Supplementary Information for
ZAP isoforms regulate unfolded protein response and epithelial-mesenchymal transition

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Supplementary materials and Methods

Cell culture
We cultured the HEK293T (ATCC CRL-3216), SJCRH30 (ATCC CRL-2061), MCF7 (ATCC HTB-22) and MDA-MB-231 (ATCC HTB-26) cell lines in DMEM/Hi-Glucose supplemented with 10% FBS (Hyclone, United States), 100 units/ml penicillin, and 100 μg/ml streptomycin (Gibco, United States), 37°C with 5% CO2. We added IFN-β (Peprotech #300-02BC) to a final concentration of 100 ng/ml to complete medium.

We transfected cells with plasmids using X-tremeGENE 9 (Roche #6365809001) or X-tremeGENE HP (Roche # 6366546001) at 3 μl reagent to 1 μg DNA ratio, unless indicated otherwise. We used Lipofectamine 2000 (ThermoFisher #11668019) or Lipofectamine 3000 (ThermoFisher #L3000015) to transfect cells with small RNA molecules (DsiRNA and ssASOs). We extracted RNA and protein ~48h after transfection, unless indicated otherwise.

Plasmids used
Plasmids used for immunofluorescence: mCherry-tagged RAB5 (Addgene #49201 (1)), RAB7A (Addgene #61804 (2)), TOMM20 (Addgene #55146), ER-3 (Caretilcin Signal Sequence and KDEL, Addgene #55041), PTS1 (Addgene #54520) and LAMP1 (Addgene #55073), EGFP-tagged G3BP1 (Addgene #135997) and DCP1A (Addgene #153972), mCherry-tagged RAB5, RAB7A and GFP-tagged DCP1A were gifts from G. Voeltz, and EGFP-tagged G3BP1 was a gift from A. Leung. mCherry-tagged PTS1, LAMP1, TOMM20, and ER-3 were gifts from M. Davidson. mScarlet-I tagged LCK (Addgene #98821) and LaminB1 (Addgene #98831) were gifts from Dorus Gadella.

Plasmids used for knockdown and overexpression: pLKO.1-TRC was a gift from D. Root (Addgene #10878 (3)). Plasmid expressing scramble shRNA was a gift from D. Sabatini (Addgene #1864 (4)). pCGT7-PTBP1 or pCGT7-hnRNPA1 from (5) for overexpression of PTBP1 and hnRNPA1.

Plasmid for CRISPR: PX458 was a gift from Feng Zhang (Addgene #μ138 (6)).

ZAP splicing minigenes
We amplified the sequences of (i) ZAP exon 8 and 300 bp of flanking intron 8; and (ii) 300 bp of the end of intron 8 till exon 10 from human genomic DNA (gDNA, Promega, #G0341) and inserted them into linearized pcDNA3.1+ plasmid, using Infusion Cloning Kit (Clontech, #011614) to generate a long ZAP splicing minigene (ZAPmg1). Then, we removed the internal sequences of intron 9, leaving 2095 and 482 bp at the 5’ and 3’ ends of intron 9, respectively, to generate a short minigene (ZAPmg2 or ZAPmg). We introduced point mutations into the short ZAPmg at I9-5’ss or iPAS using two-insert Infusion Cloning reaction. We verified all the plasmids by sequencing (1st BASE, Singapore) and aligned the sequences with the reference sequence from NCBI. We calculated the MAXENT score for 5’ss as in (7).

To express the minigene, we mixed 200 ng of individual minigene plasmids with 800 ng of pUC19 plasmid to make up 1 μg for transfection into the previously seeded 2x10^5 HEK293T cells, using X-tremeGENE 9 (Roche, #6365809001) at 3:1 ratio. We harvested RNA ~ 48h after transfection. We used pcDNA3.1+ plasmid as negative control (mock).

Generation of ZAP(L/S) knockout cells
We generated the ZAP(L/S) KO cells using CRISPR/Cas9-mediated deletion of genomic DNA on HEK293T as in (8). To remove ZAPS, we deleted the ZAP-iPAS. To knock out ZAPL, we deleted exon 10 to generate an unstable ZAPL containing a premature stop-codon. Briefly, we
transfected HEK293T with a pair of guide RNAs containing PX458 plasmids followed by selection of GFP-positive cells at 24h and 48h after transfection. We plated the selected cells into 96-well by limiting dilution (~1 cell/well). We extracted genomic DNA, RNA and proteins from the grown colonies for genotyping, RT-qPCR and western blot as indicated.

**U1-decoy and suppressor-U1**
We mutated the pN/S6 suppressor-U1 plasmid (9) to generate suppressors base-pairing perfectly to ZAP I9-5’ss (S1) or the 5’ss-like sequence located between ZAP I9-5’ss and iPAS (S2). Primers in Table S1. We transfected HEK293T cells with 700ng of either control (pUC19), U1-decoy (U1 D) or mutated U1-decoy (Mut U1 D) plasmid (9), suppressor-U1 S1 or S2 plasmids. We co-transfected 100ng of GFP-expressing plasmid (pmaxGFP) and added 200ng of pcDNA3.1+ to make up total of 1μg of DNA for transfection. For experiments with ZAPmg2, we used 200ng of ZAPmg instead of pcDNA3.1+.

**RNA extraction and RT-qPCR**
We isolated the total RNA with RNA Puriflex Mini prep kit (Invitrogen #12183020), and DNase-treated with RQ1 RNase-Free (Promega #9PIM610) or TURBO DNA-free DNase (Thermo Fisher Scientific #AM1907). We performed reverse transcription with M-MuLV Reverse Transcriptase (New England #M0253L), using Oligo-dT18 reverse primer (IDT, Singapore) or random hexamer (Qiagen #79236). We performed qPCR using SYBR Select (Thermos Scientific #4472908) on ABS Plus One machine and quantified using 2^−ΔΔCt (10). qPCR primers in Table S2.

**Knockdown and overexpression**
We used 100pmol of siRNA or DsRNA with 5μl of Lipofectamine 2000 in 200μl of Opti-MEM to transfect 3x10^5 HEK293T cells seeded overnight. We used 50pmol of each DsRNA/siRNA for double knockdown. We generated PLKO.1 plasmids expressing shRNAs against target genes as in (3). We transfected HEK293T cells with 1μg of total plasmid mixture (100ng of pmaxGFP and 900ng of scramble or target shRNA plasmids). Sequences of RNA oligos or shRNAs in Table S3-4. We used 800ng of control (pmaxGFP), pCGT7-PTBP1 or pCGT7-hnRNPA1 on previously plated 2x10^5 HEK293T cells. We treated cells with IFN-β for 8h or 24h before harvest.

**Immunofluorescence**
We fixed the cells with 4% formaldehyde/PBS for 20-30 min, rinsed once with PBS and washed twice with 0.1% Triton-X100 in PBS (0.1% PBST). We permeabilized the fixed cells using 0.1 or 0.5% PBST for 10min and blocked using 3% BSA/PBS for 1h. We incubated the cells with primary antibodies in 1% BSA/0.3% PBST overnight and secondary antibodies in 1% BSA/0.3% PBST for 1h, following by 3 short washes with 0.1% PBS after each incubation. After secondary antibodies, we mounted the stained cells in Fluoromount medium (Sigma, #F4680) in PBS for 30 min, followed by 3 short washes with PBS before mounting. We sealed the slides with nail polish and stored the slides in the dark at -20°C before viewing.

We used the following primary antibodies: mouse anti-PTB (1:250, Life Technologies #32-4800), mouse anti-hnRNPA1 (1:1000, Abcam #ab5832), rabbit anti-ZAP (1:1000, Abcam #ab154680). We used these following secondary antibodies: Donkey Anti-Mouse Alexa Fluor 488 (1:500, Abcam # ab150105) and Donkey Anti-Rabbit Alexa Fluor 488 (1:500, Abcam # ab150073), Donkey Anti-Rabbit Alexa Fluor 568 (1:500, Abcam #ab175470). We stained the DNA with DAPI (Sigma #D9542).

We visualized cells using a Zeiss 710 confocal microscope and analyzed the images using Zeiss Black. To access co-localization of ZAP and mentioned organelles, we used the Coloc 2 plugin of Image J to calculate the Pearson’s R value on background subtracted images (background subtraction plugin) and ROI based on threshold organelle-makers channels (threshold plugin).
RNA-sequencing and analysis

The genotypes we used for RNA-Sequencing were: (i) 3 batches of WT; (ii) 2 and 1 batches of ZAPS-KO1 and 2; and (iii) 2 and 1 batches of ZAPL-KO1 and 2, respectively. We subjected all the clones to medium-only or IFN-β treatment for 24h. We extracted the total RNA with RNeasy Mini Kit (Qiagen, #74104) and QIAshredder (Qiagen #79654), and removed the residual genomic DNA with TURBO DNase. Next, we concentrated the total DNase-treated RNA using the RNA Clean & Concentrator™-5 kit (Zymo Research #SKUR1015) and measured its concentration using the Qubit RNA BR assay kit (Thermo Fisher Scientific) and Qubit 4 fluorometer (Invitrogen). We oligo-dT purified the RNA samples with RIN (RNA integrity number) above 8.0 and sequenced them using the HiSeq 2500 System Rapid mode (Illumina) and 101bp paired-end reads at SCELSE genomic facility (NTU, Singapore).

We inspected the quality of the RNA-seq datasets using FastQC. We used STAR (2.7.0a) to align reads to the GRCh38 and GENCODE v34 (11). We derived the DEGs using RSEM (12) and Limma-voom (13), with default settings and cut-offs of $|\text{Log2FC}| \geq 0.5$ and adjusted $P$-value $\leq 0.05$ for DEGs or $|\text{Log2FC}| \geq 0.5$ and adjusted $P$-value $\leq 0.1$ for low cut-off DEGs.

We used Clusterprofiler (14) to perform GSEA using relative expression of all genes between ZAPL-KO or ZAPS-KO clones versus WT or between ZAPL/S-KO clones for terms in Hallmark (H) and Gene Ontology Cellular Component (GOCC) on the Molecular Signatures Database (15). We performed ORA for terms in Hallmark between ZAPL/S-KO clones with Enrichr (16). We downloaded the RNA-Seq samples (WT-24hpi-rep1/2, WT-untr-rep1/2, ZAP-g1-24hpi-rep1/2, ZAP-g1-untr-rep1/2, ZAP-g3-24hpi-rep1/2, ZAP-g3-untr-rep1/2) from NCBI GEO GSE159853 (17) for DEG analysis as before.

Protein extraction and western blot

We prepared the cell lysates in 0.5% NP-40 lysis buffer (0.5% NP40, 10mM Hepes pH=7.5, 50 mM KCl, 3 mM MgCl₂) or RIPA buffer (1% NP40, 0.5% Sodium deoxycholate, 0.1% SDS, 150 mM NaCl, Tris-HCl, pH 8.0, 2mM EDTA) supplemented with protease inhibitors (Pierce #A32965) and phosphatase inhibitors (Sigma #4906845001), 2mM phenylmethane sulfonyl fluoride (PMSF, Sigma #P7626) and 10mM EDTA. We cleared the cell debris by centrifuging at 14000g for 30min at 4°C. We measured protein concentration by Bradford assay. We separated the proteins by SDS-PAGE and transferred them to PVDF (Biorad) or nitrocellulose membrane (GE HealthCare) for detection with antibodies. We imaged the blots with Bio-Rad Chemidoc and quantified the density of protein bands using Bio-Rad Image Lab software. We normalized the density of the bands corresponding to the proteins of interest to the loading control’s density and calculated the %ZAPS as the relative density of the ZAPS band over the sum of the relative densities for the ZAPL and ZAPS bands.

We used these primary antibodies for immunoblotting: rabbit anti-ZAP (1:5000, Abcam #ab154680), rabbit anti-p84 (1:10000, Abcam #ab131268), mouse anti-β-actin (1:5000, Sigma #AC-15-A5441), rabbit anti-GAPDH (1:10,000, Santa Cruz #sc-25778), mouse anti-PTB (1:1000, Life Technologies #32-4800), mouse anti-hnRNPA1 (1:1000, Abcam #ab5832), rabbit anti-hnRNPA2/B1 (1:1000, Abcam #ab31645), rabbit anti-vinculin (1:20,000, Abcam #ab129002). We used these secondary antibodies: HRP-conjugated goat anti-rabbit IgG (1:10,000, Sigma #A6154) and HRP-conjugated goat anti-mouse IgG (1:10,000, Santa Cruz #sc2005).

Splice-switching Antisense Oligonucleotide (ssASO) treatment

We transfected the previously plated 3x10⁵ HEK293T or SJCRH30 cells with ssASOs in 10μl Lipofectamine 2000. Unless indicated otherwise, we used 200pmol of each ssASO. We transfected the previously plated 500,000 MDA-MB-231 or 250,000 MCF-7 cells in 6-well-plate format with 100pmol control or iPAS2 ssASOs or the combination of 50pmol I9-5 plus 50pmol I9-
3 ssASOs, using 10µl Lipofectamine 3000 plus 10µl P3000 according to the manufacturer's protocol. We applied IFN-β for 24h before harvesting protein ~48h after transfection.

Sequences of the ZAP ssASOs: iPAS1 (AGTTTACTGAGCAGTTCAAGGAG); iPAS2 (CAATGTAGTTTACTGAGCAG); I9-5 (AGCTGCTACAAATAAAGAGAAATT); I9-Cry5 (TCATCTGCTGCACATACCACTGAGG); I9-3 (TGATGGCTACAAATAAAGAGAAATT); I10-5 (GTAAAAACATCCCTATATTTC). The control ssASO (GCGACTATACGCGCAATATG) was from IDT. All ssASOs have 2’-O-methoxyethyl and phosphorothioate backbones throughout the length and were synthesized by IDT (Singapore).

Organelle trackers
We plated cells for 24h before incubation with Opti-MEM containing either 500nM ER-Tracker Red dye (Life Technologies), 50nM Lyso-Tracker Deep Red (Life Technologies) or 500nM Mito-Tracker Red CMXRos (Life Technologies) for 45min (ER/Mito Trackers) or 2h (Lyso-Tracker) at 37°C in dark. We suspended the stained cells in PBS and performed Flow Cytometry (Fortessa X20, 5-laser) with the indicated channels. We used FlowJo V10 to calculate the geometric mean fluorescent intensity (MFI) of each sample and normalized to the MFI of WT.

MTS assay
Briefly, we plated 10000 HEK293T or MCF7 cells in a total volume of 100µl in each well of 96-well plates in triplicates. We added Thapsigargin (Tg) into each well to a final concentration of 1µM for the indicated period. We added 1/10 volume of MTS reagent (CellTier 96 Aqueous One solution, Promega #G3582) for 2h in 37°C before absorbance reading at 490nm with Tecan M200Pro plate reader.

UPR inducer and specific inhibitors
We used Thapsigargin (Tg, Sigma #T7458) at final concentration of 1µM to induce ER-stress. We used 4µ8C (Sigma #SML0949) at 20µM final concentration to inhibit the IRE1 pathway while we pretreated the cells with 10µM of GSK2656157 (Abcam #ab273622) for 1h before Tg treatment to inhibit the PERK pathway.

Generation of MCF7 cells with ZAPL/S knockdown
We generated the PLKO.1 plasmids expressing shRNAs against ZAPL or ZAPS (sequences in Table S4). We generated lentiviruses by transfecting HEK293T cells with psPAX2, pMD2.G and the pLKO.1 plasmid in a molar ratio of 1:1:2, using X-tremeGENE™ HP DNA Transfection Reagent (Roche Applied Science, Merck) in a 3:1 ratio. We harvested the lentiviral particles 48h after transfection. psPAX2 (Addgene #12260) and pMD2.G (Addgene #12259) were gifts from Didier Trono. We transduced MCF7 with lentivirus by adding filtered virus supernatant in the presence of 10µg/ml polybrene (Sigma-Aldrich). We then selected the transduced cells with 2g/ml of puromycin dihydrochloride (Gibco, Thermo Fischer) for 9 days.

Migratory assay
We performed the cell migration assays in transwell chambers (Corning) and wound healing culture-insert dishes (Ibidi). We plated 70,000 and 80,000 of HEK293T and MCF7, respectively, in each side 2-chamber cell inserts and cultured the cells to confluence before removing the inserts. We imaged the cells Nikon Ti Eclipse microscopy with 10x lens for indicated time periods. We calculated the wound closure with the Analyze Particle plugin in Fiji after removing noises with the thresholding and the bandpass filtering functions. We fitted the wound closure rate using linear regression, and we compared the fitted slopes between the different genotypes with One-way ANOVA with Dunnett's test using the reported slopes.
For the transwell migration assay, we seeded 200,000 cells in the upper chamber with 0.5% FBS medium and filled the lower chamber with 20% FBS medium. We cultured the seeded cells for another 20h or 48h, followed by fixation, staining and imaging with the Zeiss Observer II microscopy, 10x lens. We took ~5 images per membrane, followed by eluting the migrated cells with 33% acetic acid and absorbance reading at 590nm wavelength. We calculated the average cell number per field for MCF7 migration due to the strong attachment of cells to the membrane edges, while we used absorbance for HEK293T migration. We recorded the absorbance at 590nm with the Tecan Infinite M200 Pro micro-plate reader (Tecan Group Ltd, Switzerland).

Native RNA-protein immunoprecipitation
We seeded 2×10⁶ HEK293T cells for 24h before treating them with IFN-β for another 24h. We lysed the cells in Polysome Lysis Buffer (PLB, 100mM KCl, 5mM MgCl₂, 10mM HEPES pH 7.4, 0.5% NP40, 2mM DTT, HALT protease inhibitor, 100U/ml RNasin and 2mM ribonucleoside-vanadyl complex). We removed nuclei and debris from the cytosolic lysate by centrifugation at 8000g at 4°C for 10min and precleared the lysate with Protein A Dynabeads (Invitrogen) for 30min at 4°C with rotation. Next, we incubated 2mg protein from the cytosolic lysate with 10μg rabbit anti-ZAP antibody (Abcam) or IgG control overnight at 4°C with rotation overnight. We incubated the lysate with 1.5mg of prewashed Protein A Dynabead for 4h at 4°C with rotation. We washed the RNA-protein-bead complexes once with NT2-crowders (25mg Ficoll PM400 (GE Healthcare), 75mg Ficoll PM70 (GE Healthcare), 2.5mg Dextran Sulphate (Sigma) in 10ml of NT2 buffer) and 4 times with NT2 buffer (0.05M Tris pH 7.0, 0.15M NaCl, 0.001M MgCl₂, 0.0005% (v/v) NP40). We collected Protein-RNA complexes in 100μl of NET2 buffer (0.00118M DTT (Sigma), 0.0176M EDTA, 100U/ml RNaseOUT (Thermo Fisher), 100U/ml recombinant RNasin (Promega) in 1X NT2 crowder), supplemented with 100μl 2X SDS-TE (0.04M Tris pH 7.5, 0.004M EDTA pH 8.0, 10% SDS). We isolated coprecipitated RNAs from IgG1–protein complexes (90%) with Phenol:Chloroform:Isoamyl alcohol (25:24:1) (Sigma), DNase-treated, reverse transcribed and analyzed them by qPCR. We isolated precipitated protein from IgG1–protein complexes (10%) with SDS-loading dyes and subjected them to western blotting.

Statistics
We performed statistical tests on GraphPad Prism. We show all data as Means±SDs, unless indicated otherwise. Comparisons were with control or mock, unless otherwise indicated by a line between two samples. In all figures, ns (statistically nonsignificant) for $P>0.05$, * for $P≤0.05$, ** for $P≤0.01$, *** for $P≤0.001$ and **** for $P≤0.0001$. 


Supplementary Figures

Fig. S1. U1-snRNA/snRNP binds to ZAP I9-5’ss to promote splicing and simultaneously suppress IPA. A, Top; Schematic of ZAP alternative splicing and IPA. Boxes, exons; black lines, ZAPS-3’UTR; grey thin boxes, ZAPL-3’UTR; black boxes, ZAPS-3’UTR; iPAS, intronic polyadenylation signal; arrows, primers (E8-9F/E10R and E11F/E12F for ZAPL; E8-9F/I9R for ZAPS; E8-9F/E9R for ZAP). See Table S2. Bottom; Schematic of ZAPL and ZAPS proteins. RBD, RNA-binding domain; ZF, Zinc-finger. B, Representative blot for HEK293T cells treated with U1-decoy (U1 D) or mutated U1-decoy (Mut U1 D). Top, schematic of U1-decoy that blocks binding of U1 to the I9-5’ss (dashed cross over thin arrow) and prevents its repression of IPA (dashed cross over thin T bar). β-act as loading control. Right graph, %ZAPS. C, RT-qPCR results for %ZAPS from mRNAs extracted from (B), normalized to reference genes (HPRT and SDHA). D, Representative blot for HEK293T cells treated with S1 or unspecific S2 suppressor-U1. Top, schematic of suppressor-U1 S1 which enhances binding of U1 to the I9-5’ss (thick arrow) and increases its repression of IPA (thick T bar). β-act as loading control. Right graph, %ZAPS. E, RT-qPCR results for %ZAPS from mRNAs extracted from (B), normalized to reference genes (HPRT and SDHA). F, U1-decoy or suppressor-U1 effects in %ZAPS from ZAPmg. GFP from co-transfected plasmid as reference gene. %ZAPS as ZAPS over sum of ZAPL and ZAPS (mRNA or protein). One-way ANOVA with Dunnett’s test; n≥3; Means±SDs; * P≤0.05, ** P≤0.01, ns P>0.05.
Fig. S2. ZAP ssASOs shift the balance between ZAPL/S. A, Blots and quantification of ZAPL/S from HEK293T cells without IFN-β, transfected with the indicated ssASOs against ZAP-iPAS. GAPDH, loading control. B, Blots and quantification of ZAPL/S from HEK293T cells transfected with the respective ssASOs and treated with IFN-β for 24h. C, Blot and quantification of ZAPL/S in of HEK293T cells transfected with ssASOs blocking splice sites of ZAP’s intron 9 and/or 10. D, Blots and quantification of ZAP from HEK293T cells transfected with ZAP I9-5 and I9-3 ssASOs at two different concentrations. The indicated amount shows the total (each ssASO added in equimolar concentration). Vinculin (Vin), loading control. E, Gel of RT-PCR products amplified from ZAPL mRNA exons 9-10 in HEK293T cells transfected with indicated ssASOs, showing an mRNA mixture for the I9-5 ssASO. Pictograms show the sequences of the respective
bands. The dotted red and black lines denote the junction between exon 10 with the cryptic 5’ss or the canonical 5’ss of exon 9, respectively. F, Gel of RT-PCR products amplified from ZAPL exons 9-10 in HEK293T cells transfected with I9-5 and I9-Cry5 ssASOs, showing a single product. G, Representative blots of ZAP proteins in SJCRH30 cell line transfected with ctrl-ssASO or ssASOs blocking ZAP-iPAS (iPAS2) or ssASOs blocking ZAP splice sites (I9-5 and I9-3). We treated cells with IFN-β for 24h as indicated by +. GAPDH, protein loading control; We quantified the density of protein bands using a densitometer, normalized to that of the loading control. %ZAPS as ZAPS over sum of ZAPL and ZAPS. ctrl, scramble ssASO; We used 200pmol ssASO in (A-C), and 200pmol ssASO for single and 100pmol ssASO for mixture in (G). n≥3. One-way ANOVA with Dunnett’s test (A-C, G) and two-tailed t test (D). Means±SDs; * P≤0.05, ** P≤0.01, *** P≤0.001; ns P>0.05.
Fig. S3. PTBP1/2 and hnRNPA1/A2 suppress ZAP IPA. A, Genome browser view of CLIP datasets in POSTAR2 illustrating the binding sites of PTBP1 (blue) and hnRNPA1 (yellow) in intron 9 and flanking exons of ZAP. B, Blots and quantification of %ZAPS protein and mRNA upon knockdown of both hnRNPA1/A2 by two shRNA mixtures (s1 and s2) in HEK293T cells treated with IFN-β for 8h. β-act as loading control, HPRT1 and SDHA as reference genes. n≥3. One-way ANOVA with Dunnett’s test; Means±SDs; * P≤0.05, ** P≤0.01, ns P>0.05. C, Blots of PTBP1 and hnRNPA1 proteins from whole-cell lysate, cytoplasmic and nuclear fractions from HEK293T cells, showing no obvious changes in protein distribution upon IFN-β treatment. Vin and p84 as cytoplasmic and nuclear markers, respectively. n≥3. D, Confocal images of PTBP1 and hnRNPA1 in unstimulated and IFN-β treated HEK293T cells at indicated time points, showing predominant nuclear localization, as indicated by overlapping of staining pattern of antibodies against PTBP1 and hnRNPA1 with DAPI. Scale bars, 5μm. n≥10 images/condition.
Fig. S4. Generation of ZAPL-KO and ZAPS-KO HEK293T clones. A, Schematic depicting the gRNA pairs to delete ZAP iPAS (gRNA L1 or L2 paired with gRNA R1) and ZAP exon 10 (E10) (gRNA L1 paired with gRNA R1 or R2). L, left; R, right; gRNA, guide RNA. B-C, Blots of ZAP proteins from indicated genotypes under untreated (B) or IFN-β treated cells (C), demonstrating the absence of ZAPS and ZAPL in the corresponding ZAPS-KO or ZAPL-KO clones (except for ZAPS-KO L2-R1 clone 13). Vinculin (Vin) as loading control. #, uncharacterized isoform; ##,
might correspond to ZAPM (18); both not quantified in (D-E). **D-E**, Quantification of ZAP proteins in (B) and (C), respectively. ND, non-detected; F-G, RT-qPCR results of ZAPL/S from indicated genotypes in untreated (F) or IFN-β treated (G), showing significant down-regulation of ZAPL/S in corresponding ZAPS-KO or ZAPL-KO cells. **HPRT1** and **SDHA** as reference genes. **H-I,K**, Pictograms showing the sequences of genomic DNA in indicated genotypes. **J,L-M**, Alignment of TA-cloned sequences from genomic DNA in KOs with heterogenous mutations. All showed the deletions of ZAP-iPAS or exon 10. **N**, RT-qPCR results of ZAP (all isoforms) in indicated genotypes, relative to the untreated condition. **HPRT1** and **SDHA** as reference genes. n≥3, Means±SDs; One-way ANOVA with Dunnett’s test; *P≤0.05, **P≤0.01, ***P≤0.001, ****P≤0.0001, ns P>0.05.
Fig. S5. Heat map displaying the DEGs of ZAPL/S KOs. We derived the DEGs between either KO and WT in both unstimulated and IFN-β treated conditions and the DEGs for the same genotypes between two conditions. Red color highlights the DEGs ($P \leq 0.05$ and $|\text{Log2FC}| \geq 0.5$) between the ZAPL-KO or the ZAPS-KO versus WT cells. Blue color highlights the genes in the list of 389 ISGs (19). Green color highlights the genes which are both DEGs between the ZAPL-KO or the ZAPS-KO versus WT cells and are found in the list of 389 ISGs (19).
Fig. S6. Different host transcripts targeted by ZAPL/S. A-D, Volcano plots showing the transcriptome-wide log2 fold change (Log2FC) of expression between the different ZAPL/S-KO versus WT in IFN-β treated (A-B) or untreated (C-D) cells, obtained by RNA-Seq of total polyadenylated RNAs (n=3/genotype). Red dots represent DEGs (P≤0.05 and |Log2FC|≥0.5). The dotted vertical lines mark the |Log2FC| of 1 (outer lines) and 0.5 (inner lines), while the dotted horizontal lines mark the -log10 (adjusted P-value) of 1 (P=0.1, lower lines) and 1.30 (P=0.05, upper line). E-H, RT-qPCR results for DEGs found in A (E), B (F), C (G) and D (H). We performed RT-qPCR on RNAs extracted from both KOs and WT to check for the transcript specificity by the loss of either ZAPL or ZAPS. We normalized the gene expression to two reference genes (HPRT1 and SDHA), and we normalized the expression of the KOs to that in the WT, whose Log2FC is set at 0. The dotted horizontal lines mark the |Log2FC| value of 1. Note that the qPCR validation of TIMP3 without IFN-β treatment is in Fig. S10A. I, Blots of ZAP protein from eluates after IP with anti-ZAP antibody in nRIP experiment, using whole cell lysate from IFN-β treated WT, ZAPS-KO or ZAPL-KO cells. GAPDH as loading control. J-O, RT-qPCR data measuring the abundance of TRAILR4, GET1, MYEF2, NT5DC2, ZNF121, and HSPA8 respectively performed.
on eluates from (I). n≥3; Means±SDs; One-way ANOVA with Turkey’s test (E-H) and two-tailed t test (J-O); * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, **** $P \leq 0.0001$, ns $P > 0.05$. 

Fig. S7. ZAPL and ZAPS localize to membrane or cytosol, respectively. A-F, Immunofluorescent images of cells with indicated genotypes stained with anti-ZAP antibody (green) and mCherry or mScarlet tagged markers of mitochondria TOMM20 (A), early endosome RAB5 (B), late endosome RAB7 (C), lysosome LAMP1 (D), peroxisome PTS1 (E) and inner nuclear membrane (F). G-H, Immunofluorescence images of cells from indicated genotypes stained with anti-ZAP antibody (red) and overexpressed GFP tagged markers of SG G3BP1 (G) or PB DCP1A (H) in Sodium arsenite-stressed cells. In (H), the middle and left panels showed the enlarged views of white dotted outlines in the rightmost panels. Arrows, ring-like structures surrounding PBs. Scale bars, 5 μm. n≥10 images from two independent experiments.
Fig. S8. ZAPL/S-KO cells upregulate different GOCC terms. A-B, Running Normalized Enrichment Score (NES) plots of the enriched terms from GSEA for GOCC using relative expression of all genes in ZAPS-KO versus ZAPL-KO cells, in untreated (A) or IFN-β treated (B) samples, showing a decrease in membrane/secretory and increase in cytosolic/nuclear components in ZAPS-KO cells compared to ZAPL-KO cells.
Fig. S9. ZAPL/S regulate UPR. A, Running NES plots showing the distribution of the genes for the UPR hallmark in GSEA between ZAPL-KO versus WT in untreated condition. B, Heatmaps displaying expression of core enrichment genes of the gene set of (A). Red highlights the genes validated in Fig. 5B. C, Mean fluorescence intensity (MFI) of live HEK293T cells stained with Lyso-tracker (APC) for lysosomes or Mito-Tracker (PE-CF594) for mitochondria. D, RT-qPCR results showing expression of CHOP, upon 6h treatment with Thapsigargin (Tg) in the absence or presence of PERK-inhibitor (GSK2656157). We normalized Ct values to reference genes (HPRT1 and SDHA) and further normalized to untreated WT. The dotted horizontal lines mark the \(|\log_2 FC|\) value of 1. n \(\geq 3\); Means±SDs; One-way ANOVA with Dunnett's test (C-E). * \(P \leq 0.05\), ** \(P \leq 0.01\), ns \(P > 0.05\).
Fig. S10. ZAPL/S regulate EMT. A, RT-qPCR results for factors from the EMT hallmark in Fig. 5A. We normalized the Ct values to the reference genes (HPRT1 and SDHA), and then further normalized to the respective WT. n≥3; Means±SDs; One-way ANOVA with Dunnett’s test; * P≤0.05, ** P≤0.01. The dotted horizontal lines mark the |Log2FC| value of 1. B, Running NES plots showing the distribution of the genes for the hallmark of EMT in GSEA between ZAPL-KO versus WT in untreated condition. C, Heatmaps displaying expression of core enrichment genes of the gene set of (B). Red highlights the genes validated in (A). D, Representative blots showing the protein levels of ZAP isoforms for the indicated knockdown MCF7 clones. GAPDH, loading control. n≥3.
Full western blots S1 to S3

**Fig. 1C**

**Fig. 1D**

**Fig. 2A**

**Fig. 2B**

**Fig. 2C**

**Fig. S3D**

**Full western blots S1.** Uncropped western blots from the indicated Figures and Supplementary Figures. The black boxes highlight the cut sites of the membranes to blot with different primary antibodies. The red boxes highlight the membrane parts in the Figures or Supplementary Figures.
Full western blots S2. Uncropped western blots from indicated Supplementary Figures. The black boxes highlight the cut sites of the membranes to blot with different primary antibodies. The red boxes highlight the membrane parts in the Supplementary Figures.
Full western blots S3. Uncropped western blots from indicated Supplementary Figures. The black boxes highlight the cut sites of the membranes to blot with different primary antibodies. The red boxes highlight the membrane parts in the Supplementary Figures.
### Tables S1 to S4

#### Table S1. Oligonucleotides used in cloning.

| Name                                      | Sequence                                                                 | Remark                                                                 |
|-------------------------------------------|--------------------------------------------------------------------------|------------------------------------------------------------------------|
| pcDNA3 –EcoRV                             | TGGTGGTGGAATTCTGCAGATATCAAGACAAACGGAAAAATTCAAACG                         | To amplify 5’ end fragment of ZAPmgs (common)                          |
| ZAP (E8-I8) 1R                            | AAGACACCATAGCATGGCGACGCTGAAG                                               | To amplify fragment 1 of ZAPmg1 (long)                                  |
| ZAP (I8-E9-I9-E10) pcDNA3-NotI 2R         | CTGCCATGCTATGGTGTTGGTCTTTTATTTG                                          | To amplify fragment 2 clone ZAPmg1                                      |
| ZAP mg2 R1                                | AAATCTTTCTATGTCTACTAGTTGGAAGCTAAC                                         | To amplify fragment 1 of ZAPmg2 (short)                                 |
| ZAP-E9 Seq F                              | GGATGATTACGACAAAACATAGCTTCC                                                |                                                                      |
| ZAP-iPas aggaaa R1                        | TGTTCATGAACATGTAGAGTTTCATGGTCATGCAGTTC iPAS aggaaa mutation              |                                                                      |
| ZAP Exin9 5’ss AGA>CAG R2                 | GGCACTTCATTGGCCCTCTCTCTCTCTCAGGGGAGATAC                                  | Mutate U1 suppressor to S1                                              |
| ZAP Exin9 5’ss G5c F2                     | CTCTCAGAGCAGGTCTCTCTCAGGGGAGATAC                                         | Mutate U1 suppressor to S2                                              |
| Name                | Sequence                                       | Remark                                      |
|---------------------|-----------------------------------------------|---------------------------------------------|
| ZAP Ex8-9 F2        | GCCTCTCCACTCCTTTCTTCT                         |                                             |
| ZAP5 In9 R2         | GGCTCCAGATTCCGATGTGAC                       | (20)                                        |
| ZAPL Ex10 R1        | CAGTTAAAAGACACTGACAGGTC                      |                                             |
| ZAP Ex8-9 F2        | GCCTCTCCACTCCTTTCTTCT                        | To detect total ZAP mRNA                   |
| ZAP Ex9 R2          | CACTGAGGCACAAATGTTGG                        |                                             |
| ZAPL E11 F1         | GAACTCAAGCTCTGCTGGATAAA                      | To detect ZAPL in ZAPL-KO cells            |
| ZAPL E12 R1         | TCCTCCACATTCCATAGG                          |                                             |
| pmax GFP F3         | ATCGAGAGATGACGAGGACGCGC                      | To detect GFP from pmaxGFP                 |
| pmax GFP R3         | GAAGCCGTGGCCATCACCTTTGAAG                    |                                             |
| HPRT1 F             | TGACACTGGAAACAAATGCA                        | RTPriemerDB ID 8098                       |
| HPRT1 R             | GGTCTCCCTACCAGCAAGCT                       |                                             |
| SDHA F              | TGGGAACACAGGATGACCATCTGG                     | (21)                                        |
| SDHA R              | CCACACACTGCATCAATTTG                        |                                             |
| IF6 F               | CCGTTACAGCACACATCTGG                        |                                             |
| IF6 R               | TCAGTCTGGTCTCTCAG                           | (22)                                        |
| CRISPLD1 F          | TGC CCA AGA GTA TAC TGT CCA                 |                                             |
| CRISPLD1 R          | GAT TTC GAA CCA CTA CAG CA                  |                                             |
| TNFRSF10D F         | TTAATGTCCTGCTCTCAGG                         |                                             |
| TNFRSF10D R         | TCTGATCATAGATGATGCTG                       | (23)                                        |
| TPM2 F              | GAGAGTCTGCTGCAAGTTG                        | Origen hp206833                            |
| TPM2 R              | GAGGATGCTGATGTCATGAGTGG                     |                                             |
| COL4A1 F            | TGTTACGCCTGCACCTGGAGAC                     | Origen hp205624                            |
| COL4A1 R            | GGTACAGCACTCCAGGTCCTCT                      |                                             |
| ERAP2 F             | GAAAGCTTCTATACCAAGGGAG                     |                                             |
| ERAP2 R             | CCACAAATCTCATATCCTTACG                     | (25)                                        |
| LINC00641 F2        | CAGGCTATGACAGAACGCC                        |                                             |
| LINC00641 R2        | CCAGTTGGTCTCTGCAATTG                       | (26)                                        |
| MYEF2 F3            | CAGGAGACAGCTCCCTGATA                       |                                             |
| MYEF2 R3            | GGCTGCAAATTTACTGAGCT                      | Harvard RT-qPCR bank                       |
| LGALS3BP F2         | GCCAGCAGATCTACAGATGAGTGG                    |                                             |
| LGALS3BP R2         | GTCTAGGACTTGGACTACGAC                      | Origen hp208684                            |
| TIMP3 F2            | CGGATCACTGCTGGTGG                          |                                             |
| TIMP3 R2            | GTAGCCAGGGTGTAACCGAA                      | (27)                                        |
| BNIP3 F2            | TCAGCATGAGGAACAGGCAGGT                     | Origen hp207424                            |
| BNIP3 R2            | GAGGATGGTGCAAGCCTCCTCCA                    |                                             |
| GET1 F              | ACCGGCGCTGCAGCTTCCTTCA                     |                                             |
| GET1 R              | TTATGCGTCCGCCGCTGTATCCA                    | Origen hp207914                            |
| PRXL2A F            | GCGATGTAAGAACACAGATCAG                    |                                             |
| PRXL2A R            | TCTCTCTCCATGTGGAGCGCC                     | Origen hp215884                            |
| NT5DC2 F            | CTTCACGCTAGCGAGAATGG                       |                                             |
| NT5DC2 R            | CCGTCAGCTGCTCTGGAGATAG                    | (28)                                        |
| SNAI2 F             | ATCTGCGGCAAGGCGGTTCCTCA                   | Origen hp206658                            |
| SNAI2 R             | GAGCCCTAGATTGGAGCCGCT                     |                                             |
| DAB2 F              | CTCTGCTCAGCTCCTCAGAC                      | Origen hp205244                            |
| DAB2 R              | GTTCTGAGACGGGAGAGCGAA                     |                                             |
| 4E-BP1 F            | CACCCAGCTTTGGAGAGGGAGG                     | Origen hp207459                            |
| 4E-BP1 R            | CTTCTGATGCTGATCCACACGT                    |                                             |
| ATF4 (CREB2) F      | TTCTCCAGCGCAAAAGTGAAG                     | Origen hp205494                            |
| ATF4 (CREB2) R      | CTCCACATCCAAATCTGTCCCG                    |                                             |
| Gene        | Forward Primer | Reverse Primer | Organism hp211033 |
|-------------|----------------|----------------|-------------------|
| TTC37 (SKI3) F | GACACTGATGCTGAATCTGGAGC |             |                   |
| TTC37 (SKI3) R | CCATTTTGGCGTCCAGCCTTTG |             |                   |
| XBP1s F     | CTGAGTCGAGAATCAGGCTCAG |             |                   |
| XBP1s R     | ATCCATGGGGAGATTTTCTGG |             |                   |
| CHOP F      | AGAACCAGGAAACGGAAACAGA |             |                   |
| CHOP R      | TCTCCTTCACTGCGCTGCTTT |             |                   |
| BiP F       | TTCTTCAACCTATCAGAAAACCTC |             |                   |
| BiP R       | TTCTGCTGTATCCCTTCCACCAGT |             |                   |
| EDEM1 F     | CAAGTGTTGGTACGCCACG |             |                   |
| EDEM1 R     | AAAGAAGGCTTCCATCGGTC |             | (29)              |
Table S3. DsiRNAs used for knockdown.

| Name                  | Sequence                  | Company       |
|-----------------------|---------------------------|---------------|
| Negative control DsiRNA | —                         | IDT (#51-01-14-04) |
| hnRNPA1 DsiRNA        | CAGCUGAGGAAGCUCUUC        | IDT, USA      |
|                       | CUCCAAUGAAGACUUC          |               |
| hnRNPA2 DsiRNA        | GGAACAGUUCGUAAGCU        | IDT, USA      |
|                       | AAUAAAGACUUACGGA         |               |

Sequences represent RNA, except that DNA nucleotides are shown as dN (dA, dT, dG, dC).
| Name           | Target gene | Sequence (sense) | Reference       |
|---------------|-------------|-----------------|-----------------|
| PTBP1/2 s1    | PTBP1       | AACTTCATCATTCCAGAGA | Adapted from (30) |
|               | PTBP2       | CCCTAGATGGTCAGAATATT | TRCN0000001111  |
| PTBP1/2 s2    | PTBP1       | AATGACAAAGAGCCGTGACTAC | Adapted from (31) |
|               | PTBP2       | GCTGTTATCATTCCCTTGGTTA |
| hnRNPA1/A2 s1 | hnRNPA1     | AGATATTTTGGTGGCGATT | TRCN0000006586  |
|               | hnRNPA2     | GCTTCTCTATTTGGCAGGTG | TRCN0000001058  |
| hnRNPA1/A2 s2 | hnRNPA1     | AACAATCGTCTTCAATTT  | TRCN0000235097  |
|               | hnRNPA2     | GGAACAGTCCGTAAGGCTTT | Adapted from (32) |
| ZAPS          | ZAPS-shR    | CGATGAGAAGATGCTTGAAGCA | Adapted from (33) |
| ZAPL          | ZAPL-shR    | GAATTTACTTGCAAAAGATG | Adapted from (24) |
Legends for Datasets S1 to S5

Dataset S1 (separate file). DEGs ($P \leq 0.05$ and $\mid \text{Log2FC} \mid \geq 0.5$) from ZAPL-KO or ZAPS-KO relative to WT HEK293T cells, identified from our RNA-Seq dataset. DEGs from ZAP-KO relative to WT HUVEC cells by reanalyzing GSE159853 dataset.

Dataset S2 (separate file). Result of GSEA using relative expression of all genes between IFN-β treated and untreated ZAPS-KO versus ZAPL-KO cells for terms in GOCC using C5 MSigDB. Relevant terms are highlighted in red.

Dataset S3 (separate file). Low cut-off DEGs ($P \leq 0.1$ and $\mid \text{Log2FC} \mid \geq 0.5$) between ZAPS-KO relative to ZAPL-KO cells identified from our RNA-Seq datasets.

Dataset S4 (separate file). Result of ORA GOCC using low cut-off DEGs ($P \leq 0.1$ and $\mid \text{Log2FC} \mid \geq 0.5$) between ZAPS-KO vs ZAPL-KO cells.

Dataset S5 (separate file). Result of GSEA using relative expression of all genes between untreated ZAPL-KO versus untreated WT cells for terms in MSigDB Hallmarks. Relevant terms highlighted in red.
Legends for Movies S1 to S6

**Movie S1 (separate file).** Collective migration of HEK293T *WT* cells. We recorded the images every 15min over 8h and exported as videos of 5fps (frame per second).

**Movie S2 (separate file).** Collective migration of HEK293T *ZAPS-KO1* cells. We recorded the images every 15min over 8h and exported as videos of 5fps (frame per second).

**Movie S3 (separate file).** Collective migration of HEK293T *ZAPL-KO1* cells. We recorded the images every 15min over 8h and exported as videos of 5fps (frame per second).

**Movie S4 (separate file).** Collective migration of MCF7 cells transfected with Scramble shRNA. We recorded the image sequences every 15min over 18h and exported as videos of 5fps.

**Movie S5 (separate file).** Collective migration of MCF7 cells transfected with shRNA against ZAPS. We recorded the image sequences every 15min over 18h and exported as videos of 5fps.

**Movie S6 (separate file).** Collective migration of MCF7 cells transfected with shRNA against ZAPL. We recorded the image sequences every 15min over 18h and exported as videos of 5fps.
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