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Supplementary Materials for

Tankyrase-mediated ADP-ribosylation is a regulator of TNF-induced death

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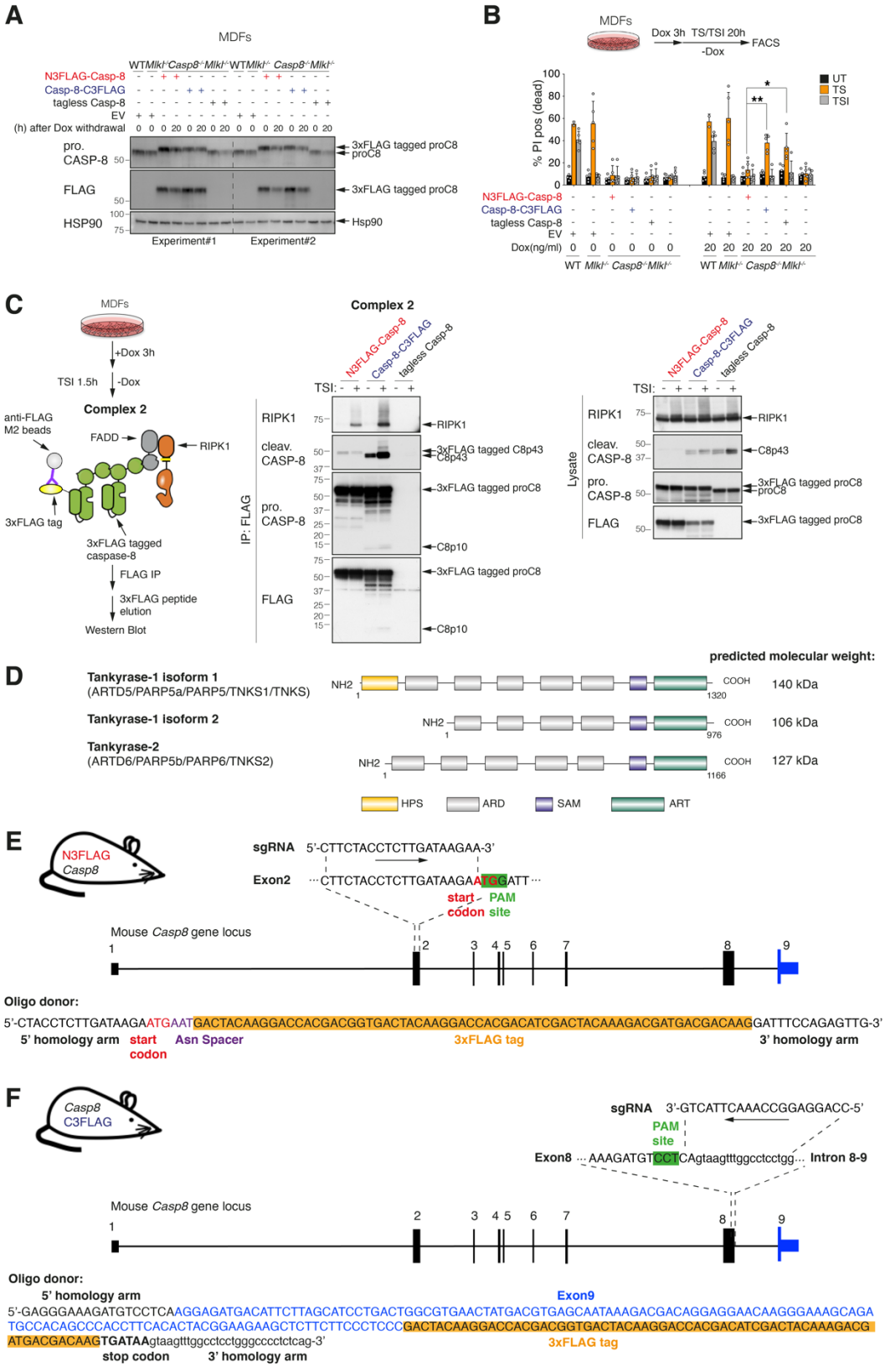
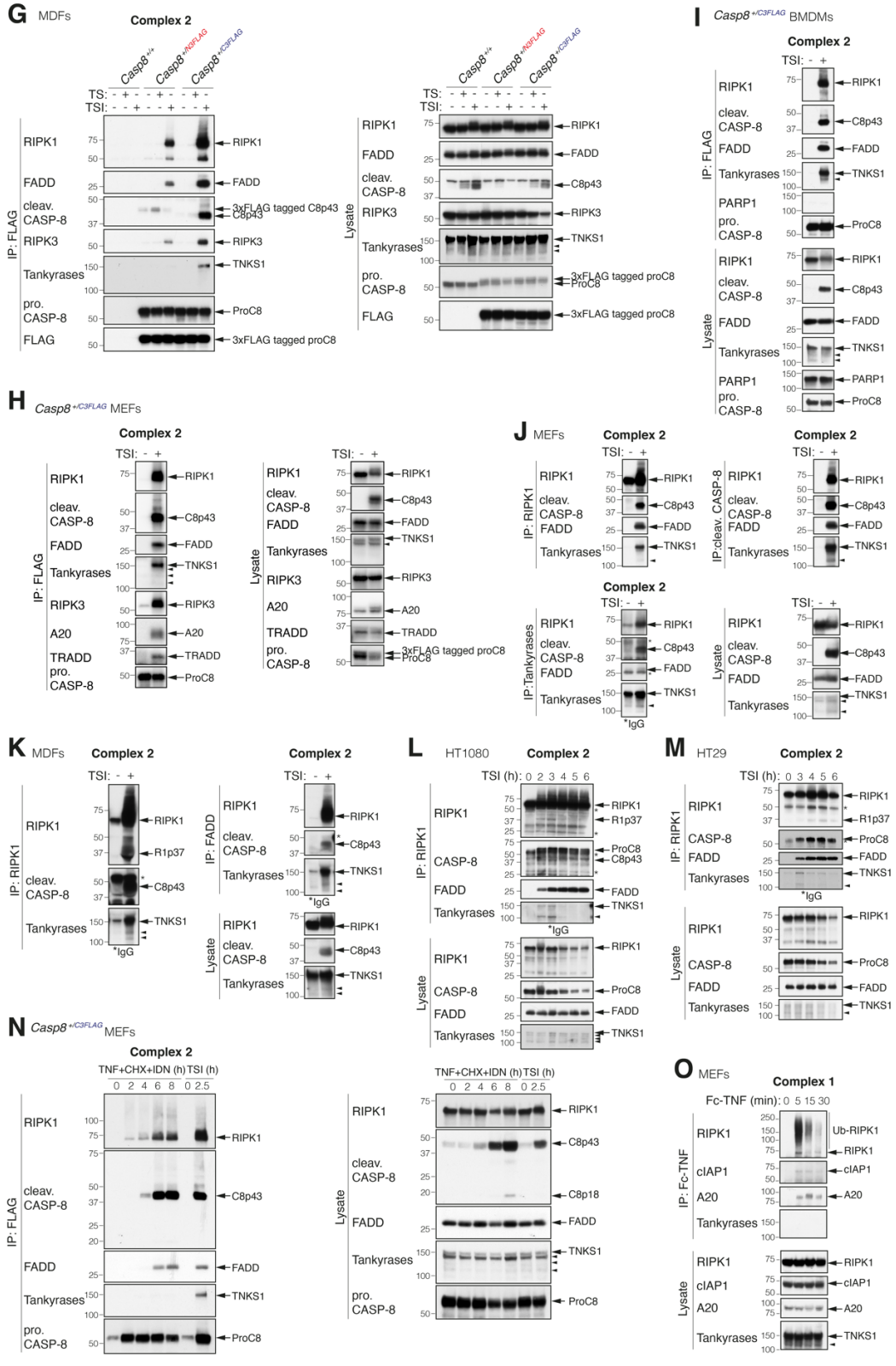
The PDF file includes:

Other Supplementary Material for this manuscript includes the following:

Supplementary data

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Casp8+/C3FLAG
TS:
TSI:
IP: FLAG
RIPK1
FADD
cleav. CASP-8
RIPK3
Tankyrases
pro. CASP-8
FLAG
3xFLAG tagged C8p43
C8p43
TNKS1
ProC8
3xFLAG tagged proC8
Lysate
H Casp8+/C3FLAG MEFs
A20
TRADD
I Casp8+/C3FLAG BMDMs
PARP1
J MEFs
IP: RIPK1
IP:cleav. CASP-8
IP:Tankyrases
*IgG
K MDFs
IP: RIPK1
IP: FADD
R1p37
L HT1080
TSI (h): 0 2 3 4 5 6
M HT29
N Casp8+/C3FLAG MEFs
TNF+CHX+IDN (h)
C8p18
O MEFs
Complex 1
Fc-TNF (min): 0 5 15 30
IP: Fc-TNF
Ub-RIPK1
cIAP1

Fig. S1. Tankyrase-1 is a novel interactor of native TNFR1 complex 2

A, Western blot analysis of cell lysates from *Casp8*$^{-/-}$.*Mlkl*$^{-/-}$ MDFs expressing doxycycline (Dox)-inducible N- (red) or C- (blue) 3x FLAG tagged murine caspase-8 or tagless caspase-8. Wild-type (WT) or *Mlkl*$^{-/-}$ MDFs expressing an empty vector (EV) were used as controls. Cells were treated with 20 ng/mL Dox for 3 hours and then Dox was withdrawn. Samples were harvested 0 hour or 20 hours after Dox withdrawal for Western blot analysis.

B, Level of cell death assessed by propidium iodide (PI) positive cells. Cells were pre-treated with 20 ng/mL Dox for 3 hours followed by stimulation with TNF (100 ng/mL) + Smac-mimetic compound A (500 nM) (TS) ± caspase inhibitor IDN-6556 (5 μM) for 20 hours in the absence of Dox. Graph shows mean ± SEM, n=3 independent experiments. Comparisons were performed with a Student's t test whose values are denoted in the figures as *$p \leq 0.05$ and **$p \leq 0.01$.

C, Left, schematic depicting the anti-FLAG immunoprecipitation. Right, TNF-induced complex 2 immunoprecipitation using anti-FLAG M2 affinity beads. Western blot analysis of complex 2 from *Casp8*$^{-/-}$.*Mlkl*$^{-/-}$ MDFs expressing Dox-inducible N- or C- 3x FLAG tagged murine caspase-8 or tagless caspase-8 using the indicated antibodies. Cells were treated with 20 ng/mL Dox for 3 hours followed by stimulation with TNF (100 ng/mL) + Smac-mimetic (500 nM) + caspase inhibitor (5 μM) (TSI) for 1.5 hours in the absence of Dox before being subjected to anti-FLAG immunoprecipitation. Caspase inhibitor was used to stabilize complex 2.

D, Schematic comparison of the domain architecture of the murine TNKS1, TNKS1 isoform2 and TNKS2. Domains are: HPS: histidine, proline and serine-rich region; ARD: ankyrin repeat domains; SAM: sterile α-motif; ART: ADP-ribosyltransferase catalytic domain. ARDs provide binding sites for interaction between tankyrases and other proteins. The SAM domain mediates protein-protein interactions, form homo- and hetero-oligomers and also binds to DNA, RNA and lipids. SAM domain is also critical for optimal catalytic activity. The ART domain is responsible for the ADP-ribosyltransferase activity.

E-F, Schematic representation of the generation of *Casp8*^{N3FLAG} (**E**) or *Casp8*^{C3FLAG} (**F**) mice using CRIPSR/Cas9 technology. For N-3x FLAG tagged caspase-8 knock-in mice, an Asn Spacer was introduced into the oligo donor to ensure successful gene translation. For C-3x FLAG tagged caspase-8 knock-in mice, the PAM site was in exon 8 and an oligo donor composed of protein coding region of exon 9 with a 3x FLAG tag followed by two stop codons were designed because there was no usable PAM site at the last exon (exon 9) of *Casp8* gene and a *Casp8* pseudogene known as Gm20257 showed ~133 bp of sequence identity to *Casp8* exon 9 and was nearby on the same chromosome (chromosome 1).

G-I, TNF-induced complex 2 immunoprecipitation using anti-FLAG M2 affinity beads. Western blot analysis of complex 2 and lysates from *Casp8*$^{+/+}$, *Casp8*$^{+/N3FLAG}$ and *Casp8*$^{+/C3FLAG}$ MDFs **(G)** *Casp8*$^{+/C3FLAG}$ MEFs **(H)** or *Casp8*$^{+/C3FLAG}$ BMDMs **(I)** using the indicated antibodies is shown. Cells were treated with TNF (100 ng/mL) + Smac-mimetic (500 nM) with or without caspase inhibitor (5 μM) for 1.5 hours before being subjected to anti-FLAG immunoprecipitation. Caspase inhibitor was used to stabilize complex 2.

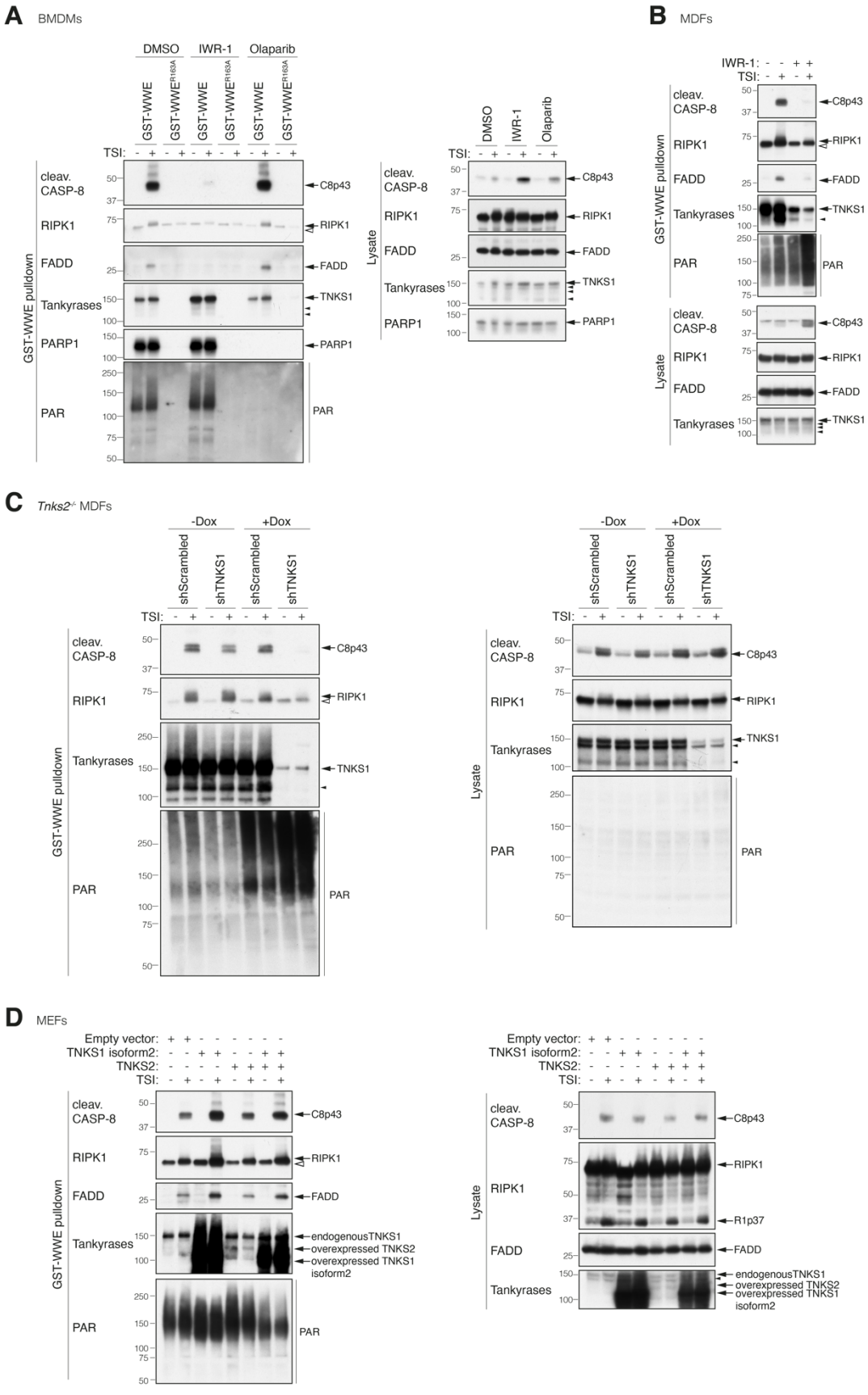
J-K, TNF-induced complex 2 immunoprecipitation. WT MEFs (**J**) or MDFs (**K**) were treated with TSI (as in **G-I**) to induce complex 2 assembly. The lysates were immunoprecipitated with anti-RIPK1 or anti-cleaved caspase-8 or anti-FADD or anti-tankyrase. Western blot analysis using the indicated antibodies is shown.

L-M, TNF-induced complex 2 immunoprecipitation using anti-RIPK1. Western blot analysis of complex 2 and lysates from HT1080 (**L**) and HT29 (**M**) cells using the indicated antibodies is shown. Cells were treated with TSI (as in **G-I**) for indicated time points.

N, TNF-induced complex 2 immunoprecipitation using anti-FLAG M2 affinity beads. Western blot analysis of complex 2 and lysates from *Casp8*$^{+/C3FLAG}$ MEFs using the indicated antibodies is shown. Cells were treated with TNF (100 ng/mL) + Smac-mimetic (500 nM) + caspase inhibitor (5 μM) (TSI) for 2.5 hours or TNF (100 ng/mL) + cycloheximide (CHX) (1 μg/mL) + caspase inhibitor (5 μM) (TNF+CHX+IDN) for the indicated time points, followed by immunoprecipitation with anti-FLAG M2 affinity beads.

O, TNF-induced complex 1 immunoprecipitation. WT MEFs were treated with Fc-TNF (1 μg/mL) for the indicated time points, followed by immunoprecipitation with protein A Sepharose and Western blot analysis.

Filled arrowheads alone denote bands between 100 kDa and 150 kDa detected by anti-tankyrase which might indicate TNKS1 isoform 2 (106 kDa) or TNKS2 (127 kDa). Double bands around 150kDa in anti-tankyrase blots indicate full length TNKS1 (upper band, 150kDa) and an undefined TNKS1 isoform (lower band). *indicate IgG chains.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RIPK1
FADD
Tankyrases
PARP1
PAR
C8p43
TNKS1
Lysate
B
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IWR-1:
C
Tnks2-/- MDFs
-Dox
+Dox
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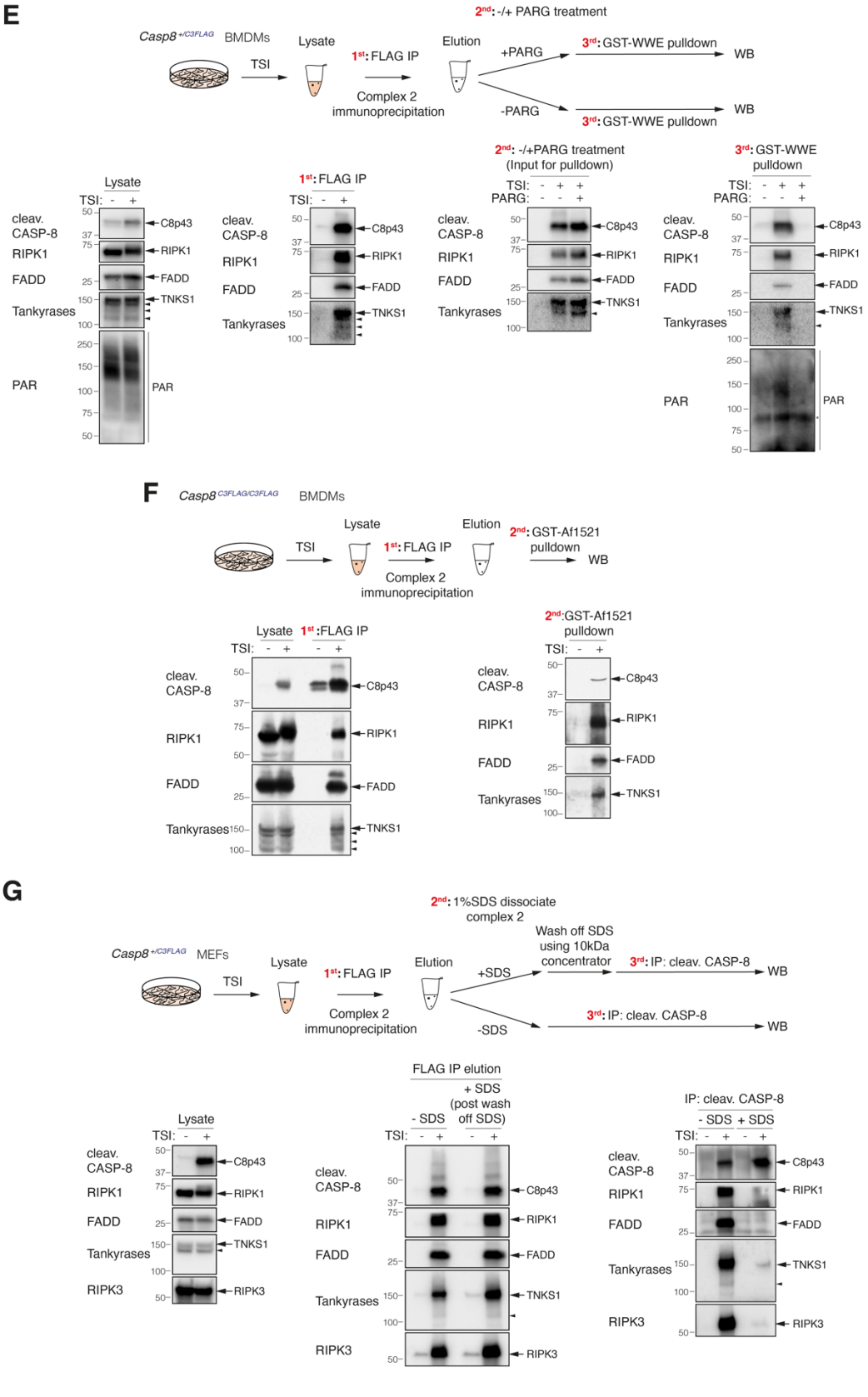
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Casp8 +/C3FLAG BMDMs
TSI
Lysate
1st: FLAG IP
Complex 2 immunoprecipitation
Elution
2nd: -/+ PARG treatment
+PARG
-PARG
3rd: GST-WWE pulldown
WB
Lysate
TSI: - +
cleav. CASP-8
RIPK1
FADD
Tankyrases
PAR
C8p43
TNKS1
1st: FLAG IP
2nd: -/+PARG treatment (Input for pulldown)
PARG: - - +
3rd: GST-WWE pulldown
F
Casp8 C3FLAG/C3FLAG BMDMs
2nd: GST-Af1521 pulldown
2nd:GST-Af1521 pulldown
G
Casp8 +/C3FLAG MEFs
2nd: 1%SDS dissociate complex 2
Wash off SDS using 10kDa concentrator
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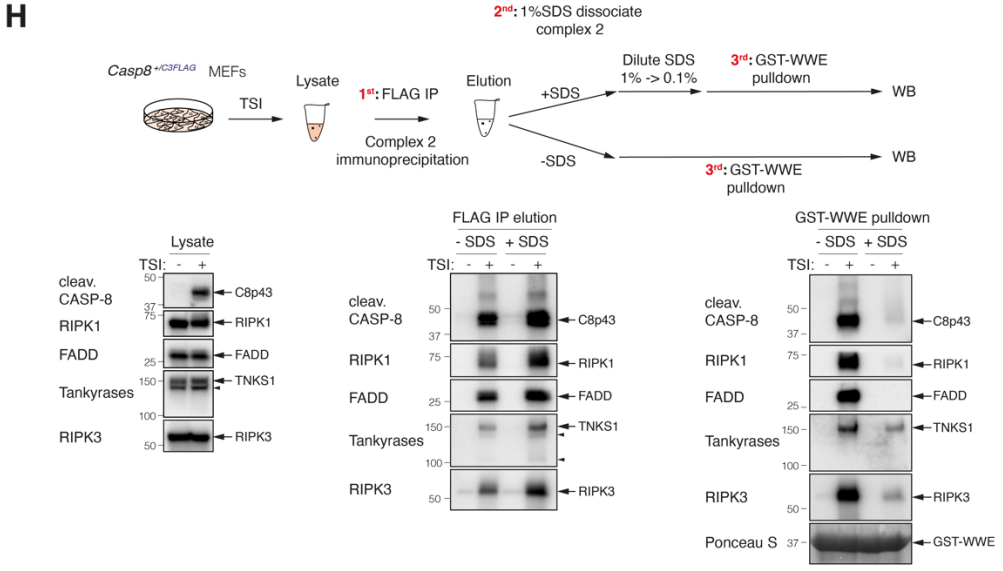
FLAG IP elution
+ SDS (post wash off SDS)
- SDS
IP: cleav. CASP-8
RIPK3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TSI
Lysate
1st: FLAG IP
Complex 2 immunoprecipitation
Elution
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-SDS
Dilute SDS 1% -> 0.1%
3rd: GST-WWE pulldown
WB
3rd: GST-WWE pulldown
Lysate
FLAG IP elution
GST-WWE pulldown
- SDS + SDS
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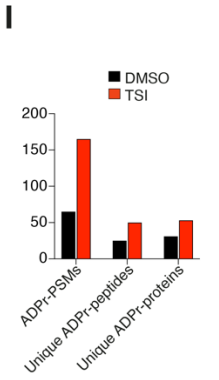
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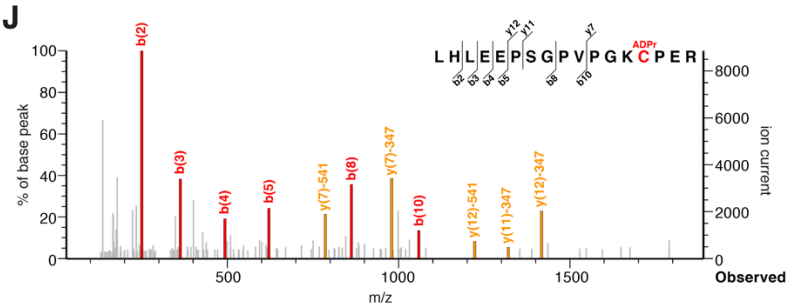
Unique ADPr-peptides
Unique ADPr-proteins


J
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m/z
Observed
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b(3)
b(4)
b(5)
y(7)-541
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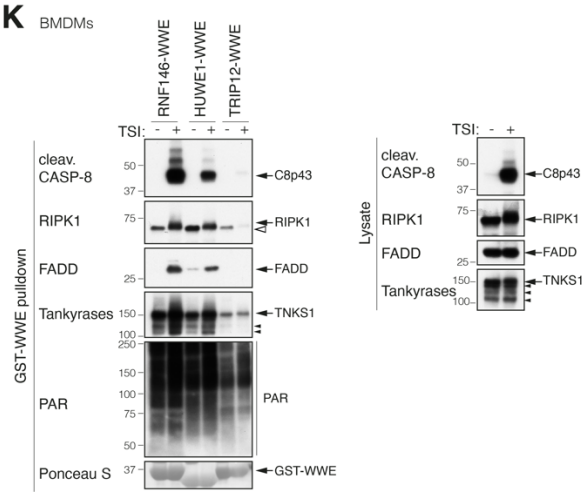
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Lysate
cleav. CASP-8
RIPK1
FADD
Tankyrases
PAR
Ponceau S
C8p43
TNKS1
GST-WWE

Fig. S2. Complex 2 is PARylated

A, GST-WWE and GST-WWE^{R163A} pull-down of stimulated WT BMDMs lysates. Cells were treated with TNF (10 ng/mL) + Smac-mimetic (250 nM) + caspase inhibitor (5 μM) (TSI) ± tankyrase inhibitor IWR-1 (5 μM) or ± PARP1/2 inhibitor olaparib (1 μM) for 1.5 hours before being subjected to GST pull-down. Western blot analysis using the indicated antibodies is shown. Anti-PAR (MABC547, Sigma) was used to recognize PAR chains.

B, GST-WWE pull-down of stimulated WT MDFs lysates. Cells were treated with TNF (100 ng/mL) + Smac-mimetic (500 nM) + caspase inhibitor (5 μM) (TSI) for 1.5 hours ± tankyrase inhibitor IWR-1 (10 μM) before being subjected to GST pull-down. Western blot analysis using the indicated antibodies is shown. Anti-PAR (MABC547, Sigma) was used to recognize PAR chains.

C, GST-WWE pull-down of stimulated *Tnks2*$^{-/-}$ MDFs lysates. *Tnks2*$^{-/-}$ MDFs expressing Dox-inducible Scrambled shRNA or TNKS1 shRNA were pre-treated with ± Dox (1 μg/mL) for 48 hours before being stimulated with TSI (as in **B**) ± Dox (1 μg/mL) for 1.5 hours. Cell lysates were subjected to GST-WWE pull-down. Western blot analysis using the indicated antibodies is shown. Anti-PAR (MABC547, Sigma) was used to recognize PAR chains.

D, GST-WWE pull-down of stimulated WT MEFs lysates. WT MEFs expressing Dox-inducible murine TNKS1 (isoform 2) or/and TNKS2 or empty vector were pre-treated with Dox (20 ng/mL) overnight before being stimulated with TSI (as in **B**) for 1.5 hours. Cell lysates were subjected to GST-WWE pull-down. Western blot analysis using the indicated antibodies is shown. Anti-PAR (MABC547, Sigma) was used to recognize PAR chains.

E, Enrichment of PARylated complex 2 using GST-WWE in a sequential pull-down analysis. *Casp8*$^{+/C3FLAG}$ BMDMs were treated with TSI (as in **B**) and complex 2 was immunoprecipitated using anti-FLAG M2 affinity beads. Immunoprecipitants were eluted with 3x FLAG peptides followed by ± PARG treatment at 37 °C for 3 hours before being subjected to GST-WWE pull-down. FLAG IP eluate ± PARG treatment samples (input for GST-WWE pull-down) were taken for Western blot analysis to show that PARG didn't cause degradation of complex 2 components. Western blot analysis of lysates and sequential pull-down using the indicated antibodies is shown. Anti-PAR (Poly/Mono-ADPRibose (E6F6A) Rabbit mAb #83732, Cell Signaling Technology) was used to recognize PAR chains.

F, Enrichment of PARylated complex 2 using GST-Af1521 in a sequential pull-down analysis. *Casp8*$^{C3FLAG/C3FLAG}$ BMDMs were treated with TSI (as in **B**) and complex 2 was immunoprecipitated using anti-FLAG M2 affinity beads. Immunoprecipitants were eluted using 3x FLAG peptides followed by GST-Af1521 pull-down. Western blot analysis of lysates and sequential pull-down using the indicated antibodies is shown.

G, Dissociation of complex 2 using 1% SDS. *Casp8*$^{+/C3FLAG}$ MEFs were treated with TSI (as in **B**) and complex 2 was immunoprecipitated using anti-FLAG M2 affinity beads. Immunoprecipitants were eluted using 3x FLAG peptides followed by incubation with 1% SDS (final concentration) at 25 °C for 4 hours. SDS was then washed off using a 10 kDa concentrator (Millipore) to minimize the potential disruption of immunoprecipitation in the following step and a sample was Western blotted to ensure that SDS didn't affect complex 2 components (middle panel). The SDS-treated FLAG IP eluate was then subjected to anti-cleaved caspase-8 IP and FLAG IP eluate without SDS treatment was used as control. Anti-cleaved caspase-8 IP suggested that most of complex 2 was dissociated in 1% SDS as compared with no SDS control.

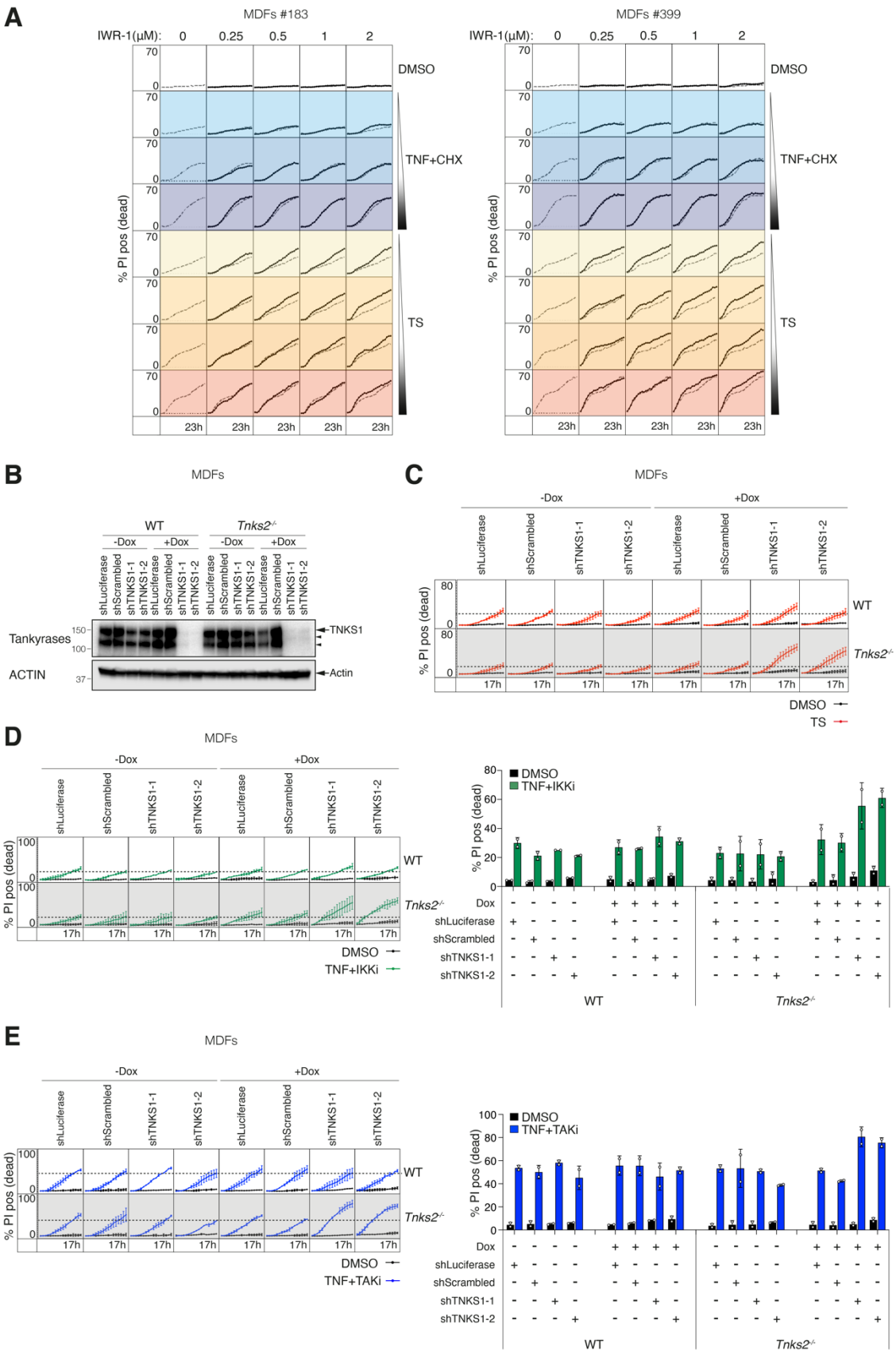
H, GST-WWE pull-down of disassembled complex 2. Complex 2 was purified and dissociated as in **G**. A sample was Western blotted to ensure that SDS didn't affect complex 2 components (middle panel). SDS was diluted from 1% to 0.1% to minimize the potential disruption of pull-down in the following step. The SDS-treated FLAG IP eluate was then subjected to GST-WWE pull-down and FLAG IP eluate without SDS treatment was used as control. Ponceau S staining of the purified proteins and their quantities used in the pull-down assay is shown.

I, TSI treatment results in global ADP-ribosylome changes in BMDMs lysates. Numbers of ADP-ribosylated peptide spectral matches (ADPr-PSMs), unique ADP-ribosylated peptides and unique ADP-ribosylated proteins identified in the untreated (DMSO) and TSI-treated cells are shown, respectively.

J, Spectrum of RIPK3 ADP-ribosylated peptide, with ADP-ribose on C360, is identified in BMDMs lysates following TSI stimulation.

K, GST-HUWE1, -TRIP12 and -RNF146 WWE pull-down stimulated WT BMDMs lysates. Cells were treated with TSI (as in **B**) and lysates were subjected to GST-WWE pull-down assays. Western blot analysis using the indicated antibodies is shown. Ponceau S staining of the purified proteins and their quantities used in the pull-down assay is shown. Anti-PAR (MABC547, Sigma) was used to recognize PAR chains.

Filled arrowheads alone indicate potential tankyrase species. Double bands around 150kDa in anti-tankyrase blots indicate full length TNKS1 (upper band, 150kDa) and an undefined TNKS1 isoform (lower band). Empty arrowheads alone denote unmodified RIPK1 that is purified non-specifically by Sepharose GST-WWE.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TS
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TNKS1
ACTIN
Actin
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TNF+TAKi

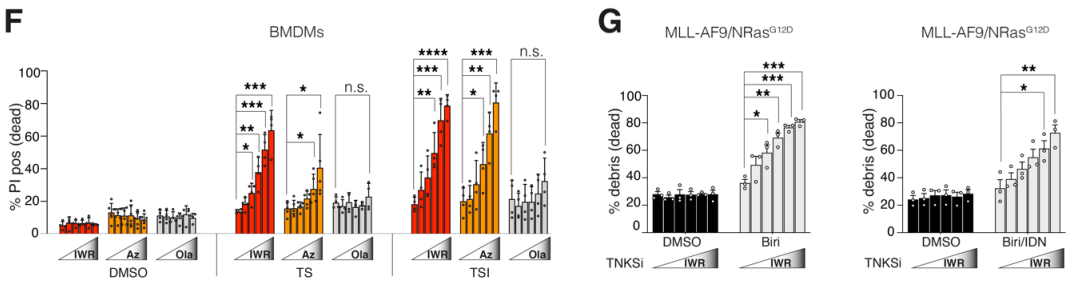

Fig. S3. Tankyrases protect from TNF-induced cell death

A, Cell death of WT MDFs, monitored by time-lapse imaging of PI staining (dead cells) over 23 hours. WT MDFs were treated with DMSO, TNF+cycloheximide (TNF+CHX) or TNF+Smac-mimetic (TS) (rows) ± tankyrase inhibitor IWR-1 (columns) for 23 hours. TNF: 50 ng/mL. Smac-mimetic: 50 nM, 100 nM, 250 nM, 500 nM. CHX: 0.25 µg/mL, 0.5 µg/mL, 1 µg/mL. IWR-1: 250 nM, 500 nM, 1 µM, 2 µM. Cell death was quantified by PI uptake and time-lapse imaging every 1 hour using IncuCyte. % PI positive (dead) was obtained by normalizing PI count to total cell number (SPY505-DNA cell nuclear stain). Dashed lines denote the % PI positive (dead) without IWR-1 treatment for reference. The results from two independent MDFs are shown.

B, Western blot analysis of TNKS1 knockdown efficiency in WT or *Tnks2*$^{-/-}$ MDFs expressing Dox-inducible shLuciferase, shScrambled or two independent TNKS1 shRNA. Cells were pre-treated with ± Dox (1 µg/mL) for 48 hours and then subjected to Western blot analysis. Filled arrowhead alone indicates potential tankyrase species.

C, Cell death monitored by time-lapse imaging of PI staining (dead cells) over 17 hours. WT MDFs or *Tnks2*$^{-/-}$ MDFs expressing Dox-inducible shLuciferase, shScrambled or two independent TNKS1 shRNAs were pre-treated with ± Dox (1 µg/mL) for 48 hours followed by TNF (50 ng/mL) + Smac-mimetic (10 nM) (TS) ± Dox (1 µg/mL) for 17 hours. Cell death was quantified by PI uptake and time-lapse imaging every 1 hour using IncuCyte. % PI positive (dead) was obtained by normalizing PI count to total cell number (SPY700-DNA cell nuclear stain). Dashed lines denote % PI positive (dead) in cells where the shRNA is not induced for reference. Graph shows mean ± SEM, n=4 independent experiments generated from two independent MDFs.

D-E, Left, Cell death monitored by time-lapse imaging of PI staining (dead cells) over 17 hours. WT MDFs or *Tnks2*$^{-/-}$ MDFs expressing Dox-inducible shLuciferase, shScrambled or two independent TNKS1 shRNAs were pre-treated with ± Dox (1 µg/mL) for 48 hours followed by indicated stimulations ± Dox (1 µg/mL) for 17 hours. Cells were treated with TNF (50 ng/mL) combined with IKK inhibitor (IKKi; 250 nM) (**D**), TAK1 inhibitor (TAKi; 100 nM) (**E**). Cell death was quantified by PI uptake and time-lapse imaging every 1 hour using IncuCyte. % PI positive (dead) was obtained by normalizing PI count to total cell number (SPY700-DNA cell nuclear stain). Dashed lines denote % PI positive (dead) in cells where the shRNA is not induced for reference. Right, % PI positive (dead) at 17 hours indicated stimulations was plotted to generate bar graph. Graphs show mean ± SD, n=2 independent MDFs.

F, Amount of cell death assessed by PI positive cells by flow cytometry. WT BMDMs were treated with TNF (10 ng/mL) + Smac-mimetic (500 nM) (TS) or TNF (10 ng/mL) + Smac-mimetic (10 nM) + caspase inhibitor (5 µM) (TSI) ± tankyrase inhibitor IWR-1 or ± Az6102 or ± PARP1/2 inhibitor olaparib for 16 hours. IWR-1: 250 nM, 500 nM, 1 µM, 2 µM, 5 µM. Az6102: 125 nM, 250 nM, 500 nM, 1 µM, 2 µM. Olaparib: 62.5 nM, 125 nM, 250 nM, 500 nM, 1 µM. Graph shows

mean ± SEM, n = 4-5 independent BMDMs. Comparisons were performed with a Student's t test whose values are denoted in the figures as *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, ****p ≤ 0.0001 and n.s.= no significance.

G, Amount of cell death assessed by percentage of cell debris by flow cytometry. MLL-AF9/NRas^{G12D} leukemic cells were treated with Smac-mimetic birinapant (500 nM) ± tankyrase inhibitor IWR-1 (250 nM, 500 nM, 1 μM, 2 μM, 5 μM) for 15 hours or Smac-mimetic birinapant (20 nM) + caspase inhibitor IDN-6556 (5 μM) ± IWR-1 (250 nM, 500 nM, 1 μM, 2 μM, 5 μM) for 7 hours. Graph shows mean ± SEM, n=3 independent MLL-AF9/NRas^{G12D} leukemic cells. Comparisons were performed with a Student's t test whose values are denoted in the figures as *p ≤ 0.05, **p ≤ 0.01 and ***p ≤ 0.001.

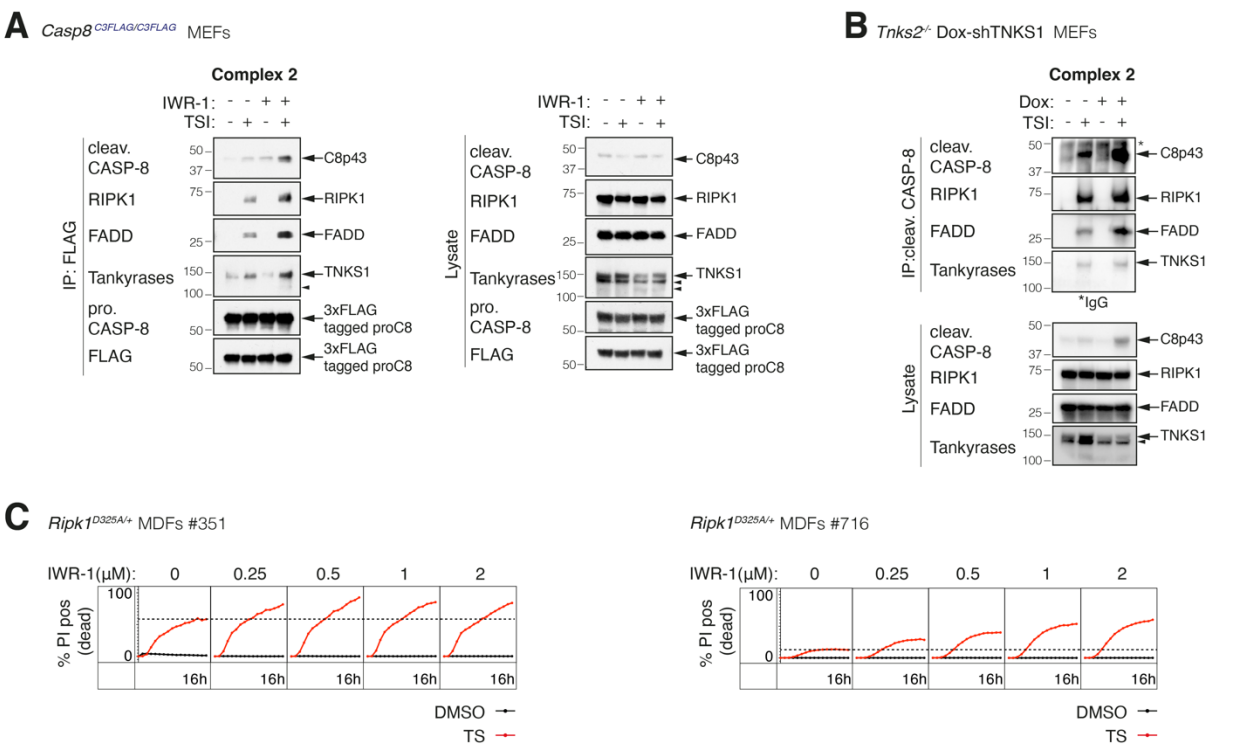

Fig. S4. Tankyrases limit complex 2 assembly

A, TNF-induced complex 2 immunoprecipitation using anti-FLAG M2 affinity beads. Western blot analysis of complex 2 and lysates from *Casp8*$^{C3FLAG/C3FLAG}$ MEFs using the indicated antibodies is shown. Cells were treated with TNF (100 ng/mL) + Smac-mimetic (50 nM) + caspase inhibitor (5 μM) (TSI) ± tankyrase inhibitor IWR-1 (5 μM) for 2 hours before being subjected to anti-FLAG immunoprecipitation.

B, TNF-induced complex 2 immunoprecipitation using anti-cleaved caspase-8 antibody. Western blot analysis of complex 2 and lysates from *Tnks2*$^{-/-}$ MEFs expressing Dox-inducible TNKS1 shRNA using the indicated antibodies is shown. Cells were pre-treated with ± Dox (1 μg/mL) for 48 hours followed by TNF (100 ng/mL) + Smac-mimetic (250 nM) + caspase inhibitor (5 μM) (TSI) for 2 hours before being subjected to anti-cleaved caspase-8 immunoprecipitation.

C, Cell death monitored by time-lapse imaging of PI staining over 16 hours using IncuCyte. *Ripk1*$^{D325A/+}$ heterozygote MDFs were treated with TNF (50 ng/mL) + Smac-mimetic (10 nM or 100 nM) (TS) ± tankyrase inhibitor IWR-1 (250 nM, 500 nM, 1 μM, 2 μM) for 16 hours. Cell death was quantified by PI uptake and time-lapse imaging every 1 hour using IncuCyte. % PI positive (dead) was obtained by normalizing PI count to cell confluency. Dashed lines denote the % PI positive (dead) without IWR-1 treatment for reference. The results from two independent MDFs are shown.

Filled arrowheads alone indicate potential tankyrase species. Double bands around 150kDa in anti-tankyrase blots indicate full length TNKS1 (upper band, 150kDa) and an undefined TNKS1 isoform (lower band). *indicate IgG chains.

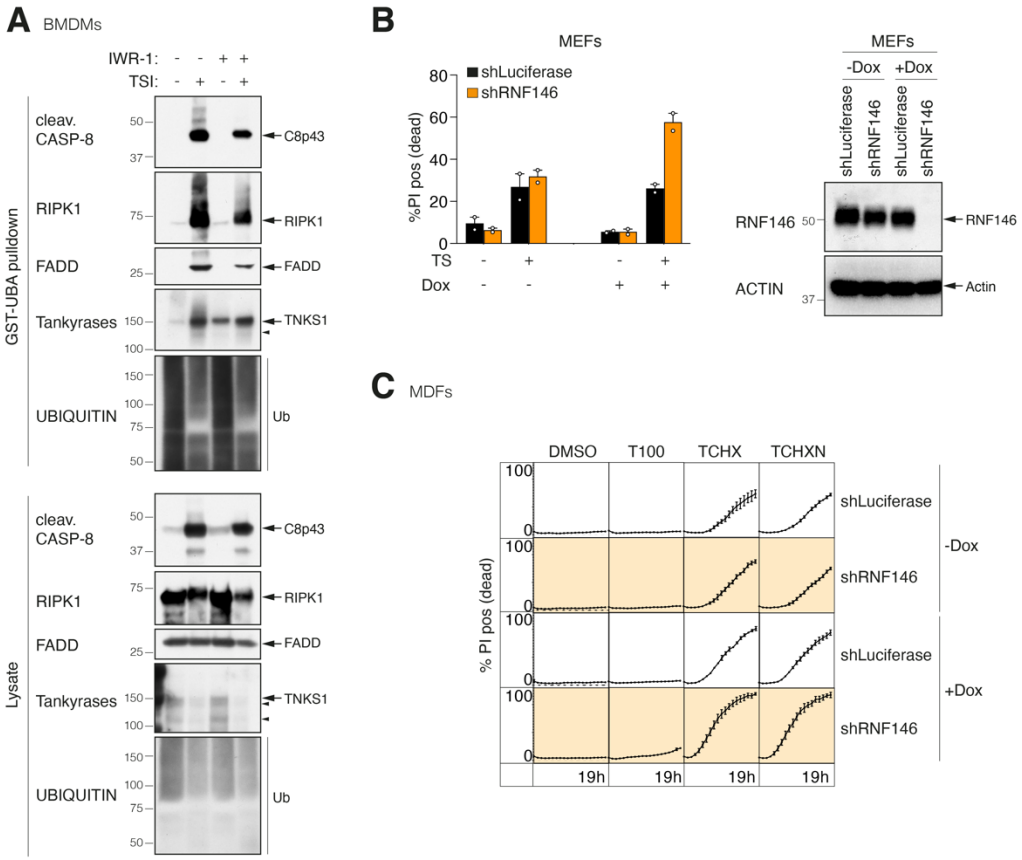

Fig. S5. The tankyrase-RNF146 axis regulates the stability of complex 2 and TNF-induced death

A, GST-UBA pull-down of stimulated WT BMDM lysates. Cells were treated with TNF (100 ng/mL) + Smac-mimetic (500 nM) + caspase inhibitor (5 μM) (TSI) for 1.5 hours ± IWR-1 (5 μM) before being subjected to GST-UBA pull-down. Western blot analysis using the indicated antibodies is shown.

B, Level of cell death assessed by PI positive cells. WT MEFs expressing Dox-inducible shLuciferase or shRNF146 were pre-treated with ± Dox (1 μg/mL) for 48 hours. Cells were then subjected to Western blot analysis or treated with TNF (100 ng/mL) + Smac-mimetic (25 nM) (TS) ± Dox (1 μg/mL) for another 12 hours. Graph shows mean ± SD throughout, n = 2 independent MEFs.

C, Cell death monitored by time-lapse imaging of PI staining over 19 hours. WT MDFs expressing GFP tagged Dox-inducible shLuciferase or shRNF146 were pre-treated with ± Dox (1 μg/mL) for 48 hours, followed by indicated stimulations ± Dox (1 μg/mL) for another 19 hours. T100 denotes 100 ng/mL TNF; T denotes 50 ng/mL TNF; CHX denotes 1 μg/mL cycloheximide; N denotes 10 μM Nec-1s. Cell death was quantified by PI uptake and time-lapse imaging every 1 hour using IncuCyte. % PI positive (dead) was obtained by normalizing PI count to total cell number (GFP positive cells). Graph shows mean ± SEM, n=3 independent MDFs.

Filled arrowheads alone indicate potential tankyrase species. Double bands around 150kDa in anti-tankyrase blots indicate full length TNKS1 (upper band, 150kDa) and an undefined TNKS1 isoform (lower band).

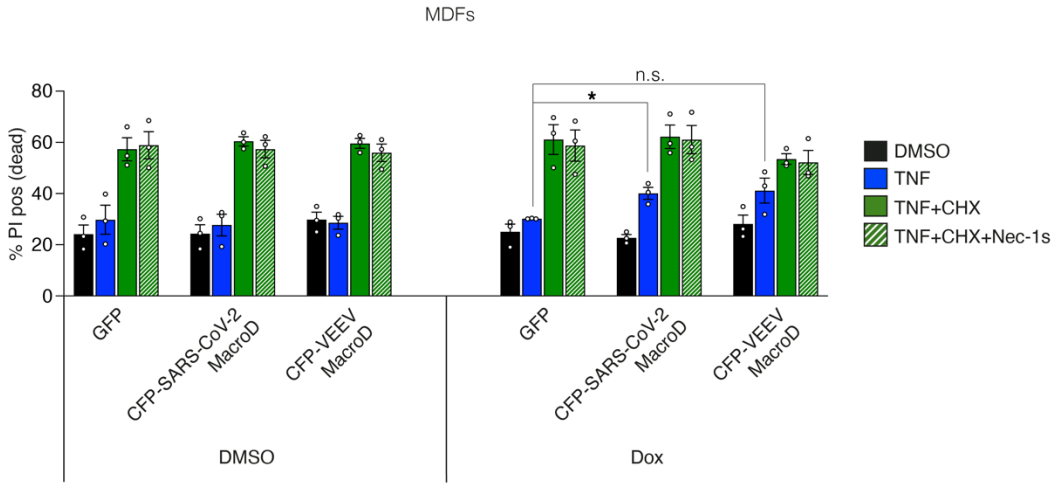

Fig. S6. Viral macrodomains had no effect on TNF+CHX-induced death

Level of cell death assessed by PI positive cells. WT MDFs expressing Dox-inducible GFP or CFP-SARS-CoV-2 macrodomain or CFP-VEEV macrodomain were pre-treated with ± Dox (10 ng/mL) for 9 hours. Cells were then treated with either a high dose of TNF (100 ng/mL) or a low dose of TNF (T; 50 ng/mL) combined with cycloheximide (CHX; 1 µg/mL) ± Nec-1s (10 µM) in the absence of Dox for another 20 hours, and amount of cell death was assessed by PI staining and flow cytometry. Graph shows mean ± SEM, n=3 independent MDFs. Comparisons were performed with a Student's t test whose values are denoted as *p ≤ 0.05 and and n.s.= no significance.

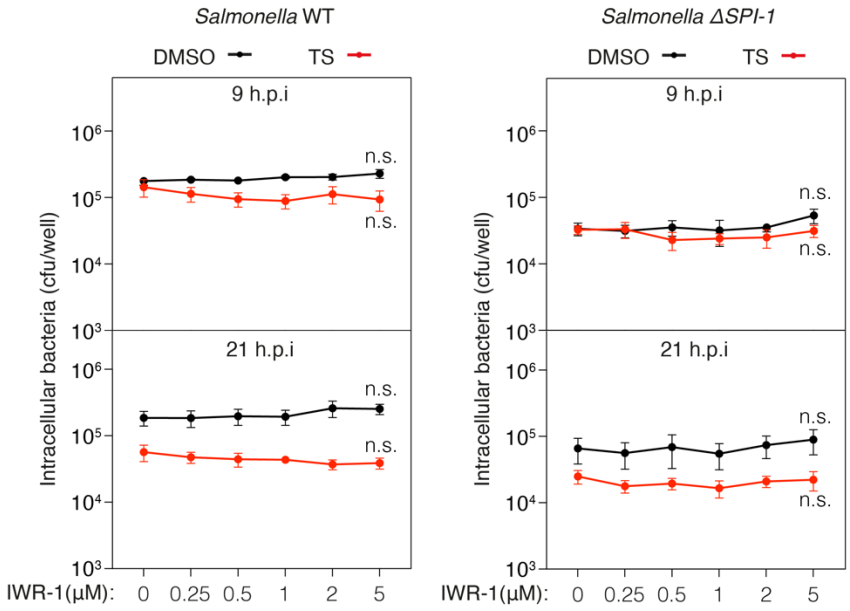

Fig. S7. Tankyrases protect against the cytotoxic effect of TNF under infection condition

Intracellular bacteria enumeration of *Salmonella* infected cells. iBMDMs were infected with Salmonella SL1344 WT or *ΔSPI-1* (MOI : 2), treated with TNF (10 ng/mL) + Smac-mimetic (250 nM) (TS) ± IWR-1 (250nM, 500nM, 1 μM, 2 μM, 5 μM) at 3 hours post infection (h.p.i), then intracellular bacteria were enumerated at 9 h.p.i and 21 h.p.i. Graph shows mean ± SEM, n=3 independent experiments. Comparisons were performed with a Student's t test whose values are denoted as n.s.= no significance.

MOI; multiplicity of infection. cfu; colony forming units.

Table S1. MS raw data for C-terminally 3x FLAG tagged caspase-8 IP

Gene name	Unique peptides	Unique sequence coverage	Log2 FC TSI-untreated	P value (-Log10)	Visual verification of peptide distribution
Ripk1	26	54.9	3.144	0.000165	Y
Tnks	11	12.6	1.717	0.003173	Y
Fpgs	6	18.3	1.576	0.012165	N
Ripk3	14	42.4	1.442	0.030146	Y
Vps52	6	9.1	1.342	0.060197	N
Tnfaip3	7	10.3	1.236	0.018105	Y
Tab1	9	31.3	0.785	0.183953	N
Immt	1	47.2	0.781	0.140838	N
Tradd	6	26.8	0.554	0.399367	Y
Dnajc13	35	20.6	0.547	0.247936	N
Mbnl1	6	23.6	0.504	0.371307	N
Tu52	5	10.8	0.458	0.440472	N
Fadd	7	50.2	0.445	0.535684	Y
Huwe1	40	14.4	0.365	0.514900	N
Ncbp1	10	15.1	0.354	0.556543	N
Carkd	1	27.9	0.295	0.527826	N
Ilk	16	40.7	0.263	0.431446	N
Dars	32	67.5	0.169	0.521317	N
Casp8	45	82.9	0.144	0.888903	N/A
Akap12	30	30.6	0.121	0.848520	N
Nab1	3	11.9	0.059	0.899417	N
Fam129a	16	21.3	0.022	0.969615	N
Fam45a	7	22.4	0.002	0.997329	N
Rraga	1	22.4	-0.023	0.960796	N
Apaf1	8	7.9	-0.028	0.955331	N
Slc1a5	4	11.5	-0.068	0.904336	N
Urod	7	30.8	-0.069	0.889015	N
Fbxo38	11	12.8	-0.121	0.774825	N
Kti12	6	30.5	-0.158	0.740669	N
Copg2	11	20.9	-0.173	0.688017	N
Prmt3	6	13.6	-0.208	0.717812	N

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Continued

Gene name	Unique peptides	Unique sequence coverage	Log2 FC TSI-untreated	P value (-Log10)	Visual verification of peptide distribution
Rragc	5	13.1	-0.375	0.451706	N
Tmem173	8	31.5	-0.385	0.507883	N
Cth	3	10.3	-0.745	0.124432	N
Rint1	5	9.9	-0.774	0.068940	N
Hsdl1	5	21.5	-0.851	0.220485	N
Hacd3	4	14.6	-1.131	0.053466	Y
Cwc22	5	7.7	-1.355	0.041065	N

Casp8$^{-/-}$*.Mlkl*$^{-/-}$ MDFs expressing Dox-inducible C-terminally 3x FLAG tagged caspase-8 (caspase-8 C3FLAG) were treated with 20 ng/mL Dox for 3 hours followed by TNF (100 ng/mL) + Smac-mimetic (500 nM) + IDN-6556 (5 μM) (TSI) for 1.5 hours in the absence of Dox before being subjected to anti-FLAG immunoprecipitation. Untreated *Casp8*$^{-/-}$*.Mlkl*$^{-/-}$ MDFs expressing Dox-inducible tagless caspase-8 were used as negative control. Immunoprecipitants were eluted by FLAG peptide and then subjected to Mass Spectrometry analysis. Proteins shown in this table were filtered by requiring a P value <0.05 in a pairwise comparison between the caspase-8 C3FLAG in either the untreated or TSI-treated samples and the untreated tagless caspase-8 negative control.

Y: a clear visual difference in the peptide distribution between untreated and TSI treated samples

N: not a strong visual difference in the peptide distribution between untreated and TSI treated samples

N/A: not applicable

Table S2. ADP-ribosylome ± TSI stimulation in BMDMs lysates

BMDMs were treated with TNF (100 ng/mL) + Smac-mimetic (500 nM) + caspase inhibitor (5 μM) (TSI) for 1.5 hours. Lysates were prepared in 6M GdnHCl. Identification of ADP-ribosylated peptides was performed and all relevant data have been deposited to the ProteomeXchange Consortium via the PRIDE (http://www.ebi.ac.uk/pride) partner repository with the data set identifier PXD032131.