RESEARCH ARTICLE

Figures and figure supplements

SRSF6 balances mitochondrial-driven innate immune outcomes through alternative splicing of BAX

Allison R Wagner *et al*.

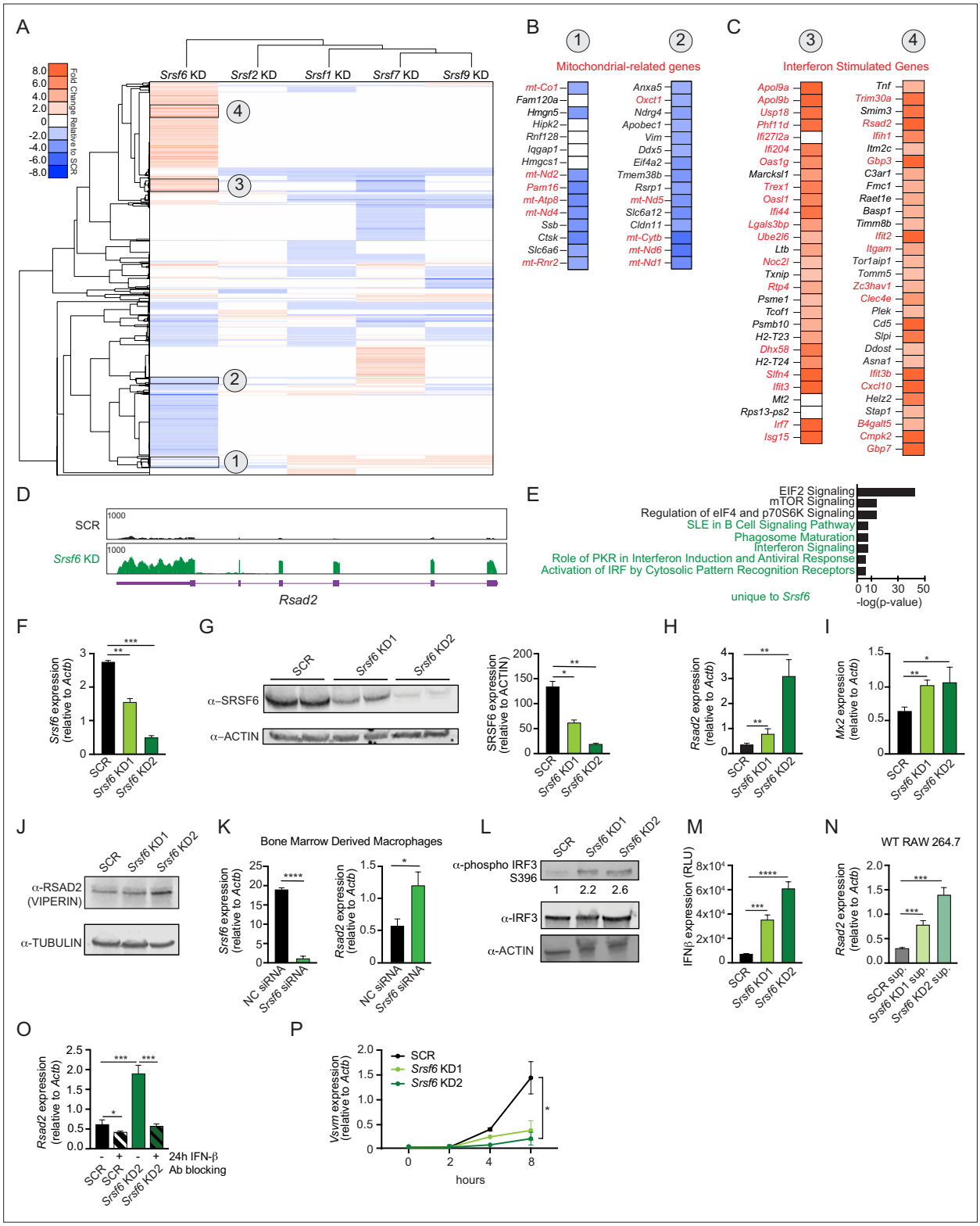

Figure 1. SRSF6 controls basal type I interferon expression in macrophages. (**A**) Heatmap of differentially expressed genes after knockdown of *Srsf1*, *2*, *6*, *7*, and *9* in RAW 264.7 macrophage-like cell lines (RAW MΦ) relative to a scramble (SCR) shRNA control. (**B**) Differential gene expression of mitochondria related genes (red) in *Srsf6* KD RAW MΦ. (**C**) As in B but highlighting ISGs (red). (**D**) Integrative Genomics Viewer (IGV) tracks of *Rsad2* from *Srsf6* KD macrophage RNA seq. (**E**) Ingenuity Pathway Analysis showing canonical pathways from *Srsf6* KD RAW MΦ RNA seq. Green indicates pathways unique to SRSF6. (**F**) RT-qPCR of *Srsf6* in *Srsf6* KD RAW MΦ. (**G**) Immunoblot of SRSF6 in *Srsf6* KD RAW MΦ. (**H**) RT-qPCR of *Rsad2* in *Srsf6*

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KD RAW MΦ. (**I**) RT-qPCR of *Mx2* in *Srsf6* KD RAW MΦ. (**J**) Immunoblot of RSAD2 (VIPERIN) in *Srsf6* KD RAW MΦ. (**K**) RT-qPCR of *Srsf6* and *Rsad2* in *Srsf6* siRNA KD BMDMs compared with a negative control (NC) siRNA control. (**L**) As in G but for phosphorylated IRF3 and total IRF3. Numbers indicate densiometric measurements of pIRF3 (LICOR). (**M**) Protein quantification of extracellular IFNβ in *Srsf6* KD RAW MΦ measured by relative light units (RLU). (**N**) RT-qPCR of *Rsad2* in WT RAW MΦ incubated with SCR or *Srsf6* KD RAW MΦ supernatants for 24 h. (**O**) RT-qPCR of *Rsad2* in *Srsf6* KD RAW MΦ given IFNβ neutralizing antibody treatment for 24 h. (**P**) VSV replication in *Srsf6* KD RAW MΦ at 0, 2, 4, 8 hr post infection (MOI = 1) measured by RT-qPCR of *Vsvm*. All data are compared with a SCR control unless indicated. Data are expressed as a mean of three or more biological replicates with error bars depicting SEM. Statistical significance was determined using two tailed unpaired student's *t* test. *=$p < 0.05$, **=$p < 0.01$, ***=$p < 0.001$, ****=$p < 0.0001$.

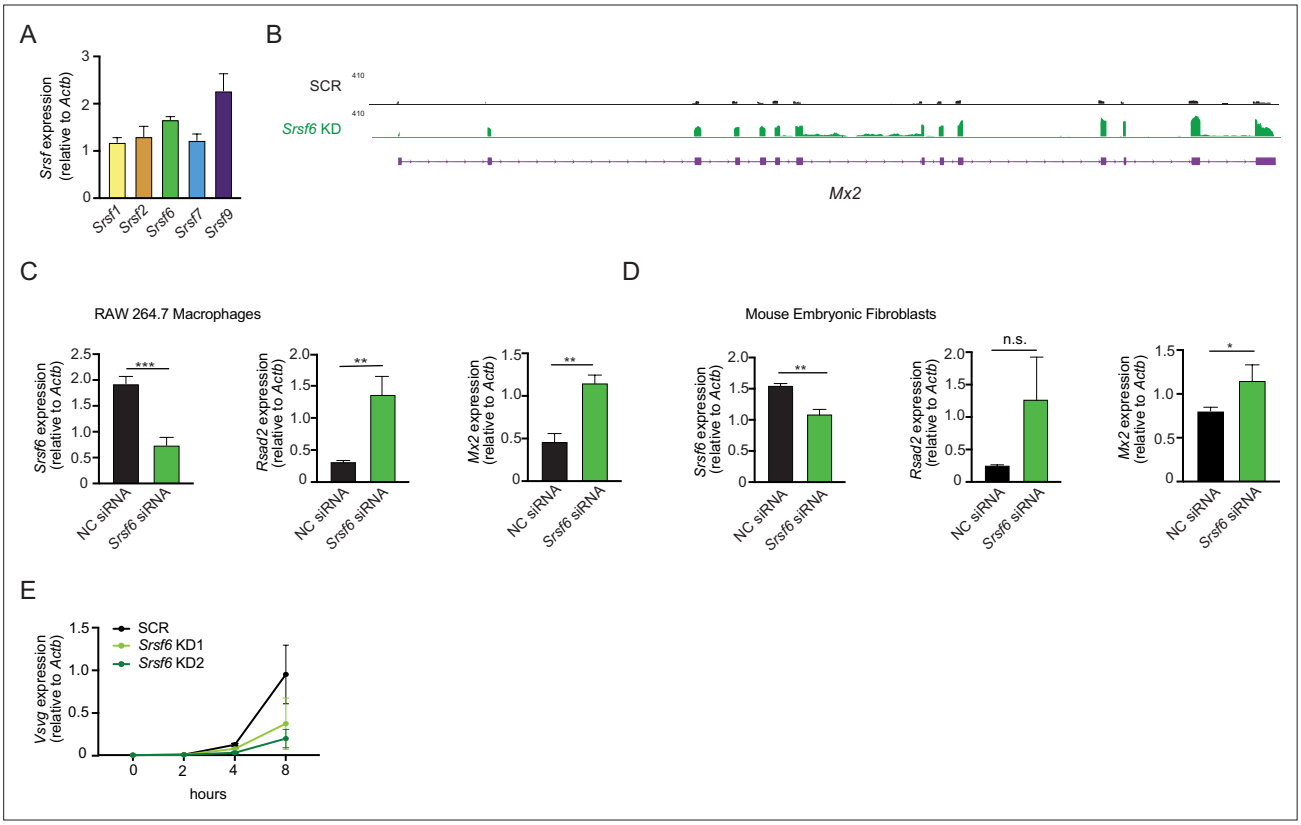

Figure 1—figure supplement 1. Loss of SRSF6 upregulates interferon stimulated genes. (**A**) RT-qPCR of *Srsf* in RAW MΦ. (**B**) Integrative Genomics Viewer (IGV) tracks of *Mx2* in *Srsf6* KD RAW MΦ. (**C**) RT-qPCR of *Srsf6, Rsad2,* and *Mx2* in *Srsf6* siRNA KD RAW MΦ. (**D**) As in C but in MEFs. (**E**) VSV replication in *Srsf6* KD RAW MΦ at 0, 2, 4, 8 hr post infection (MOI = 1) measured by RT-qPCR of *Vsvg*. All data is compared with a SCR control unless indicated. Data are expressed as a mean of three or more biological replicates with error bars depicting SEM. Statistical significance was determined using two tailed unpaired student's *t* test. *=p < 0.05, **=p < 0.01, ***=p < 0.001, ****=p < 0.0001.

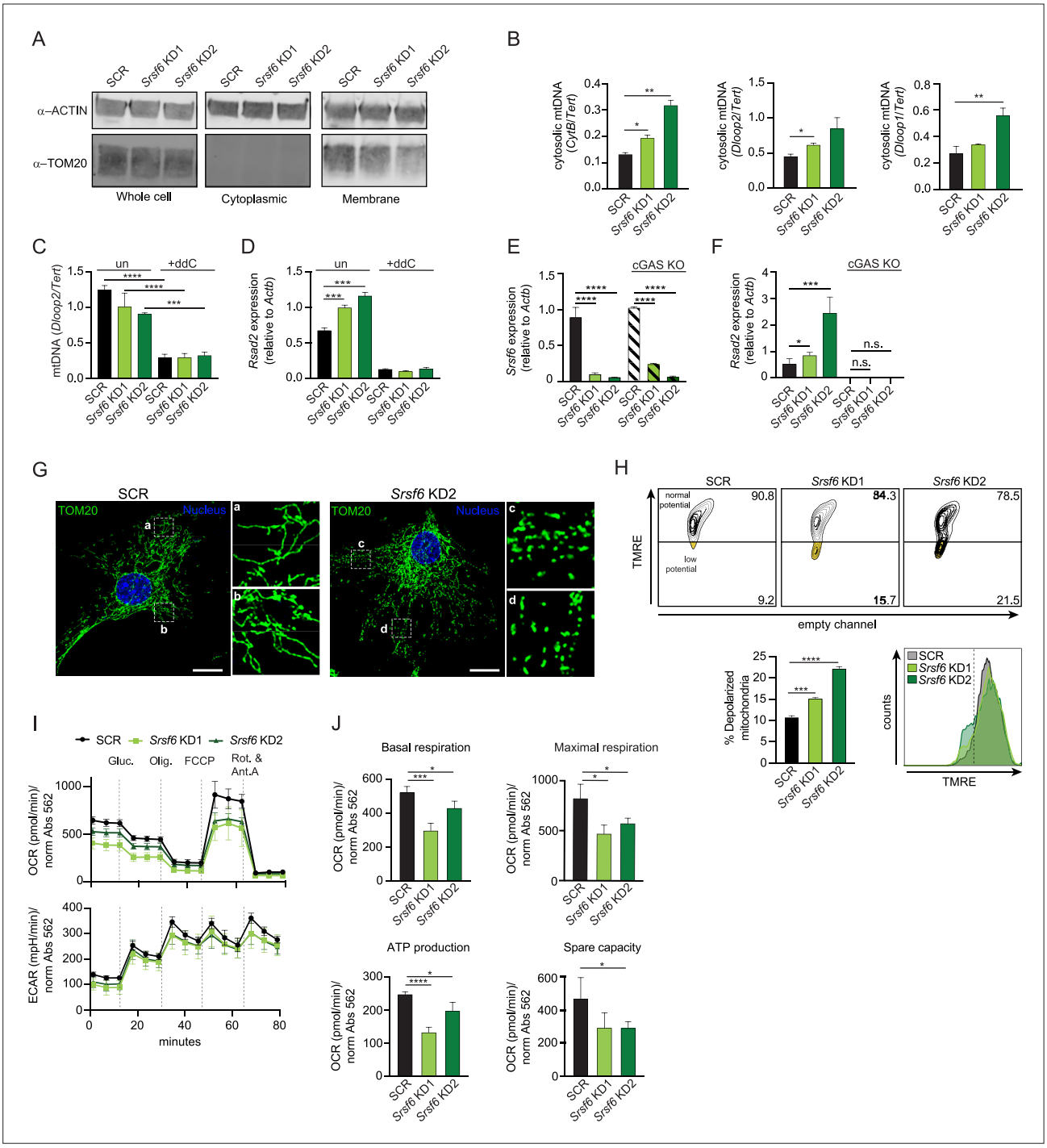

Figure 2. SRSF6 limits cytosolic mtDNA release by maintaining mitochondrial homeostasis. (**A**) Immunoblot of mitochondria (TOM20) in total, cytoplasmic, and membrane fractions of *Srsf6* KD RAW MΦ. (**B**) RT-qPCR of mtDNAs *CytB*, *Dloop1*, *Dloop2* relative to nuclear DNA *Tert* in cytosolic fractions of *Srsf6* KD RAW MΦ. (**C**) RT-qPCR of total mtDNA *Dloop2* relative to nuclear DNA *Tert* in *Srsf6* KD and SCR control RAW MΦ with or without mtDNA depletion for 8 days. (**D**) As in C but measuring *Rsad2*. (**E**) RT-qPCR of *Srsf6* in cGAS KO RAW MΦ. (**F**) As in E but measuring *Rsad2*. (**G**) Immunofluorescence microscopy images visualizing mitochondria in *Srsf6* KD MEFs immunostained with TOM20. Scale bar = 10 μm. (**H**) Mitochondria membrane potential measured by TMRE staining of *Srsf6* KD RAW MΦ. (**I**) Oxygen consumption rate (OCAR) and Extracellular acidification rate (ECAR) measured by Seahorse in *Srsf6* KD RAW MΦ. (**J**) Basal respiration, maximal respiration, ATP production, and spare capacity of *Srsf6* KD RAW MΦ determined by OCAR analysis. All data are compared with a SCR control unless indicated. Data are expressed as a mean of three or more biological replicates with error bars depicting SEM. Statistical significance was determined using two tailed unpaired student's *t* test. *=p < 0.05, **=p < 0.01, ***=p < 0.001, ****=p < 0.0001.

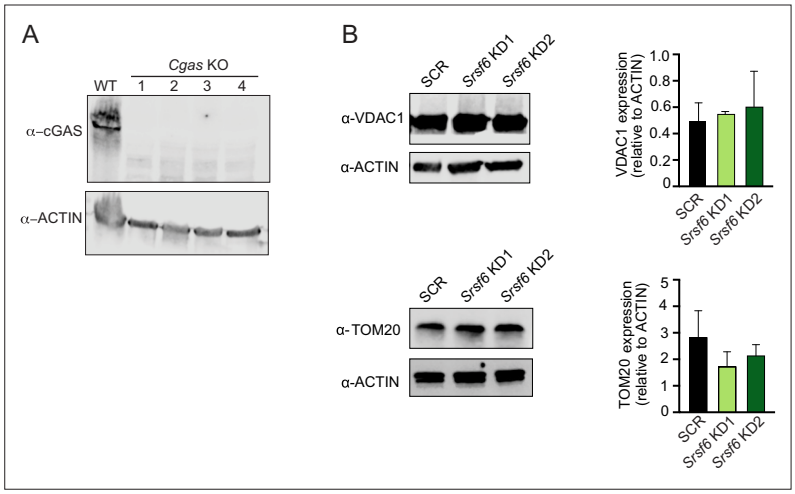

Figure 2—figure supplement 1. Mitochondrial protein levels in *Srsf6* KD macrophages.
(**A**) Immunoblot of cGAS in WT and *cGAS* KO RAW MΦ. cGAS lanes are from multiple protein preparations. (**B**) Immunoblots of VDAC1 (top) and TOM20 (bottom) in *Srsf6* KD RAW MΦ. Data are expressed as a mean of three or more biological replicates with error bars depicting SEM. Statistical significance was determined using two tailed unpaired student's *t* test. *=p < 0.05, **=p < 0.01, ***=p < 0.001, ****=p < 0.0001.

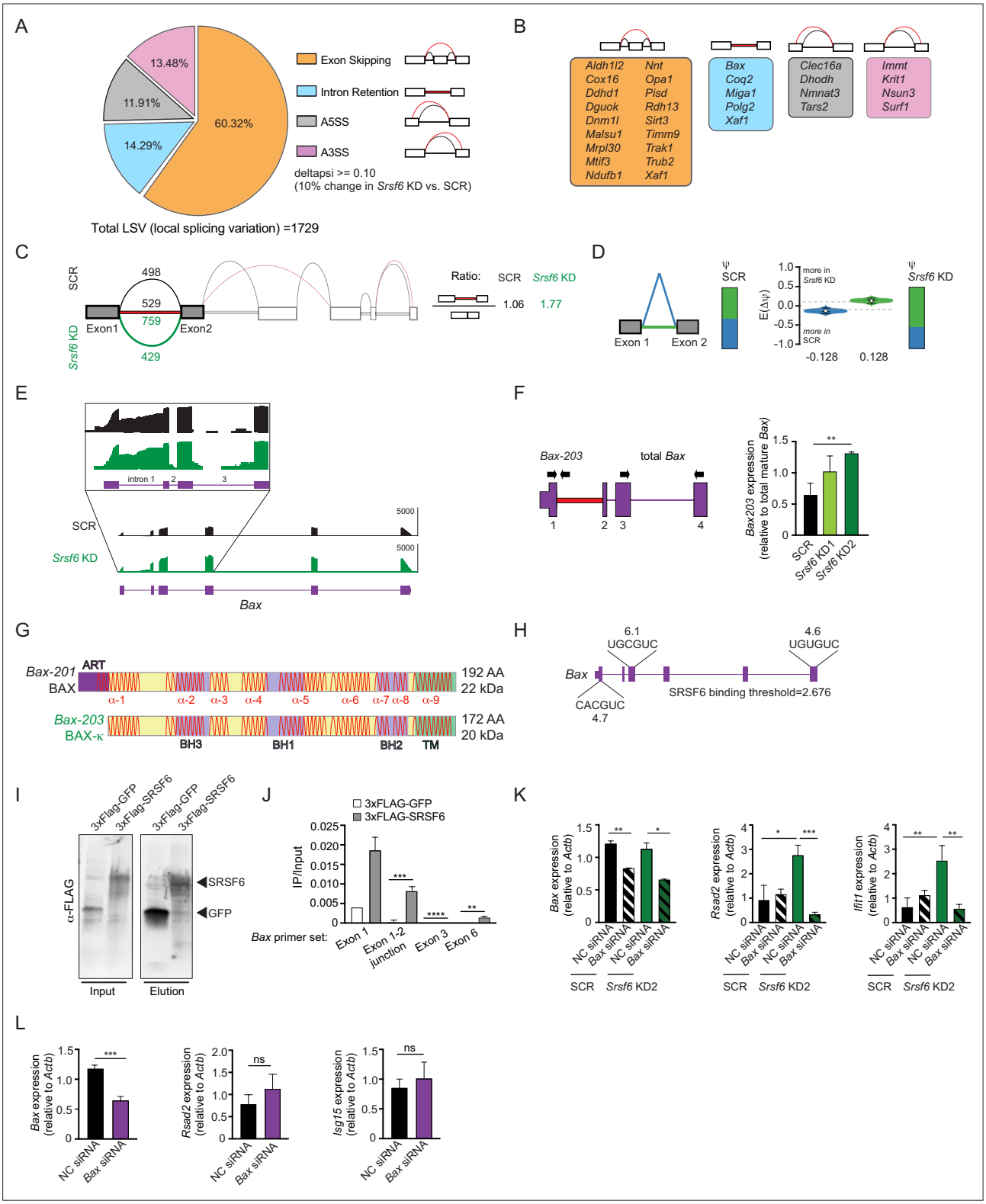

Figure 3. SRSF6 controls alternative splicing of the mitochondrial apoptotic factor BAX. (**A**) Percentages of alternative splicing (AS) events in *Srsf6* KD RAW MΦ (deltapsi ≥ 0.1). (**B**) Categorization of alternative splicing events in mitochondria genes differentially expressed in *Srsf6* KD RAW MΦ. Red lines are AS events and black lines are WT events. (**C**) Splice graph of *Bax* in SCR (top) and *Srsf6* KD (bottom) RAW MΦ generated by MAJIQ/VOILA. Intron 1 retention reads relative to exon1-2 junction reads in each genotype shown on right. (**D**) MAJIQ Ψ quantification of junctions as illustrated in (**C**) from SCR (left) and *Srsf6* KD (right) RAW MΦ. Intron retention displayed in green; intron removal displayed in blue. (**E**) Integrative Genomics Viewer (IGV)

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tracks of *Bax*, highlighting exon 1 to exon 3. Zoom-in (top) uses a log scale to facilitate appreciation of the intron reads. (**F**) RT-qPCR of *Bax*203 relative to mature *Bax* expression in *Srsf6* KD RAW MΦ. Primers shown on schematic. (**G**) Schematics of BAX and BAX-κ proteins. Alpha-helical domains shown as red lines. ART = apoptosis regulatory targeting domain (***Goping et al., 1998***). (**H**) Diagram of predicted *Srsf6* binding sites in *Bax* pre-mRNA with predicted binding strength scores (from ESE Finder). (**I**) CLIP Immunoblot of 3xFLAG-GFP and 3xFLAG-SRSF6 constructs expressed in RAW MΦ for 24 h. (**J**) CLIP RT-qPCR of 3xFLAG-GFP and 3xFLAG-SRSF6 RT-qPCR of *Bax* exon 1, exon1-2 junction, exon 3, and exon 6. Data shown as IP relative to input. (**K**) RT-qPCR of *Bax*, *Rsad2,* and *Isg15* in *Srsf6* KD RAW MΦ with *Bax* KD via siRNA transfection. (**L**) RT-qPCR of *Bax*, *Rsad2,* and *Isg15* in transient *Bax* KD RAW MΦ. All data are compared with a SCR control unless indicated. Data are expressed as a mean of three or more biological replicates with error bars depicting SEM. Statistical significance was determined using two tailed unpaired student's *t* test. *=p < 0.05, **=p < 0.01, ***=p < 0.001, ****=p < 0.0001.

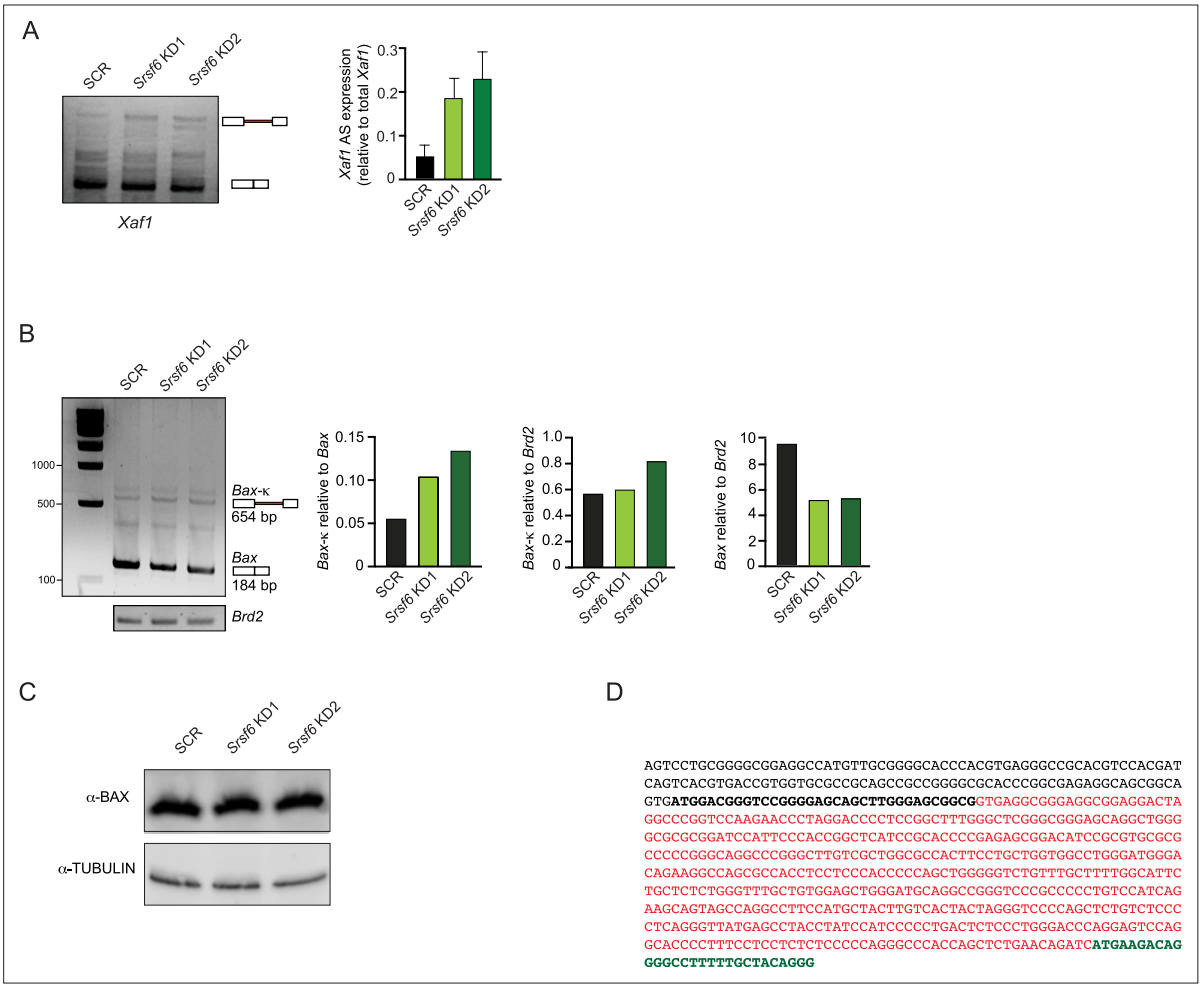

Figure 3—figure supplement 1. Loss of SRSF6 impacts alternative splicing of transcripts with known roles in mitochondrial biology. (**A**) Semi-quantitative RT-PCR of *Xaf1* in *Srsf6* KD RAW MΦ with quantification. (**B**) Semi-quantitative RT-PCR of *Bax* and *Brd2* (control) in *Srsf6* KD RAW MΦ with densiometric quantification (LICOR) on right. Gel shown is representative of n>3. (**C**) Immunoblot of BAX in *Srsf6* KD RAW MΦ. (**D**) mRNA sequence of *Bax*201 with *Bax*203 truncated isoform (red). All data are compared with a SCR control unless indicated. Data are expressed as a mean of three or more biological replicates with error bars depicting SEM. Statistical significance was determined using two tailed unpaired student's *t* test. *=p < 0.05, **=p < 0.01, ***=p < 0.001, ****=p < 0.0001.

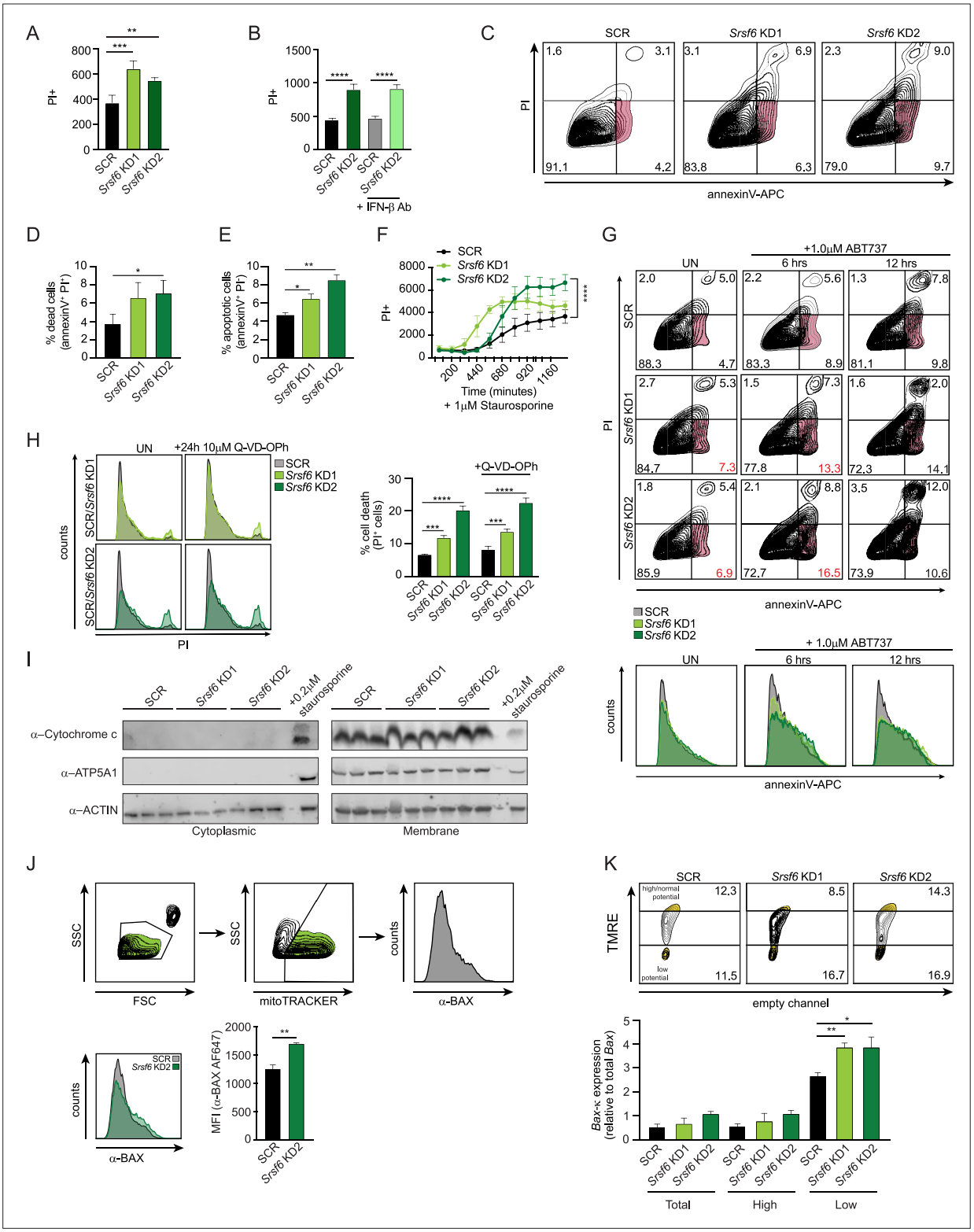

Figure 4. Loss of SRSF6 sensitizes macrophages to caspase-independent apoptotic cell death. (**A**) Cell death in *Srsf6* KD RAW MΦ measured by live cell imaging of propidium iodide (PI) staining. (**B**) Cell death in *Srsf6* KD RAW MΦ treated with IFN-β neutralizing antibody. (**C**) Apoptotic cell death measured by flow cytometry using APC conjugated annexin V (annexinV-APC) and propidium iodide (PI) dyes in *Srsf6* KD RAW MΦ. (**D**) Quantification of dead cells in *Srsf6* KD RAW MΦ from C. (**E**) Quantification of apoptotic cells in *Srsf6* KD RAW MΦ from C. (**F**) Cell death over a time course in *Srsf6* KD RAW 264.7 cells treated with 1 μM staurosporine. (**G**) Apoptotic cell death over a time course measured by flow cytometry using annexinV-APC and PI in *Srsf6* KD RAW MΦ treated with 1 μM ABT737. Histograms display annexinV-APC single stain in *Srsf6* KD. Red numbers indicate annexinV+/

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PI- cells in *Srsf6* KDs. (**H**) Histogram showing cell death after caspase inhibition by flow cytometry in *Srsf6* KD RAW MΦ. Cell death quantification (right). (**I**) Immunoblot of cytochrome c in cytoplasmic and membrane fractions of *Srsf6* KD RAW 264.7 cells. SCR cells treated with 0.2 μM staurosporine for 24 h used as a positive control. (**J**) Schematic of mitoFLOW workflow (top). Histogram showing BAX accumulation on *Srsf6* KD RAW MΦ isolated mitochondria (bottom) (**K**) Mitochondria membrane potential measured by TMRE staining of *Srsf6* KD RAW MΦ (top). RT-qPCR of BAX-κ relative to mature *Bax* expression in total, high, and low mitochondria membrane potential cell populations. All data are compared with a SCR control unless indicated. Data are expressed as a mean of three or more biological replicates with error bars depicting SEM. Statistical significance was determined using two tailed unpaired student's *t* test. *=p < 0.05, **=p < 0.01, ***=p < 0.001, ****=p < 0.0001.

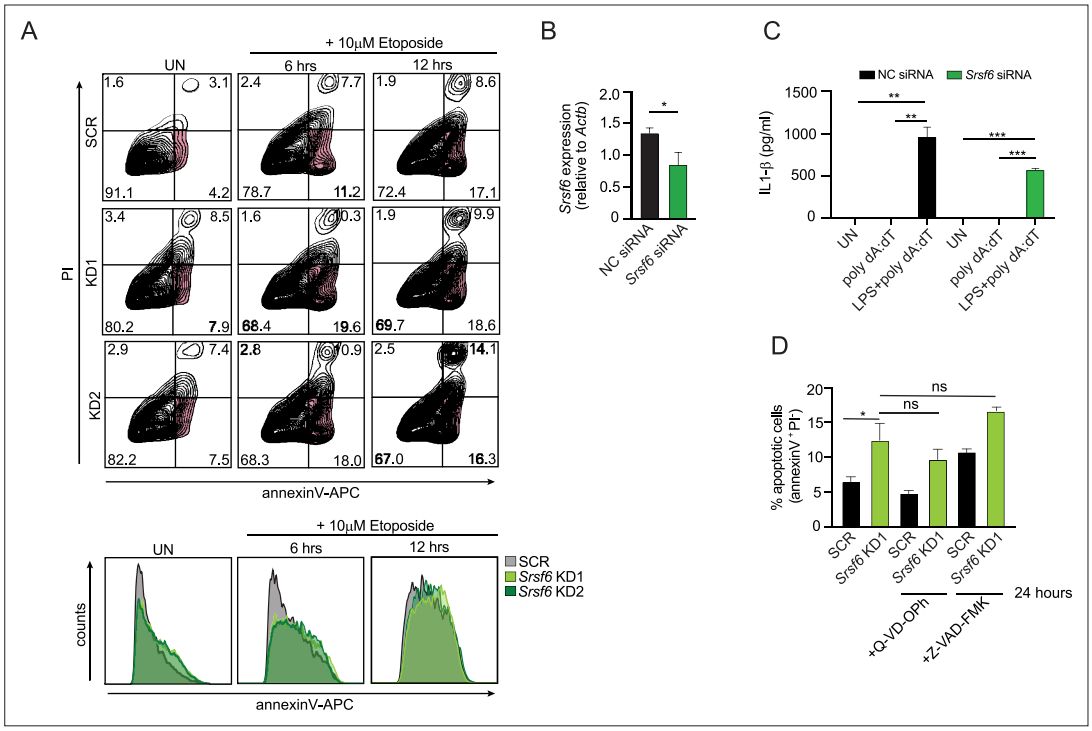

Figure 4—figure supplement 1. *Srsf6* KD cells are sensitive to cell death agonists. (**A**) Apoptotic cell death over a time course measured by flow cytometry using annexinV-APC and PI in *Srsf6* KD RAW MΦ treated with 10 μM etoposide. Histograms display annexinV-APC single stain in *Srsf6* KD RAW MΦ. (**B**) RT-qPCR of *Srsf6* in *Srsf6* siRNA KD BMDMs. (**C**) Extracellular IL-1β in negative siRNA control and *Srsf6* KD BMDMs untreated and inflammasome treated with LPS 3 hr, poly dA:dT 4 hr by ELISA with AIM2 inflammasome stimulated positive control (LPS/ poly dA:dT). (**D**) Apoptotic cells (AnnexinV+/PI-) quantification in *Srsf6* KD RAW MΦ treated with caspase inhibitors Q-VD-OPh and Z-VAD-FMK for 24 hr. All data are compared with a scramble control unless indicated. Data are expressed as a mean of three or more biological replicates with error bars depicting SEM. Statistical significance was determined using two tailed unpaired student's *t* test. *=p < 0.05, **=p < 0.01, ***=p < 0.001, ****=p < 0.0001.

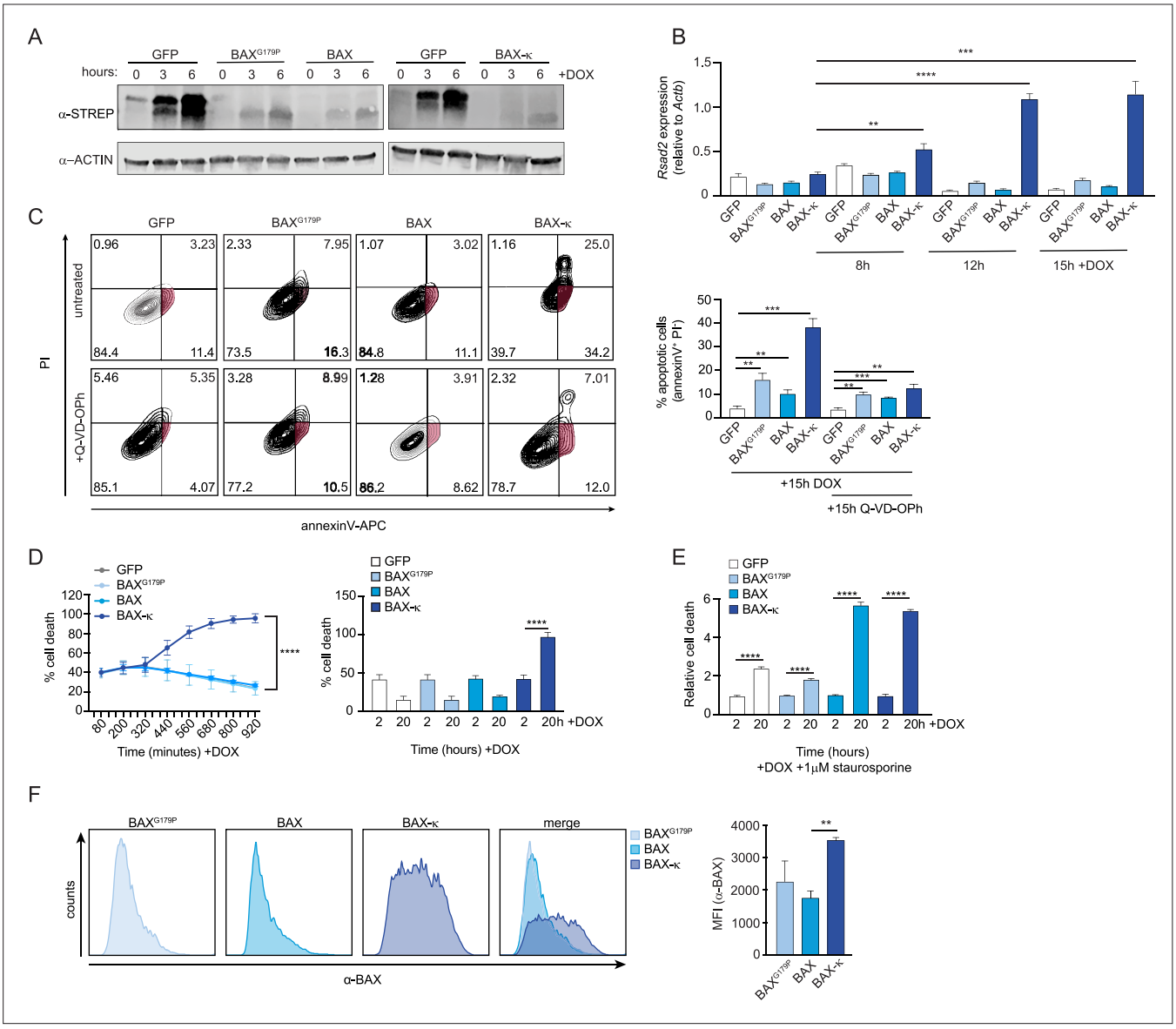

Figure 5. Expression of BAX-κ promotes type I IFN expression and cell death in macrophages. (**A**) Immunoblot of strep tagged BAX^{G179P}, BAX, and BAX-κ inducible RAW MΦ expressed over a time course after addition of doxycycline (DOX). (**B**) Expression of *Rsad2* over a time course of 8, 12, and 15 hr after DOX induction in GFP, BAX^{G179P}, BAX, and BAX-κ-expressing RAW MΦ by RT-qPCR. (**C**) Apoptotic cell death measured by flow cytometry using annexinV-APC and PI in GFP, BAX^{G179P}, BAX, and BAX-κ inducible macrophages expressed for 15 hr with 1 μg DOX and caspase inhibitor (10 μM Q-VD-OPh). Apoptotic cells (AnnexinV+/PI-) quantification (right). (**D**) Cell death over a time course after DOX induced expression of GFP, BAX^{G179P}, BAX, and BAX-κ. Starting and ending cell death (PI+) shown as a bar graph on right. (**E**) Relative cell death measured by PI incorporation at 2 and 20 hr after DOX-induced expression of GFP, BAX^{G179P}, BAX, and BAX + addition of 1 μM staurosporine. (**F**) Histogram showing BAX accumulation on 20 h DOX-induced GFP, BAX^{G179P}, BAX, and BAX isolated mitochondria. Data are expressed as a mean of three or more biological replicates with error bars depicting SEM. Statistical significance was determined using two tailed unpaired student's *t* test. *=p < 0.05, **=p < 0.01, ***=p < 0.001, ****=p < 0.0001.

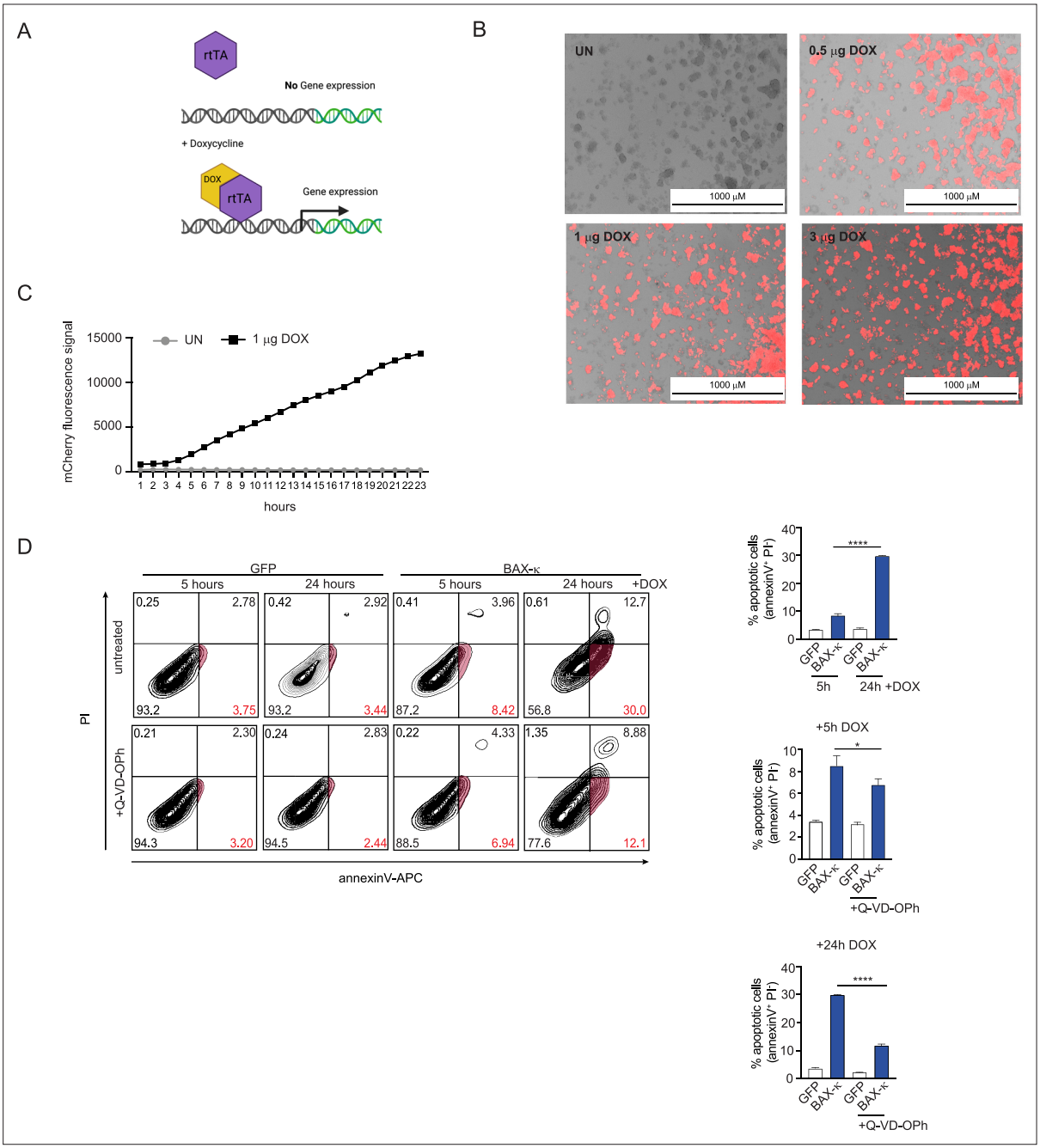

Figure 5—figure supplement 1. Bax- κ expression induces cell death. (**A**) Schematic of DOX activation of transactivator to induce construct expression (**B**) Immunofluorescence/DIC microscopy images visualizing mCherry doxycycline-inducible RAW MΦ stimulated with 0.5 µg, 1.0 µg, and 3.0 µg of DOX for 15 hr. (**C**) mCherry fluorescence over a time course measured using a Lionheart XF analyzer +/-1.0 µg DOX. (**D**) Apoptotic cell death measured by flow cytometry using annexinV-APC and PI in GFP and BAX- κ inducible macrophages expressed for 5 and 24 hr with 1 µg DOX and caspase inhibitor (10 µM Q-VD-OPh). Red numbers indicate apoptotic cells (AnnexinV+/PI-) quantification (right). Data are expressed as a mean of three or more biological replicates with error bars depicting SEM. Statistical significance was determined using two tailed unpaired student's *t* test. *=p < 0.05, **=p < 0.01, ***=p < 0.001, ****=p < 0.0001.

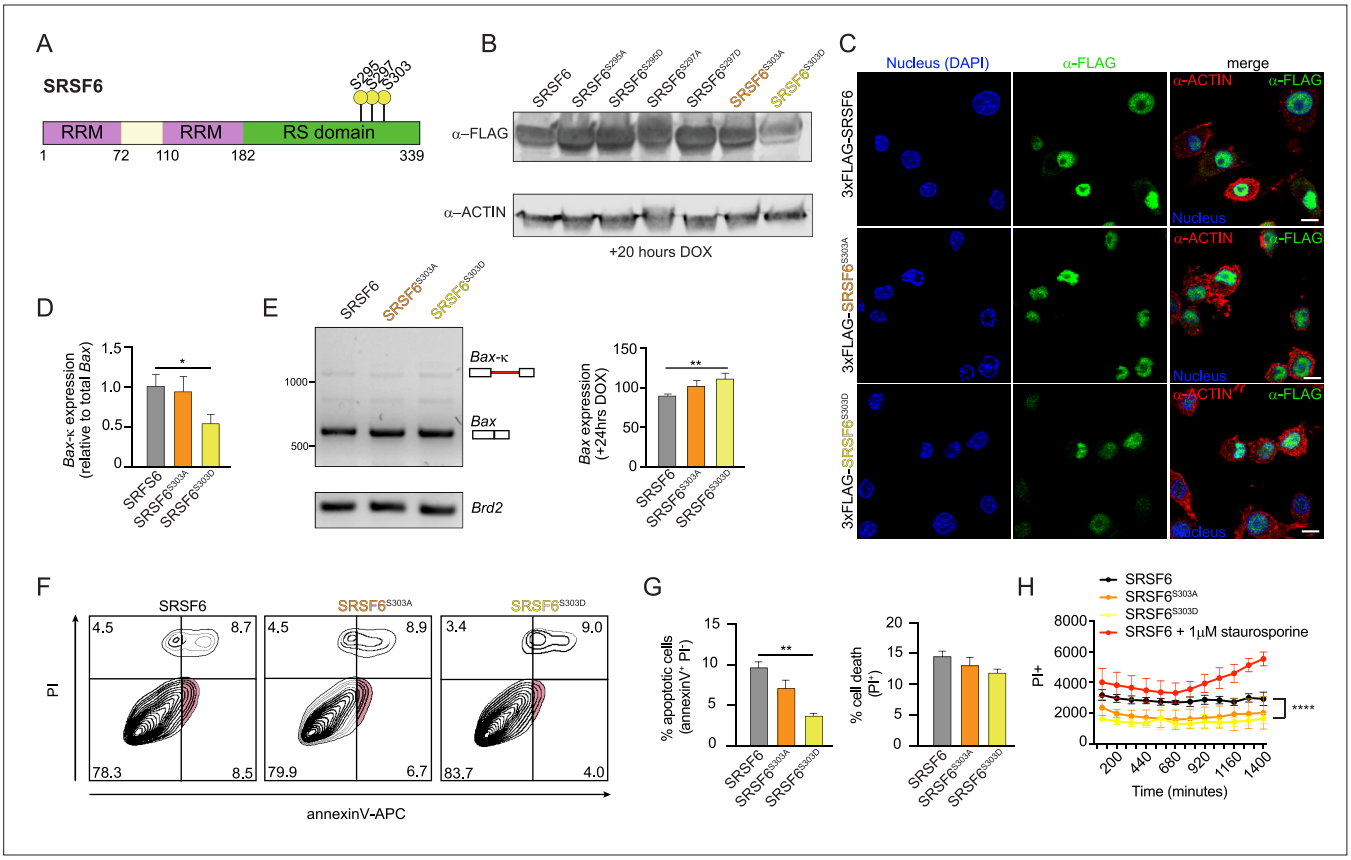

Figure 6. Phosphorylation of SRSF6 at S303 promotes splicing of *Bax* to limit *Bax-* κ expression and prevent cell death. (**A**) Diagram of differentially phosphorylated residues in SRSF6 according to ***Budzik et al., 2020***. (**B**) Immunoblot of FLAG tagged SRSF6, SRSF6^{S295A}, SRSF6^{295D}, SRSF6^{S297A}, SRSF6^{S297D}, SRSF6^{S303A}, and SRSF6^{S303D} inducible RAW MΦ expressed for 24 hr after DOX induction. (**C**) Immunofluorescence microscopy images visualizing 3x-FLAG tagged SRSF6, SRSF6^{S303A}, and SRSF6^{S303D} inducible RAW MΦ expressed for 24 hr after DOX induction. Scale bar = 10 μm. (**D**) RT-qPCR of *Bax*203 in FLAG-tagged SRSF6, SRSF6^{S303A}, and SRSF6^{S303D} inducible RAW MΦ after DOX induction for 24 hr. (**E**) Semi-quantitative RT-PCR of *Bax* and *Brd2* (control) in FLAG-tagged SRSF6, SRSF6^{S303A}, and SRSF6^{S303D} inducible RAW MΦ expressed for 24 hr after DOX induction with quantification of multiple independent experiment. Representative gel shown. (**F**) Apoptotic cell death measured by flow cytometry using annexinV-APC and PI in FLAG tagged SRSF6, SRSF6^{S303A}, and SRSF6^{S303D} inducible RAW MΦ expressed for 24 hr after DOX induction. (**G**) % apoptotic cells and % dead cells from D. (**H**) Cell death over a time course in FLAG tagged SRSF6, SRSF6^{S303A}, and SRSF6^{S303D} inducible RAW MΦ. FLAG-tagged SRSF6 inducible RAW MΦ were treated with 1 μM staurosporine as a positive control. Data are expressed as a mean of three or more biological replicates with error bars depicting SEM. Statistical significance was determined using two tailed unpaired student's *t* test. *=p < 0.05, **=p < 0.01, ***=p < 0.001, ****=p < 0.0001.

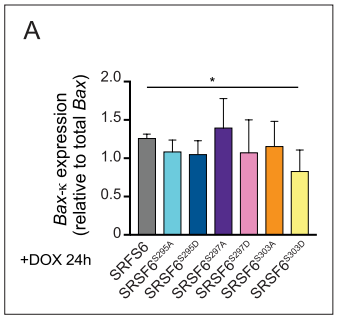

Figure 6—figure supplement 1. Expression of SRSF6-S303D reduces Bax-κ expression. (**A**) RT-qPCR of *Bax*203 in FLAG tagged SRSF6, SRSF6^{S295A}, SRSF6^{S295D}, SRSF6^{S297A}, SRSF6^{S297D}, SRSF6^{S303A}, and SRSF6^{S303D} doxycycline-inducible RAW MΦ expressed for 24 hr after DOX induction. Data are expressed as a mean of three or more biological replicates with error bars depicting SEM. Statistical significance was determined using two tailed unpaired student's *t* test. *=p < 0.05, **=p < 0.01, ***=p < 0.001, ****=p < 0.0001.

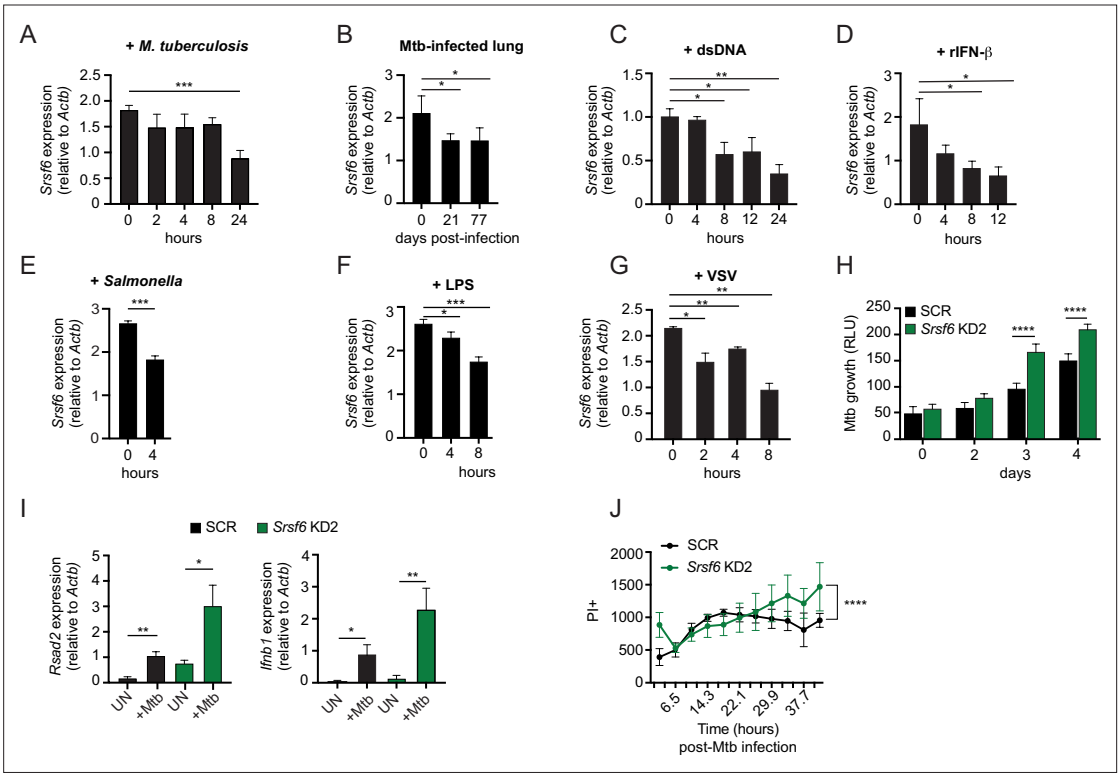

Figure 7. Modulation of SRSF6 expression contributes to innate immune control of the intracellular bacterial pathogen *M. tuberculosis*. (**A**) RT-qPCR of *Srsf6* in RAW MΦ infected with *M. tuberculosis* (Mtb) (MOI = 5) over a time course. (**B**) RT-qPCR of *Srsf6* in Mtb-infected mouse lung samples over a time course of *in vivo* infection. (**C**) RT-qPCR of *Srsf6* in RAW MΦ treated with 1 µg double stranded DNA (dsDNA). over a time course. (**D**) As in C but treated with recombinant IFN-β (rIFN-β). (**E**) RT-qPCR of *Srsf6* in *S. enterica* (Typhimurium) infected RAW MΦ (MOI = 5) at 0 and 4 hr. (**F**) As in C but treated with LPS. (**G**) RT-qPCR of *Srsf6* in VSV infected RAW MΦ (MOI = 1) over a time course. (**H**) Mtb *luxBCADE* growth in *Srsf6* KD RAW MΦ measured by relative light units (RLUs) over a time course (MOI = 1). (**I**) RT-qPCR of *Rsad2* and *Ifnb1* in *Srsf6* KD RAW MΦ infected with Mtb at (MOI = 10), 4 hr post-infection. (**J**) Cell death over a time course in SCR and *Srsf6* KD RAW MΦ infected with Mtb at (MOI = 5). All data are compared with a SCR control unless indicated. Data are expressed as a mean of three or more biological replicates with error bars depicting SEM. Statistical significance was determined using two tailed unpaired student's *t* test. *=p < 0.05, **=p < 0.01, ***=p < 0.001, ****=p < 0.0001.