**Supplementary Material - File Descriptions**

** FIG S1**

Codon-optimization of ZAP does not affect ZAP protein levels. HEK 293T cells were transfected with either pEF empty vector (ev), pEF1-ZAP-S-myc (WT), or pEF1-ZAP-S-myc codon-optimized (opt.) (upper panel) or with pEF1-ZAP-L-myc WT or codon-optimized (opt.) expression constructs (lower panel). Expression levels of ZAP were determined by immunoblotting using a ZAP-specific antibody. Actin served as the loading control. Download [FIG S1, TIF file, 0.1 MB](https://mbio.asm.org/content/mbio/12/3/e02683-20/DC1/embed/inline-supplementary-material-1.tif?download=true).

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** FIG S2**

The untagged version of ZAP-L can control HCMV replication. (A) Schematic representation of the protein domains of the two main isoforms of ZAP (long ZAP-L and short ZAP-S) as described in [Fig. 1A](https://mbio.asm.org/content/12/3/e02683-20/figures-only#F1). The CaaX prenylation motif, only present in ZAP-L, is indicated with a green arrow. C, cysteine; aa, aliphatic amino acids; X, one of several amino acids. (B, C) WT, ZAP KO, or ZAP KO HFF-1 cells reconstituted with myc-tagged ZAP-S or untagged ZAP-L were generated as described in [Fig. 2C](https://mbio.asm.org/content/12/3/e02683-20/figures-only#F2). As the control, WT and ZAP KO HFF-1 cells were transduced with lentiviruses harboring empty vector (ctrl). (B) HCMV genome copy numbers were determined as shown in [Fig. 2A](https://mbio.asm.org/content/12/3/e02683-20/figures-only#F2). Briefly, cells were infected with HCMV (MOI 0.1) for 2 h. Both cells and supernatant were harvested at 1, 3, and 5 days postinfection (dpi), followed by DNA extraction and measurement of viral genome copies by qPCR. HCMV copy numbers/ml are displayed as bar plots showing the mean ± S.D. of one independent experiment performed with biological triplicates. (C) Cells were infected by centrifugal enhancement with HCMV (MOI 0.1), and lysates were analyzed at the indicated time points postinfection by immunoblotting with specific antibodies against ZAP, HCMV UL44, and actin. Quantifications of UL44 band intensities normalized to actin are indicated and represented as bar plots. WT (in gray) or ZAP KO (in red) transduced with ctrl, empty vector; S, ZAP-S-myc; L, ZAP-L untagged. Significant changes were calculated using unpaired two-sided Student’s *t* tests; n.s., not significant; *\*P <*0.05; *\*\*\*P <*0.001. Download [FIG S2, TIF file, 0.4 MB](https://mbio.asm.org/content/mbio/12/3/e02683-20/DC2/embed/inline-supplementary-material-2.tif?download=true).

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** DATA SET S1**

Proteome analyses. Download [Data Set S1, XLSX file, 7.7 MB](https://mbio.asm.org/content/mbio/12/3/e02683-20/DC3/embed/inline-supplementary-material-3.xlsx?download=true).

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** DATA SET S2**

Transcriptome analyses (RNA-seq). Download [Data Set S2, XLSX file, 12.7 MB](https://mbio.asm.org/content/mbio/12/3/e02683-20/DC4/embed/inline-supplementary-material-4.xlsx?download=true).

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** FIG S3**

ISG expression is similar in HCMV-infected WT and ZAP KO cells. WT and ZAP KO HFF-1 cells were untreated or infected by centrifugal enhancement with HCMV (MOI 0.1). Total RNA was extracted at 24 hpi, and lysates were subjected to total transcriptome analysis. Represented are log2 transformed fold changes at 24 hpi compared to untreated cells of WT and ZAP KO (g3) cell lines, calculated using edgeR and plotted against each other. ISGs are depicted in red. hpi, hours postinfection. Download [FIG S3, TIF file, 0.1 MB](https://mbio.asm.org/content/mbio/12/3/e02683-20/DC5/embed/inline-supplementary-material-5.tif?download=true).

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** FIG S4**

Relative temporal expression levels of HCMV genes above a reasonable expression threshold at 8, 24, and 72 hpi. WT, control, and two independent ZAP KO HFF-1 cell lines were left untreated, mock-treated, or infected by centrifugal enhancement with HCMV (MOI 0.1). Total RNA was extracted at 8, 24, and 72 hpi, and lysates were subjected to total transcriptome analysis. Expression of HCMV genes was quantified from the RNA-sequencing analysis and relative temporal expression levels calculated by dividing per-sample normalized expression values (fpkm) to the sum of these values from the same gene over six samples of the same cell line. Based on these values, genes were grouped using unsupervised clustering, and the clusters, representing kinetic classes, were ordered from immediate-early (top) to late (bottom). In addition, shown to the right is the kinetic classification from Weekes et al. ([32](https://mbio.asm.org/content/12/3/e02683-20/figures-only#ref-32)) (immediate-early, early, late), where available, or if the gene codes for a noncoding RNA. UL138 and RNA2.7 are marked with an asterisk (\*). Download [FIG S4, TIF file, 1.8 MB](https://mbio.asm.org/content/mbio/12/3/e02683-20/DC6/embed/inline-supplementary-material-6.tif?download=true).

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** DATA SET S3**

SLAM-sequencing. Download [Data Set S3, XLSX file, 4.2 MB](https://mbio.asm.org/content/mbio/12/3/e02683-20/DC7/embed/inline-supplementary-material-7.xlsx?download=true).

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** FIG S5**

The presence of ZAP-S and ZAP-L leads to reduced *TNFRSF10D* and *ZMAT3* cellular transcripts levels. WT, ZAP KO, and ZAP KO HFF-1 cells expressing either ZAP-S (blue) or ZAP-L (green) were mock-treated or infected by centrifugal enhancement with HCMV (MOI 0.1). At 24 hpi, total RNA was extracted, and qRT-PCR for *TNFRSF10D* and *ZMAT3* mRNA was performed. Cellular mRNA expression normalized to *GAPDH* is displayed as bar plots showing the mean ± S.D. of experimental duplicates. One representative of two independent experiments is shown. hpi, hours postinfection. Download [FIG S5, TIF file, 0.2 MB](https://mbio.asm.org/content/mbio/12/3/e02683-20/DC8/embed/inline-supplementary-material-8.tif?download=true).

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** DATA SET S4**

eCLIP-sequencing. Download [Data Set S4, XLSX file, 2.5 MB](https://mbio.asm.org/content/mbio/12/3/e02683-20/DC9/embed/inline-supplementary-material-9.xlsx?download=true).

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** FIG S6**

ZAP binds to cellular transcripts with no apparent specific motif. (A and B) Empirical cumulative distributions of log2 fold changes comparing KO and WT in total RNA (A) or newly synthesized RNA (B) in untreated samples are shown. Genes are stratified according to the number of detected ZAP binding sites in eCLIP data (“weak” binding sites are defined as having <5× enrichment over input RNA). *P* values are from a two-sided Kolmogorov-Smirnov test comparing against genes without binding sites, and the numbers of genes per stratum are indicated. (C) YTTCC motif identified by DREME in 1,268 out of 2,158 binding sites 1 to 50 nt downstream of the main cross-linking site and in 729 out of 2,158 shuffled control sequences. (D) AGRA motif identified by DREME in 990 out of 2,158 binding sites 1 to 50 nt downstream of the main cross-linking site and in 698 out of 2,158 shuffled control sequences. (E) GCYGCYGC motif identified by DREME in 269 out of 2,158 binding sites 1 to 50 nt downstream of the main cross-linking site and in 83 out of 2,158 shuffled control sequences. Download [FIG S6, TIF file, 0.5 MB](https://mbio.asm.org/content/mbio/12/3/e02683-20/DC10/embed/inline-supplementary-material-10.tif?download=true).

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