## Appendix:



## Appendix Supplementary Methods

ZBP1-Zα1α2mut transgenic mice

5' and 3' homology arms were PCR amplified from C57BL/6 Bac clones RP24-289A19 and RP24-299I12. The N46A (AAT to GCT) and Y50A (TAC to GCC) mutations were introduced into exon 2 in the 5' homology arm. The N122A (AAC to GCC) and Y126A (TAT to GCT) mutations were introduced into exon 3 in the 3' homology arm. The homology arms were cloned into the targeting vector using AscI and PspXI (5' homology arm) and KpnI and NotI (3' homology arm) restriction sites. DTA cloned upstream of the 5' homology arm was used for negative selection. A Neo cassette is inserted between the homology arms and is flanked by two LoxP-sites. The Neo cassette contains a Cre-recombinase gene driven by the testes specific promotor of angiotensin converting enzyme, which self-deletes the cassette when going through male germline (Bunting *et al*, 1999). The linearized vector was electroporated into ES cells (C57Bl/6), followed by drug selection, PCR screening, and Southern Blot confirmation. Out of 139 drug-resistant clones, 6 were confirmed positive for the targeting construct. Two clones were

selected for blastocyst injection, followed by chimera production. Four heterozygous founder males from each ES-cell line were selected for breeding to C57Bl/6 females to generate Neo-deleted *Zbp1*$^{+/Z_{\alpha}1_{\alpha}2}$ offspring. Sequencing and genotyping primers are listed in Appendix Table S1. ZBP1-Zα1α2 primary MEFs were isolated from E12.5-14.5 embryos from heterozygous breeding pairs.

Virus production and infection

Cells were infected with a MOI of 0.001 and 2 hours later the inoculum was washed away. Virus was harvested 2 days after 100% CPE was reached. Virus was concentrated by centrifugation at 30,000 g for 1 hour and viral stocks were sonicated before use. Viral titres were determined by plaque assay on immortalised Zbp1-/- MEFs. Cells were infected for 2 hours in DMEM containing 10% FCS, after which the viral inoculum was washed away and fresh medium was added to the cells. For measuring viral genome replication, MCMV was incubated with 10 U/ml DNase I (Turbo DNase I, ThermoFisher Scientific) for 30 min at 37□C in 1X reaction buffer diluted in PBS. 2 hours after infection with DNase-treated virus, cells were washed several times to remove the inoculum and fresh medium was provided. 50 μM ganciclovir (GCV, Merck) was added to the cells at the time of infection. MCMV was UV inactivated by exposing to 250 mJ/cm2 UV-C (254nm; Spectrolinker XL-1500, Spectroline). For *in vivo* infections, 8-12 week old sex-matched mice were inoculated with $2x10^6$ pfu MCMV-M45$^{WT}$ or MCMV-M45$^{mutRHIM}$ by intraperitoneal injection. 5 days post infection, mice were sacrificed and spleens were harvested and homogenised with glass beads (Sigma Aldrich) in 1 ml DMEM containing 10% FCS on a FastPrep F120 instrument (Thermo Fisher). Debris was removed by centrifugation (300 g, 5 min) and virus-containing supernatant was titrated in duplicate on primary MEFs.

(PAR)-CLIP

7.5 x $10^6$ NIH3T3 cells expressing wild type or mutant ZBP1 were seeded into 15 cm diameter tissue culture dishes and one plate per sample was used as input. For PAR-CLIP, cells were treated with 4-thiouridine (Sigma Aldrich) or 6-thioguanosine (Carbosynth) at the time of MCMV-M45$^{mutRHIM}$ infection (MOI 3). 8 hour later cells were washed, overlaid with 5 ml PBS and crosslinked with 150 mJ/cm$^2$ UV-A (365 nm; Stratalinker 2400, Stratagene) for PAR-CLIP or 150 mJ/cm$^2$ UV-C (254 nm; Spectrolinker XL-1500, Spectroline) for CLIP. Cells were lysed for 20 min. in 1 ml RIPA buffer (50 mM Tris.HCl pH 7.5, 150 mM NaCl, 1% NP-40, 0.1% SDS, 0.5% deoxycholate). Lysates were incubated for 5 min. at 37°C on a shaker (1100 rpm) with Turbo DNase I (Ambion) followed by a 5 min. incubation with RNase A (Affymetrix) at 37°C at 1100 rpm. Lysates were transferred to ice and RNase A was inactivated with 12.5 µl RNasin Plus (Promega). Lysates were centrifuged for 20 min at 17,000 g and supernatant was used as input. Samples were immunoprecipitated with 6 µg of anti-FLAG (Sigma Aldrich, clone M2) covalently linked to magnetic beads (Dynabeads antibody coupling kit, Life Technologies, 30 µg anti-FLAG per mg beads). After 2 hours, beads were washed twice with high salt wash buffer (50 mM Tris.HCl pH 7.5, 1 M NaCl, 1% NP-40, 0.1% SDS, 0.5% deoxycholate) and once with PNK buffer (20 mM Tris.HCl pH 7.5, 10 mM $MgCl_2$, 0.2% Tween-20). The beads were incubated for 3 min. at 65°C on a shaker (1100 rpm) with 100 µl urea cracking buffer (50 mM Tris.HCl pH 7.5, 6 M Urea, 1% SDS, 25% PBS) to denature RNA-protein complexes and to remove contaminating protein-RNA complexes. Supernatant was separated from the beads and transferred to 1 ml of T-20 buffer (50 mM Tris.HCl pH 7.5, 150 mM NaCl, 0.5% Tween-20, 0.1 mM EDTA). The protein-RNA complexes were immunoprecipitated a second time using 5 µg of

anti-FLAG (Sigma Aldrich, clone M2) coupled to protein G magnetic beads (Dynabeads, Life Technologies). After 2 hours, beads were washed twice with high salt wash buffer and twice with PNK wash buffer. RNA was the labelled on-bead with T4 PNK (NEB) and in 40 µl reaction volume (1 µl T4 PNK, 4 µl 10X PNK buffer, 0.5 µl RNasin Plus, 1 µl γ-32P-ATP; Hartmann Analytic) for 5 min at 37°C at 1100 rpm. The T4 PNK reaction mix was removed and the ZBP1-RNA complexes were eluted from the beads by incubating for 10 min. at 70°C in 20 µl NuPAGE LDS sample buffer (Life Technologies) with 50 mM DTT. Samples were loaded on 4-12% Bis-Tris SDS-PAGE gels (Life Technologies) and transferred on nitrocellulose membranes using a wet-blot transfer system (Bio-Rad). To visualize the ZBP1-RNA complexes, the blots were exposed via autoradiography.

ZBP1 purification

Ten 15 cm diameter tissue culture dishes seeded with TLA HEK293T cells were each transfected with 5 µg pLenti6.3/TO/V5-DEST-ZBP1-3xFLAG using FuGENE 6 (Promega). 48 hours post-transfection cells were lysed in hypotonic buffer (10 mM Tris.HCl pH 7.4, 10 mM KCl, 1.5 mM $MgCl_2$) supplemented with protease inhibitor cocktail (Cell Signalling). Lysates were centrifuged at 100,000 g and supernatant was filtered through a 0.45 um PVDF-membrane and applied on a HiTrap heparin HP column (GE Healthcare) equilibrated in buffer B (20 mM Tris.HCl pH 7.4, 0.02% CHAPS and 0.5 mM DTT) and eluted with 0.5 M NaCl in buffer B. ZBP1-3xFLAG was then immunoprecipitated using anti-FLAG-coupled magnetic beads (Sigma Alrich, clone M2), washed 3 times in buffer C (50 mM Tris.HCl pH 7.4, 100 mM NaCl, 10% glycerol, 0.5% NP-40 (Igepal CA-630, Sigma), 0.5 mM EDTA, 0.5 mM EGTA). ZBP1-3xFLAG was eluted three times from the beads using 0.1 mg/ml 3xFLAG-peptide (Sigma Aldrich) in

50mM Tris.HCl pH 7.4 150mM NaCl 0.02% CHAPS. Finally, the eluate was concentrated using an Amicon Ultra-15 MWCO 10kDa tube.

**Appendix Table S1: Primers for genotyping and cloning**

| Name | Sequence (5' to 3') | Purpose |
|---|---|---|
| gtZBP1Za1Za2-fwd | TGGCTTCAAATCTGAGCTTGGC | Genotyping Zbp1-Zα1Zα1 mutant mice |
| gtZBP1Za1Za2-rvs | CTATCTGGAGCTGAATGAAGGCA | |
| Zbp1-Exon2-fwd | TGACCACTGTAACCAATGCCACCT | PCR primers for sequencing of Exon 2 (Zα1) |
| Zbp1-Exon2-rvs | GACCTGAAACCTGACCTTCCCAAC | |
| Zbp1-Exon3-fwd | TGGTGTCGTCGTTCCTGTCTGCTT | PCR primers for sequencing of Exon 3 (Zα2) |
| Zbp1-Exon3-rvs | CTCGTGGCTCTTAATGTGCGTGTAG | |
| mZBP1fwd-Kozak | GCCGCCACCATGGCAGAAGCTCCTGTTG | TOPO-TA cloning in pCR8/GW/TOPO |
| mZBP1rvs-3xFLAG | CTTGTCATCGTCATCCTTGTAATCGATGTCATGATCTTTATAATCACCGTCATGGTCTTTGTAGTCTTGCTTGCTCAGTCCTGTG | |
| Mut-Zα1-fwd | CCCAAGAAAACCCTCGCTCAAGTCCTTGCCCGCCTGAAGAAGGAG | Overlap PCR for Zα1 mutation |
| Mut-Zα1-rvs | CTCCTTCTTCAGGCGGGCAAGGACTTGAGCGAGGGTTTTCTTGGG | |
| Mut-Zα2-fwd | ACAGCCAAAGAAGTGGCCCCACTCCTGGCTTCCATGAGAAATAAG | Overlap PCR for Zα2 mutation |
| Mut-Zα2-rvs | CTTATTTCTCATGGAAGCCAGGAGTGGGGCCACTTCTTTGGCTGT | |
| M36fwd-Kozak | AGGAGGACAGCTATGTATGAGCAAGAGGAACAAC | TOPO-TA cloning in pCR8/GW/TOPO |
| M36rvs | TCGATATCCCCGTGTCATC | |

**Appendix Table S2: Primers and probes for (RT)-qPCR**

| Gene | Assay Probe ID / Sequence (5' to 3') | Assay type |
|---|---|---|
| *Ifi44* | Mm00505670_m1 (Applied Biosystems) | Taqman |
| *Ifit1* | Mm00515153_m1 (Applied Biosystems) | Taqman |
| *Oasl1* | Mm00455081_m1 (Applied Biosystems) | Taqman |
| *Ifnb* | Mm00439552_s1 (Applied Biosystems) | Taqman |
| *Gapdh* | Cat. no. 4352932E (Applied Biosystems) | Taqman |
| *gB-fwd* | AGGGCTTGGAGAGGACCTACA | Taqman |
| *gB-rvs* | GCCCGTCGGCAGTCTAGTC | |
| *gB-probe* | 5'FAM_AGCTAGACGACAGCCAACGCAACGA_3'TAMRA | |
| *M36-fwd* | GTGTGATGAAGGAAAGTACGTC | SYBR green |
| *M36-rvs* | CTGGAAGAAGGACACTAGACTG | |
| *IE3-fwd* | ATGTCGCCAACAAGATCCTC | SYBR green |
| *IE3-rvs* | ATATCTATGTTCATCTCGGGTCCT | |

**Appendix Table S3: Antibodies used for Western blot**

| Antigen | Supplier | Host | Cat. number | Dilution |
|---|---|---|---|---|
| Mouse ZBP1 | A. Pichlmair | Rabbit polyclonal | | 1:2000 |
| Mouse ZBP1 | Adipogen (clone Zippy-1) | Mouse monoclonal | AG-20B-0010 | 1:2000 |
| Mouse RIPK3 | Novus Biologicals | Rabbit polyclonal | NBP1-77299 | 1:2000 |
| Mouse MLKL | Millipore (clone 3H1) | Rat monoclonal | MABC604 | 1:1000 |
| Mouse MLKL | Sigma Aldrich (for trimer and oligomers) | Rabbit polyclonal | SAB1302339 | 1:2000 |
| Mouse P-S345 MLKL | Abcam (EPR9515(2)) | Rabbit monoclonal | ab196436 | 1:2000 |
| Mouse ISG15 | A. Pichlmair | Rabbit polyclonal | | 1:2000 |
| MCMV IE1 | Q. Tang | Mouse monoclonal | | 1:1000 |
| HA-tag | sc-7392 (clone F-7) | Mouse monoclonal | sc-7392 | 1:5000 |
| FLAG-tag | Sigma Aldrich (clone M2) | Mouse monoclonal (HRP-coupled) | A8592 | 1:20,000 |
| V5-tag | Life Technologies | Mouse monoclonal (HRP-coupled) | R96125 | 1:2000 |
| Beta-actin | Sigma Aldrich | Mouse monoclonal (HRP-coupled) | A3854 | 1:500,000 |

**Appendix Table S4: siRNA sequences**

| siRNA | Sequence (5’ to 3’) / cat. No. |
|---|---|
| IE3-1-sense | CUAAGAAGCAUAAGAACAAUU |
| IE3-1-antisense | UUGUUCUUAUGCUUCUUAGUU |
| IE3-2-sense | GAAGAUAAGUCCAGGAAGUUU |
| IE3-2-antisense | 5'P_ACUUCCUGGACUUAUCUUCUU |
| IE3-3-sense | GGAUGAAGAUGAUGAGGAUUU |
| IE3-3-antisense | AUCCUCAUCAUCUUCAUCCUU |
| IE3-4-sense | GUGAGUAGUGGGAGUGAUAUU |
| IE3-4-antisense | UAUCACUCCCACUACUCACUU |
| Control siRNA | ON-TARGETplus Non-targeting Control Pool |