A critical role for IL-1β in DNA damage-induced mucositis

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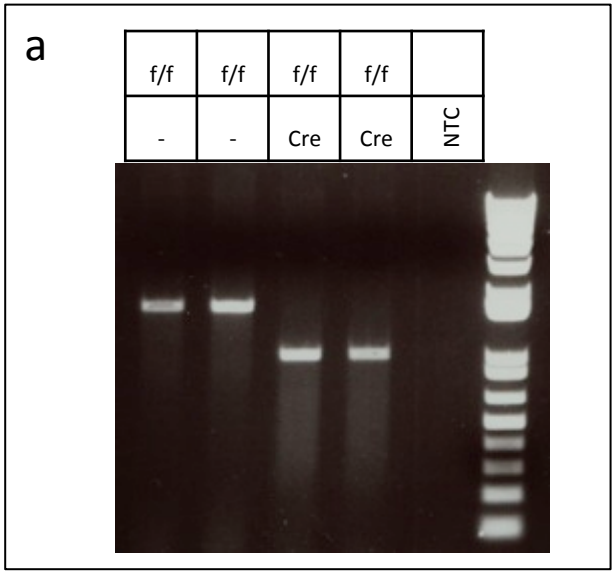
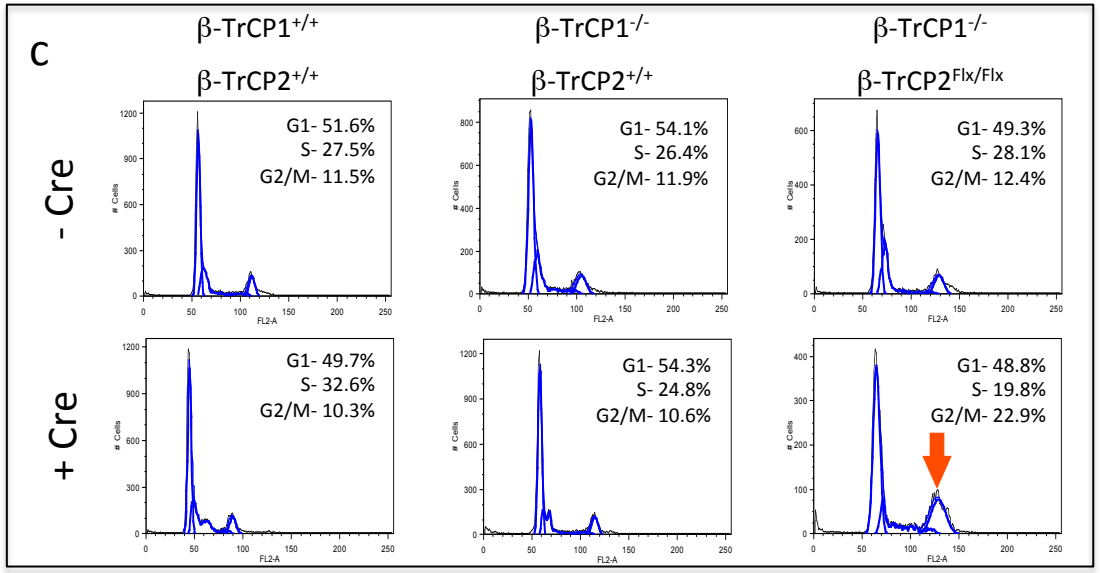
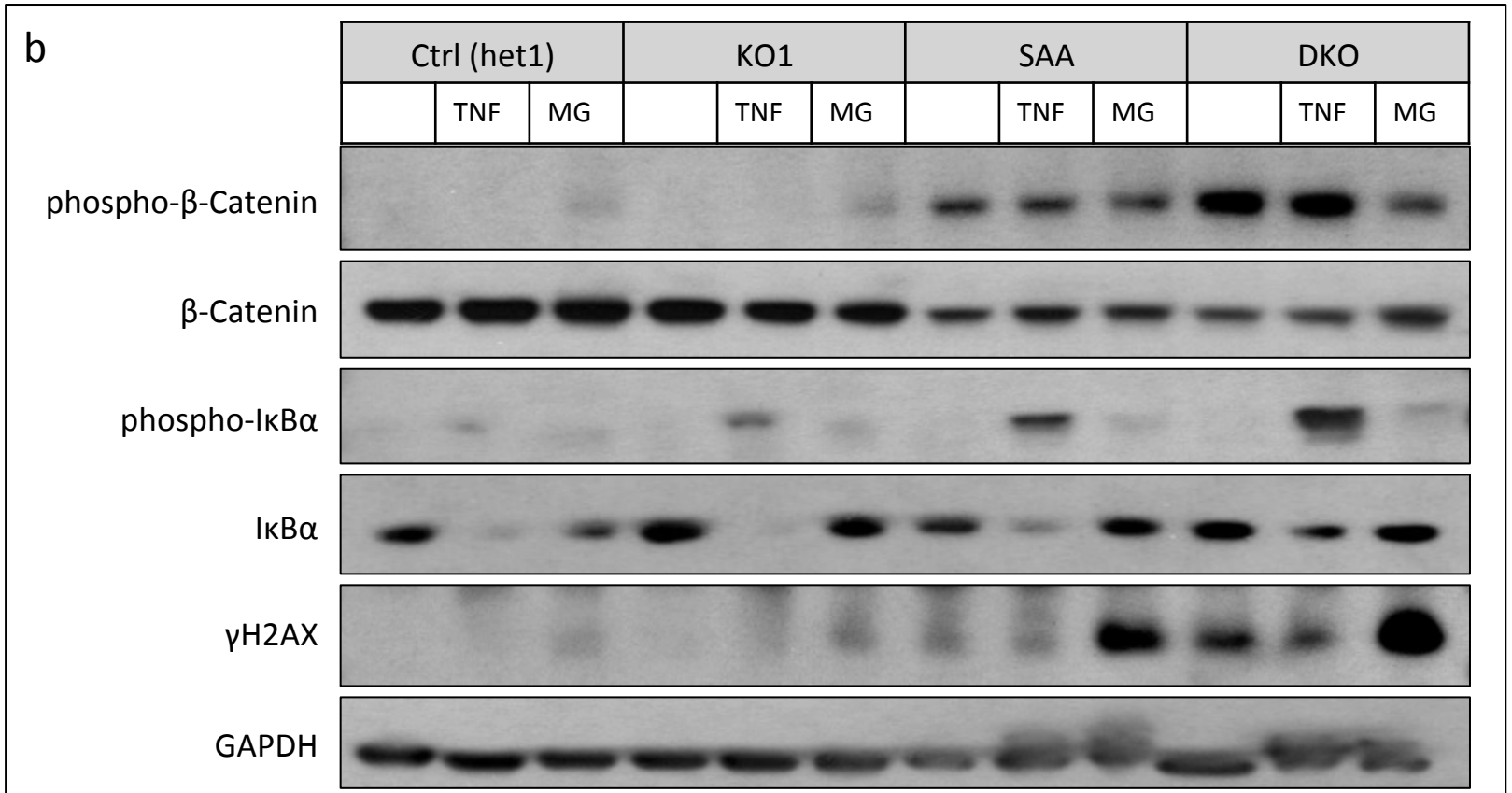
Supplementary Figures

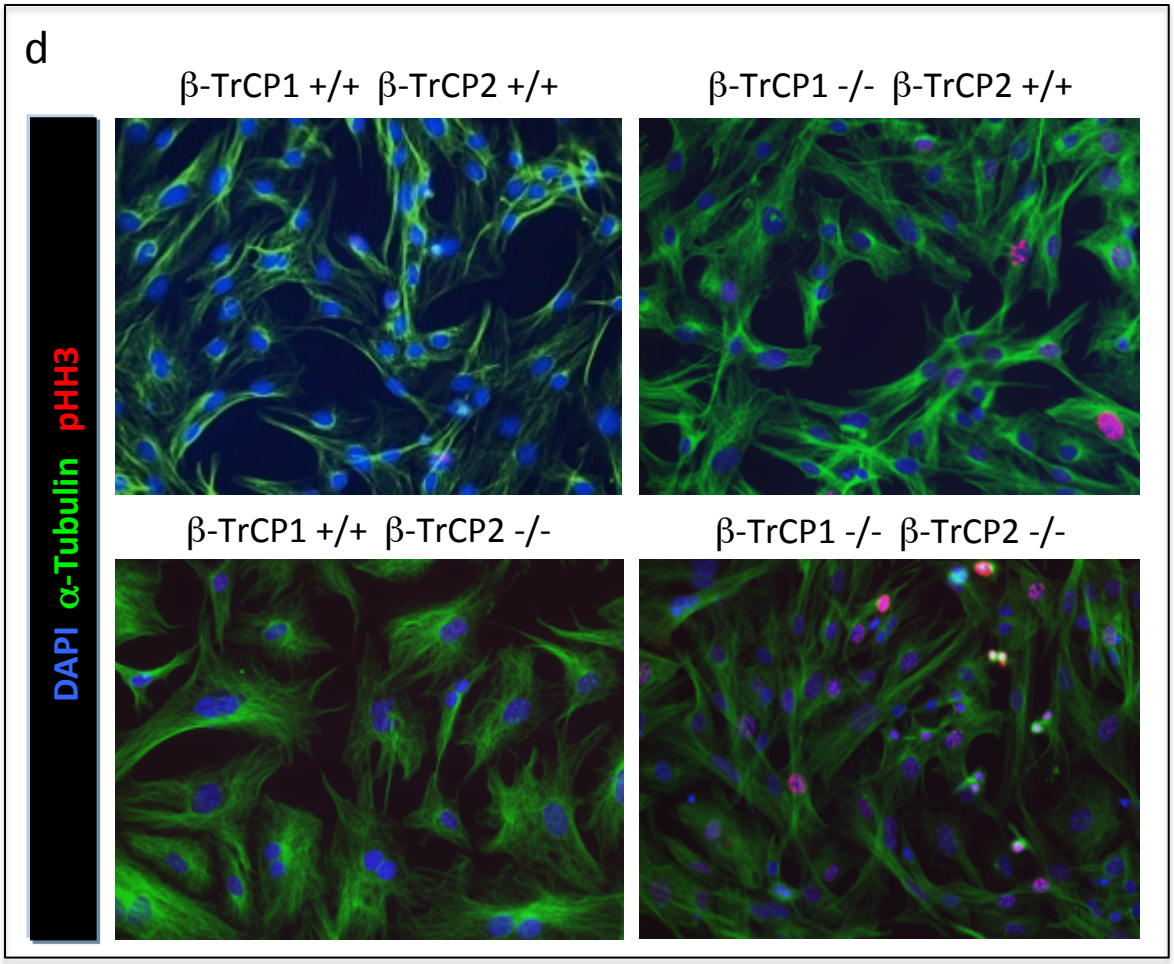
Supp Fig. 1


a
f/f
f/f
f/f
f/f
-
-
Cre
Cre
NTC

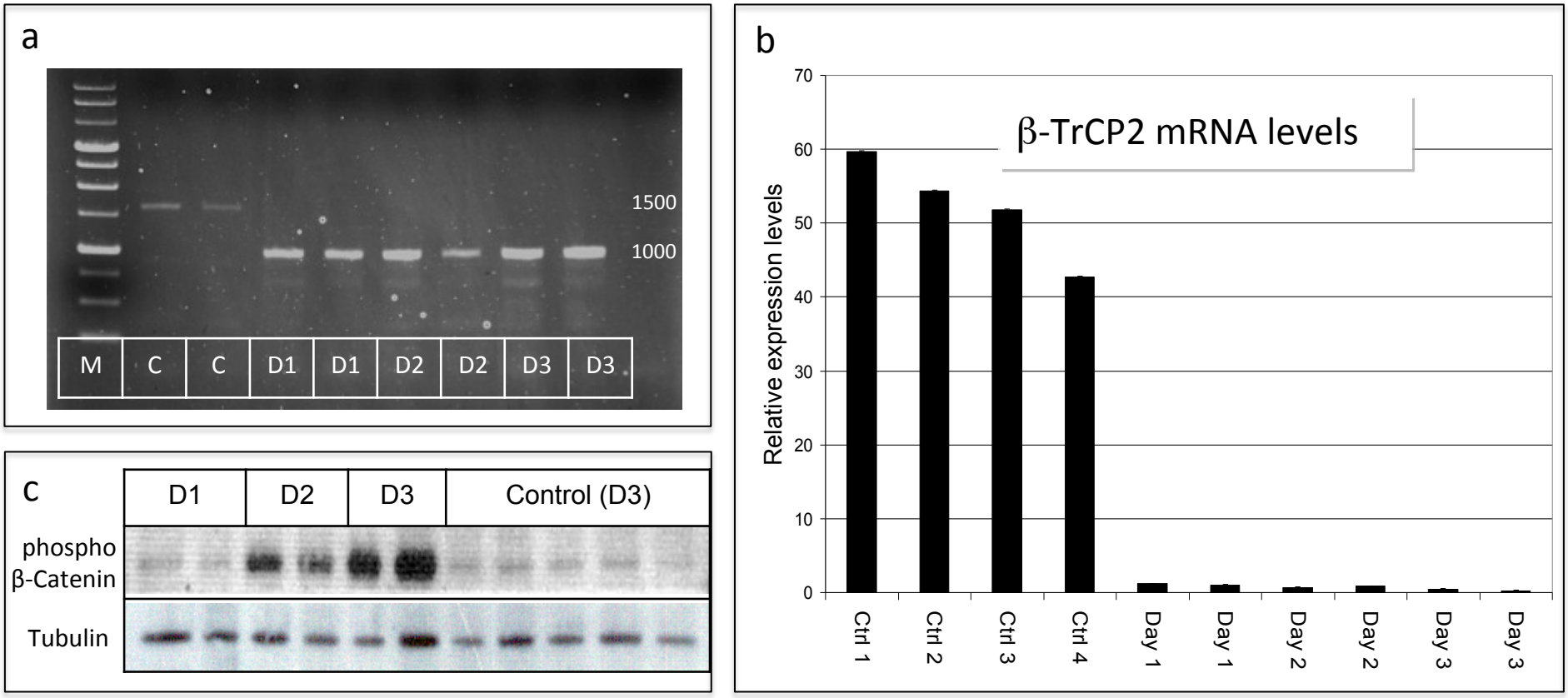

c
β-TrCP1+/+
β-TrCP1-/-
β-TrCP1-/-
β-TrCP2+/+
β-TrCP2+/+
β-TrCP2Flx/Flx
- Cre
+ Cre
G1- 51.6%
S- 27.5%
G2/M- 11.5%
G1- 54.1%
S- 26.4%
G2/M- 11.9%
G1- 49.3%
S- 28.1%
G2/M- 12.4%
G1- 49.7%
S- 32.6%
G2/M- 10.3%
G1- 54.3%
S- 24.8%
G2/M- 10.6%
G1- 48.8%
S- 19.8%
G2/M- 22.9%


b
Ctrl (het1)
KO1
SAA
DKO
TNF
MG
phospho-β-Catenin
β-Catenin
phospho-IκBα
IκBα
γH2AX
GAPDH

Supp
Fig. 1

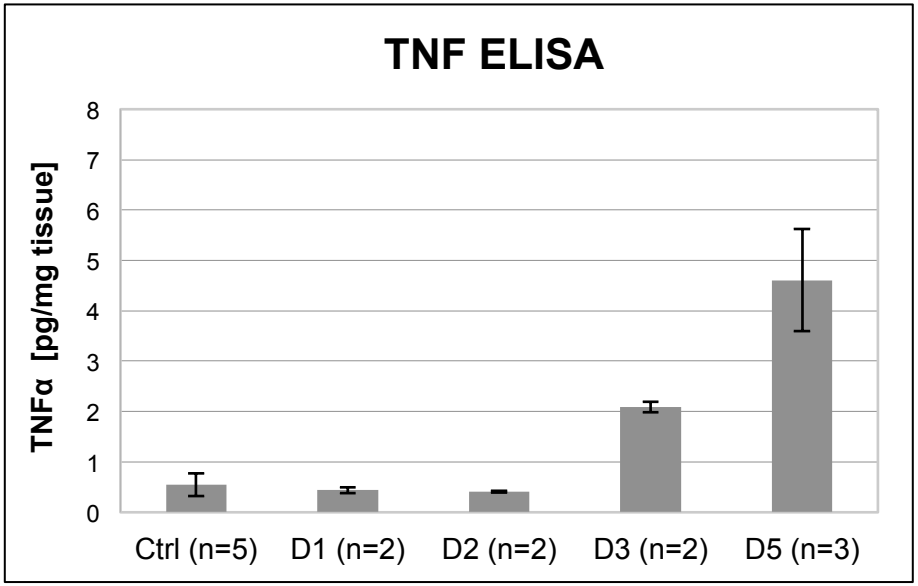

d
β-TrCP1 +/+ β-TrCP2 +/+
β-TrCP1 -/- β-TrCP2 +/+
β-TrCP1 +/+ β-TrCP2 -/-
β-TrCP1 -/- β-TrCP2 -/-
DAPI α-Tubulin pHH3

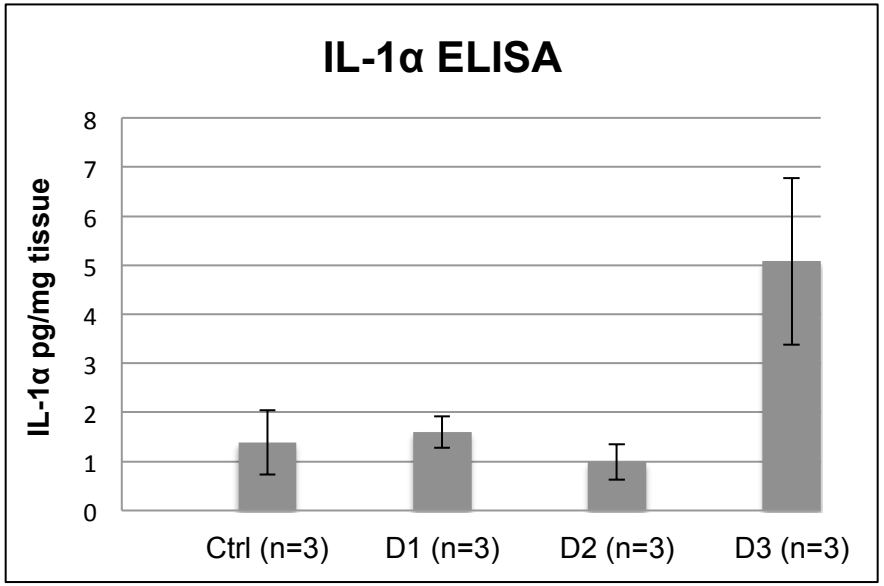
Supp Fig. 2


a
1500
1000
M
C
C
D1
D1
D2
D2
D3
D3
b
β-TrCP2 mRNA levels
Relative expression levels
70
60
50
40
30
20
10
0
Ctrl 1
Ctrl 2
Ctrl 3
Ctrl 4
Day 1
Day 1
Day 2
Day 2
Day 3
Day 3
c
D1
D2
D3
Control (D3)
phospho β-Catenin
Tubulin

Supp
Fig. 3

a


TNF ELISA
TNFα [pg/mg tissue]
8
7
6
5
4
3
2
1
0
Ctrl (n=5)
D1 (n=2)
D2 (n=2)
D3 (n=2)
D5 (n=3)


IL-1α ELISA
IL-1α pg/mg tissue
8
7
6
5
4
3
2
1
0
Ctrl (n=3)
D1 (n=3)
D2 (n=3)
D3 (n=3)

Supp
Fig. 3

b. Anti-TNF treatment (Enbrel)

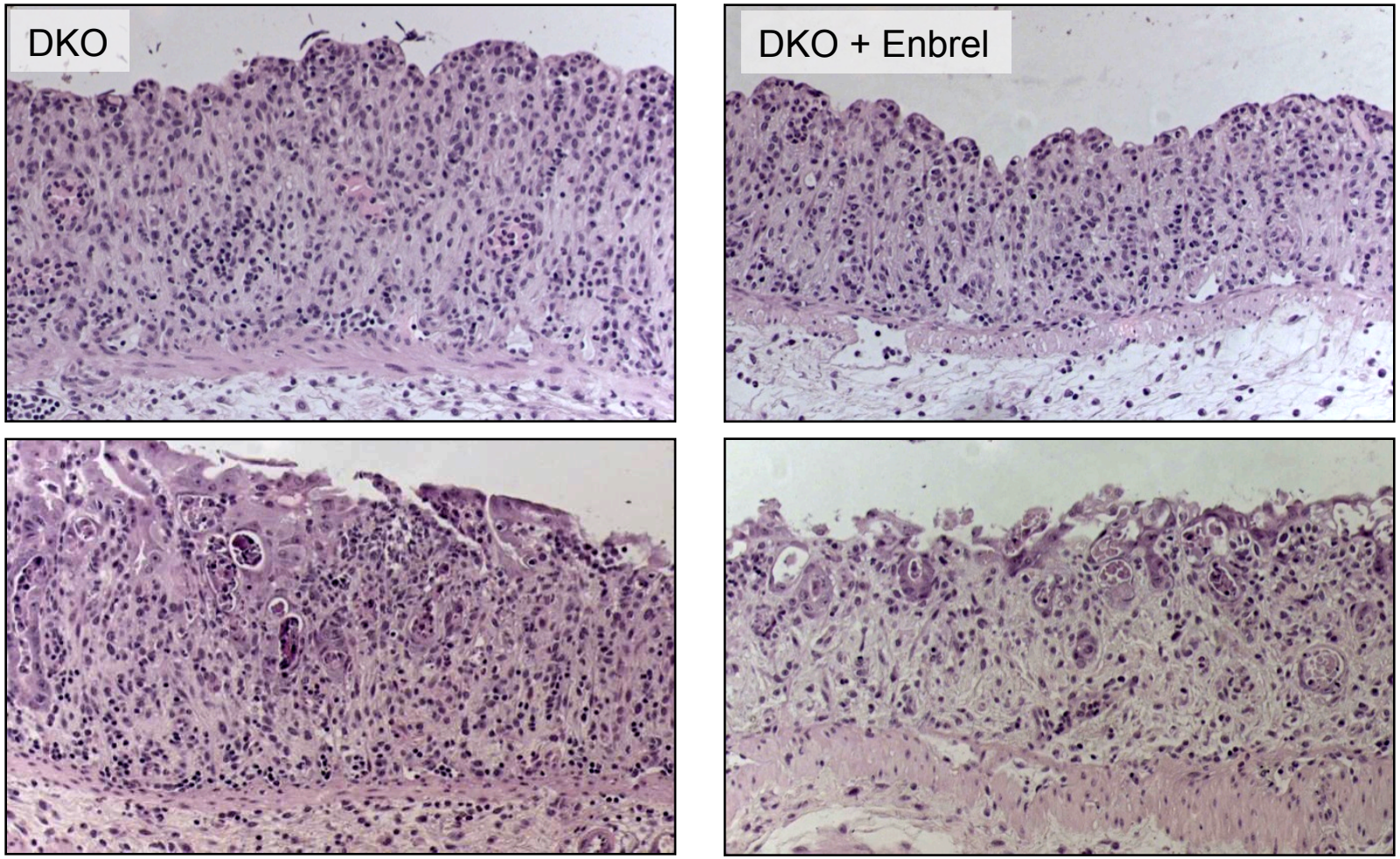


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Fig. 3

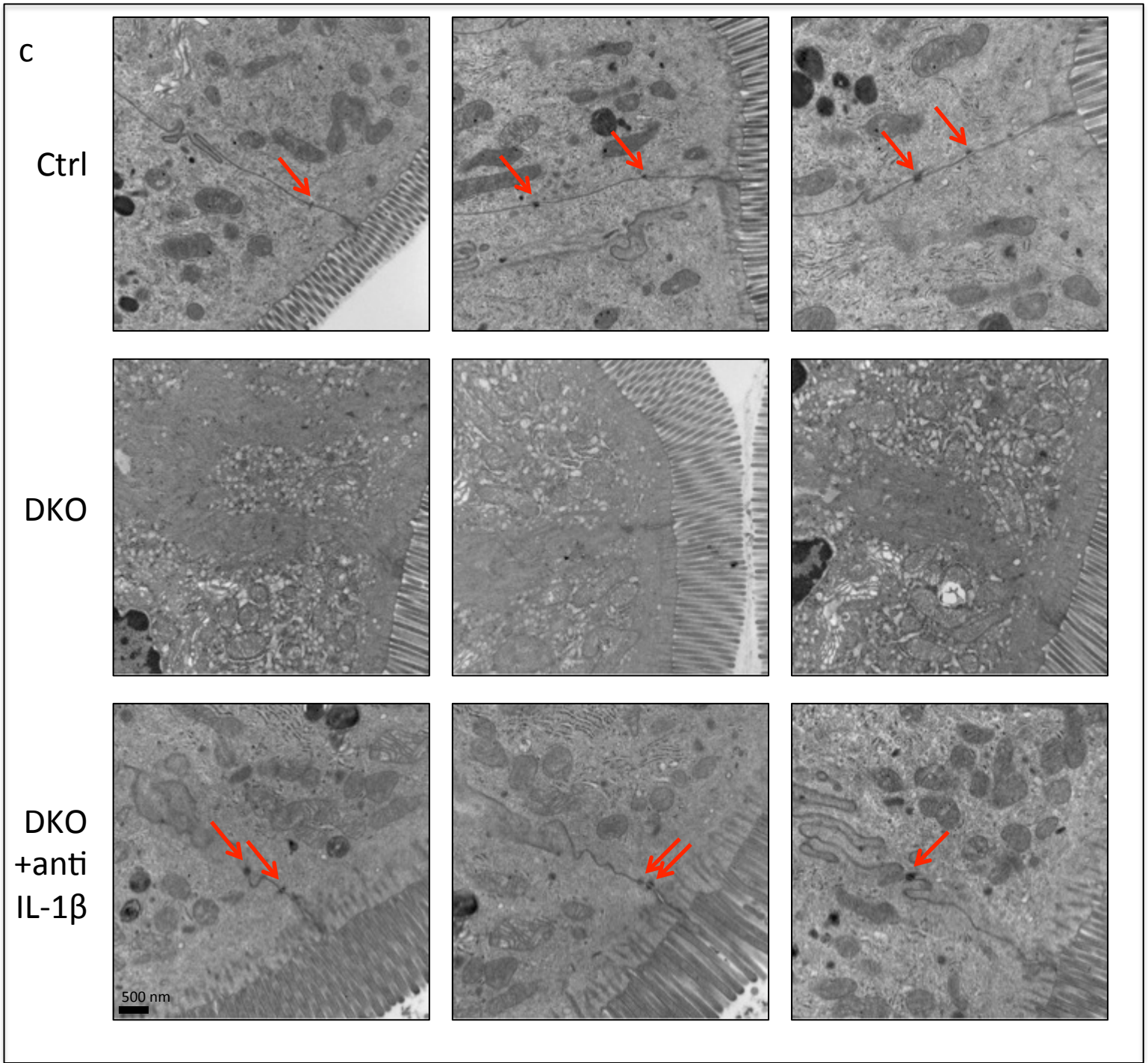

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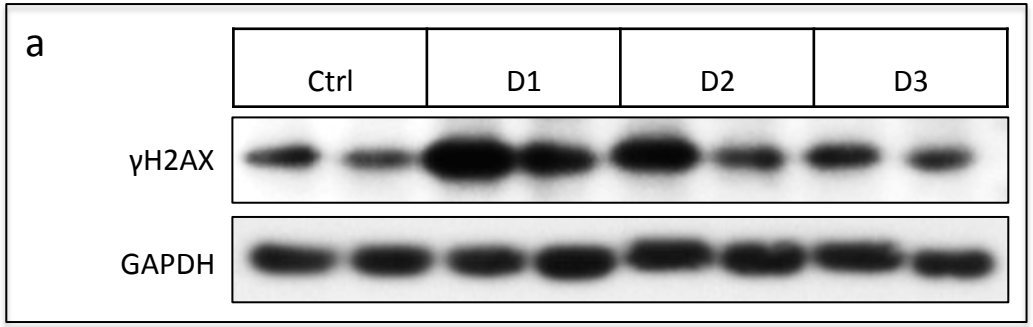
Fig. 4



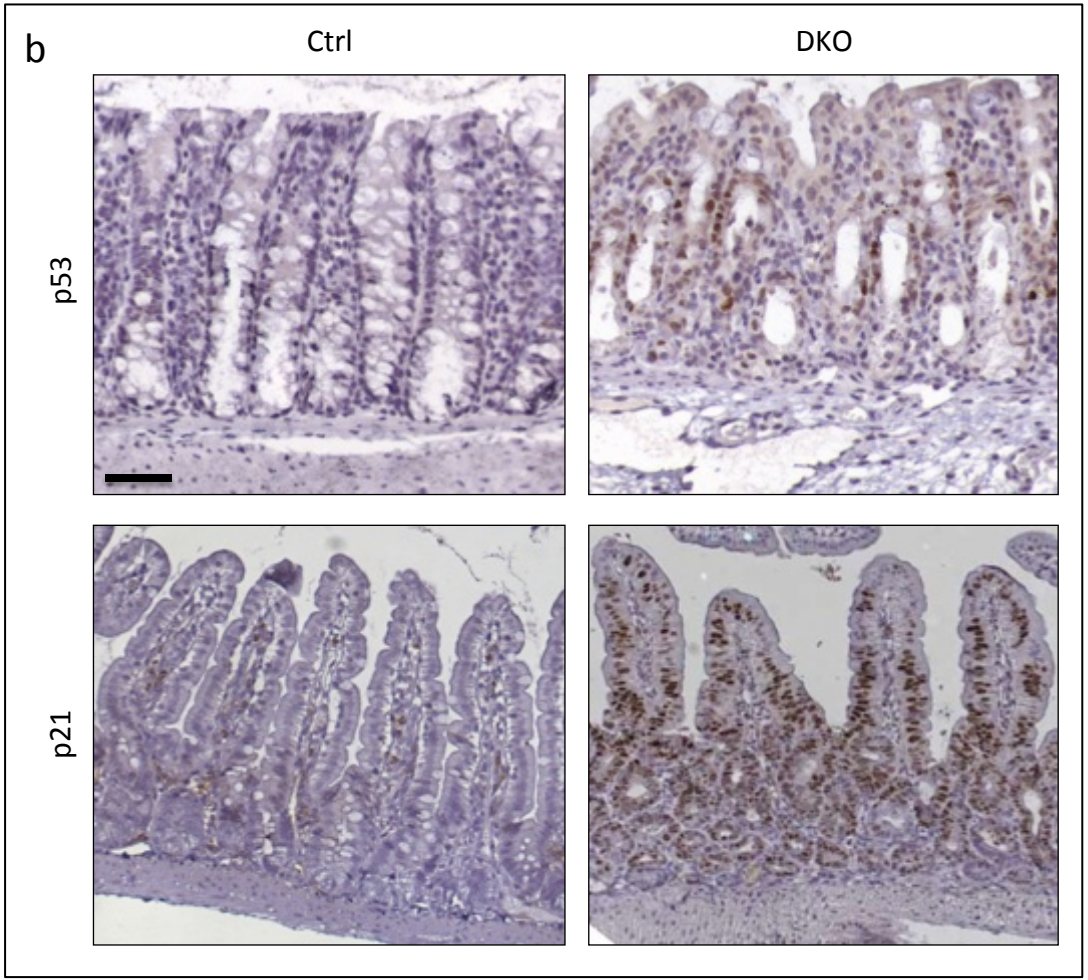


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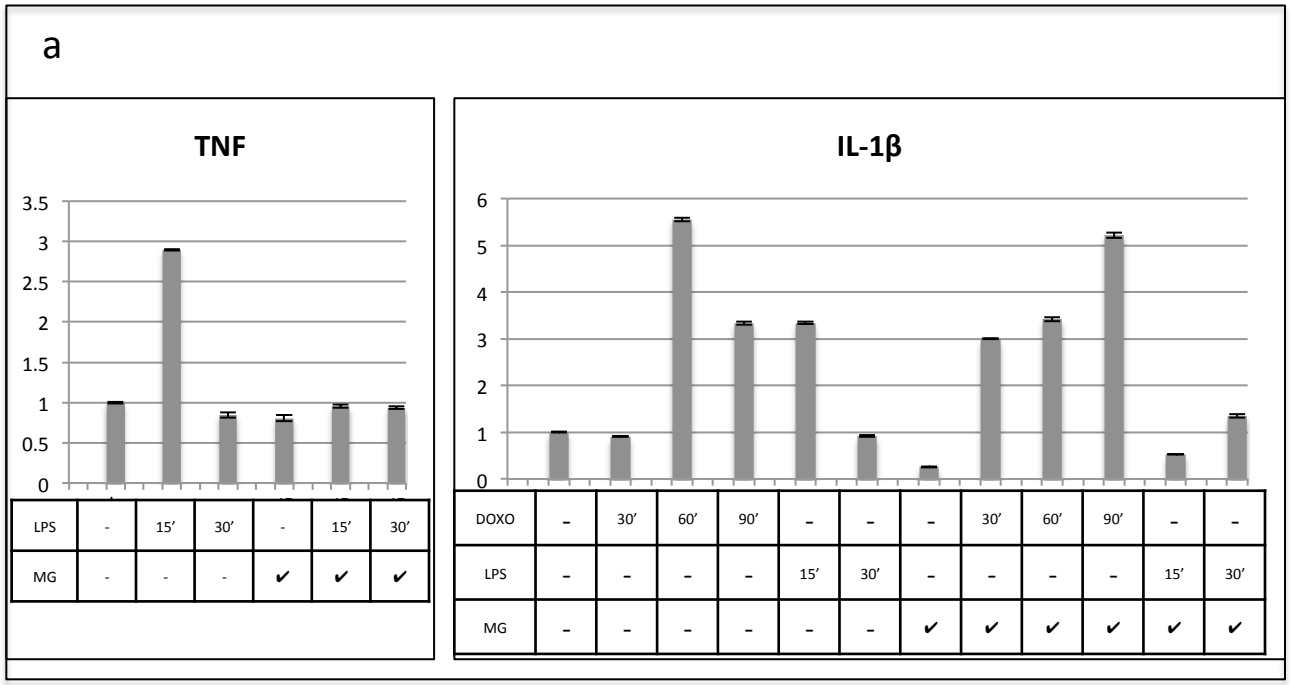
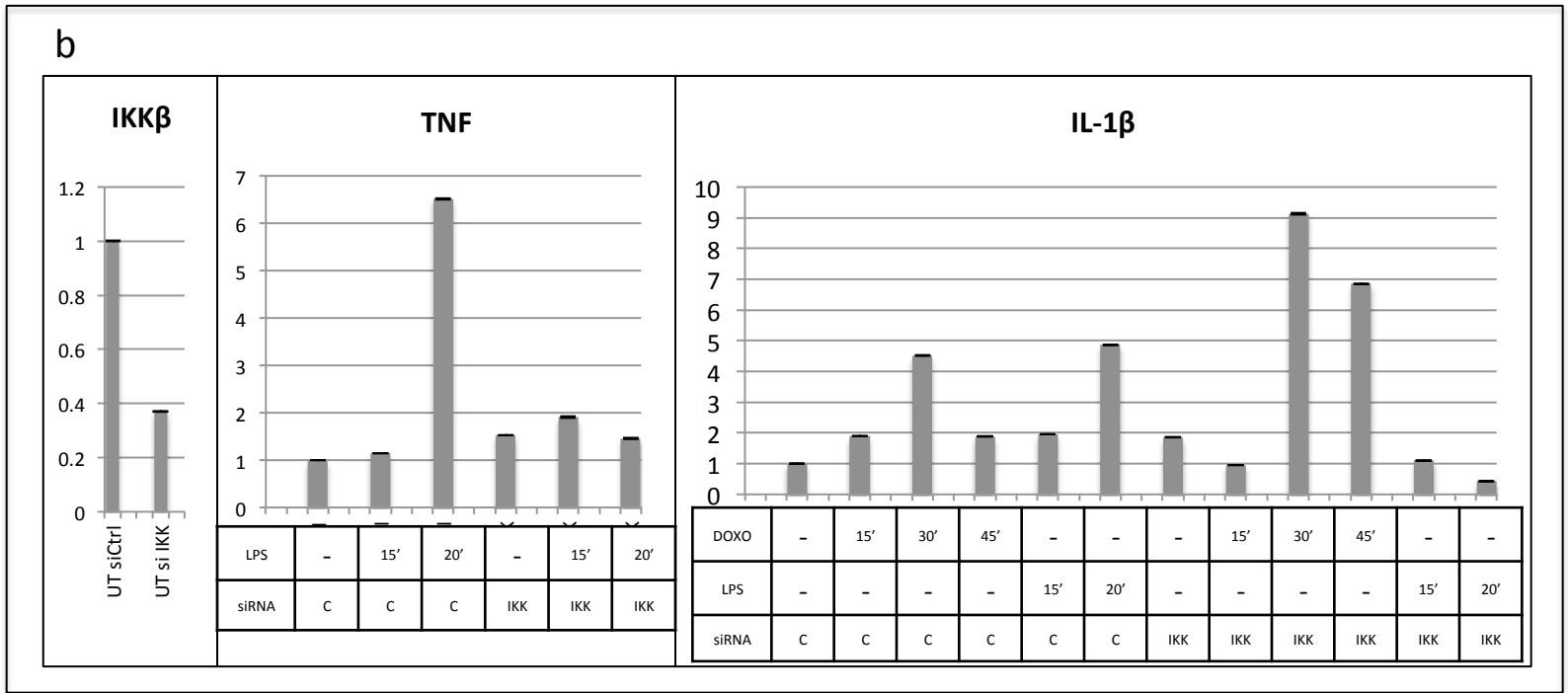
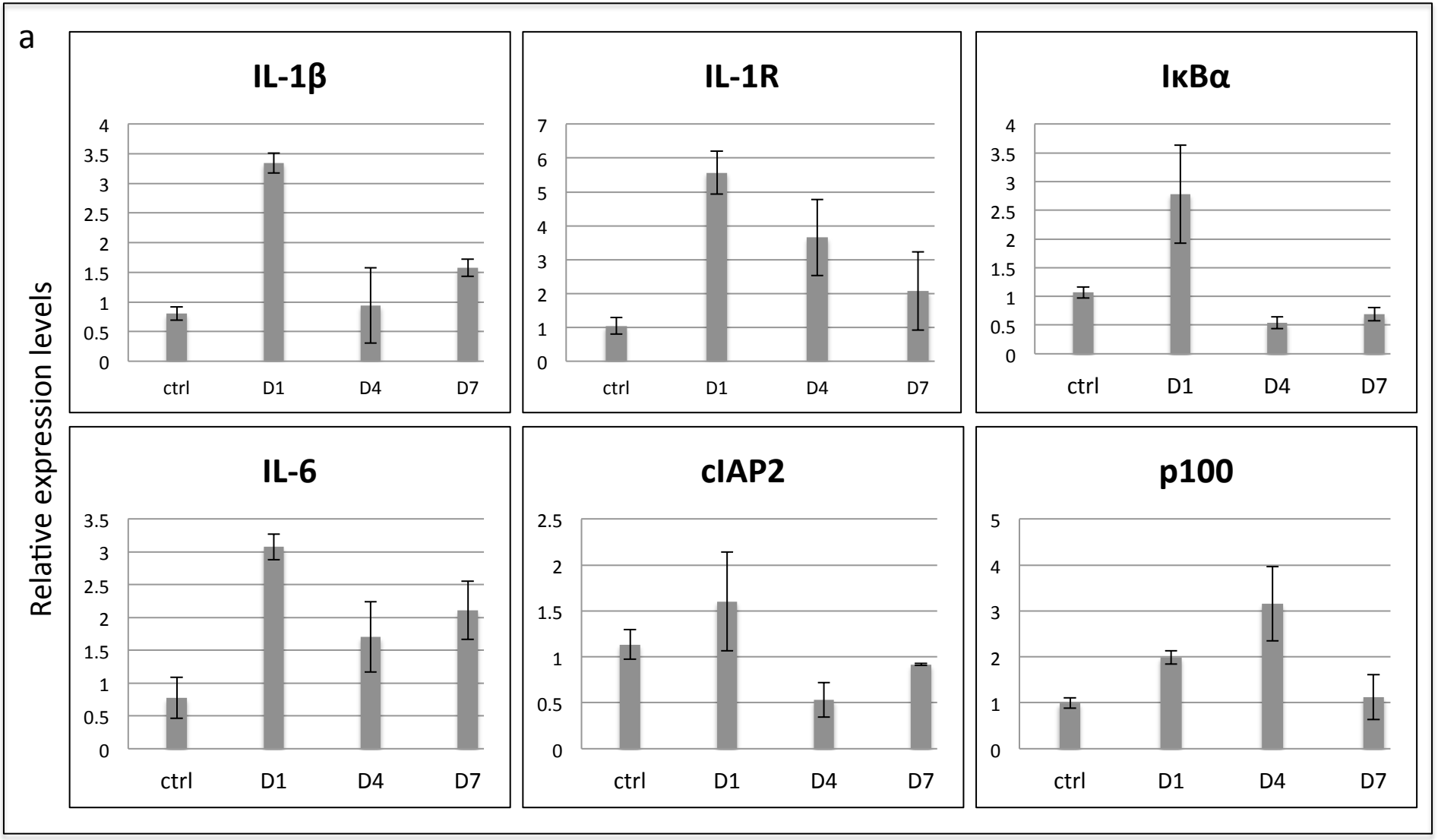
Fig. 5

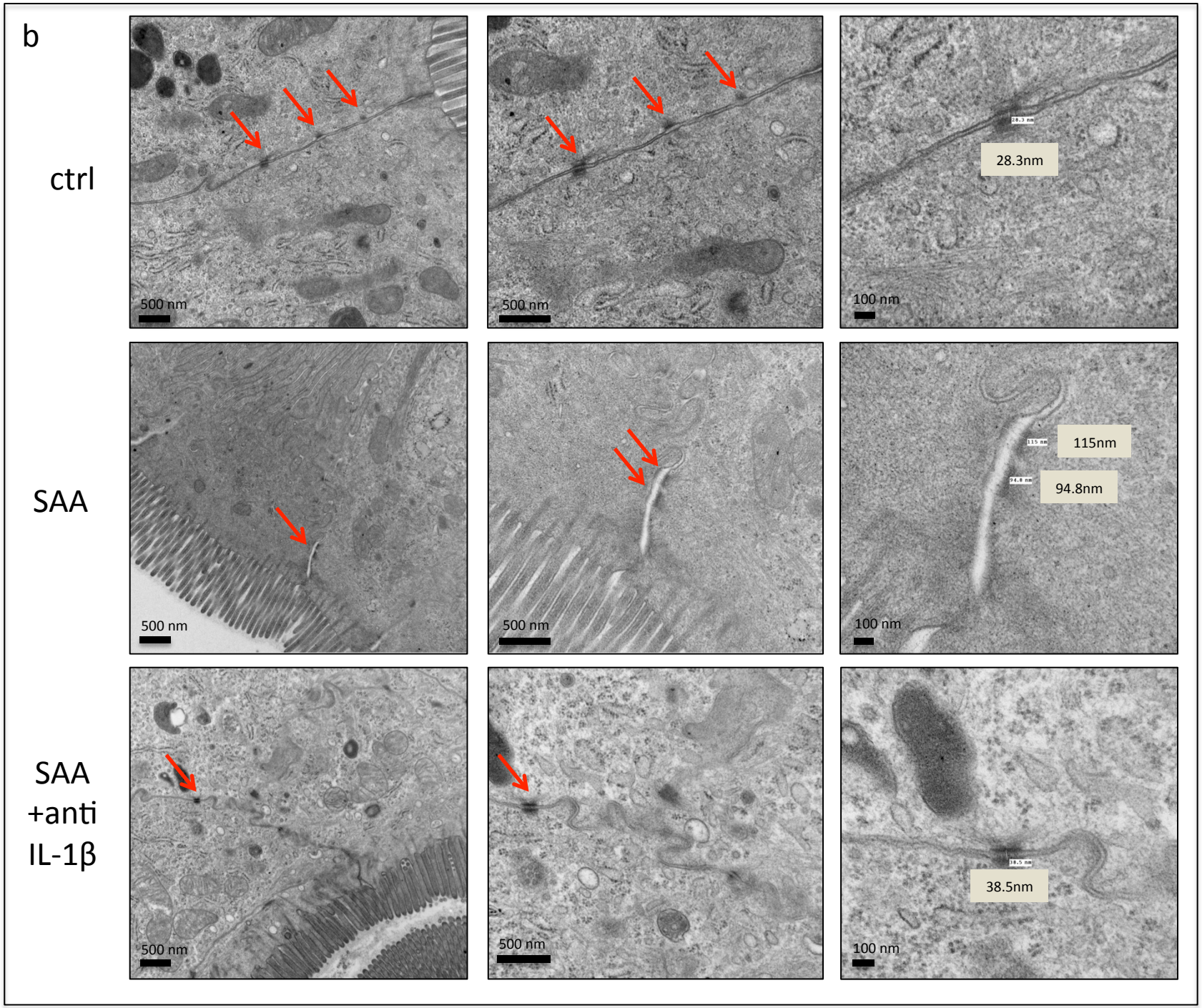

a
TNF
3.5
3
2.5
2
1.5
1
0.5
0
LPS - 15′ 30′ - 15′ 30′
MG - - - ✔ ✔ ✔
IL-1β
6
5
4
3
2
1
0
DOXO – 30′ 60′ 90′ – – – 30′ 60′ 90′ – –
LPS – – – – 15′ 30′ – – – – 15′ 30′
MG – – – – – – ✔ ✔ ✔ ✔ ✔ ✔


b
IKKβ
1.2
1
0.8
0.6
0.4
0.2
0
UT siCtrl
UT si IKK
TNF
7
6
5
4
3
2
1
0
LPS – 15′ 20′ – 15′ 20′
siRNA C C C IKK IKK IKK
IL-1β
10
9
8
7
6
5
4
3
2
1
0
DOXO – 15′ 30′ 45′ – – – 15′ 30′ 45′ – –
LPS – – – – 15′ 20′ – – – – 15′ 20′
siRNA C C C C C C IKK IKK IKK IKK IKK IKK

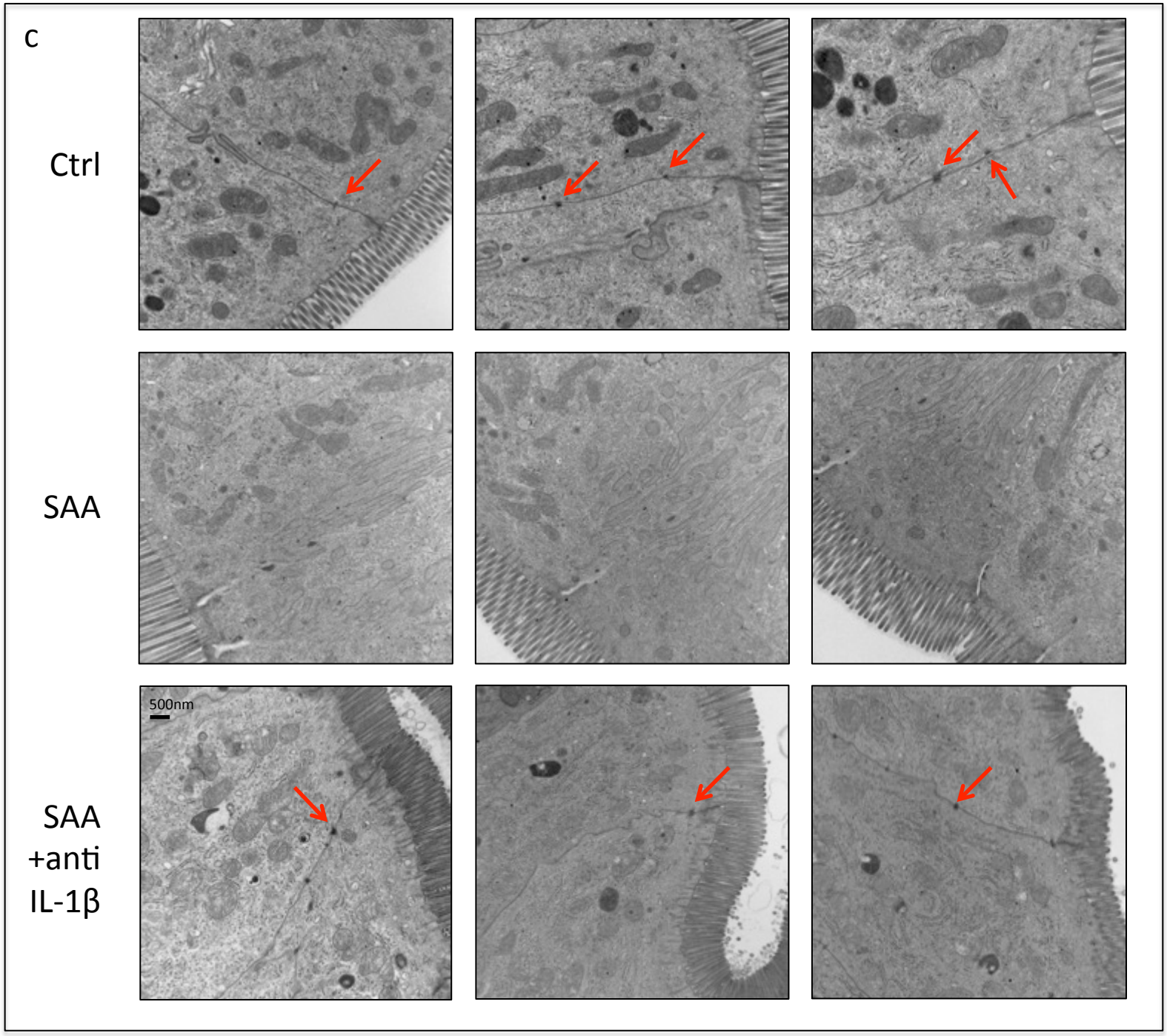
Supp
Fig. 6


a
Relative expression levels
IL-1β
IL-1R
IκBα
IL-6
cIAP2
p100
ctrl
D1
D4
D7

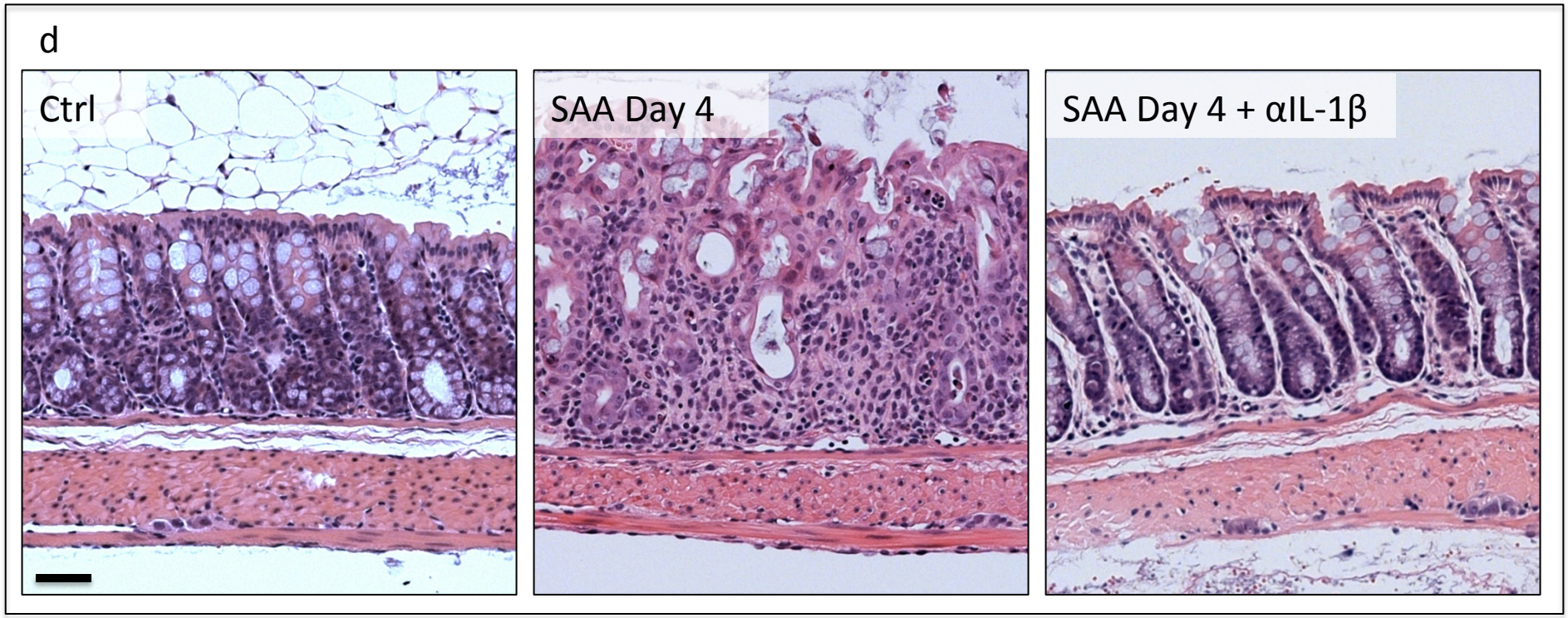
Supp Fig. 6

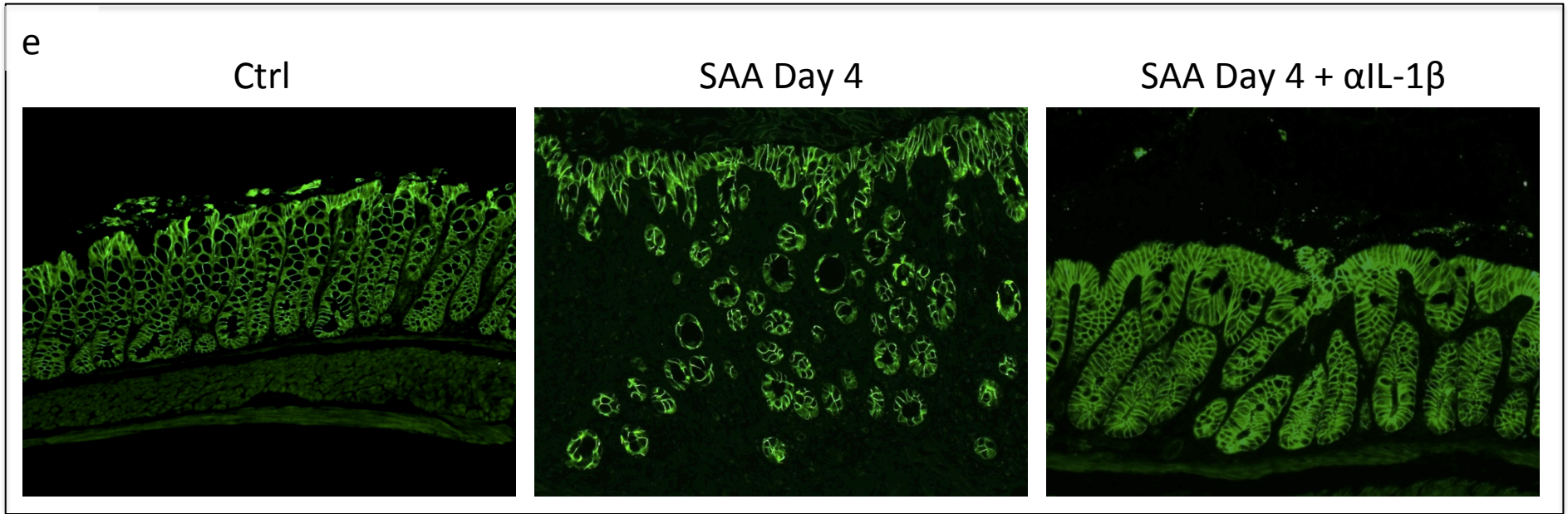

b
ctrl
SAA
SAA +anti IL-1β
500 nm
500 nm
28.3nm
100 nm
500 nm
500 nm
115nm
94.8nm
100 nm
500 nm
500 nm
38.5nm
100 nm

Supp
Fig. 6



Supplementary Fig. 6





Supplementary Figure legend

Supplementary Fig. 1

a. β-TrCP2 knockout validation by PCR. Exon 4 is flanked by Flox sequences and is deleted following Adeno-Cre infection (10^6 MOI, 48 hours) of β-TrCP2$^{f/f}$ MEFs. Undeleted band is 1500bp, deleted band is 1000bp. Table upper row indicates β-TrCP2 MEF genotype, lower row indicates Adeno-Cre infection.

b. Western blot analysis of phosphorylated β-catenin and phosphorylated IκBα, β-TrCP's bona fide substrates. MEF analyzed are Ctrl (Het1– β-TrCP1$^{+/-}$, β-TrCP2$^{f/f}$, no Cre), KO1 (β-TrCP1$^{-/-}$, β-TrCP2$^{f/f}$, no Cre), SAA (single active allele - β-TrCP1$^{+/-}$, β-TrCP2$^{f/f}$, Cre), DKO (β-TrCP1$^{-/-}$, β-TrCP2$^{f/f}$, Cre). MEFs were either left untreated, TNF treated (10nM, 15 minutes) or MG132 treated (10nM, 3.5 hours). Phosphorylated β-catenin and phosphorylated IκBα stabilized upon Cre-dependent deletion of β-TrCP2 on the background of β-TrCP1$^{+/-}$ (SAA) and more so when β-TrCP1 was completely absent (DKO). Fifth panel from top – western blot analysis of the DNA damage marker γH2AX. DNA damage is detected in SAA MEFs following MG132 treatment and in DKO MEFs. Bottom panel – GAPDH, loading control

c. FACS analysis of Propidium Iodide stained MEFs with the indicated genotypes. Cell count of G2/M MEFs is 2 fold higher in DKO cell population compared with controls.

d. IF of the mitotic marker pHH3 (red) reveals high numbers of mitotic cells in the DKO MEF population and α-Tubulin (green) discloses aberrations in the DNA-spindles axis. DNA is stained in blue by DAPI.

Supplementary Fig. 2

a. PCR results distinguish between β-TrCP2 undeleted allele (1,500bp) and exon 4-deleted allele (1,000bp). C = control mice (β-TrCP1$^{+/-}$, β-TrCP2$^{f/f}$, no Cre), 72 hours after Tamoxifen injection. D1, D2, D3 = DKO (β-TrCP1$^{-/-}$, β-TrCP2$^{f/f}$, Villin-Cre-ER^{T2}), 24, 48 and 72 hours after Tamoxifen injections, respectively. No trace of the undeleted allele in

Day 1 DKO mice indicates full deletion of exon 4 as soon as 24 hours after knockout induction.

b. qPCR of enterocytes from the indicated mice; Control mice were injected with Tamoxifen but harbor no Cre. Days 1,2,3, are 24, 48 and 72 hours after Tamoxifen injection. The deletion of exon 4 induced by Tamoxifen injection results in a truncated, unstable RNA product.

c. Western blot analysis of phosphorylated β-catenin, a well known substrate of β-TrCP. Samples were produced from enterocytes isolated from DKO mice 24, 48 and 72 hours after Tamoxifen injection and Control mice, 72 hours after injection.β-catenin is stabilized in enterocytes around 48 hours after knockout induction

Supplementary Fig. 3

a. TNF (left chart) and IL-1α (right chart) ELISA on intestinal samples of the indicated mice. An increase in TNF and IL-1α levels is observed only three days after knockout induction and is probably a later outcome of the inflammation rather than the initial cause for the barrier disruption.

b. Colon H&E staining of DKO mice treated (right panel) and untreated (left panel) with anti TNF regimen (Enbrel, or in its generic name - etanercept). The treatment had no preventive effect on the mucosa.

c. Aberrant tight junctions in DKO mice captured by electron microscopy. Additional mice for each group (control, DKO and DKO treated with neutralizing αIL-1β antibody) described in Fig. 3G. Original magnification X20,000. Red arrows indicate tight junctions.

Supplementary Fig. 4

a. Western blot analysis of γH2AX isolated enterocytes from the same mice as in Fig. 3A. A clear increase in γH2AX at day 1 that diminishes at days 2 and 3 possibly due to loss of γH2AX-positive dying cells in sample preparation procedure. GAPDH - loading control.

b. IHC reveals plentiful p53 (top right panel) and p21 (bottom right panel) positive cells in DKO intestines signifying DNA damage response in

these cells. Left panel – intestinal sections from control mice. Scale bar=100μm.

Supplementary Fig. 5

a. SW480 cells were pretreated with 10nM MG132 for 4 hours and then treated with either Doxorubicin or LPS for the indicated times. Proteasome was inhibited and no NF-κB dependent transcription was permissible following MG132 treatment as indicated by the absence of LPS induced TNF elevation (left chart). However, inhibition of NF-κB activation by MG132 did not block DNA-damage induced transcriptional induction of IL-1β (right chart).

b. SW480 cells were transfected with siRNA targeting IKKβ (Darmacon) and 48 hours later were treated with either Doxorubicin or LPS for the indicated time points. IKKβ knockdown efficiency was approximately 60% (left chart), which was sufficient for inhibition of LPS-induced TNF upregulation (middle chart), but did not suppress the elevation in mRNA expression of IL-1β following Doxorubicin-induced DNA damage (right chart). C = control siRNA. IKK = IKKβ siRNA.

Supplementary Fig. 6

a. qPCR analysis of NF-κB target genes at days 1, 4 and 7 in enterocytes of SAA mice, showing intact NF-κB response in these mice. n=3 for all groups (P values for Day 1 SAA compared to controls: IL-1β<0.0001, IL-1R=0.0021, IκBα=0.2518, IL-6=0.0024, cIAP2=0.5342, p100=0.0015).

b. Intestinal tight junctions captured by electron microscopy. Tight junctions (marked by red arrows) appear normal in control (top panel) and abnormal with wide gaps in untreated SAA mice (middle panel). However, SAA mice treated with neutralizing anti IL-1β antibody have normal tight junctions (bottom panel). The left panel magnification is X20,000, middle is X50,000, and right panel's magnification is X100,000. All mice were sacrificed on day1. Distances between

membranes in the tight junctions were measured and the measurements appear next to the junction.

c. Additional mice for each group as described for Supplementary Figure 6. b. Aberrant tight junctions in DKO mice captured by electron microscopy. (control, SAA and SAA treated with neutralizing αIL-1β antibody). Original magnification X20,000. Red arrows indicate tight junctions.

d. H&E staining of large intestine of control mouse (left), untreated SAA (middle) and SAA treated with neutralizing anti-IL-1β antibody (right) at day 4 after knockout induction. SAA mice at day 4 show severe inflammation and disrupted epithelial layer. In contrast, following anti IL-1β neutralizing antibody treatment, inflammation is diminished and tissue structure is comparable with control mice. Scale bar = 50μm.

e. Immunofluorescence staining of E-cadherin, an epithelial marker, emphasize the difference in the epithelial layer integrity of SAA treated with neutralizing anti-IL-1β antibody (right) and untreated SAA (middle) at day 4 after β-TrCP knockout induction. Control mouse on the left.