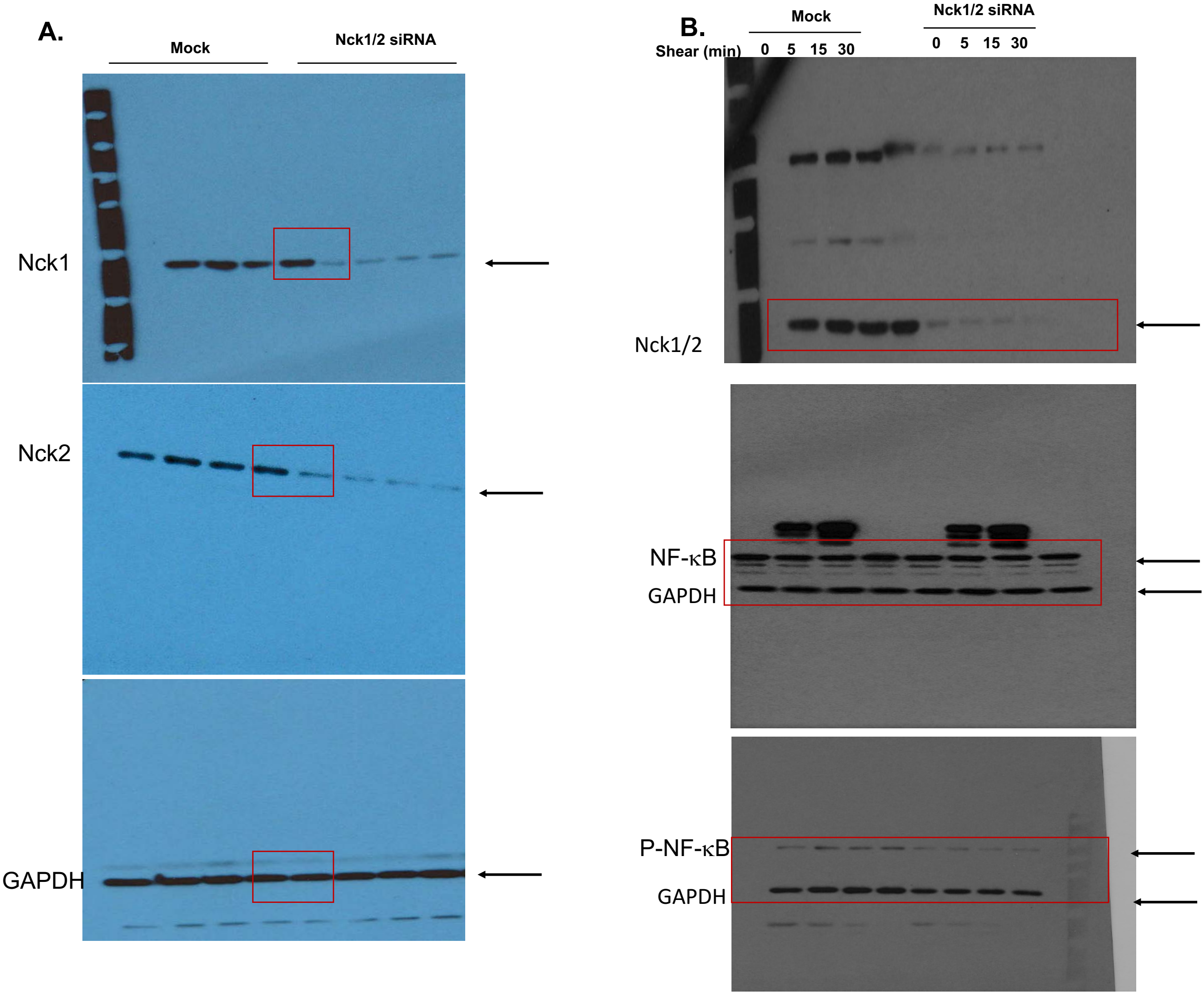

A.
Mock
Nck1/2 siRNA
Nck1
Nck2
GAPDH
B.
Mock
Nck1/2 siRNA
Shear (min)
0 5 15 30
0 5 15 30
Nck1/2
NF-κB
GAPDH
P-NF-κB
GAPDH

Figure 1.

F.

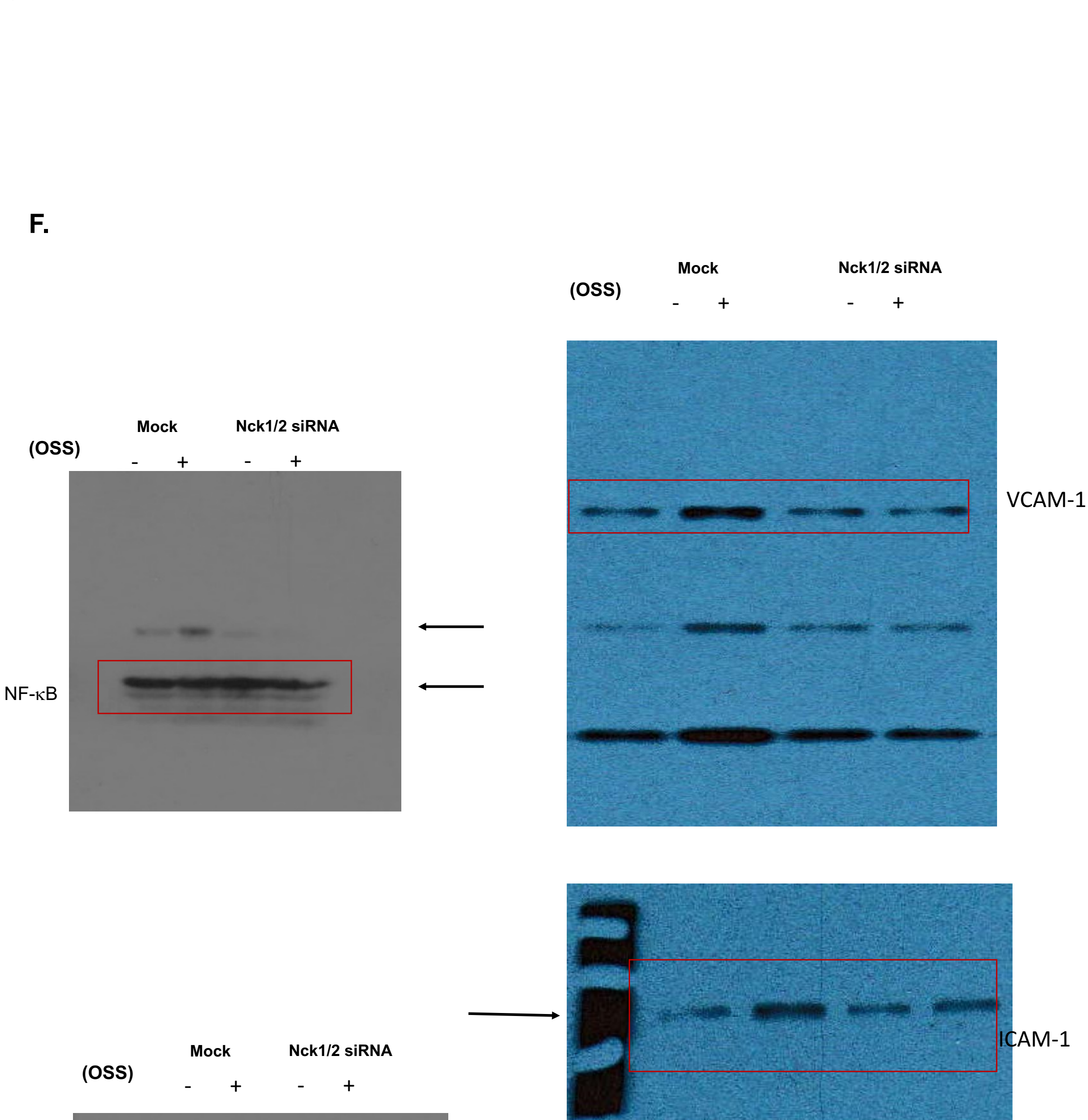
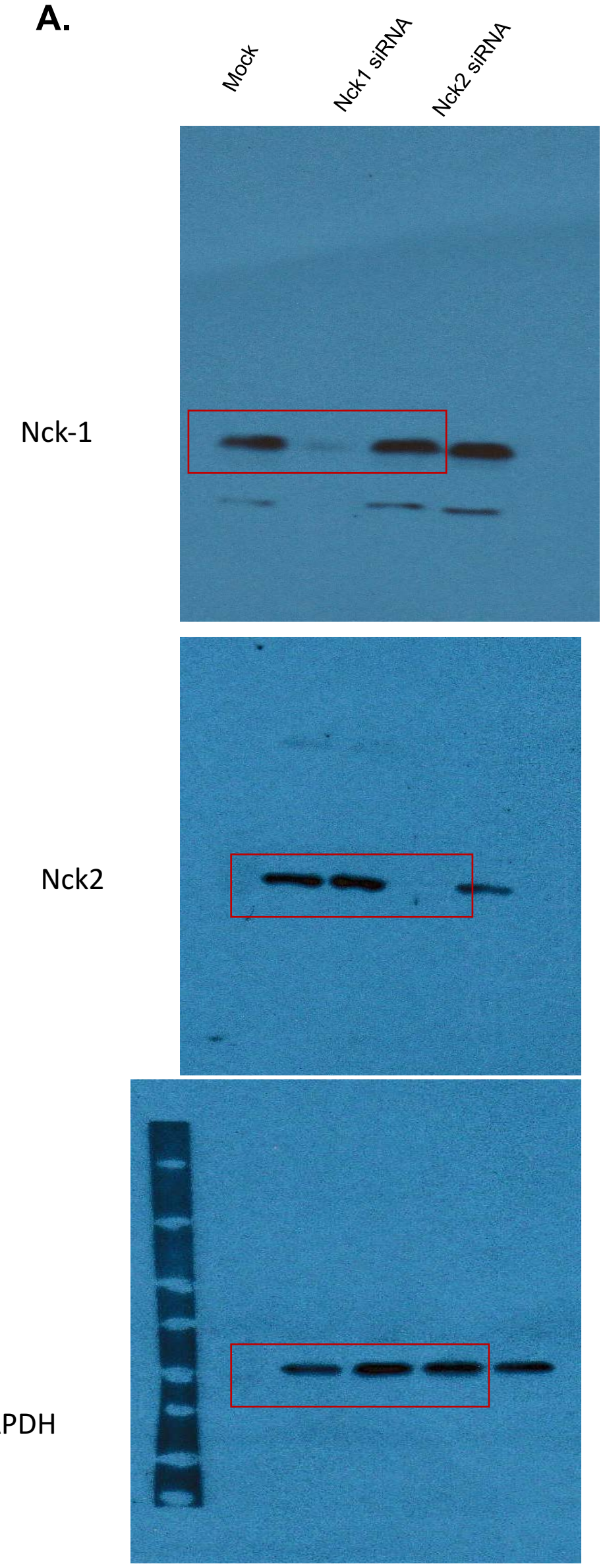

Mock
Nck1/2 siRNA
(OSS)
- + - +
NF-κB
VCAM-1
ICAM-1
(OSS)
Mock
Nck1/2 siRNA
- + - +
P-NF-κB
GAPDH

Figure 2.

A.


Mock
Nck1 siRNA
Nck2 siRNA
Nck-1
Nck2
GAPDH

B.

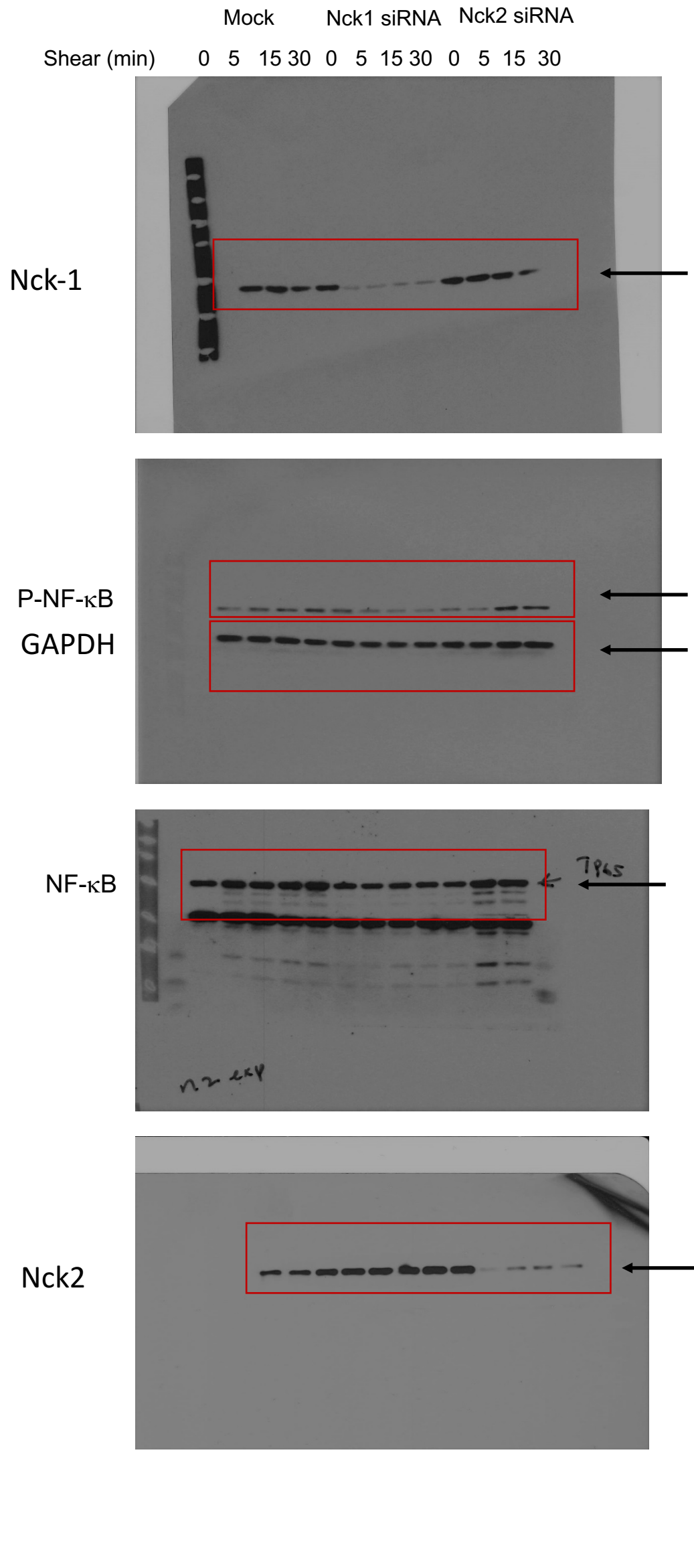

Mock
Nck1 siRNA
Nck2 siRNA
Shear (min) 0 5 15 30 0 5 15 30 0 5 15 30
Nck-1
P-NF-κB
GAPDH
NF-κB
Nck2

Figure 2F.

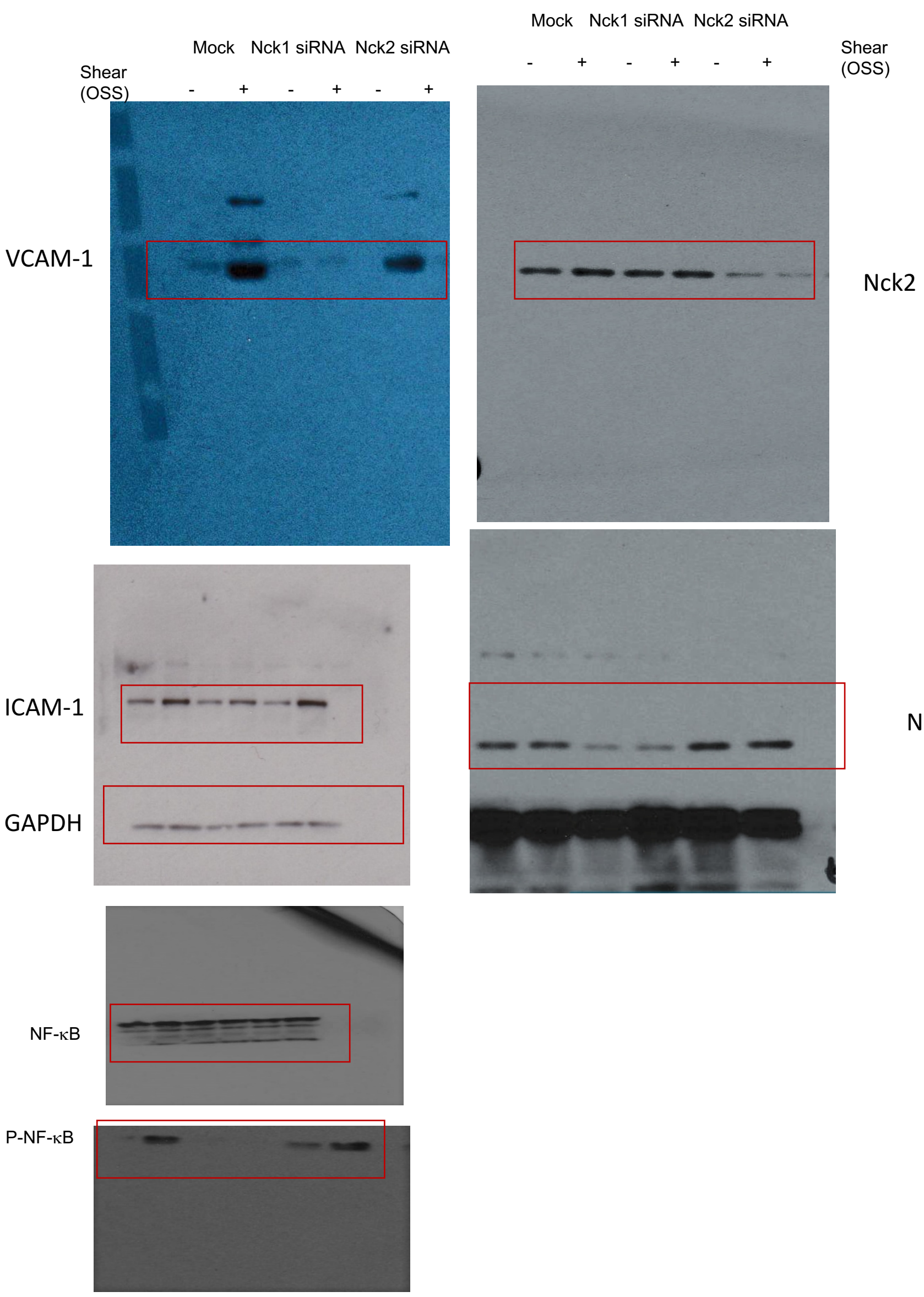
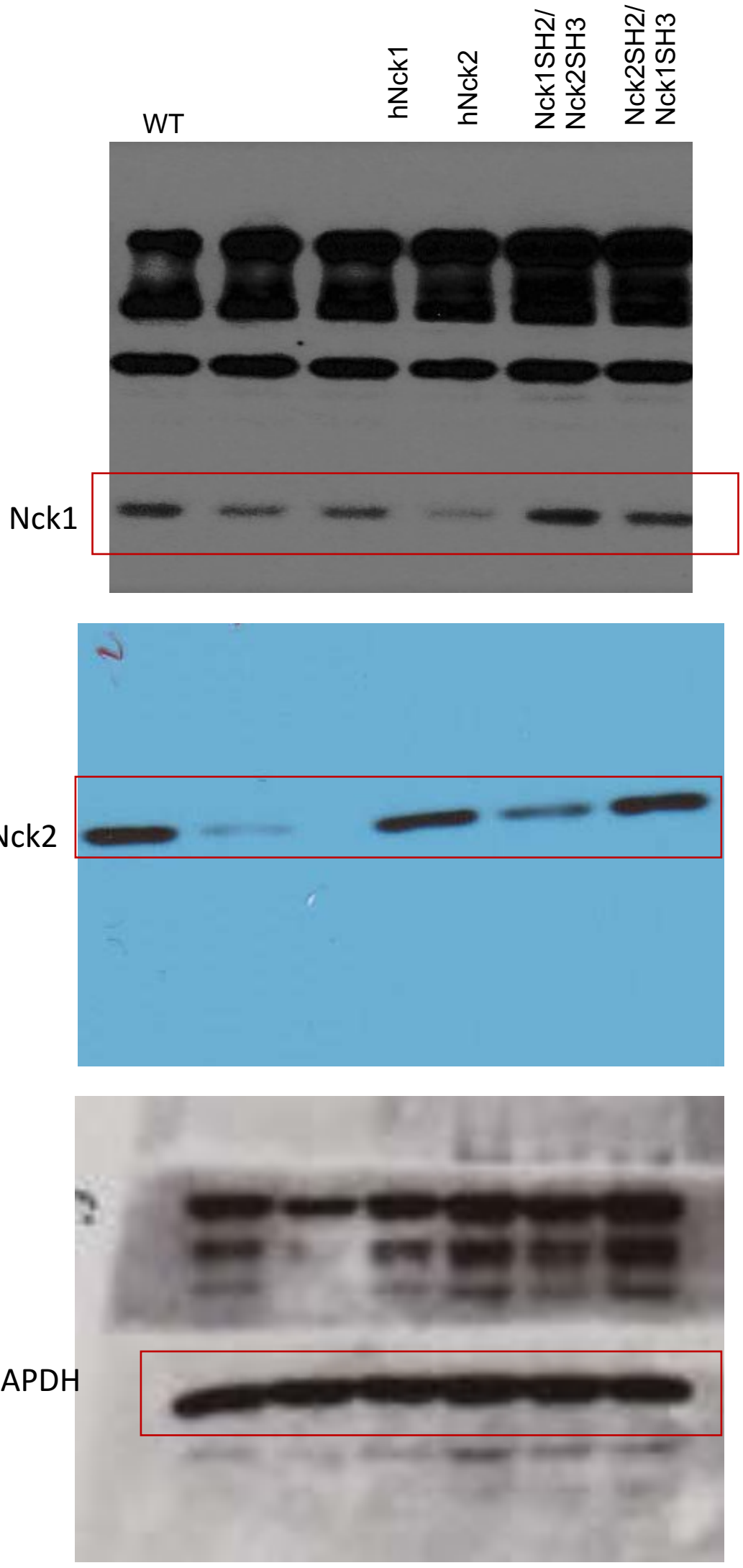
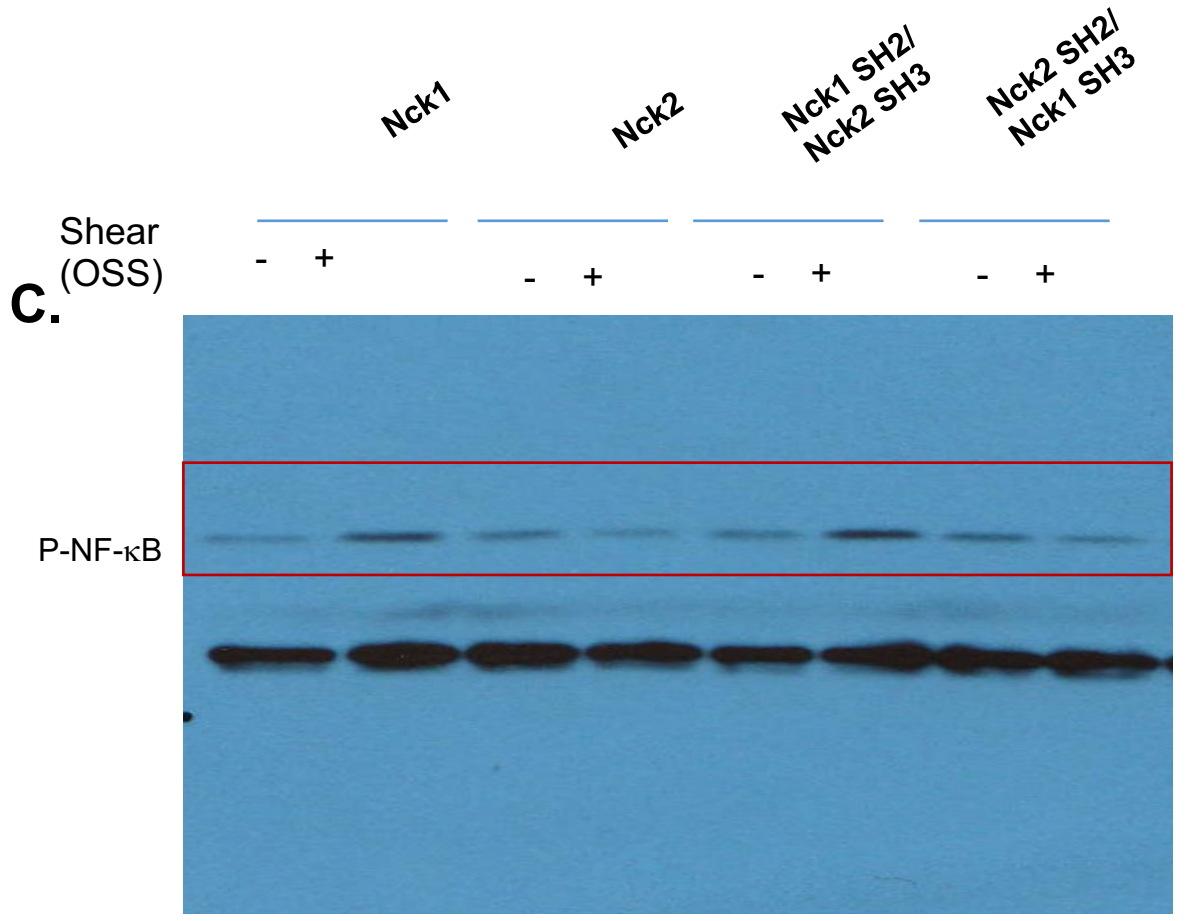

Mock
Nck1 siRNA
Nck2 siRNA
Shear (OSS)
- + - + - +
VCAM-1
ICAM-1
GAPDH
NF-κB
P-NF-κB
Shear (OSS)
- + - + - +
Nck2
Nck1

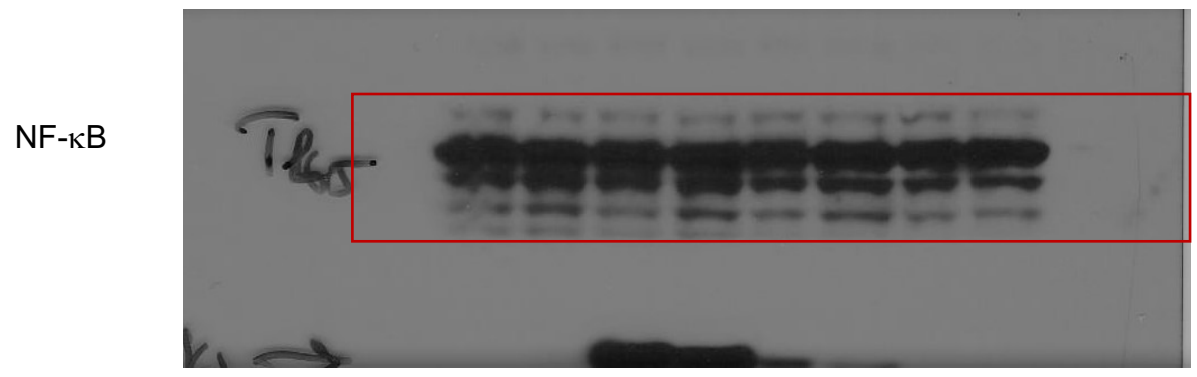
Figure 7.

B.

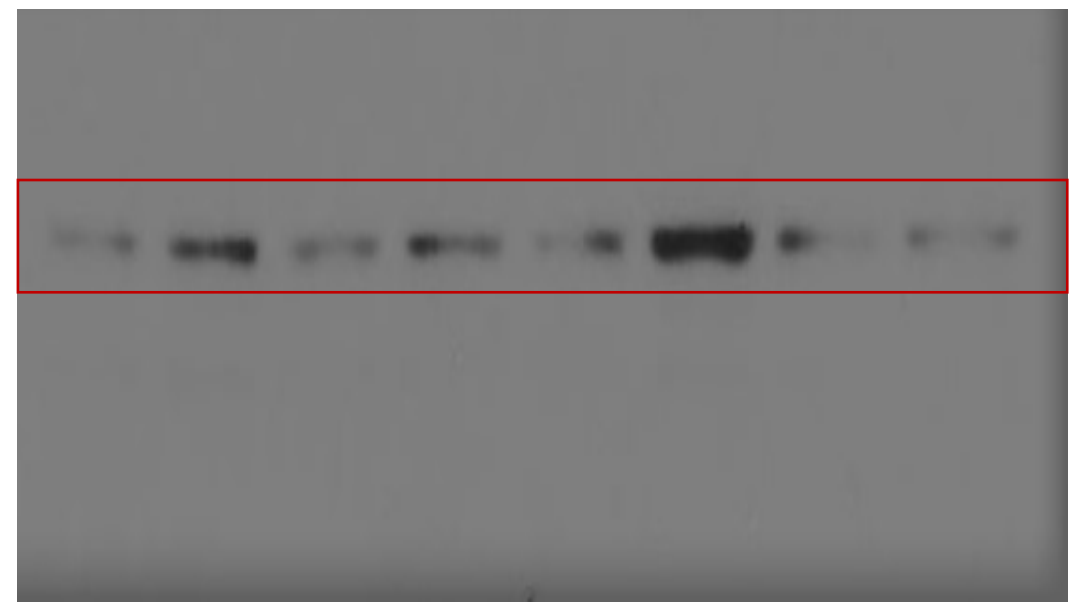

WT
hNck1
hNck2
Nck1SH2/
Nck2SH3
Nck2SH2/
Nck1SH3
Nck1
Nck2
GAPDH

C.


Nck1
Nck2
Nck1 SH2/
Nck2 SH3
Nck2 SH2/
Nck1 SH3
Shear
(OSS)
- + - + - + - +
P-NF-κB


NF-κB

D.


VCAM-1

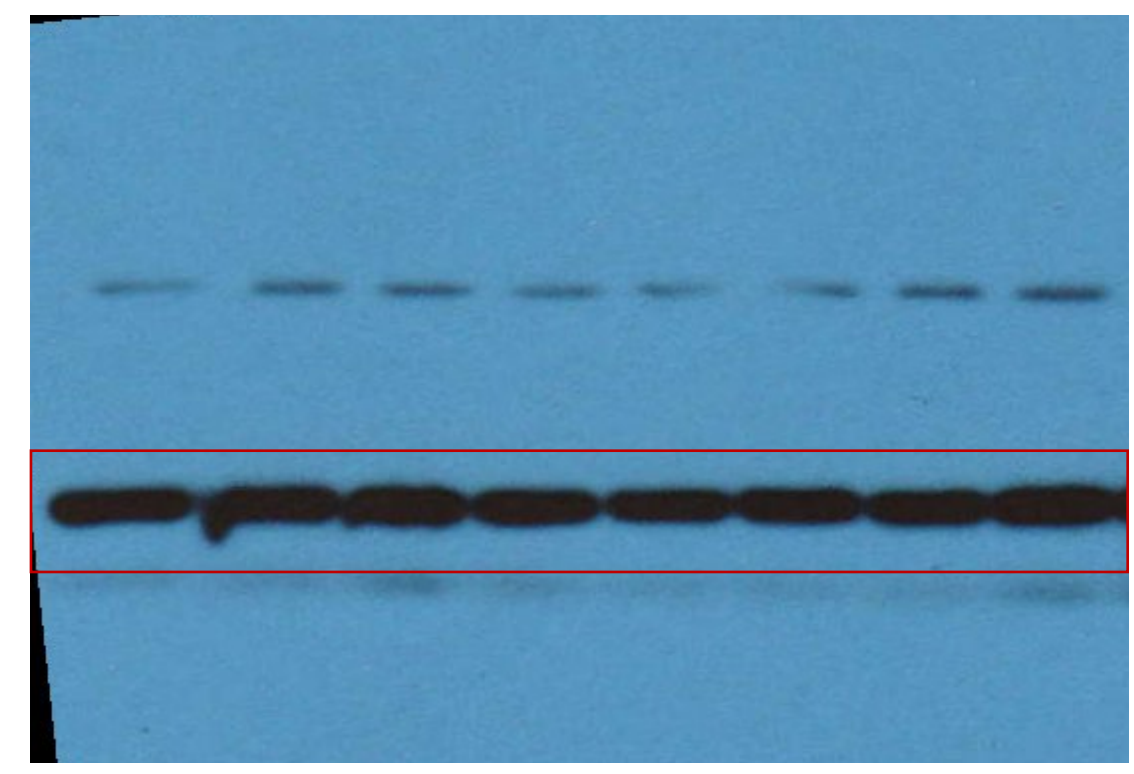
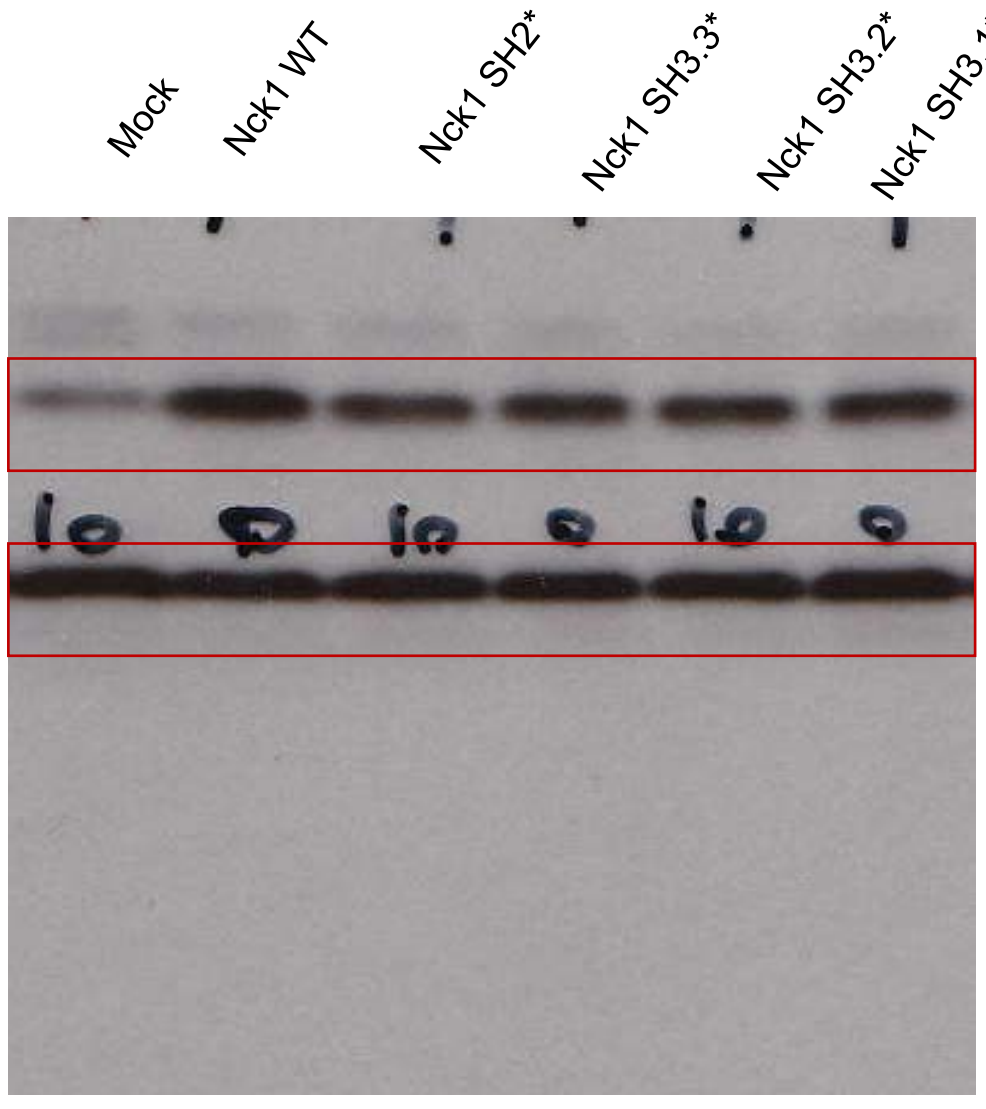

GAPDH

Figure 7.

F.


Mock
Nck1 WT
Nck1 SH2*
Nck1 SH3.3*
Nck1 SH3.2*
Nck1 SH3.1*
Nck1
GAPDH

G.

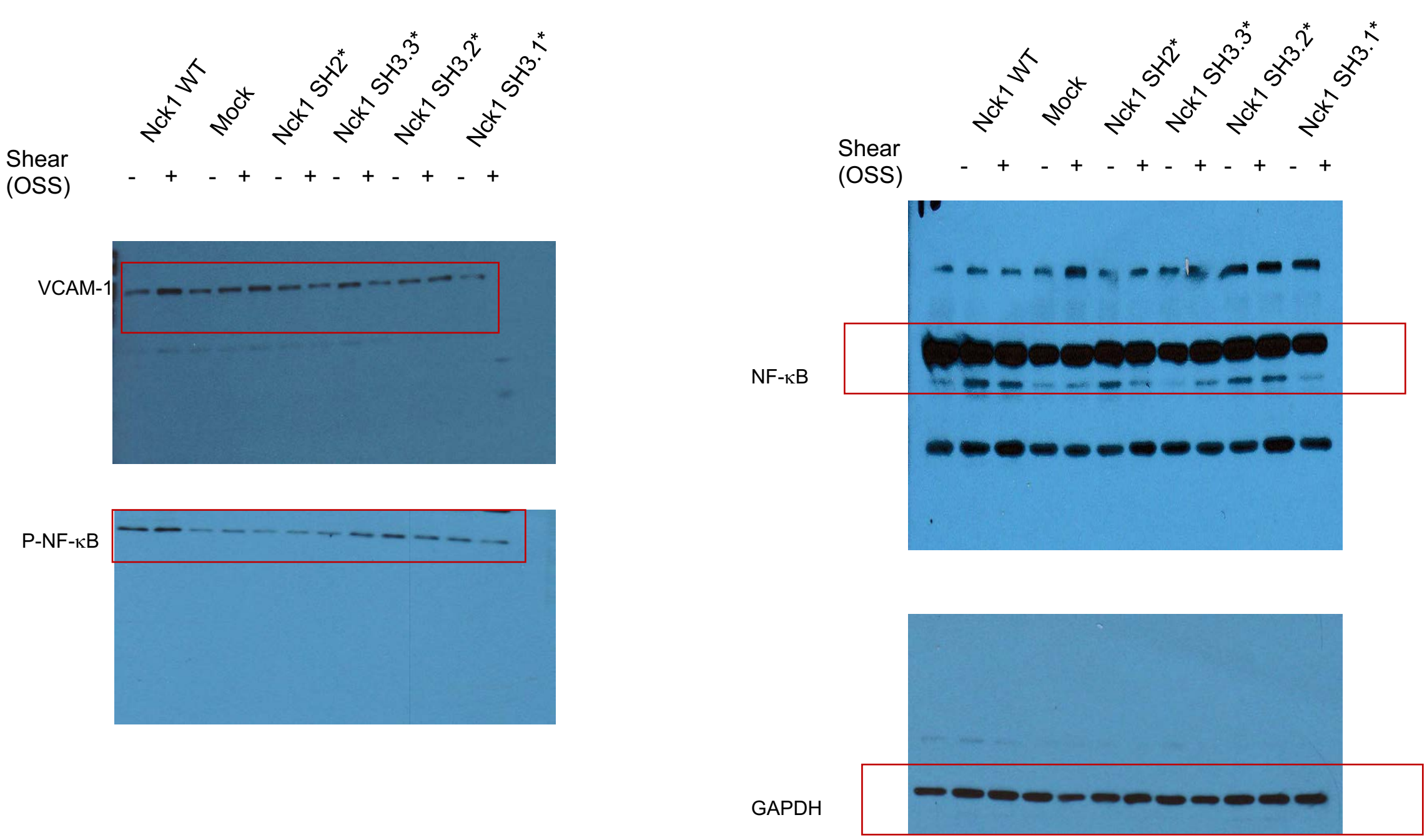
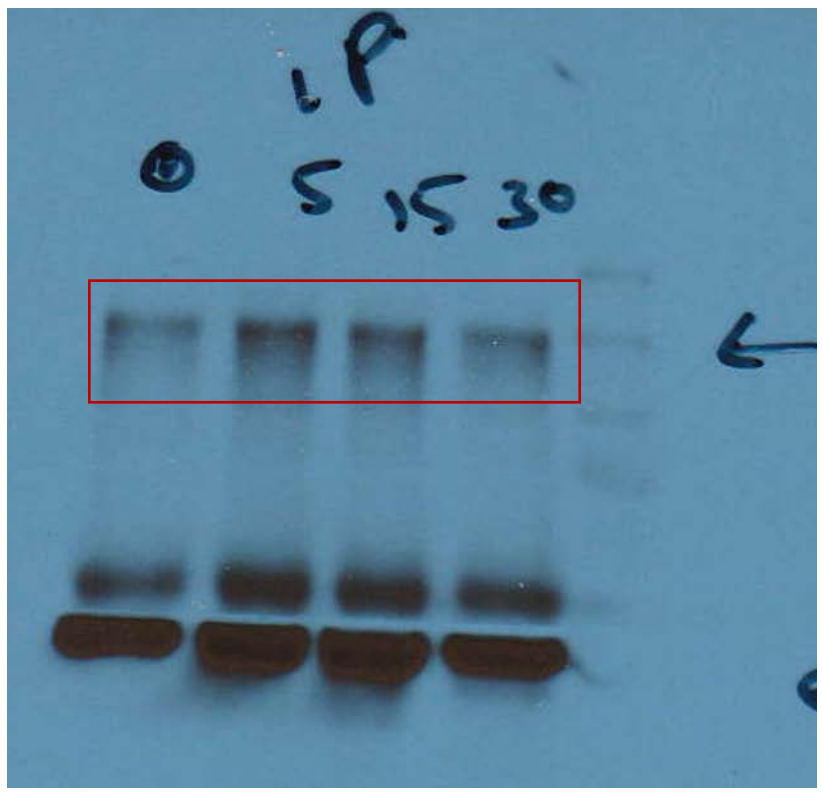
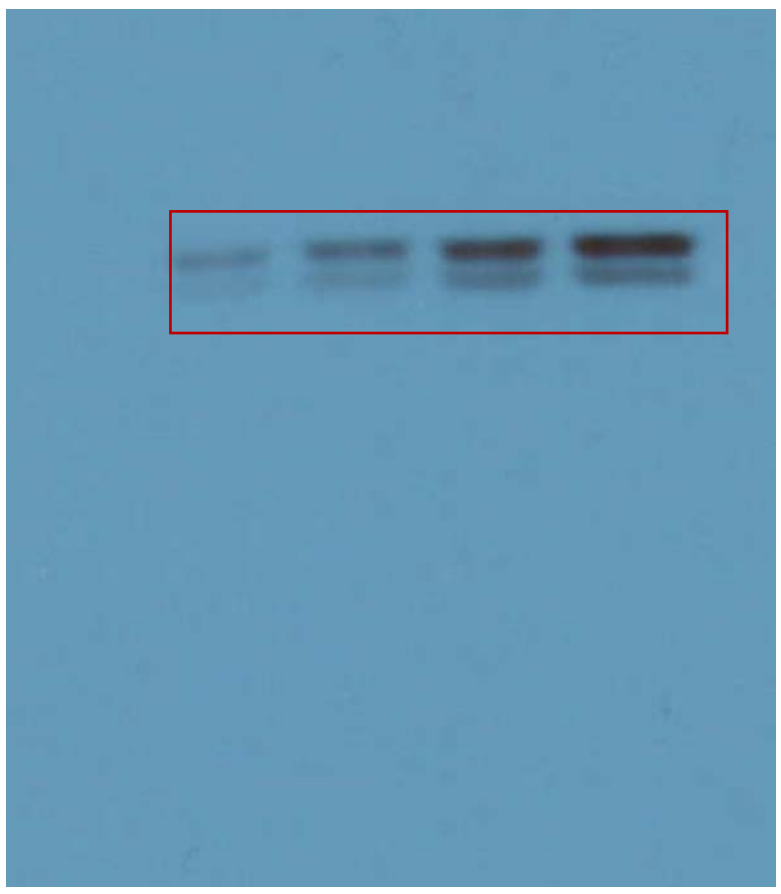

Nck1 WT
Mock
Nck1 SH2*
Nck1 SH3.3*
Nck1 SH3.2*
Nck1 SH3.1*
Shear (OSS)
- + - + - + - + - + - +
VCAM-1
P-NF-κB
NF-κB
GAPDH

Figure 8.

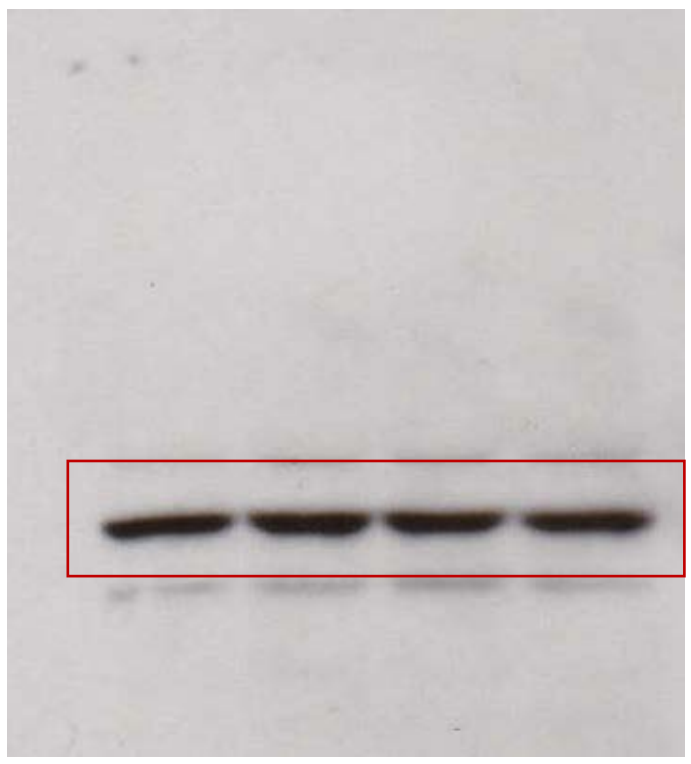
A.



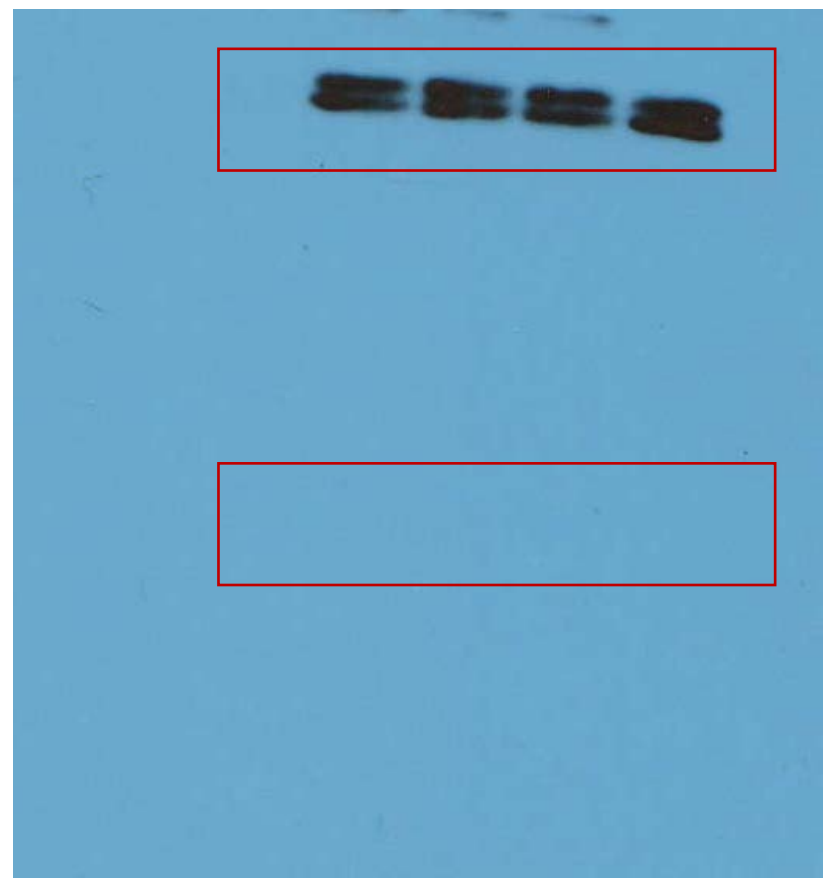
pIRAK-1



IRAK-1

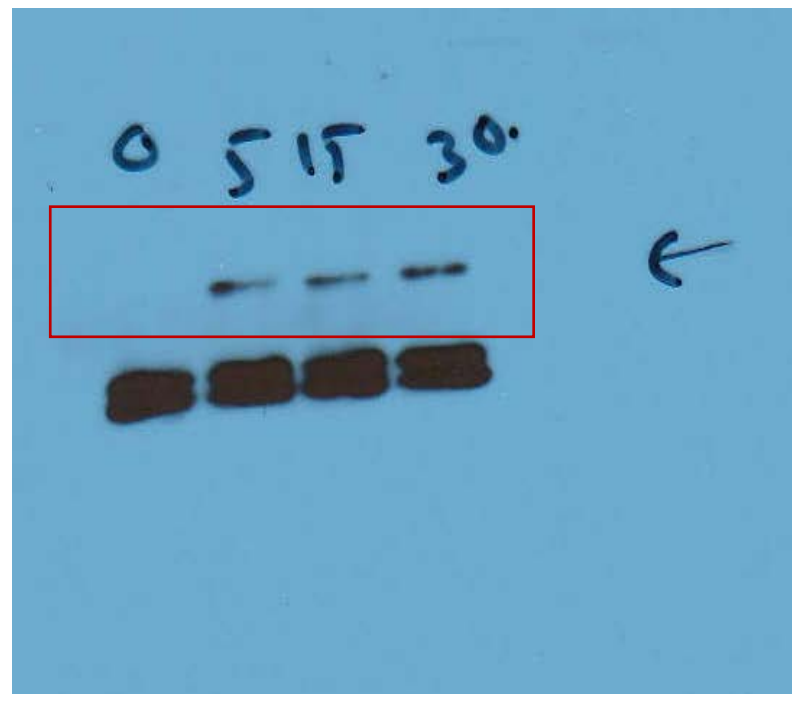


Nck1



IRAK-1

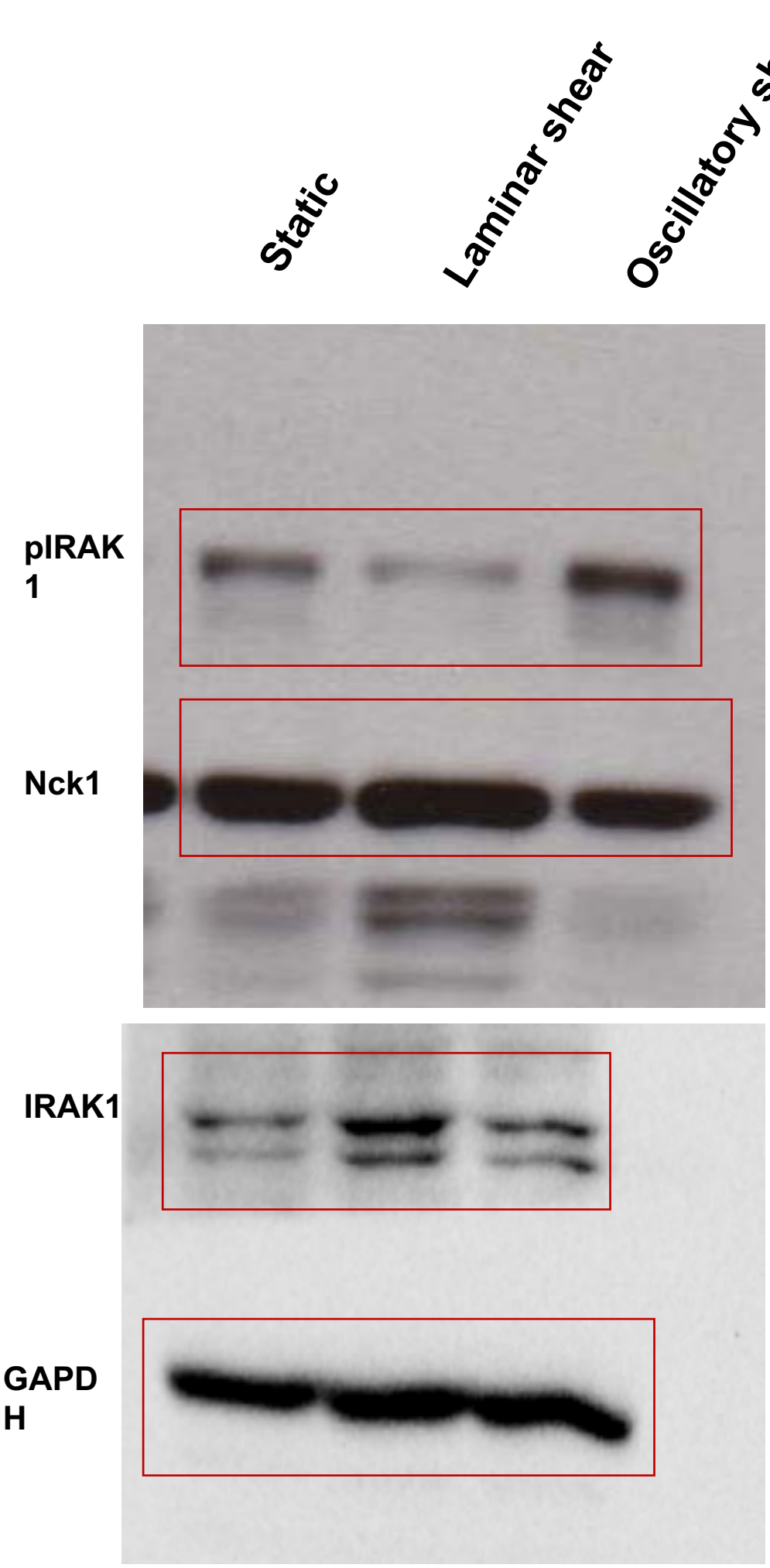
Nck2



Nck1

Figure 8.

D.


Static
Laminar shear
Oscillatory shear
pIRAK 1
Nck1
IRAK1
GAPD H

G.

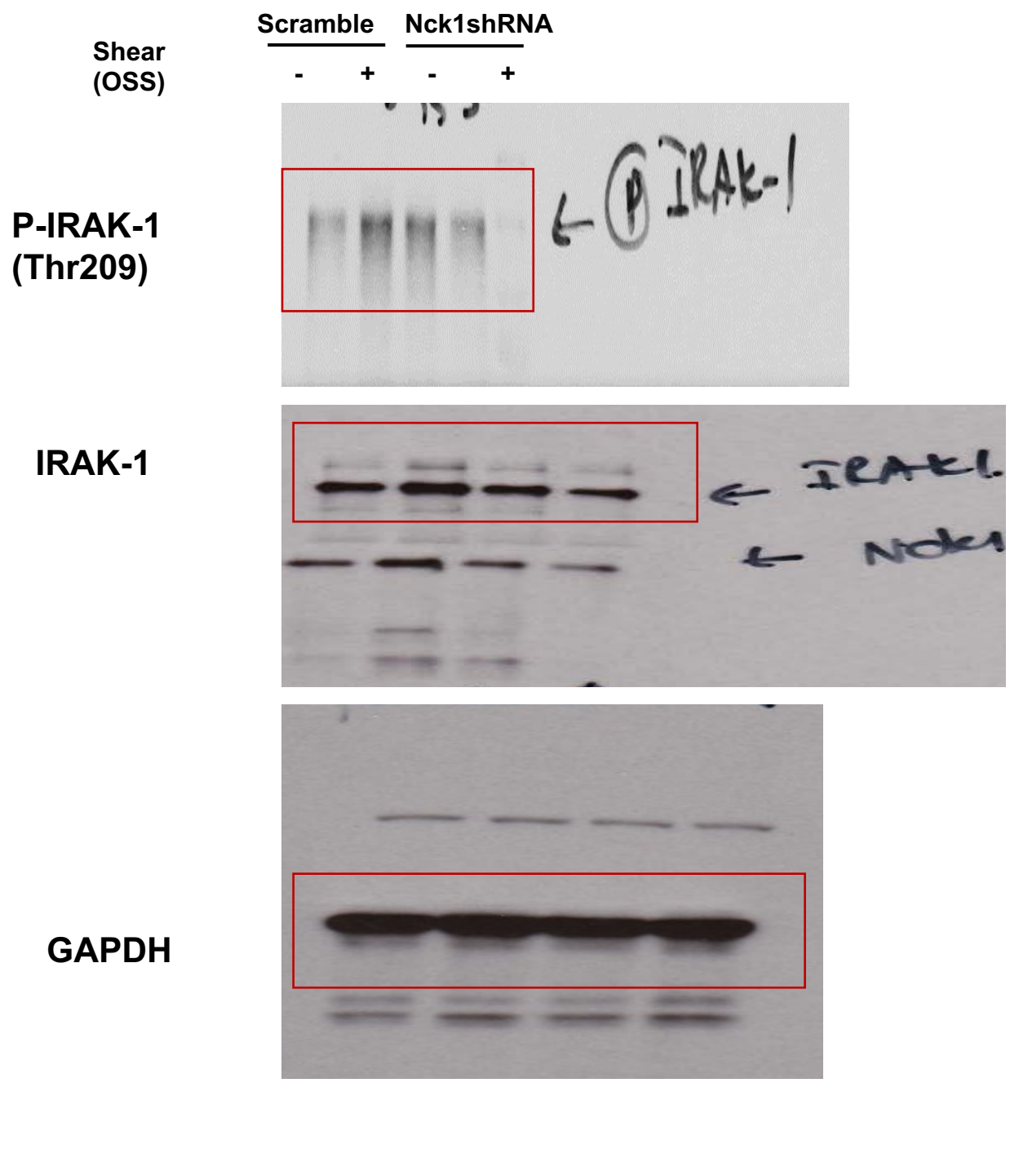
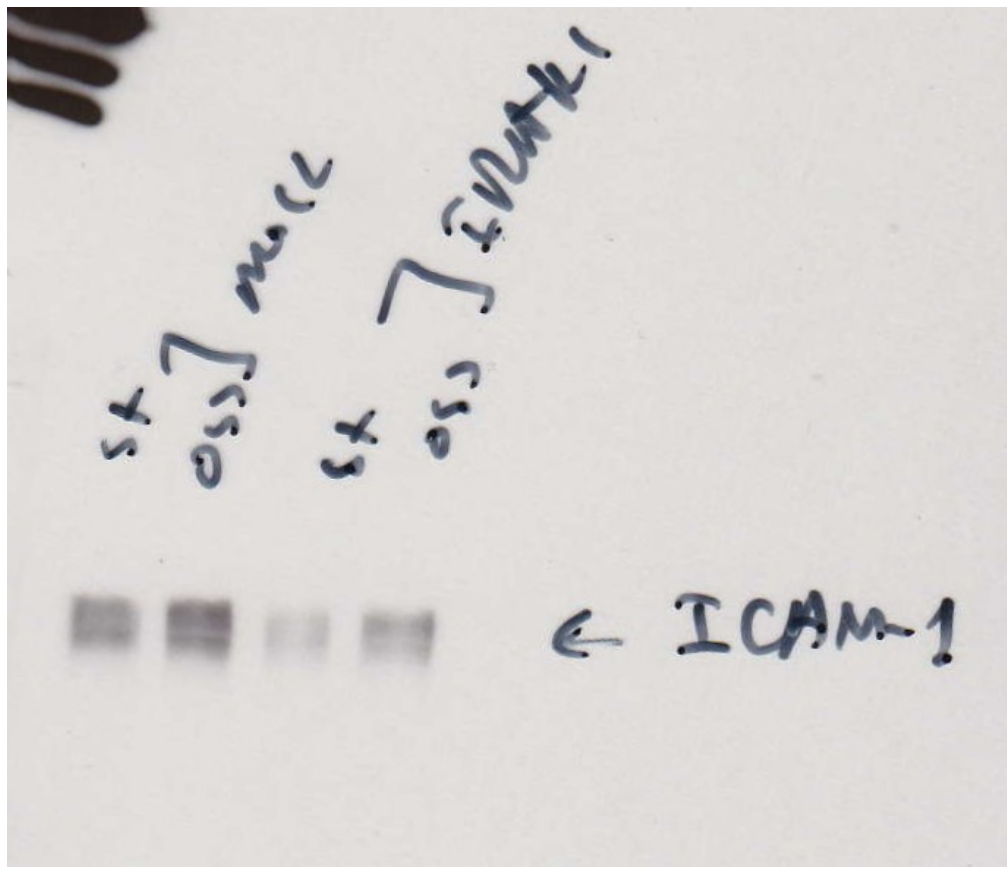
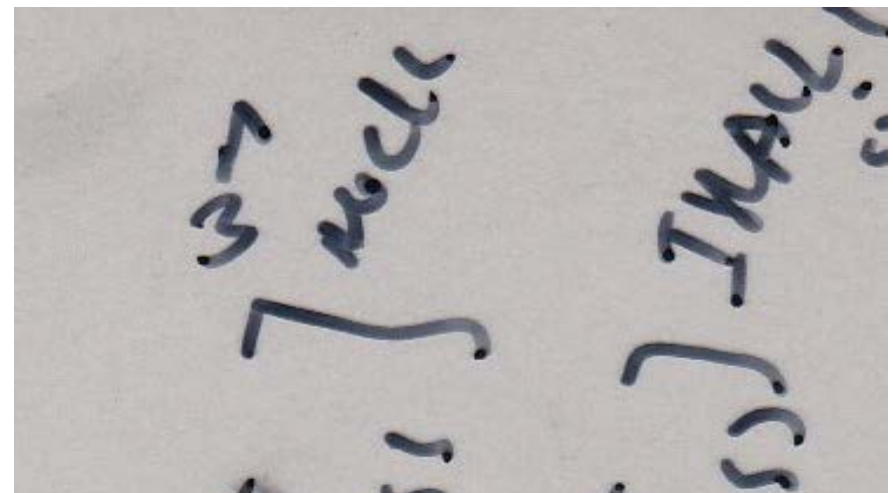
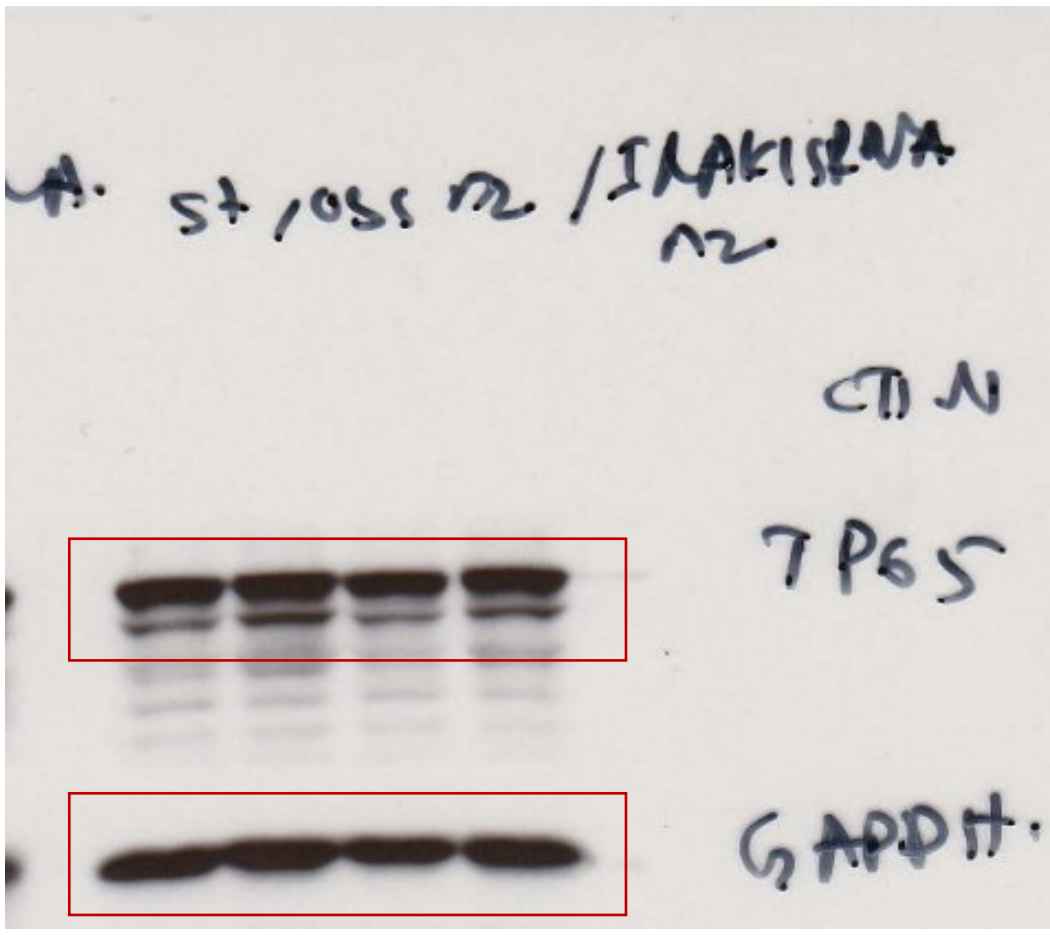
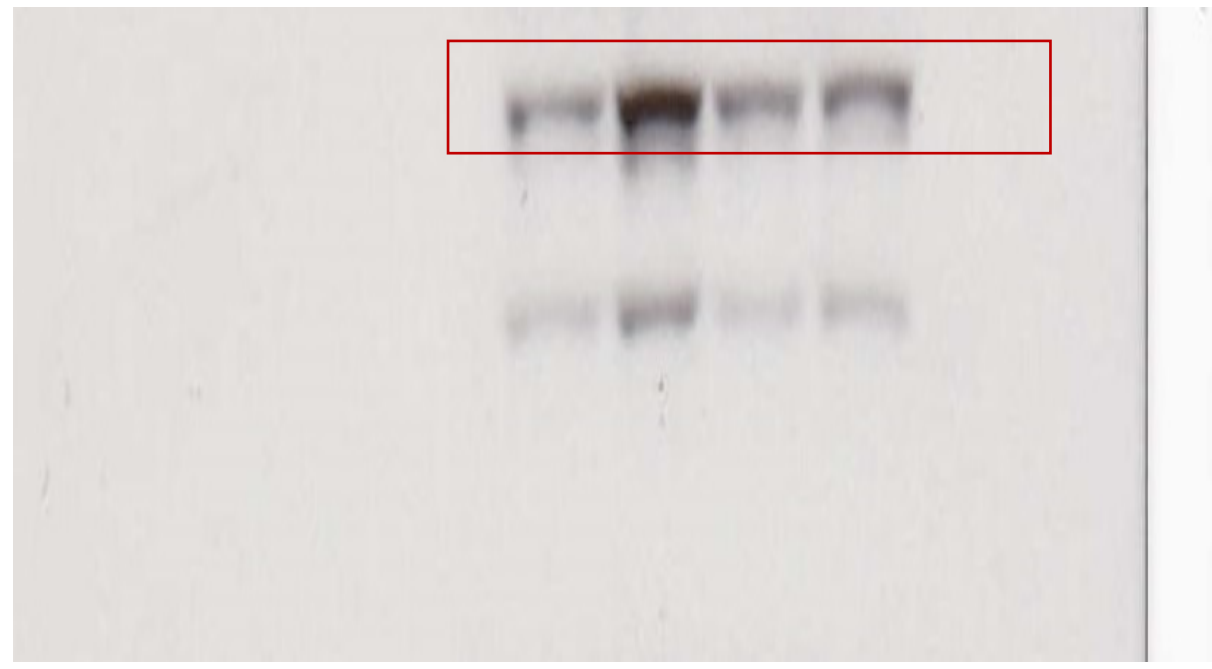

Scramble
Nck1shRNA
Shear (OSS)
- + - +
P-IRAK-1 (Thr209)
IRAK-1
GAPDH

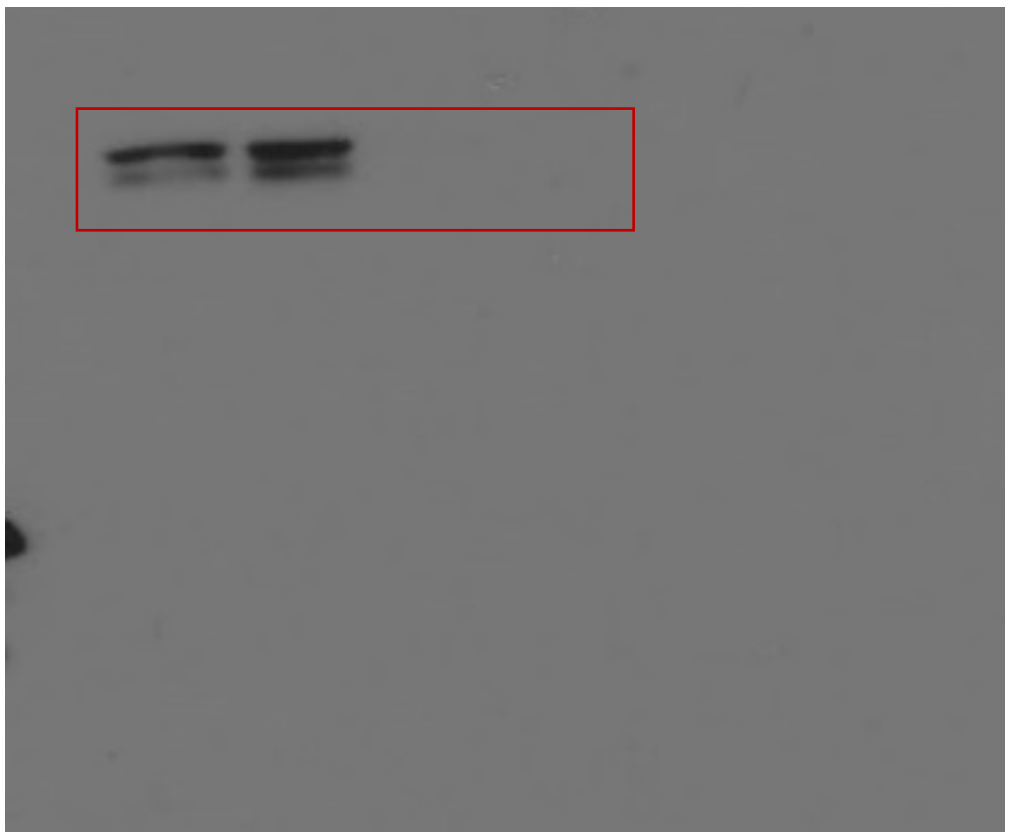
Figure 9.











Supplemental Figures

Supplemental Figure 1.

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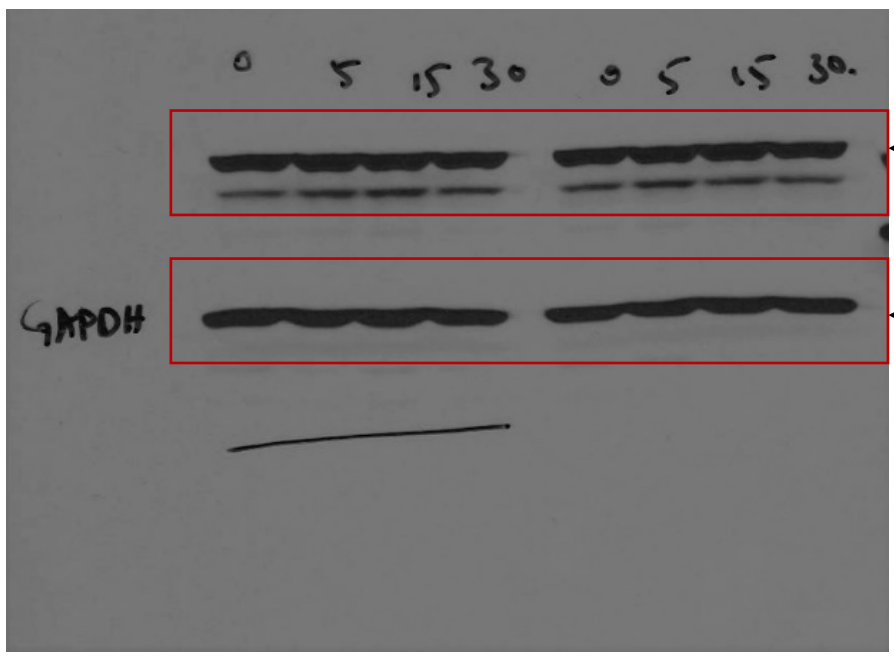
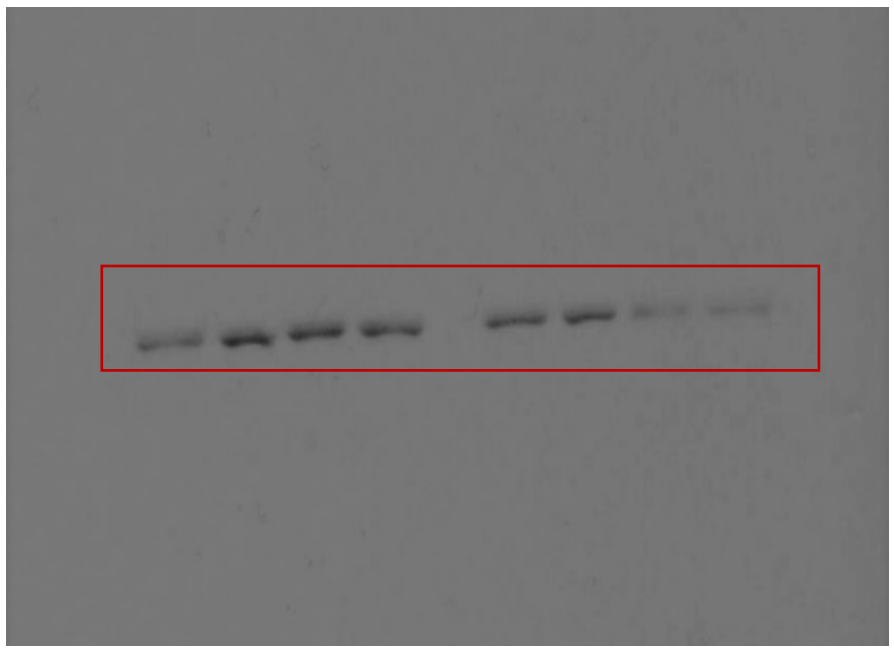


NF-κB

GAPDH



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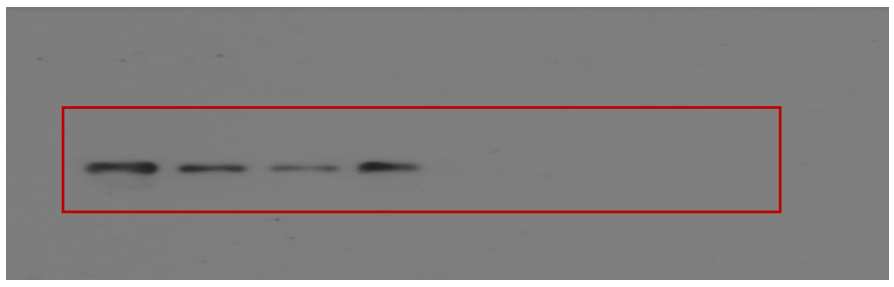

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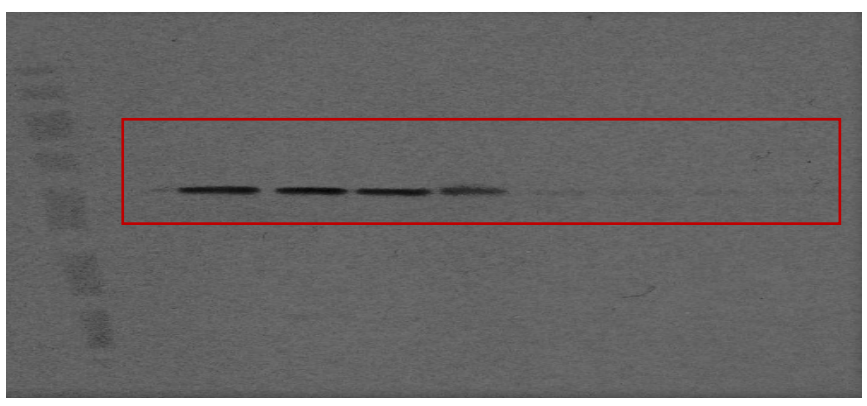

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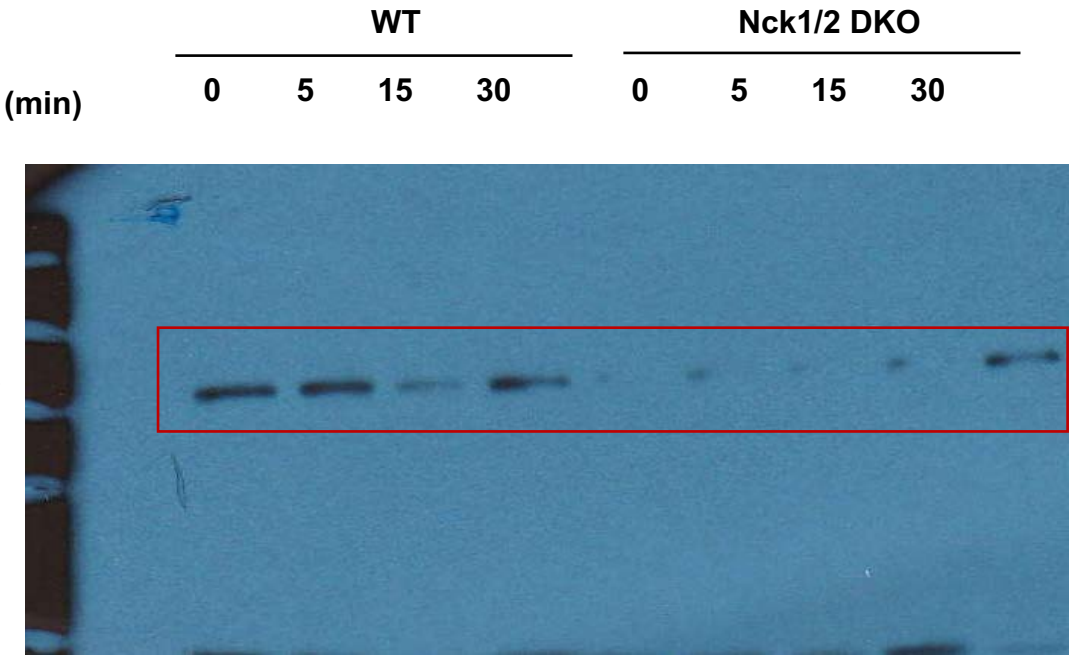
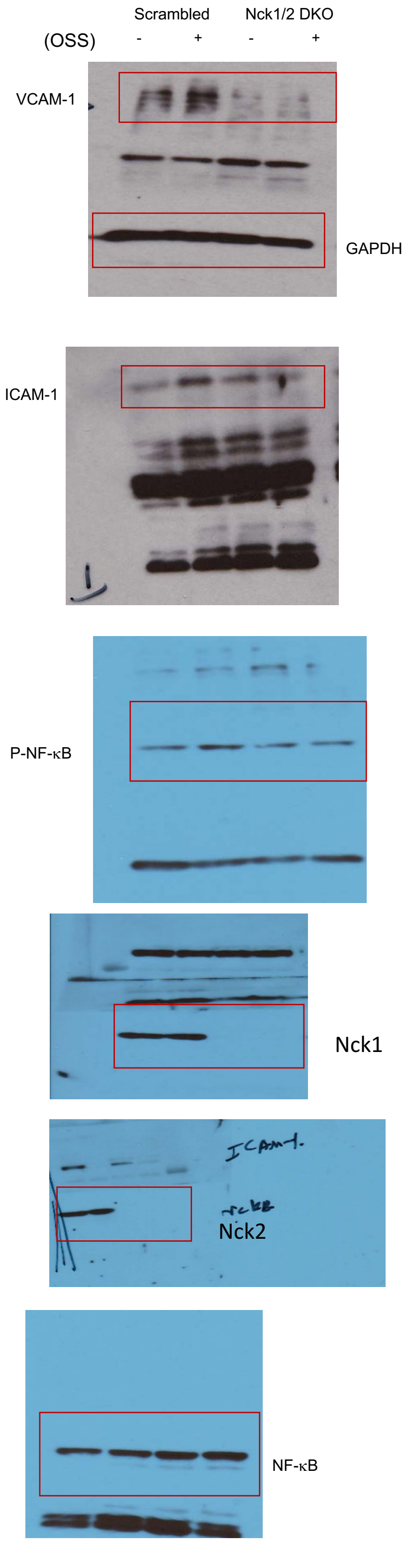
G



Supplemental Figure 1.

H



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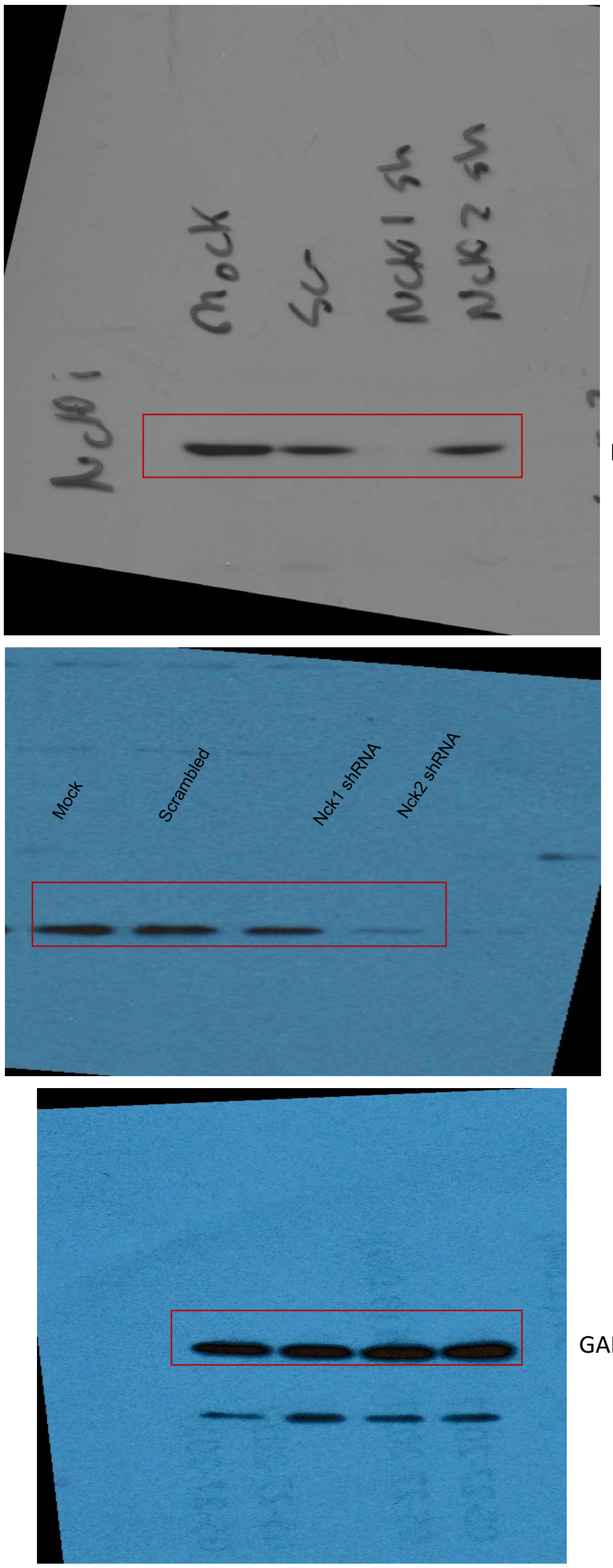

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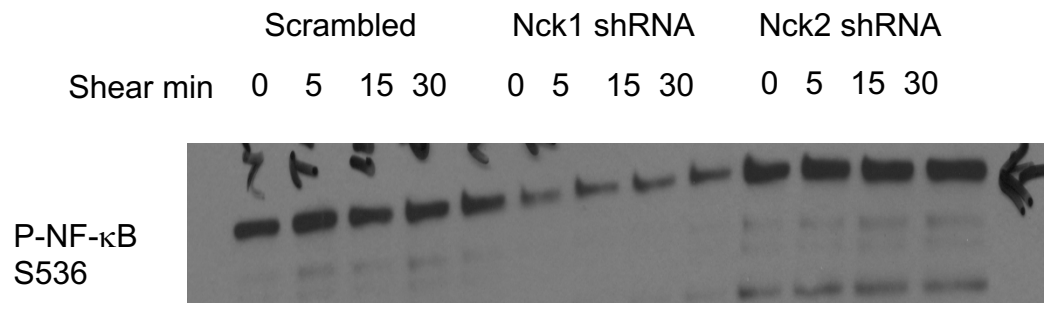
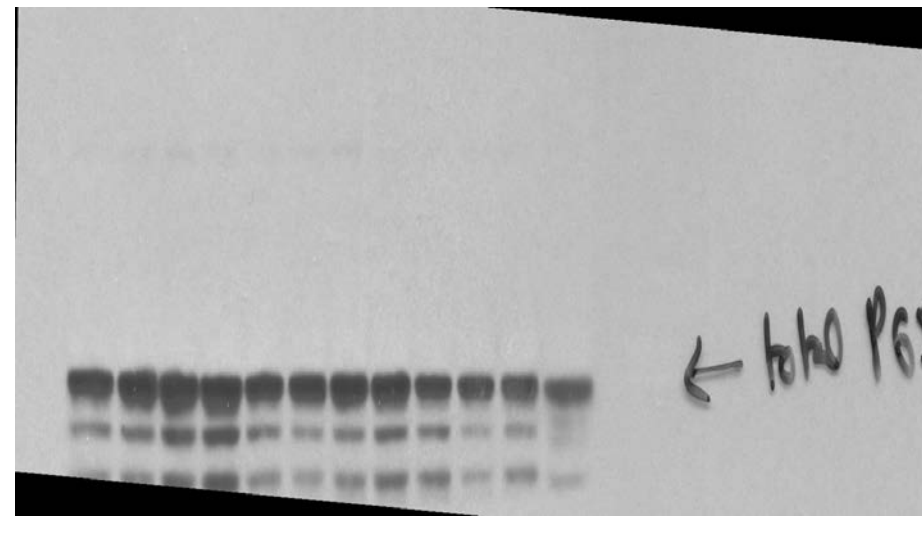
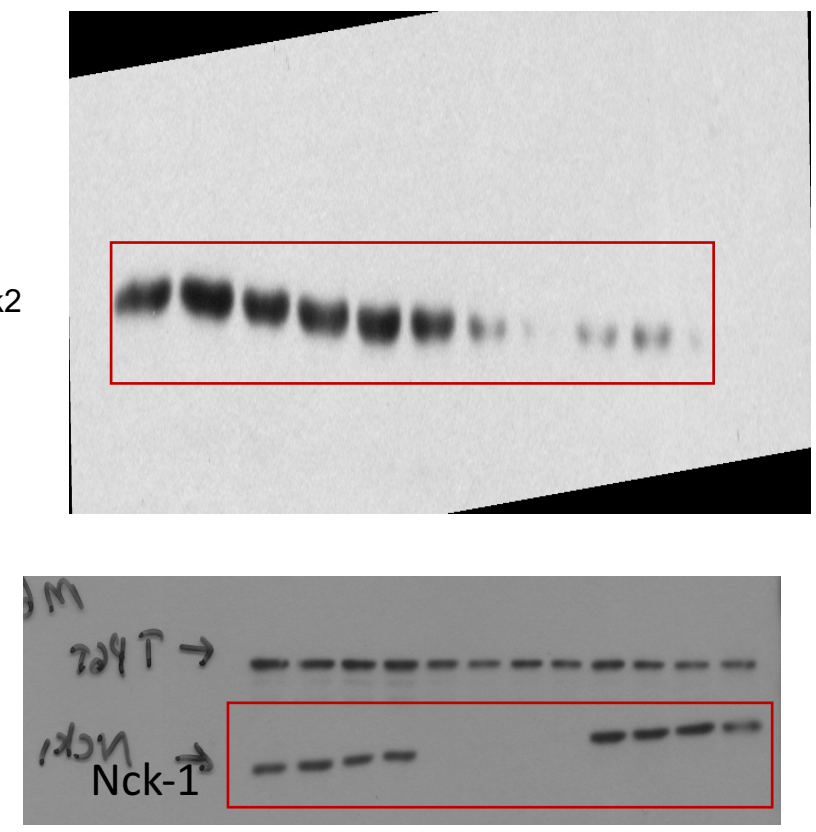
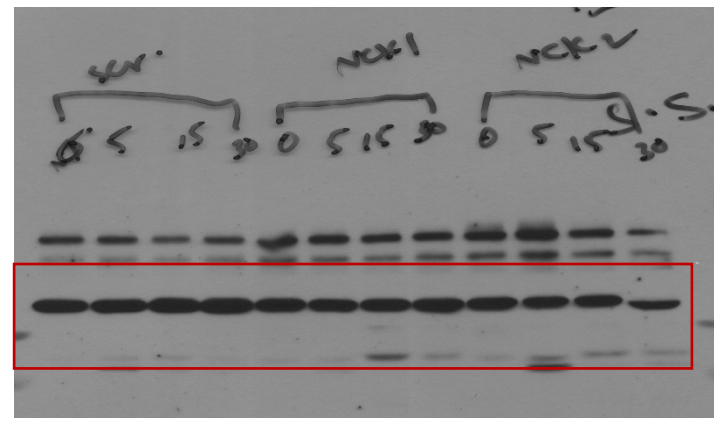


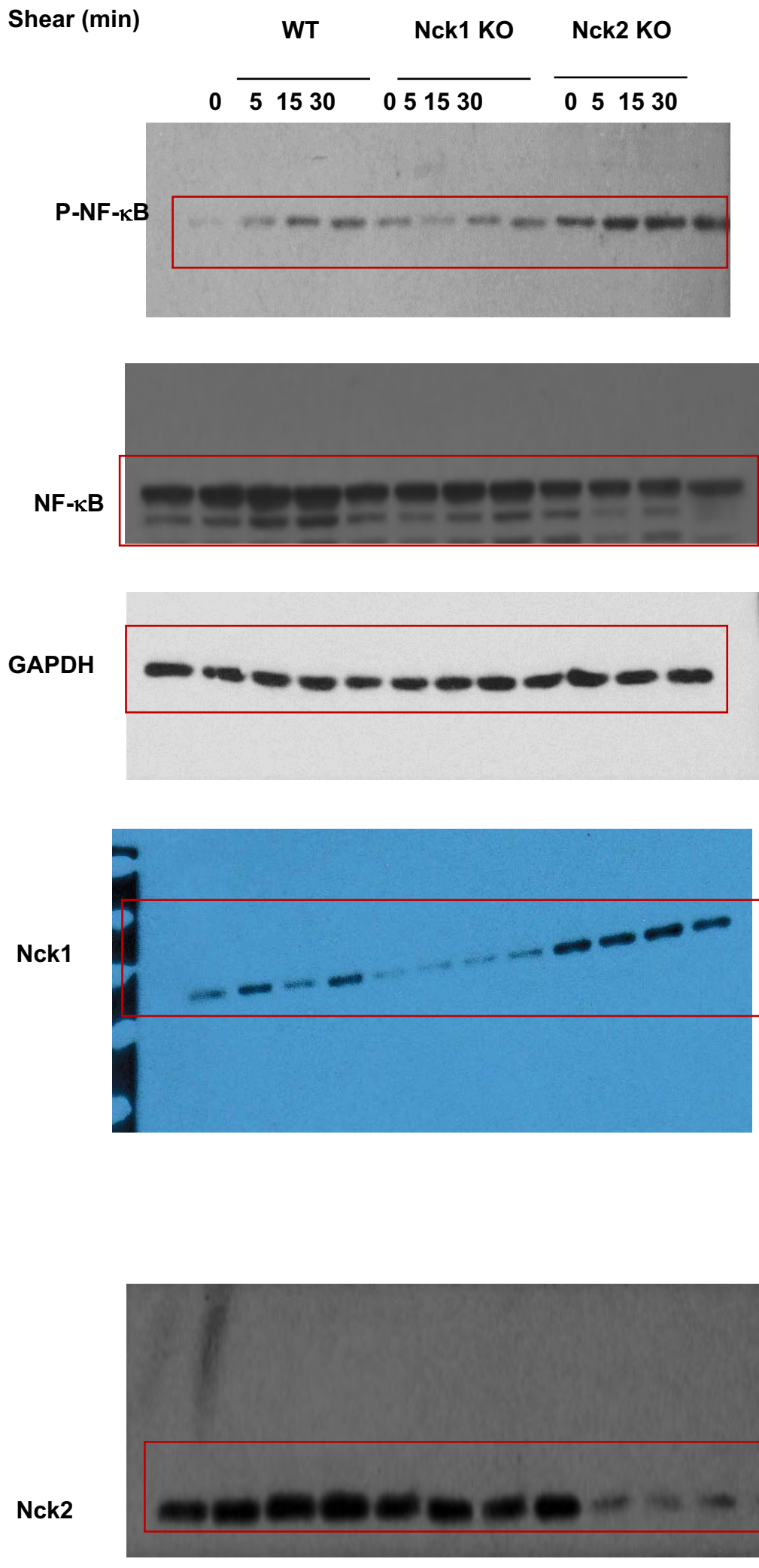






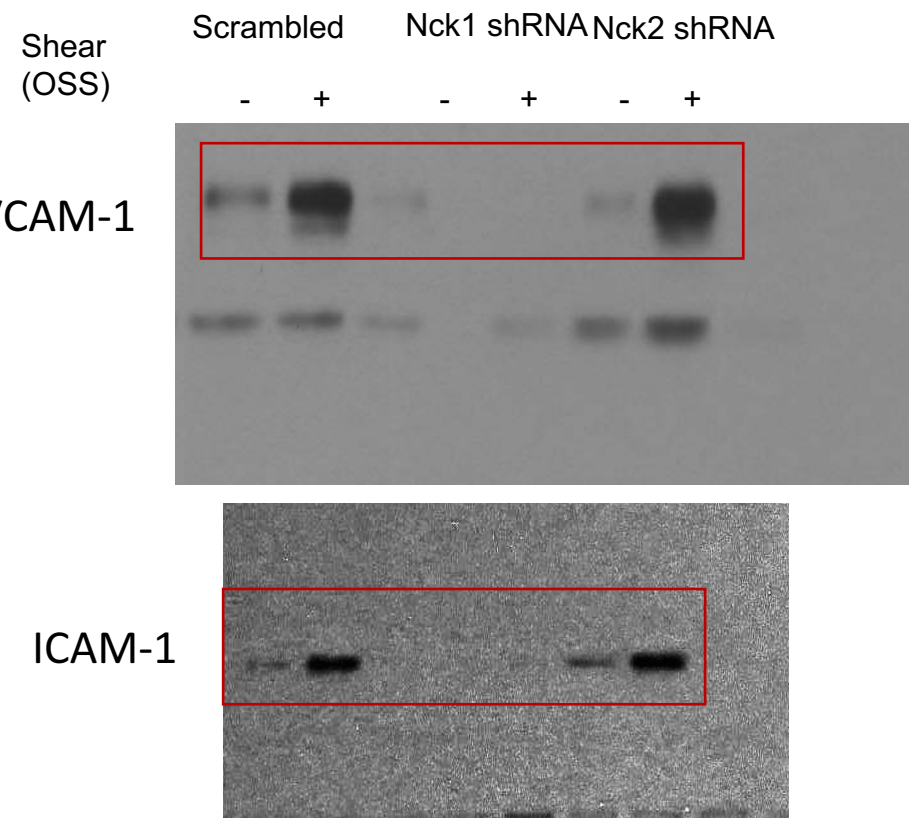
Supplemental Figure 2.

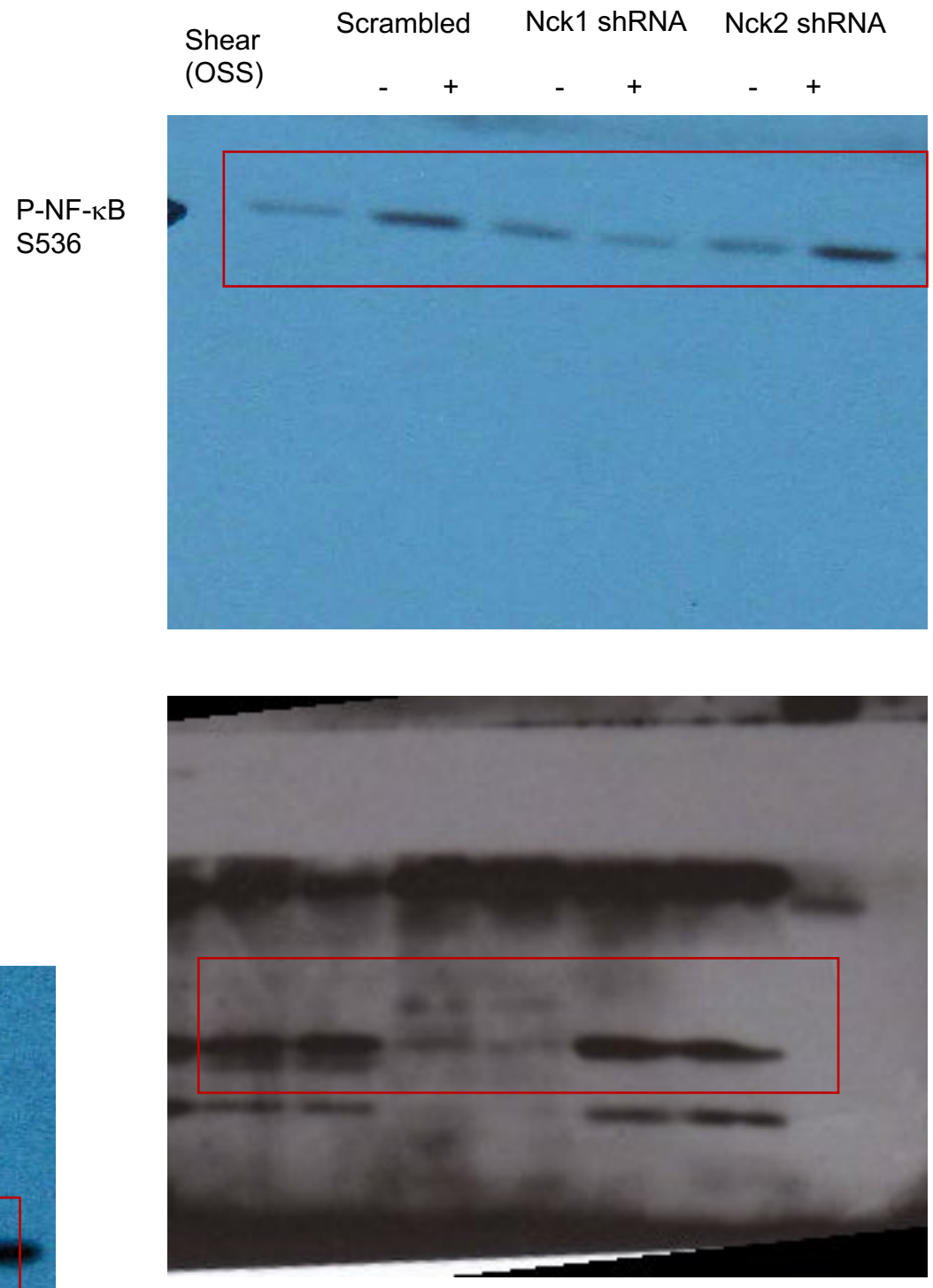
H.

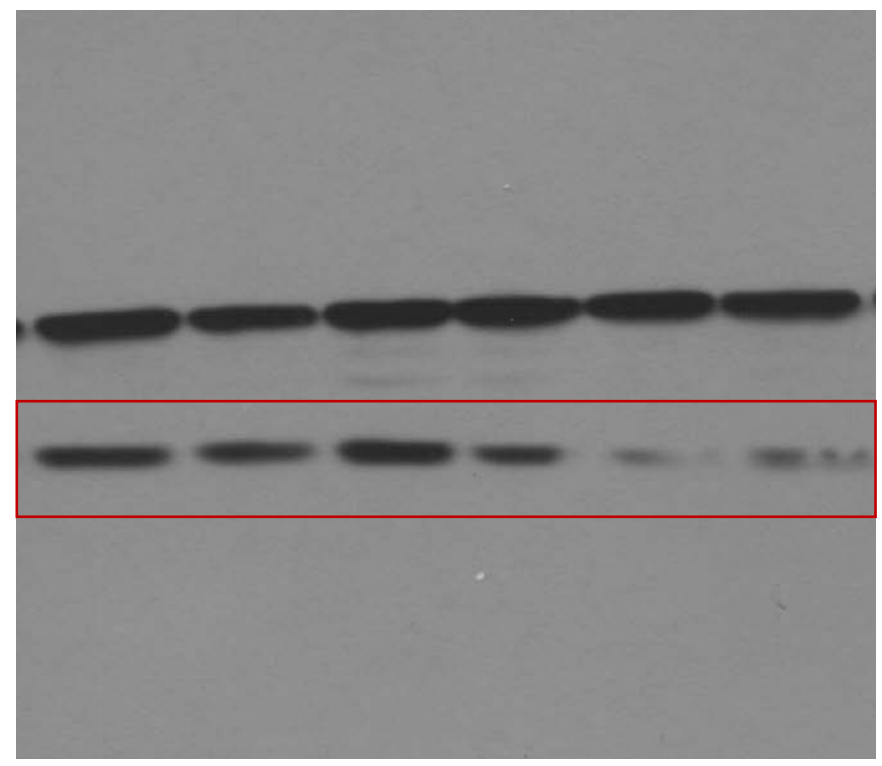


Supplemental Figure 2.

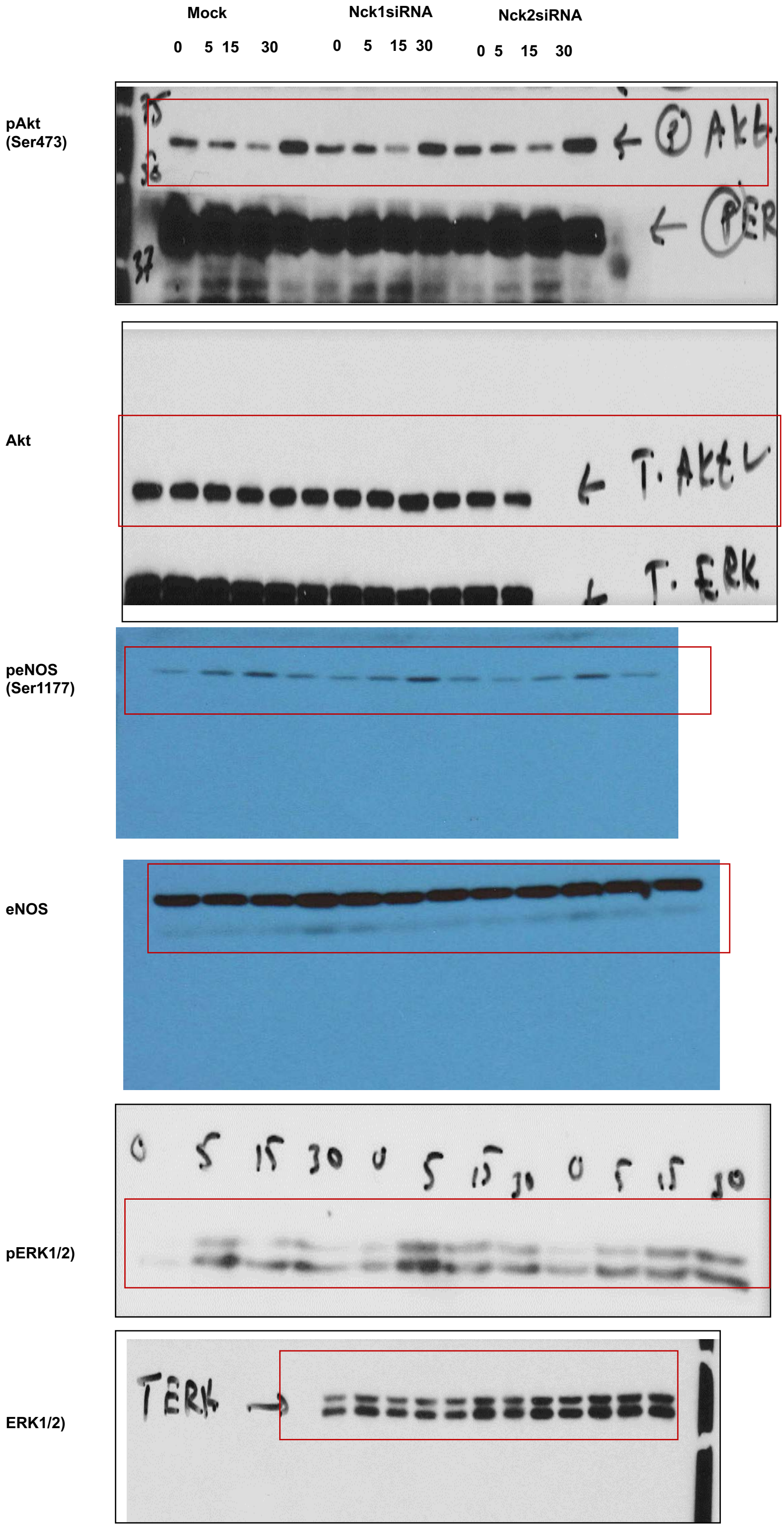
I.







Supplemental Figure 3.



Supplemental Figure 11