Supplemental Information

A Class of Viral Inducer of Degradation of the Necroptosis Adaptor RIPK3 Regulates Virus-Induced Inflammation

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Table S1: CPXV genes included in the siRNA screen. Related to Figure 3.

CPXV gene name	Identity with VACV	Query coverage	siRNA sequence used
CPXV001	None found	N/A	5' rArArCrCrUrCrUrUrUrCrUrGrArUrGrGrArGrUrCrArCrUAA 3' 5' rUrUrArGrUrGrArCrUrCrCrArUrCrArGrArArArGrArGrGrUrUrUrA 3'
CPXV002	None found	N/A	5' rArUrArCrGrArUrUrArUrGrArUrCrArCrUrArArArArUrATT 3' 5' rArArUrArUrUrUrUrArGrUrGrArUrCrArUrArArUrCrGrUrArUrCrA 3'
CPXV003	81%	93%	5' rArArGrGrArArUrCrArUrCrUrGrArArCrUrUrGrArGrGrUCA 3' 5' rUrGrArCrCrUrCrArArGrUrUrCrArGrArUrGrArUrUrCrCrUrUrGrC 3'
CPXV004	None found	N/A	5' rGrGrArArUrUrGrGrArArUrUrArGrUrArArUrCrArGrUrUTA 3' 5' rUrArArArCrUrGrArUrUrArCrUrArArUrUrCrCrArArUrUrCrCrCrA 3'
CPXV005	75%	25%	5' rGrArUrArCrArArUrCrUrCrUrUrArUrCrArUrGrUrGrGrGTA 3' 5' rUrArCrCrCrArCrArUrGrArUrArArGrArGrArUrUrGrUrArUrCrArG 3'
CPXV006	90%	16%	5' rArGrArArUrGrArArGrGrUrCrArCrArUrUrUrCrUrGrUrCAA 3' 5' rUrUrGrArCrArGrArArArUrGrUrGrArCrCrUrUrCrArUrUrCrUrCrA 3'
CPXV007	None found	N/A	5' rCrCrArArGrArUrGrArUrArArArArArCrArUrUrUrArCrCGG 3' 5' rCrCrGrGrUrArArArUrGrUrUrUrUrArUrCrArUrCrUrUrGrGrArC 3'
CPXV008	26%	88%	5' rGrCrUrArUrGrUrUrGrArUrArArArCrUrArCrUrUrArCrATT 3' 5' rArArUrGrUrArArGrUrArGrUrUrUrArUrCrArArCrArUrArGrCrUrU 3'
CPXV009	40%	87%	5' rCrGrUrGrGrArUrGrArCrArUrCrArArArCrArUrUrGrUrCTG 3' 5' rCrArGrArCrArArUrGrUrUrUrGrArUrGrUrCrArUrCrCrArCrGrGrU 3'
CPXV010	31%	54%	5' rGrGrArUrGrArUrCrUrCrUrArUrArArCrArUrUrGrArCrUTG 3' 5' rCrArArGrUrCrArArUrGrUrUrArUrArGrArGrArUrCrArUrCrCrArU 3'
CPXV011	59%	45%	5' rCrUrUrGrGrGrArUrArUrUrCrUrGrArUrGrArArArUrCrAGA 3' 5' rUrCrUrGrArUrUrUrCrArUrCrArGrArArUrArUrCrCrCrArArGrArU 3'
CPXV012	None found	N/A	5' rGrUrCrCrUrUrArCrCrArUrUrGrGrUrArUrArArArCrArACA 3' 5' rUrGrUrUrGrUrUrUrArUrArCrCrArArUrGrGrUrArArGrGrArCrArU 3'
CPXV013	29%	61%	5' rGrArUrGrArUrArCrUrUrArUrUrCrUrArGrUrArArArUrUGG 3' 5' rCrCrArArUrUrUrArCrUrArGrArArUrArArGrUrArUrCrArUrCrArU 3'
CPXV014	55%	24%	5' rGrArUrGrUrUrArUrArUrCrGrArCrUrUrCrUrGrArArUrUGA 3' 5' rUrCrArArUrUrCrArGrArArGrUrCrGrArUrArUrArArCrArUrCrUrG 3'
CPXV015	None found	N/A	5' rCrUrGrArUrArCrCrArCrUrArCrArArArArArArGrUrACA 3' 5' rUrGrUrArCrUrUrUrUrUrUrGrUrArGrUrGrGrUrArUrCrArGrGrA 3'
CPXV016	24%	60%	5' rGrUrGrArArGrArUrGrArUrArUrUrCrArUrUrArArArCrCrAAC 3' 5' rGrUrUrGrGrUrUrUrArArUrGrArUrArUrCrArUrCrUrUrCrArCrUrU 3'
CPXV017	34%	90%	5' rArGrArUrArArUrArArArCrArArUrGrUrArUrCrArArCrUGA 3' 5' rUrCrArGrUrUrGrArUrArCrArUrUrGrUrUrUrArUrUrArUrCrUrCrA 3'
CPXV018	None found	N/A	5' rArUrArGrGrUrGrArUrGrArArArUrUrUrUrCrUrUrArCrCGT 3' 5' rArCrGrGrUrArArGrArArArArUrUrUrCrArUrCrArCrCrUrArUrCrA 3'
CPXV019	26%	53%	5' rGrUrGrArGrArArUrCrArGrUrArUrGrArUrArGrUrUrArCGA 3' 5' rUrCrGrUrArArCrUrArUrCrArUrArCrUrGrArUrUrCrUrCrArCrArA 3'
CPXV020	31%	85%	5' rGrGrArUrGrUrArArArUrUrArCrArArUrUrGrArUrArUrCTA 3' 5' rUrArGrArUrArUrUrCrArArUrUrGrUrArArUrUrUrArCrArUrCrCrArU 3'
CPXV025	94%	35%	5' rArGrArArUrUrGrArArCrGrArGrArUrGrArArArArArArATA 3' 5' rUrArUrUrUrUrUrUrUrCrArUrCrUrCrGrUrUrCrArArUrUrCrUrUrG 3'
CPXV031	54%	78%	5' rCrUrGrArUrCrUrCrArCrUrArArUrGrUrGrArUrArGrArGAA 3' 5' rUrUrCrUrCrUrArUrCrArCrArUrUrArGrUrGrArGrArUrCrArGrUrA 3'
CPXV045	75%	47%	5' rArGrArArUrUrCrArGrUrArUrCrUrArGrArGrArUrArUrGrGAT 3' 5' rArUrCrCrArUrArUrCrUrCrUrArGrArUrArCrUrGrArUrUrCrUrGrG 3'
CPXV051A	None found	N/A	5' rGrUrGrGrArUrGrCrArArUrArArArArUrGrUrUrUrArArArCAT 3' 5' rArUrGrUrUrUrArArArCrArUrUrUrArUrUrGrCrArUrCrCrArCrCrG 3'
CPXV058	None found	N/A	5' rGrUrArArUrGrArArArUrUrArUrUrCrArCrUrArUrArUrCAG 3' 5' rCrUrGrArUrArUrArGrUrGrArArUrArArUrUrUrCrArUrUrArCrCrA 3'
CPXV063	None found	N/A	5' rGrUrGrGrArUrGrCrArArUrArArArArUrGrUrUrUrArArArCAT 3' 5' rArUrGrUrUrUrArArArCrArUrUrUrArUrUrGrCrArUrCrCrArCrCrG 3'
CPXV078A	None found	N/A	5' rGrUrCrCrArUrGrUrUrGrUrCrUrArUrUrUrCrCrArArCrAGG 3' 5' rCrCrUrGrUrUrGrGrArArArUrArGrArCrArArCrArUrGrGrArCrGrC 3'
CPXV096	None found	N/A	5' rCrUrUrCrUrUrCrArCrUrGrArArCrUrCrUrGrArArArArATA 3' 5' rUrArUrUrUrUrUrCrArGrArGrUrUrCrArGrUrGrArArArGrUrG 3'
CPXV116	None found	N/A	5' rGrGrCrGrUrUrUrArArArUrArUrArGrArCrArUrUrArUrCAA 3' 5' rUrUrGrArUrArArUrGrUrCrUrArUrArUrUrUrArArArCrGrCrCrArU 3'
CPXV141	84%	100%	5' rGrArArCrArArGrArUrGrArCrArArGrArArGrArArUrArCTA 3' 5' rUrArGrUrArUrUrCrUrUrCrUrUrGrUrCrArUrCrUrUrGrUrUrCrArG 3'
CPXV152A	None found	N/A	5' rCrArArGrArUrArCrArCrGrArGrGrUrCrArArUrArArUrAAT 3' 5' rArUrUrArUrUrArUrUrGrArCrCrUrCrGrUrGrUrArUrCrUrUrGrGrA 3'

CPXV159	85%	100%	5' rGrCrArGrUrArArUrArCrUrArGrCrArArGrArArArGrArUAA 3' 5' rUrUrArArUrCrUrUrUrCrUrUrGrCrUrArGrUrArUrUrArCrUrGrCrArU 3'
CPXV160	None found	N/A	5' rArUrCrArUrUrGrCrUrArArArUrUrUrUrUrCrArArUrArGTA 3' 5' rUrArCrUrArUrUrGrArArArArArUrUrUrArGrCrArArUrGrArUrGrC 3'
CPXV166	87%	100%	5' rCrArArArGrCrArUrUrCrUrArCrUrGrUrUrGrUrUrArArATA 3' 5' rUrArUrUrUrArArCrArArCrArGrUrArGrArArUrGrCrUrUrUrGrGrC 3'
CPXV191	74%	33%	5' rGrUrGrGrArUrArCrUrUrUrGrGrUrGrGrArArUrArGrArUGA 3' 5' rUrCrArUrCrUrArUrUrCrCrArCrCrArArArGrUrArUrCrCrArCrArU 3'
CPXV192	None found	N/A	5' rGrCrCrArUrGrArUrCrArUrGrUrUrArCrCrArUrArCrArUCA 3' 5' rUrGrArUrGrUrArUrGrGrUrArArCrArUrGrArUrCrArUrGrGrCrUrA 3'
CPXV197	94%	42%	5' rGrGrArGrGrArGrArArUrGrArUrArCrArGrUrArArArUrCTT 3' 5' rArArGrArUrUrUrArCrUrGrUrArUrCrArUrUrCrUrCrCrUrCrCrC 3'
CPXV203	78%	30%	5' rGrCrUrArArArGrArUrUrUrCrUrArUrUrCrCrArArArArGAA 3' 5' rUrUrCrUrUrUrUrGrGrArArUrArGrArArArUrCrUrUrUrArGrCrCrU 3'
CPXV204	91%	32%	5' rGrGrArArUrCrArUrUrGrCrUrArArArArGrArArUrGrUrCAA 3' 5' rUrUrGrArCrArUrUrCrUrUrUrUrArGrCrArArUrGrArUrUrCrCrArC 3'
CPXV213	92%	38%	5' rArUrGrArArUrGrUrGrGrUrArArArArGrArArUrUrArCrUTT 3' 5' rArArArGrUrArArUrUrCrUrUrUrUrArCrCrArCrArUrUrCrArUrGrU 3'
CPXV214	None found	N/A	5' rArUrGrArUrGrArUrCrArGrArCrArArCrUrCrArArArUrUAC 3' 5' rGrUrArArUrUrUrGrArGrUrUrGrUrCrUrGrArUrCrArUrCrArUrGrG 3'
CPXV215	93%	24%	5' rCrArGrCrUrCrUrGrCrArUrArCrUrArUrUrCrUrUrCrArUGA 3' 5' rUrCrArUrGrArArGrArArUrArGrUrArUrGrCrArGrArGrCrUrGrArU 3'
CPXV219	None found	N/A	5' rArGrCrArUrUrGrArArUrGrArCrArUrGrUrUrUrArArGrGAA 3' 5' rUrUrCrCrUrUrArArArCrArUrGrUrCrArUrUrCrArArUrGrCrUrUrC 3'
CPXV220	31%	94%	5' rGrArArUrCrArUrCrUrGrUrUrUrUrArArArArArCrCrUAT 3' 5' rArUrArGrGrUrUrUrUrUrUrArArArArCrArGrArUrGrArUrUrCrArA 3'
CPXV221	39%	27%	5' rArGrGrUrUrUrCrUrUrUrArCrArArArCrGrArUrArArArArGTA 3' 5' rUrArCrUrUrUrArUrCrGrUrUrUrGrUrArArArGrArArArCrCrUrGrA 3'
CPXV222	40%	87%	5' rCrGrUrGrGrArUrGrArCrArUrCrArArArCrArUrUrGrUrCTG 3' 5' rCrArGrArCrArArArUrGrUrUrUrGrArUrUrGrUrCrArUrCrCrArCrGrGrU 3'
CPXV223	26%	88%	5' rGrCrUrArUrGrUrUrGrArUrArArArCrUrArCrUrUrArCrATT 3' 5' rArArUrGrUrArArArGrUrArGrUrUrUrArUrCrArArCrArUrArGrCrUrU 3'
CPXV224	None found	N/A	5' rCrCrArArGrArUrGrArUrArArArArArCrArUrUrUrArCrCGG 3' 5' rCrCrGrGrUrArArArUrGrUrUrUrUrUrArUrCrArUrCrUrUrGrGrArC 3'
CPXV225	90%	16%	5' rArGrArArUrGrArArGrGrUrCrArCrArUrUrUrCrUrGrUrCAA 3' 5' rUrUrGrArCrArGrArArArUrGrUrGrArCrCrUrUrCrArUrUrCrUrCrA 3'
CPXV226	75%	25%	5' rGrArUrArCrArArUrCrUrCrUrUrArUrCrArUrGrUrGrGrGTA 3' 5' rUrArCrCrCrArCrArUrGrArUrArArGrArGrArUrUrGrUrArUrCrArG 3'
CPXV227	81%	93%	5' rArArGrGrArArUrUrCrArUrCrUrGrArArCrUrUrGrArGrGrUCA 3' 5' rUrGrArCrCrUrCrArArGrUrUrCrArGrArUrGrArUrUrCrCrUrUrGrC 3'
CPXV228	None found	N/A	5' rArCrUrArArArArUrArUrUrArArArCrCrUrCrUrUrUrCrUGA 3' 5' rUrCrArGrArArArGrArGrGrUrUrUrArArUrArUrUrUrUrArGrUrGrA 3'
CPXV229	None found	N/A	5' rArArCrCrUrCrUrUrUrCrUrGrArUrGrGrArGrUrCrArCrUAA 3' 5' rUrUrArGrUrGrArCrUrCrCrArUrCrArGrArArArGrArGrGrUrUrUrA 3'

Table S2. Q-PCR and genotyping primers. Related to Figure 7.

Ccl2	5'-aggtgtcccaaagaagctgta 5'-atgtctggacccattccttct
Cxcl2	5'-gagcttgagtgtgacgcccccagg 5'-gttagccttgcctttgttcagtatc
Ccl4	5'-ttgctcctcacgttcagatttc 5'-ggagacacgcgtcctataacta
Il6	5'-cggagaggagacttcacaga. 5'-ccagtttggtagcatccatc
Ccl8	5'- ttctttgcctgctgctcata 5'- ctcgtagcttttcagcaccc
Cxcl1	5'- cgaagtcatagccacactcaa 5'- gagcagtctgtcttctttctcc
Cxcl10	5'- tcaggctcgtcagttctaagt 5'- ccttgggaagatggtggttaag
Oas1a	5'-cagatgttcagcagtggttaga 5'- gggttcctctcttgtgtgtatg
Oasl1	5'- gtgctcaaggtactcaaggtag 5'- ctgtggaaacagctcagg
Alox15	5'- ctggatgaggagctcaagaaag 5'- ccaggtactgctgacta
C3	5'- ctggcctctggagtagatagat 5'-cagtcttcttcggtgtg
C4a	5'- tgccaggaccttcagataga 5'- tctgtggctggcatgttatag
Ptgs1	5'- aagatgggtcctggctttac 5'- ggtgatactgtcgttccagatt
Arg1	5'- acagcaaagcagacagaacta 5'- gaaaggaactgctgggataca
Tbp1	5'- caaacccagaattgttctcctt 5'- atgtggtcttcctgaatccct
Ripk3	5'-tgtcaagttatggcctactggtgcg 5'-aaccatagccttcacctcccaggat
Tnf	5'-cccactctgacccctttact 5'-tttgagtccttgatggtggt
CPXV011	5'-agtgtataatggaaatgatataaacgcc 5'-atccgctcctagttctataaatgtatc
CPXV016	5'-tcttagatccagaaaccgataacg 5'-ccctaaacaattgacggctg
CPXV017	5'-aactagatgctggcggaag 5'-tccactgccacatcaagac

Supplementary Figure 1

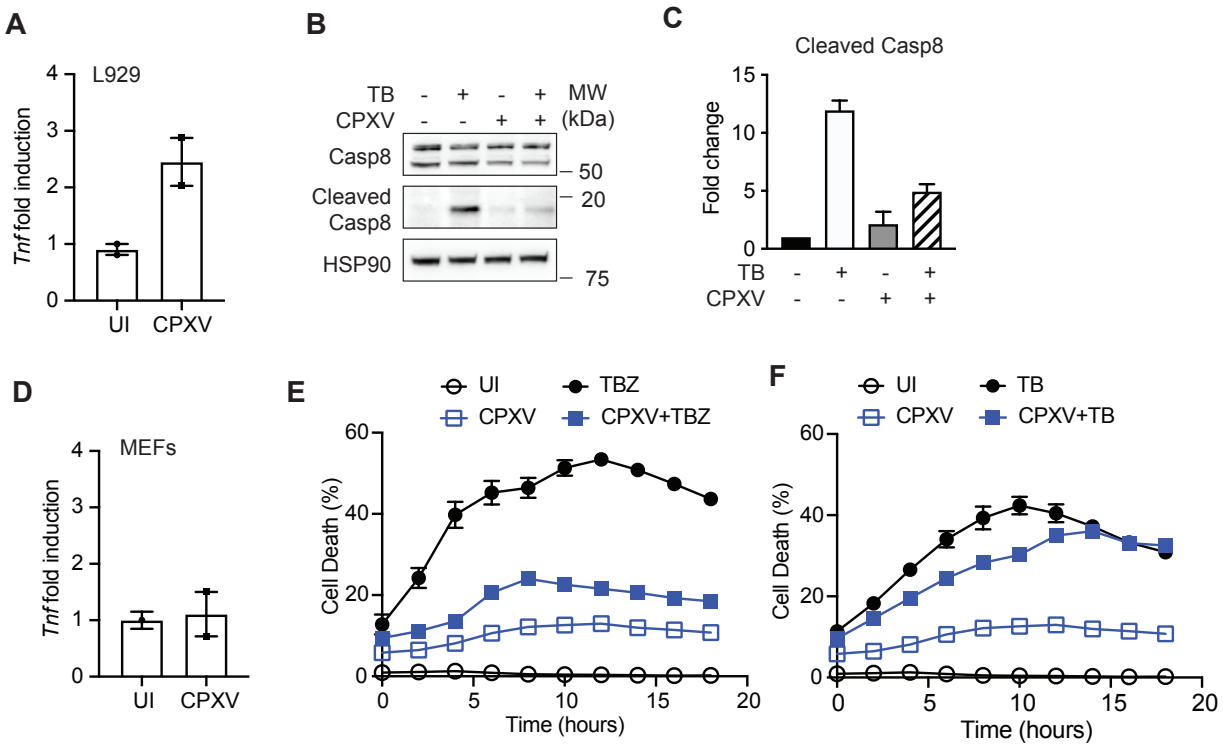

Figure S1: Cell death responses of L929 and MEFs in response to CPXV infection. Related to Figure 1. (**A**) CPXV infection induces autocrine *Tnf* expression in L929 cells. Uninfected (UI) and CPXV-infected L929 cells were harvested at 4 hours post-infection and *Tnf* expression was determined by qPCR. (**B**) Uninfected or CPXV-infected MEFs were treated with TNF and BV6 for 4 hours as indicated. Caspase 8 cleavage was determined by Western blot. (**C**) Quantification of caspase 8 cleavage in (**B**). (**D**) CPXV did not induce autocrine *Tnf* expression in MEFs. *Tnf* expression was determined 4 hours post-infection by qPCR. (**E-F**) Uninfected and CPXV-infected MEFs were treated with (**E**) TNF, BV6 and zVAD-fmk (TBZ) or (**F**) TNF and BV6 (TB). Cell death was determined by Incucyte using Yoyo-1.

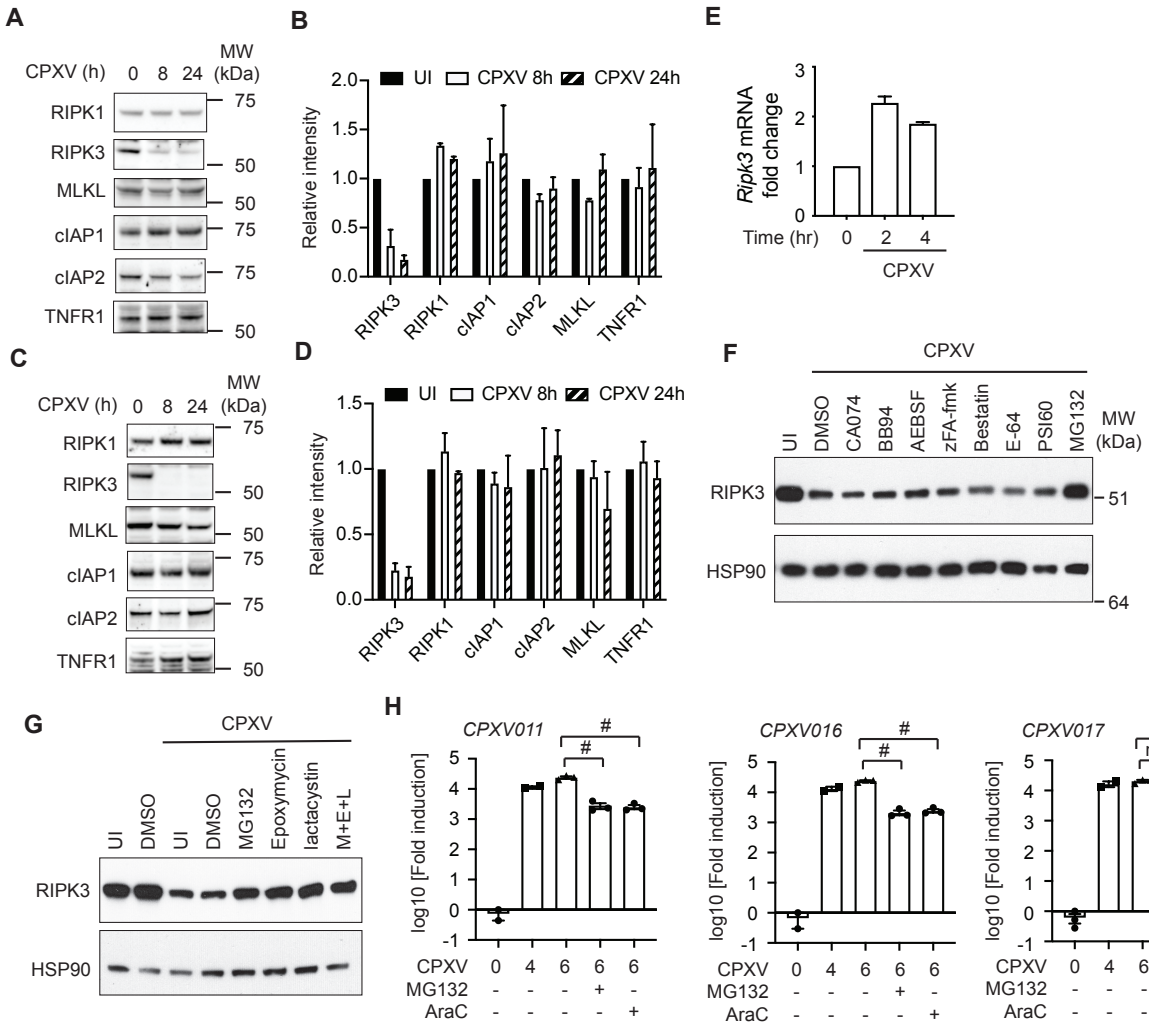
A
CPXV (h) 0 8 24
MW (kDa)
RIPK1
RIPK3
MLKL
cIAP1
cIAP2
TNFR1
75
50
50
75
75
50
B
UI
CPXV 8h
CPXV 24h
Relative intensity
RIPK3
RIPK1
cIAP1
cIAP2
MLKL
TNFR1
C
D
E
Ripk3 mRNA fold change
Time (hr) 0 2 4
CPXV
F
CPXV
UI
DMSO
CA074
BB94
AEBSF
zFA-fmk
Bestatin
E-64
PSI60
MG132
MW (kDa)
RIPK3
HSP90
51
64
G
CPXV
UI
DMSO
UI
DMSO
MG132
Epoxymycin
lactacystin
M+E+L
RIPK3
HSP90
H
CPXV011
CPXV016
CPXV017
log10 [Fold induction]
#
n.s
CPXV 0 4 6 6 6
MG132 - - - + -
AraC - - - - +

Figure S2: Effect of CPXV on RIPK3 expression in different cell lines. Related to Figure 2. (**A-B**) Expression of cell death proteins in HT29 cells infected with CPXV. (**B**) Quantification of results in (**A**) from two independent experiments. Expression level in uninfected cells at T=0 was normalized to "1". (**C-D**) Expression of cell death proteins in Colo205 in response to CPXV. (**D**) Quantification of results in (**C**) from two independent experiments. The expression level in uninfected cells at T=0 was normalized to "1". (**E**) mRNA level of *Ripk3* in response to CPXV infection was determined by qPCR. (**F-G**) Uninfected or CPXV-infected L929 cells were treated with the indicated inhibitors. RIPK3 expression was determined by Western blot. M: MG132, E: epoxomicin, L: lactacystin. (**H**) The effect of MG132 and AraC on CPXV gene expression. L929 cells infected with CPXV and treated with the indicated inhibitors were analyzed for expression of the indicated CPXV genes by qPCR.

Supplementary Figure 3

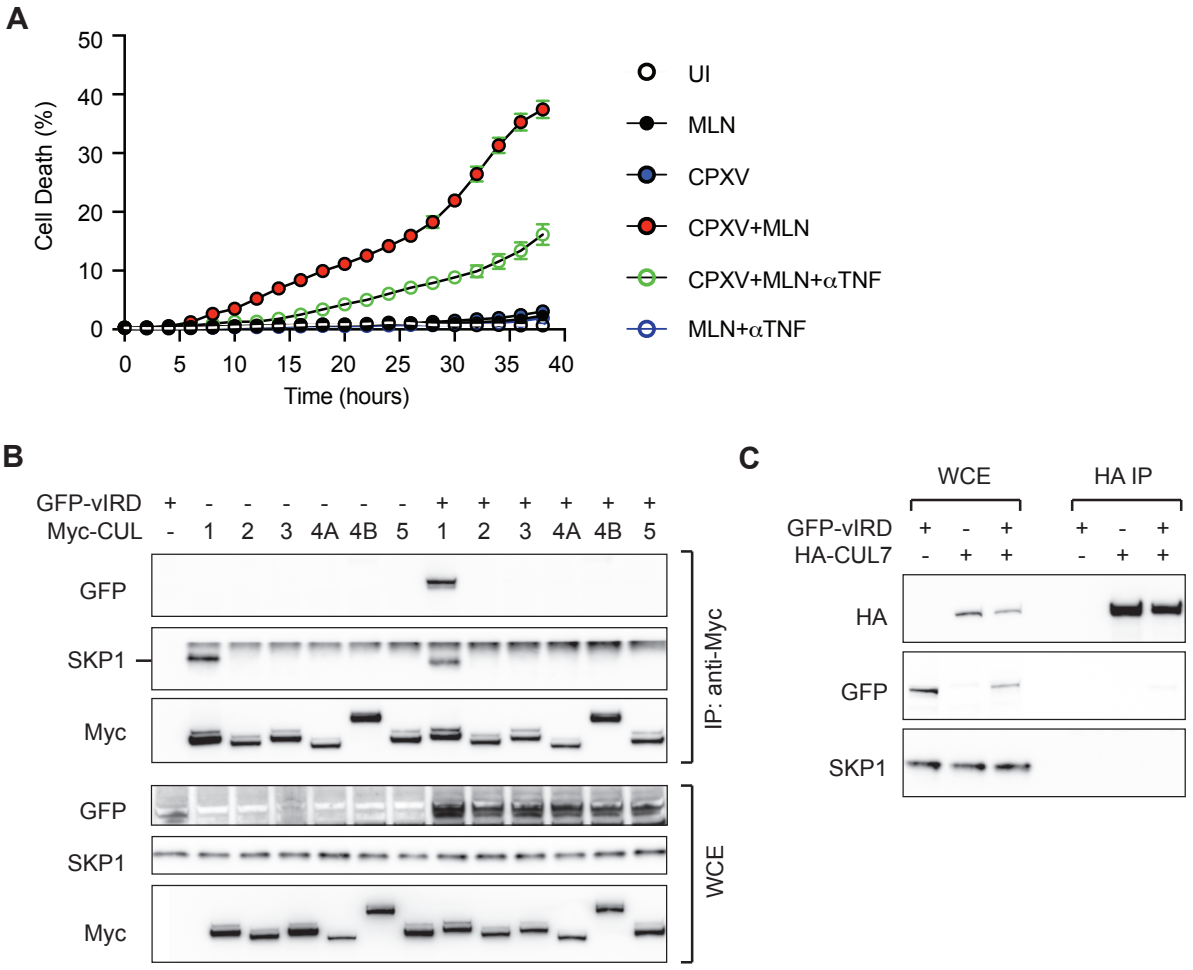

Figure S3: CUL1 is critical for vIRD-mediated necroptosis resistance. Related to Figure 3.

(**A**) Inhibition of CUL1 neddylation sensitizes CPXV-infected cells to autocrine TNF-induced necroptosis. Uninfected and CPXV-infected L929 cells were treated with MLN4924 (MLN) and/or anti-mouse TNF antibody. Cell death was determined by Incucyte live cell imaging. (**B**) vIRD interacts with CUL1. HEK293T cells were transfected with the indicated plasmids. Immunoprecipitation with Myc-tag antibody was followed by Western blot detection of GFP-vIRD. (**C**) vIRD does not interact with CUL7. HEK293T cells were transfected with the indicated plasmids. Immunoprecipitation and Western blot were performed as indicated.

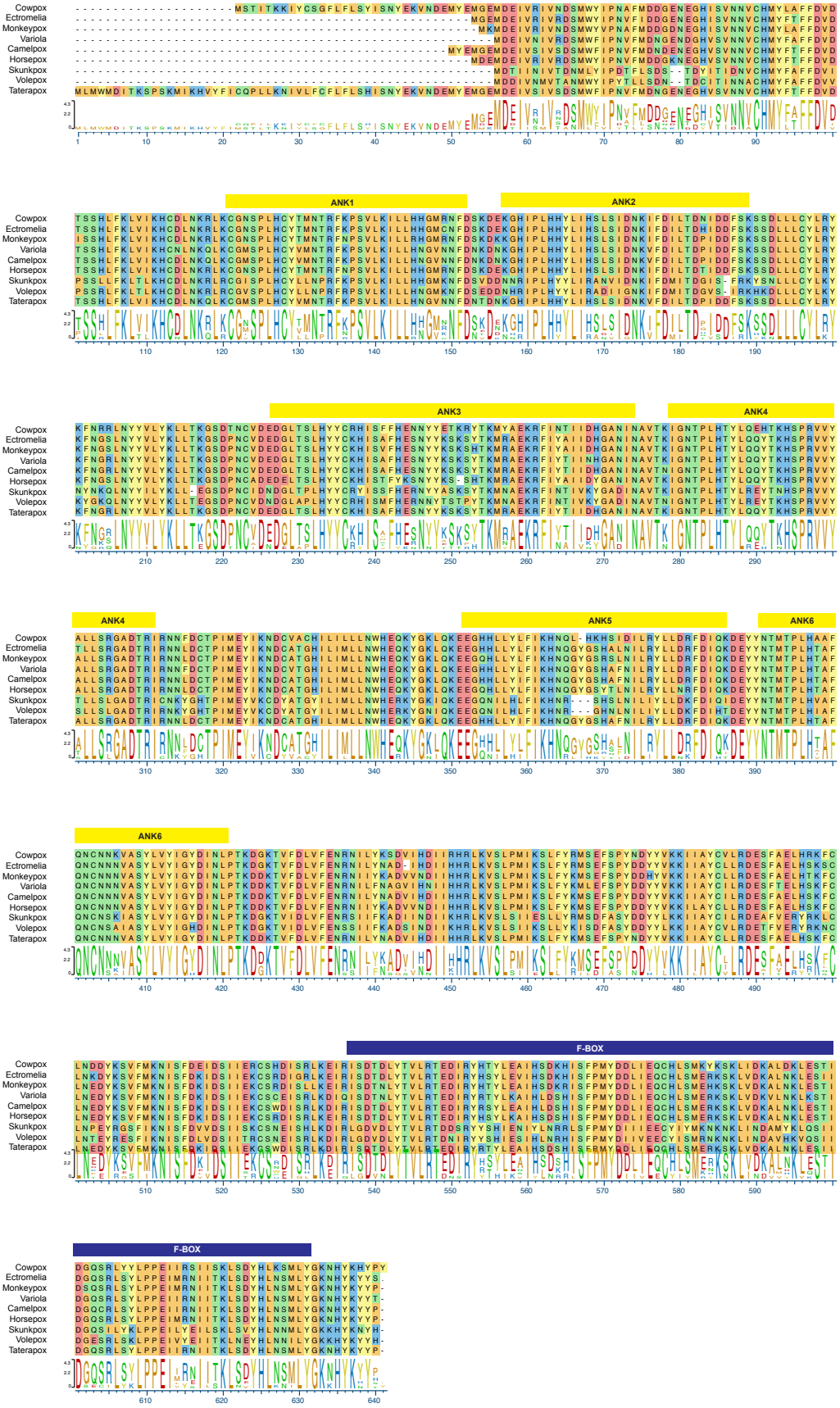
Cowpox
Ectromelia
Monkeypox
Variola
Camelpox
Horsepox
Skunkpox
Volepox
Taterapox
ANK1
ANK2
ANK3
ANK4
ANK5
ANK6
F-BOX

Figure S4: Highly conserved vIRD orthologues in orthopoxviruses. Related to Figure 5.

vIRD orthologues from cowpox, ectromelia, monkeypox, variola, camelpox, horsepox, skunkpox, volepox and taterapox were aligned using Clustal W. Individual ankyrin repeat and the F-box are highlighted. The consensus sequence is shown below the sequence alignment.

Supplementary Figure 5

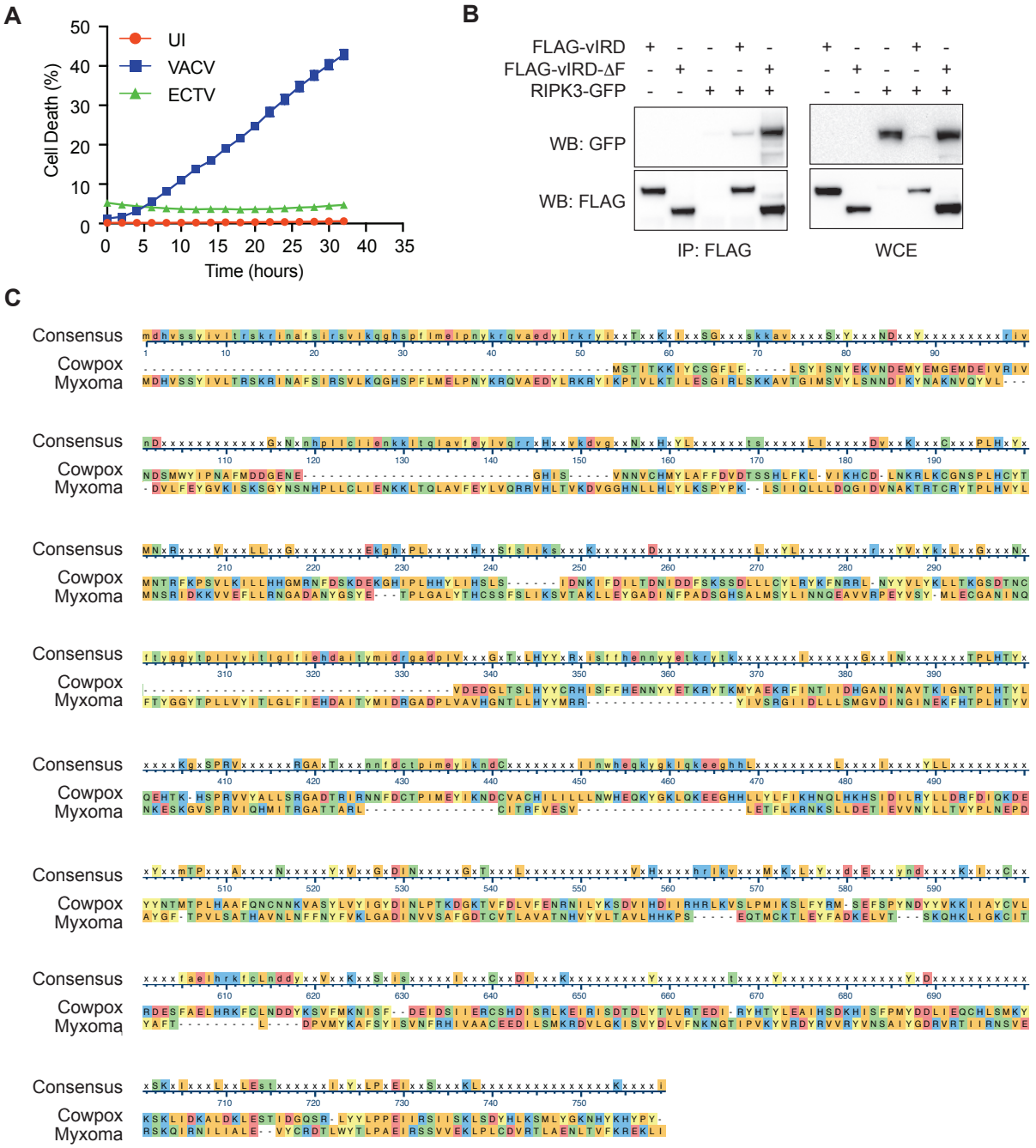

Figure S5: ECTV encodes a functional vIRD orthologue that promotes RIPK3 degradation and necroptosis resistance. Related to Figure 5. (**A**) L929 cells infected with VACV or ECTV were monitored for cell death using Incucyte. (**B**) Expression of ECTV-vIRD but not ECTV-vIRD-ΔF triggered RIPK3 degradation. HEK293T cells were transfected with the indicated plasmids. Immunoprecipitation and Western blot were performed as indicated. (**C**) Sequence alignment of CPXV-vIRD and MYXV-m148R. The alignment reveals poor sequence conservation between the two ankyrin repeat-containing proteins.

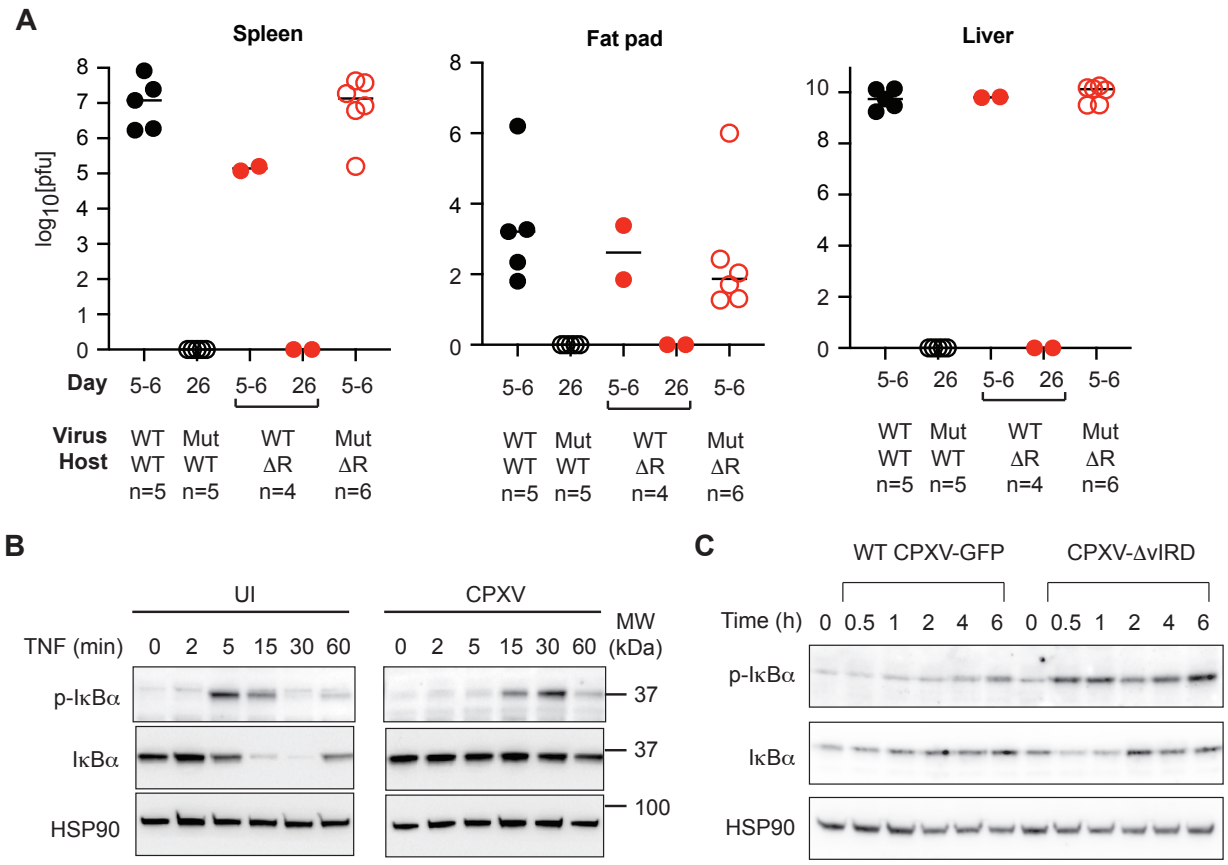

Figure S6: Role of vIRD on viral clearance and NF-κB activation. Related to Figure 6.

(**A**) Viral titers of mice infected with the indicated CPXV were monitored on day 26 or when the mice became moribund and had to be humanely euthanized. (**B**) Effect of vIRD on NF-κB. Uninfected (UI) or CPXV-infected MEFs were stimulated with TNF. IκBα phosphorylation and degradation were determined by Western blot as indicated. (**C**) Human monocytic THP-1 cells were treated with phorbol ester overnight. Cells were then infected with CPXV or CPXV-ΔvIRD for the indicated times. IκBα phosphorylation and degradation were determined by Western blot as indicated.

Supplementary Figure 7

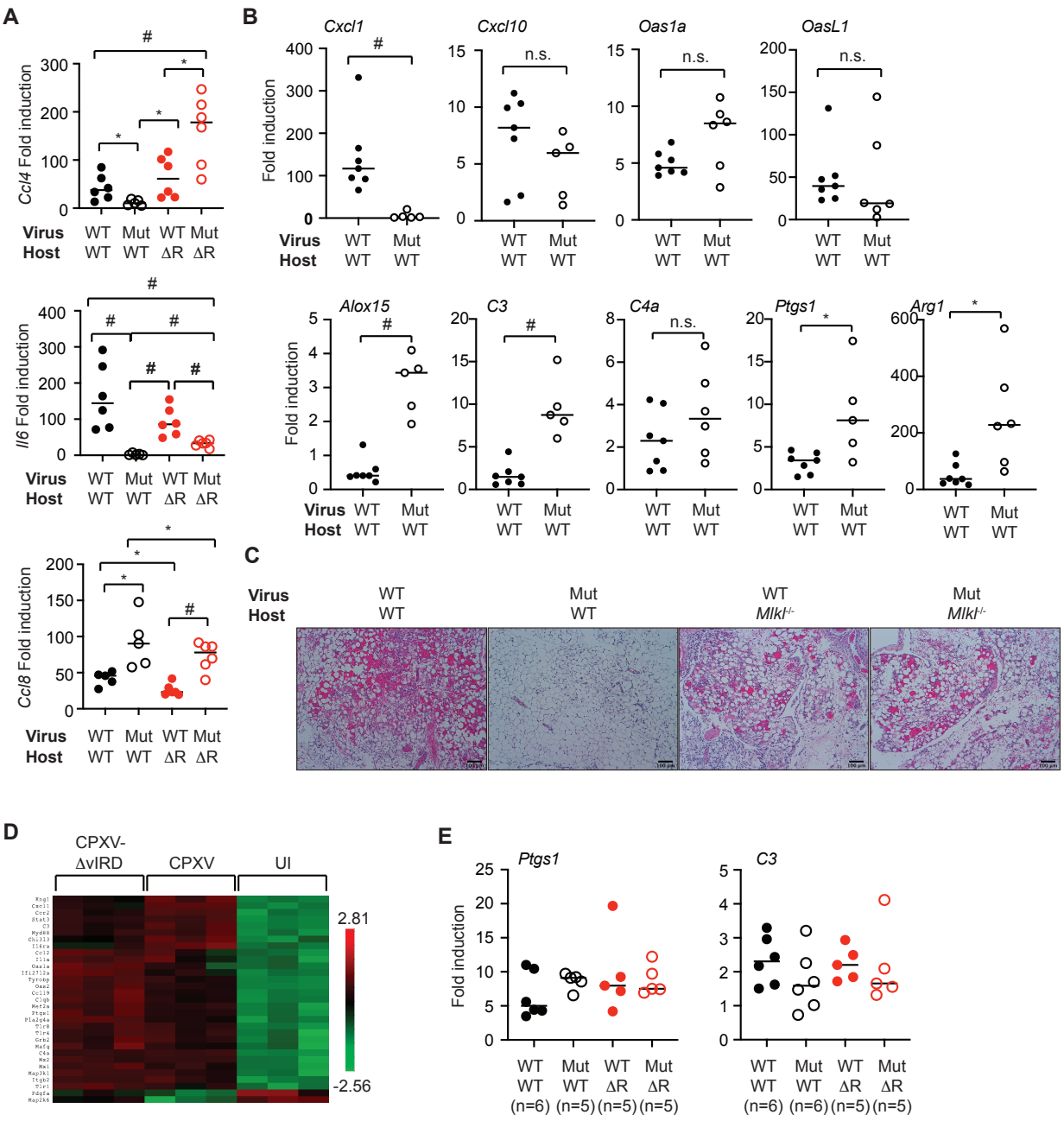

Figure S7: Tissue-specific regulation of inflammation by vIRD. Related to Figure 7.

(**A-B**) Cytokine expression in the visceral fat pad 3.5 days post-infection was determined by qPCR. (**C**) H&E staining og visceral fat pad from CPXV or CPXV-ΔvIRD infected wild type or *Mlkl*$^{-/-}$ mice. Scale bars represent 100 μm. (**D**) Heat map of the top DE genes in the liver identified by Nanostring analysis. Uninfected mice (UI) were used for comparison. (**E**) qPCR validation of *Ptgs1* and *C3*, two of the top DE genes in the liver identified in the Nanostring analysis.