Expanded View Figures

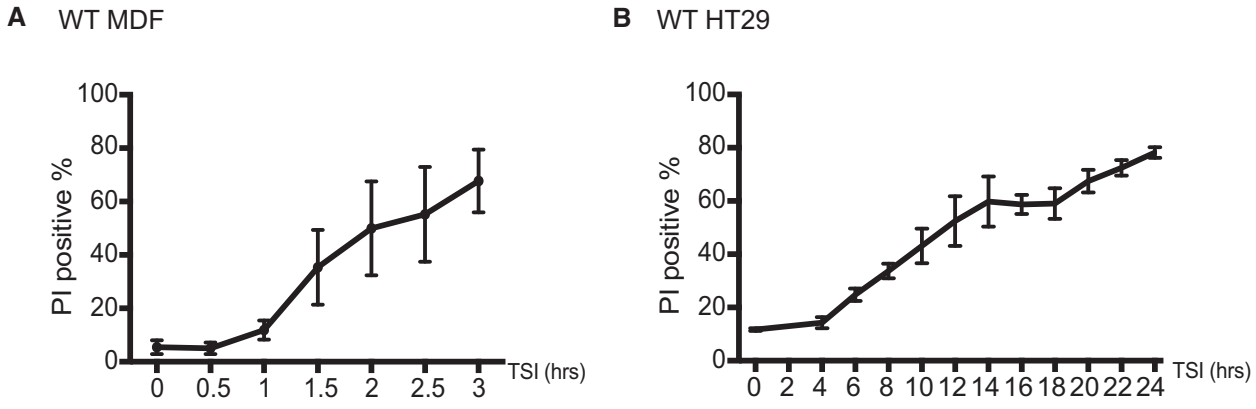


Figure EV1. Cell death time course of MDFs and HT29 cells following necroptotic stimulation.

A, B MDFs (A) and HT29 (B) cells were treated with TSI. Cell death was measured by PI staining and flow cytometry. Data are plotted as mean ± SEM of at least three independent experiments.

Data information: Propidium iodide (PI). TSI is used as a necroptotic stimulus.
Source data are available online for this figure.

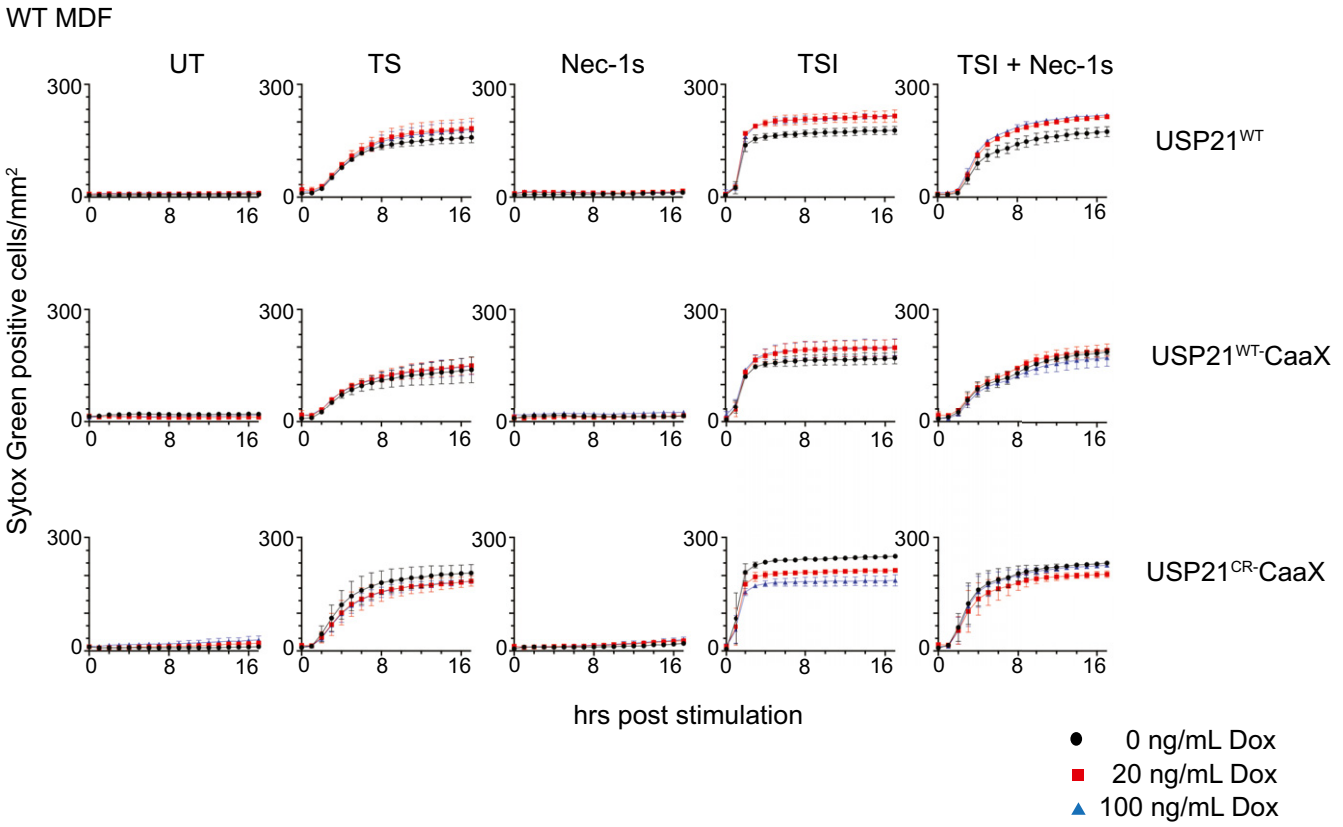


Figure EV2. USP21-CaaX expression does not alter the kinetics of TNF-induced apoptosis or necroptosis in MDFs.

WT USP21, USP21-CaaX and USP21^{C221R}-CaaX were inducibly expressed in WT MDFs by doxycycline. TS and Nec-1s were used to control for apoptotic signalling, and TSI was used to trigger necroptotic signalling. Sytox Green-positive cells were quantified in real time by live cell imaging. Data are plotted as mean ± SEM of two independent experiments.

Data information: Wild-type (WT), untreated (UT). TS and TSI are used as apoptotic or necroptotic stimuli respectively.
Source data are available online for this figure.

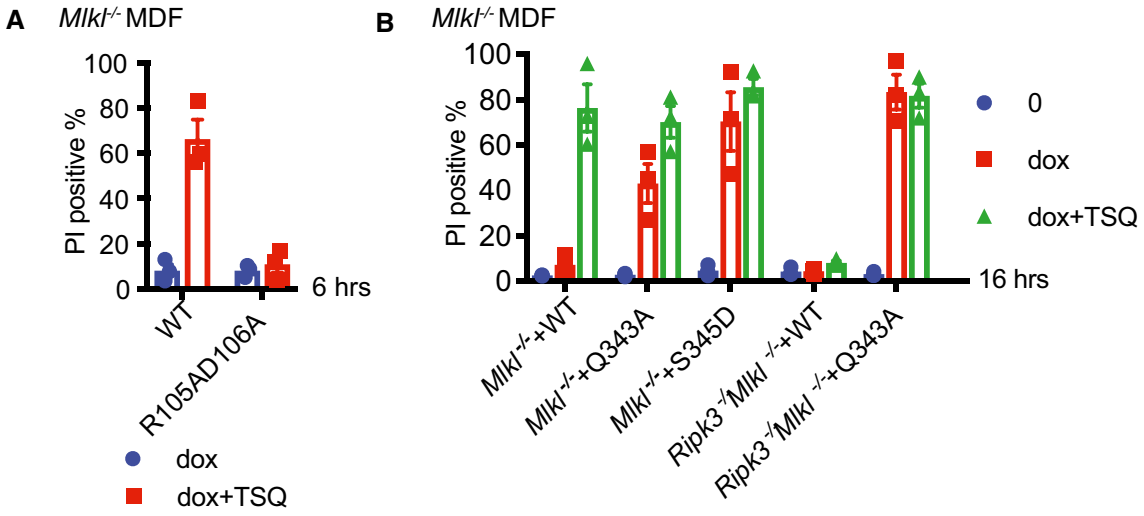


Figure EV3. MLKL oligomerization drives its necroptosis-specific ubiquitylation.

A Cell death of samples from Fig 4A was measured by PI staining based on flow cytometry. Data are plotted as mean ± SEM of three independent experiments.
B Cell death of samples from Fig 4C and D was analysed as in (A).

Data information: Propidium iodide (PI). TSQ is used as a necroptotic stimulus.
Source data are available online for this figure.

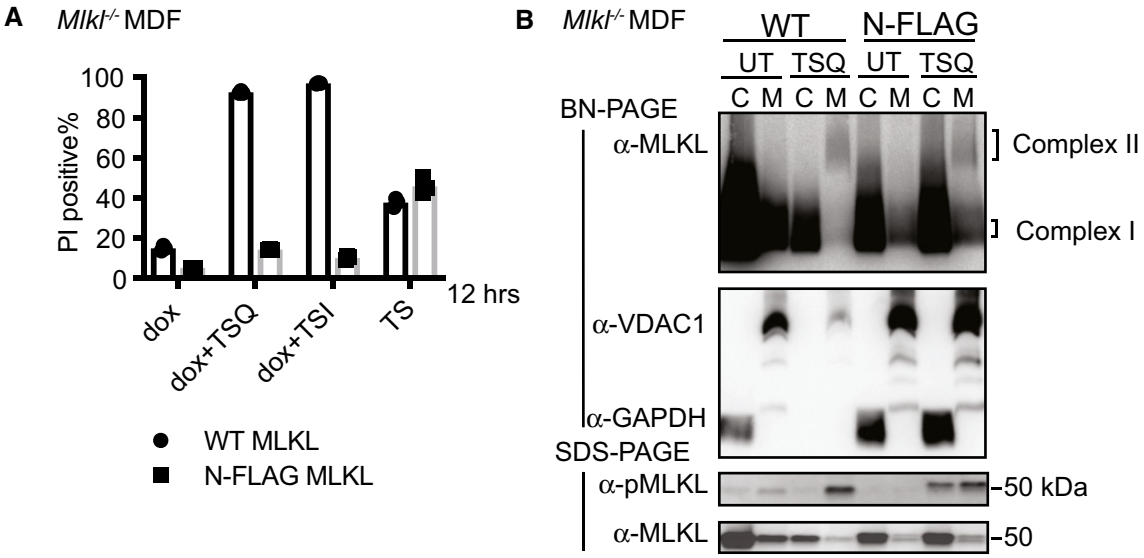
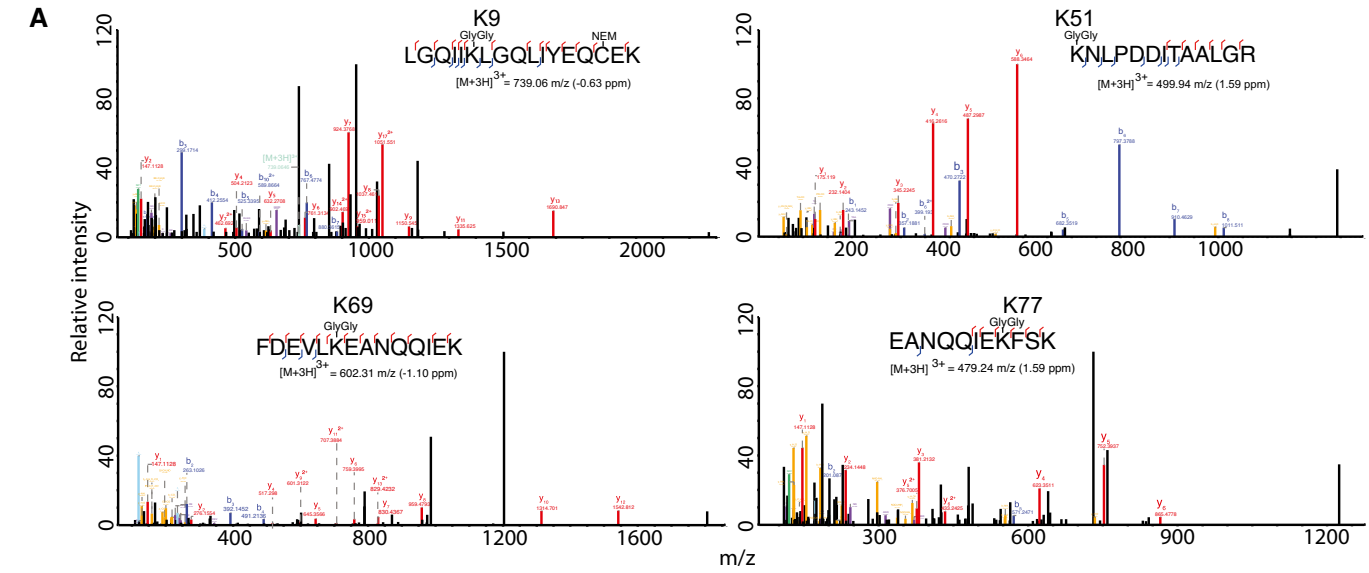
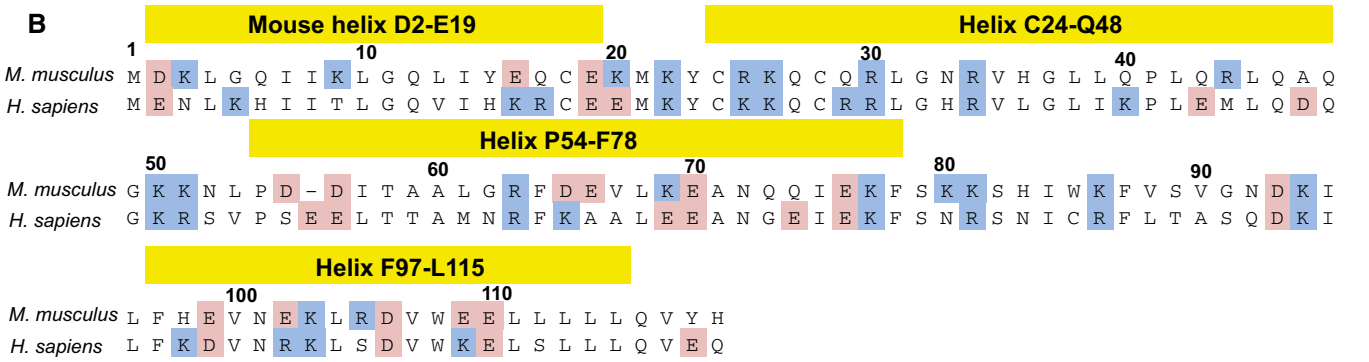


Figure EV4. N-FLAG MLKL behaves like WT MLKL but does not induce cell death following necroptotic stimulation.

A WT MLKL and N-FLAG MLKL were inducibly expressed in *Mlkl*$^{-/-}$ MDFs by doxycycline for 12 h, and cells were treated with TSI or TSQ. TS treatment controlled that the response to TS was normal. Cell death was measured by PI staining based on flow cytometry. Data are plotted as mean ± SEM of three independent experiments.
B WT MLKL and N-FLAG MLKL were inducibly expressed in *Mlkl*$^{-/-}$ MDFs by doxycycline for 6 h, and cells were untreated (UT) or treated with TSQ. Crude membrane (M) and cytosolic (C) cellular fractions were analysed by Western blot from BN-PAGE or SDS–PAGE using antibodies as indicated. Representative of three independent experiments.

Data information: Wild-type (WT). TS, TSI and TSQ are used as apoptotic or necroptotic stimuli.
Source data are available online for this figure.


A
K9
GlyGly
NEM
LGQIIKLGQLIYEQCEK
[M+3H]3+ = 739.06 m/z (-0.63 ppm)
K51
GlyGly
KNLPDDITAALGR
[M+3H]3+ = 499.94 m/z (1.59 ppm)
K69
GlyGly
FDEVLKEANQQIEK
[M+3H]3+ = 602.31 m/z (-1.10 ppm)
K77
GlyGly
EANQQIEKFSK
[M+3H]3+ = 479.24 m/z (1.59 ppm)
Relative intensity
m/z


B
Mouse helix D2-E19
Helix C24-Q48
Helix P54-F78
Helix F97-L115
M. musculus MDKLGQIIKLGQLIYEQCEKMKYCRKQCQRLGNRVHGLLQPLQRLQAQ
H. sapiens MENLKHIITLGQVIHKRCEEMKYCKKQCRRLGHRVLGLIKPLEMLQDQ
M. musculus GKKNLPD-DITAALGRFDEVLKEANQQIEKFSKKSHIWKFVSVGNDKI
H. sapiens GKRSVPSEELTTAMNRFKAALEEANGEIEKFSNRSNICRFLTASQDKI
M. musculus LFHEVNEKLRDVWEELLLLLQVYH
H. sapiens LFKDVNRKLSDVWKELSLLLQVEQ

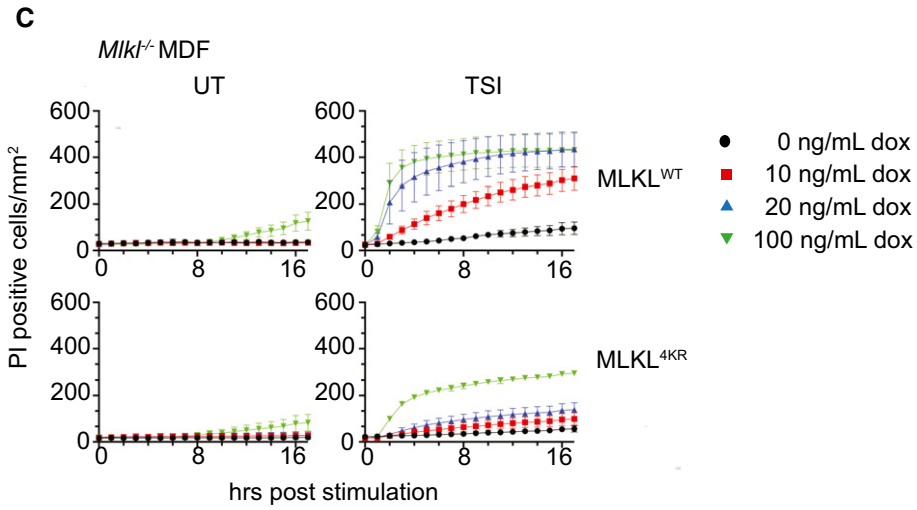

C
Mlkl-/- MDF
UT
TSI
PI positive cells/mm2
MLKLWT
MLKL4KR
0 ng/mL dox
10 ng/mL dox
20 ng/mL dox
100 ng/mL dox
hrs post stimulation

Figure EV5.

Figure EV5. Simultaneous arginine replacement of four ubiquitylation sites on the mouse MLKL 4HB domain does not prevent necroptosis-induced ubiquitylation.

A MS spectra were manually validated to confirm the identification of four Gly-Gly sites on activated MLKL.
B Alignment of mouse and human MLKL N-terminal domain. Positively charged residues are labelled in blue, and negatively charged residues are labelled in pink.
C WT and 4KR mutant MLKL were inducibly expressed in *Mlkl*$^{-/-}$ MDFs by doxycycline (at the indicated concentrations), and cells were treated ± TSI (added simultaneously) for 4 h. Sytox Green-positive cells were quantified in real time by IncuCyte S3 live cell imaging. Data are plotted as mean ± SEM of three independent experiments.

Data information: Wild-type (WT), untreated (UT). TSI is used as a necroptotic stimulus.
Source data are available online for this figure.

Figure EV6. MLKL ubiquitylation antagonizes necroptosis.

A Mouse MLKL, USP21, USP21^{C221R}, MLKL-USP21 and MLKL-USP21^{C221R} fusions were inducibly expressed in *Mlkl*$^{-/-}$ MDFs and *Ripk3*$^{-/-}$*Mlkl*$^{-/-}$ MDFs as indicated, following doxycycline addition (20 ng/ml) for 6 h ± TSI. Representative of two independent experiments.
B *MLKL*$^{-/-}$ HT29 cells stably transfected with doxycycline-inducible constructs encoding human USP21 and human USP21^{C221R} were treated with doxycycline, NSA (1 μM), TSI or combinations thereof (added simultaneously). Sytox Green-positive cells were quantified in real time by live cell imaging. Representative of two independent experiments.
C Mouse MLKL-USP21 and MLKL-USP21^{C221R} fusions were inducibly expressed in *Mlkl*$^{-/-}$ MDFs by doxycycline (10 ng/ml) for 8 h with addition of a necroptotic stimulus (TSI) for 3 h, followed by UBA pulldown and USP21 digestion. Antibody (D6E3G, Cell Signaling Technology) was used here to detect MLKL phosphorylation. Representative of two independent experiments.
D *MLKL*$^{-/-}$ HT29 cells were stably transfected with indicated doxycycline-inducible *MLKL* alleles (phospho-mimetic human MLKL mutant T357E/S358E indicated as MLKL^{TSEE}) and treated with doxycycline (100 ng/ml) ± TSI (added simultaneously). A residue band from MLKL blot is indicated by an asterisk in RIPK3 blot due to reprobing. Representative of three independent experiments.

Data information: TSI is used as a necroptotic stimulus.
Source data are available online for this figure.

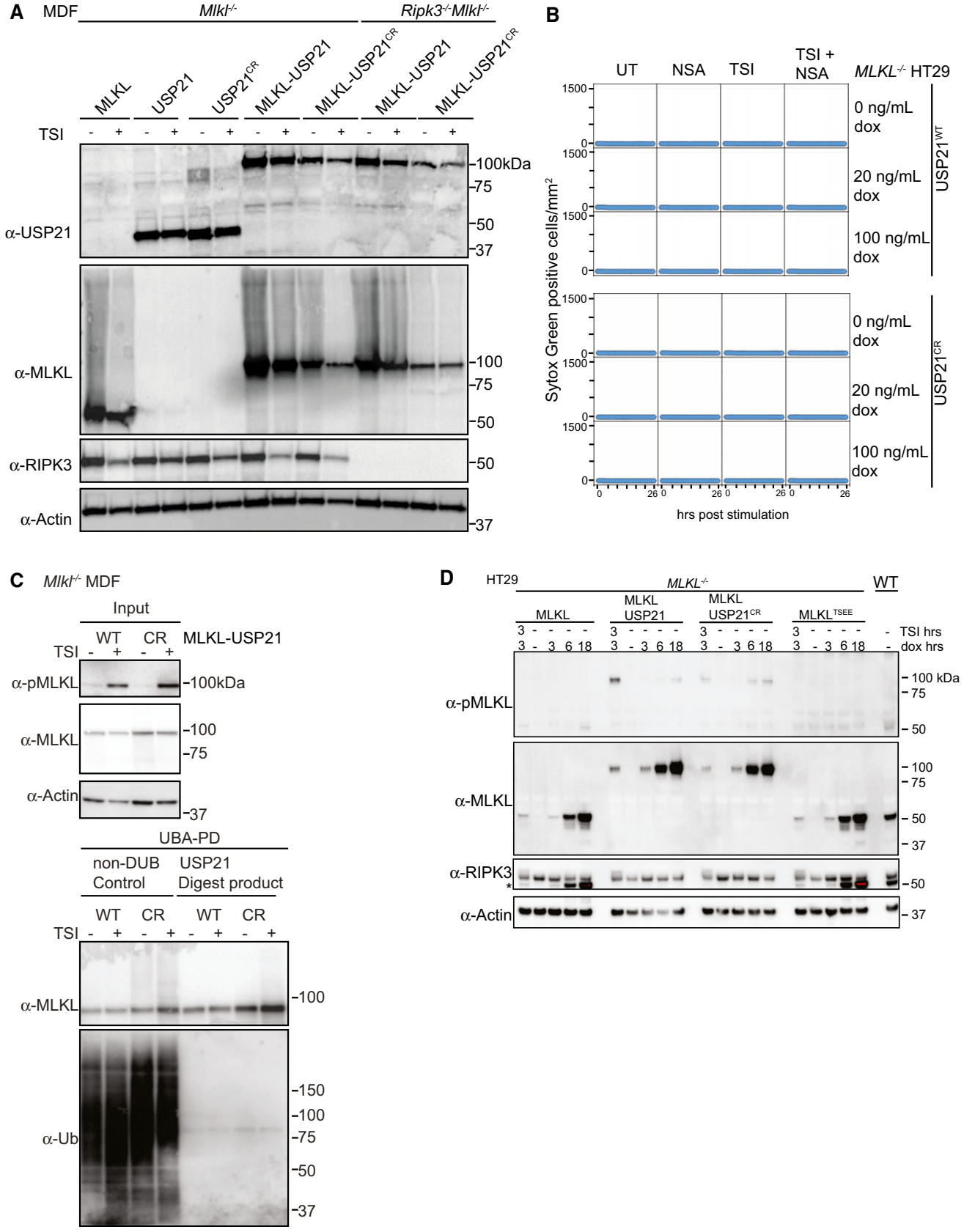

A
MDF
Mlkl-/-
Ripk3-/-Mlkl-/-
MLKL
USP21
USP21CR
MLKL-USP21
MLKL-USP21CR
MLKL-USP21
MLKL-USP21CR
TSI
α-USP21
α-MLKL
α-RIPK3
α-Actin
100kDa
75
50
37
100
75
50
50
37
B
UT
NSA
TSI
TSI + NSA
MLKL-/- HT29
0 ng/mL dox
20 ng/mL dox
100 ng/mL dox
USP21WT
USP21CR
Sytox Green positive cells/mm2
1500
0
hrs post stimulation
C
Mlkl-/- MDF
Input
WT
CR
MLKL-USP21
TSI
α-pMLKL
α-MLKL
α-Actin
100kDa
100
75
37
UBA-PD
non-DUB Control
USP21 Digest product
α-MLKL
α-Ub
100
150
100
75
50
37
D
HT29
MLKL-/-
WT
MLKL
MLKL USP21
MLKL USP21CR
MLKLTSEE
TSI hrs
dox hrs
α-pMLKL
α-MLKL
α-RIPK3
α-Actin
100 kDa
75
50
100
75
50
37
50
37

Figure EV6.