Global transcriptomics analyses reveal specialized roles for splicing regulatory proteins in the macrophage innate immune response

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Running title: Splicing regulatory factors control innate immune responses

ABSTRACT

While the signaling cascades and transcription factors that activate gene expression in macrophages following pattern recognition receptor engagement are well known, the role of post-transcriptional RNA processing in modulating innate immune gene expression remains understudied. Recent phosphoproteomics analyses revealed that members of the SR and hnRNP families of splicing regulatory proteins undergo dynamic post-translational modification in infected macrophages. To begin to test if these splicing factors play a privileged role in controlling the innate immune transcriptome, we analyzed steady state gene expression and alternatively spliced isoform production in ten SR/hnRNP knockdown RAW 264.7 macrophage cell lines following infection with the bacterial pathogen *Salmonella enterica* serovar Typhimurium (*Salmonella*). We observed that thousands of genes were up or downregulated in SR/hnRNP knockdown cells and differentially expressed genes (DEGs) varied significantly depending on the SR/hnRNP examined. We discovered that a subset of critical innate immune genes (*Nos2*, *Mx1*, *Il1a*) rely heavily on SR/hnRNPs for proper induction and/or repression, while others (*Tnf*, *Il1b*) are generally unaffected by splicing factor knockdown. We also discovered that many key immune sensors and signaling molecules are subject to regulation by alternative splicing. While our data does not provide evidence for positive correlation between a transcripts' reliance of SR/hnRNPs for proper expression and the gene's induction level, length, or intron/exon architecture, we found that many rapidly induced primary response genes are repressed by SR/hnRNPs. Consistent with SR/hnRNP proteins contributing to innate immune outcomes, knockdown of hnRNP K and U significantly enhanced the ability of RAW 264.7 macrophages to control viral replication. Based on these collective findings, we conclude that many innate immune genes have evolved to rely on one or more splicing regulatory factors to ensure the proper timing and magnitude of their induction, supporting a model wherein pre-splicing is a critical regulatory node in the innate immune response.

INTRODUCTION

When innate immune cells like macrophages sense pathogens, they undergo dramatic gene expression reprogramming and upregulate thousands of genes. Proper regulation of the timing and magnitude of innate immune gene induction is critical to ensure that the immune system is adequately engaged to fend off microbial invaders without risking deleterious outcomes associated with hyperinflammation [1-3]. While there has been great interest in the nature of macrophage

sensing and signaling events that activate a transcriptional response following an inflammatory signal, much less is known about how regulation of RNA processing steps downstream of transcription influence innate immune gene expression outcomes.

Consistent with the current “transcription-focused” paradigm of innate immune gene expression, research has categorized innate immune genes into primary and secondary response genes [4, 5]. Primary, or early response genes, are readily induced upon activation of pathogen sensing cascades. Many of these transcripts reach maximal levels as soon as 30 minutes post-pathogen sensing [6, 7]. Secondary response genes, defined on basis of their requirement for nascent protein synthesis of a transcription factor or cytokine that activates another wave of gene expression via autocrine signaling, are maximally induced with slower kinetics. The timing and degree to which genes in both primary and secondary response genes are induced relies on a number of tightly regulated mechanisms, including but not limited to, cooperative binding of transcription factors [8, 9], nucleosome occupancy and histone modification at promoters [10, 11], signal dependent interactions between transcription factor subunits [12, 13], and selective interaction with transcriptional elongation machinery [14].

On top of this dizzying transcriptional regulatory network, once innate immune transcripts are transcribed, they, like most eukaryotic transcripts, are subject to post-transcriptional regulation at the level of pre-mRNA splicing, cleavage and polyadenylation, mRNA export, and nonsense mediated decay. Several studies have demonstrated important roles for each of these RNA processing steps in modulating innate immune gene expression outcomes. Both *Salmonella*- and *Listeria*-infection promote widespread 3’UTR shortening and exon inclusion in primary human macrophages [15] and alternative splicing and nonsense mediated decay play an important role in balancing isoform abundance of key antiviral innate immune molecules like *Oas1g* [16]. Kinetic studies of gene expression in Lipid-A treated bone marrow derived macrophages show that removal of introns from pre-mRNAs and release of processed innate immune transcripts from chromatin can be significantly delayed relative to transcription [6, 7]. These findings are consistent with numerous reports over the last decade demonstrating that the majority of splicing, both intron recognition and catalysis, occurs co-transcriptionally [17-20]. While these findings argue that post-transcription regulatory mechanisms play a key role in controlling the timing and abundance of translation-competent mRNAs and thus directly influence the overall production of cytokines, chemokines, and antimicrobial proteins, we still know very little about the mechanisms that drive this regulation and the specific players involved.

Pre-mRNA splicing is being increasingly appreciated as an important regulatory node in cells undergoing stress or responding to extracellular triggers. For example, several recent

studies have described a role for global intron retention in mediating monocyte-to-macrophage differentiation, whereby unprocessed pre-mRNAs accumulate in monocytes as a way to prevent expression of inappropriate genes while allowing the cell to remain poised to generate mRNAs when differentiation signals are received [21, 22]. T cell activation is another well-studied immune related phenomenon associated with global RNA processing changes. Famously, T-cell activation-dependent exon skipping of the CD45 phosphatase is a critical regulator of T cell receptor signaling and is important for limiting T-cell hyperactivation [23]. T-cell activation also promotes alternative splicing of the MAPK kinase MKK7, driven by upregulation of the splicing factor CELF2 [24, 25]. A number of other environmental triggers including exposure to vitamins and metal ions [26], heat shock [27, 28], and UV damage [29, 30] have been shown to modulate RNA processing through a variety of mechanisms, including SRSF protein phosphorylation [27, 28, 31].

Our lab became interested in a role for SR and hnRNP proteins in regulating macrophage gene expression after several global phosphoproteomics studies revealed that proteins involved in mRNA processing are among the most differentially phosphorylated proteins in macrophages following infection with a bacterial [32, 33] or fungal pathogen [34], despite showing no significant change in protein abundance. These data led us to speculate that splicing regulatory proteins are functionalized following pathogen sensing. Consistent with this idea, we recently found that phosphorylation of one such factor—hnRNP M—at a single serine residue (S574) is sufficient to ablate its ability to repress expression of innate immune transcripts like *Il6* and *Mx1*, suggesting that post-translational modification of this splicing factor is important to allow cells to maximally induce expression of specific innate immune genes [35]. Our studies of hnRNP M suggest that other individual RNA binding proteins may also coordinate the innate immune transcriptome in unexpected ways.

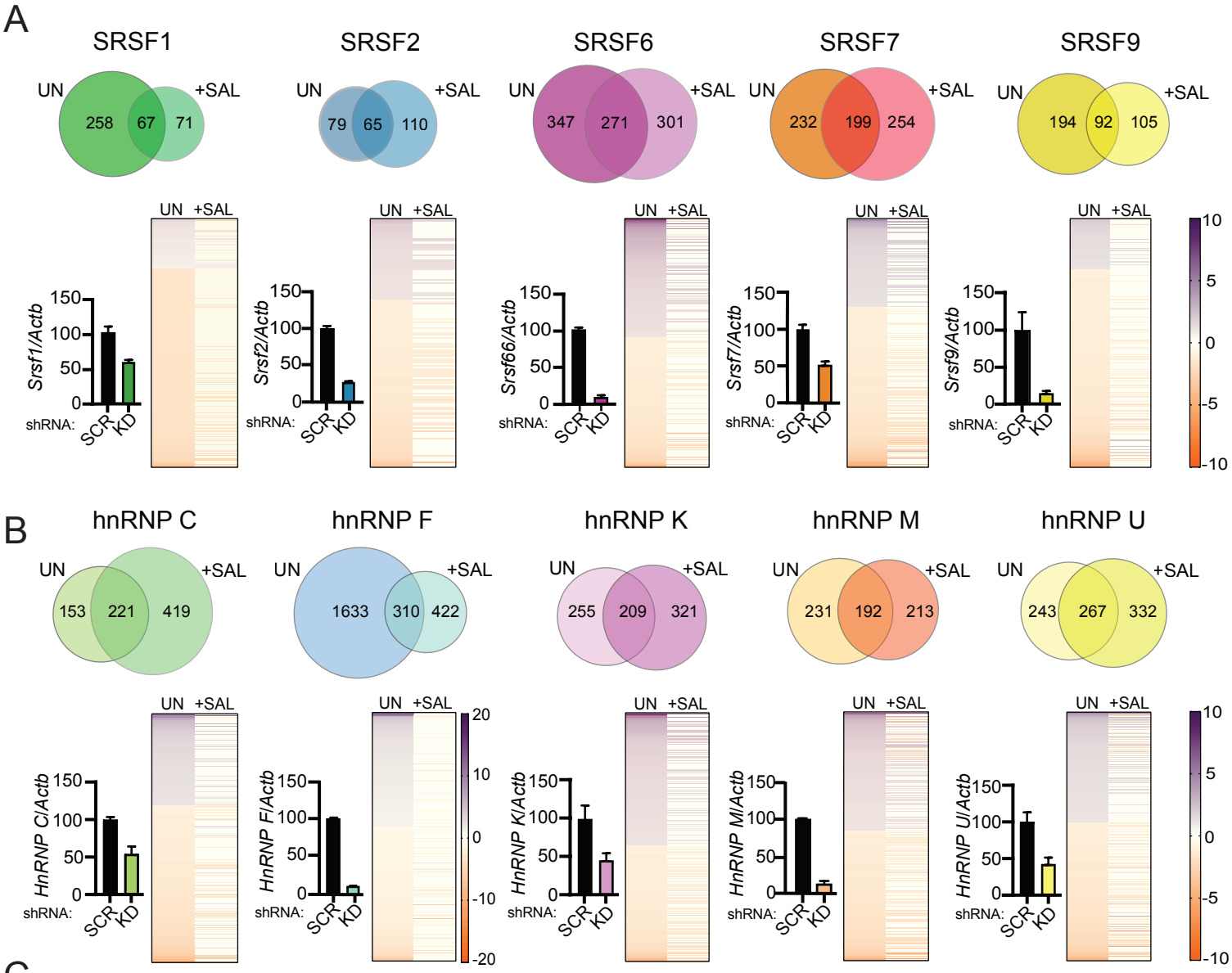
To begin to the define the contribution of splicing regulatory proteins to modulating innate immune gene expression, we took an unbiased approach and knocked down expression of ten members of the SR/hnRNP families of splicing regulatory factors. We infected these knockdown cell lines with *Salmonella enterica* serovar Typhimurium (*Salmonella*) and measured differential gene expression (DGE) and differential isoform usage (DIU) in steady state RNA at a key innate immune time point. Our analysis found that most SR/hnRNPs are unique in the cohorts of genes whose expression and splicing they control and that most DEGs are not also subject to DIU. We also discovered that certain innate immune genes (e.g. *Nos2*, *Mx1*) are more reliant on SR/hnRNPs for maintaining proper induction levels than others (e.g. *Tnf*, *Il1b*) and this reliance correlates with SR/hnRNP binding site enrichment. Notably, two of the factors we looked at, hnRNP K and hnRNP U, shared a number of DEGs and genes that underwent DIU, and loss of

both of these factors contributed to significantly enhanced control of vesicular stomatitis virus (VSV) replication, compared to SCR controls. Together, these data implicate splicing regulatory protein networks in fine-tuning the timing and magnitude of a cohort of innate immune transcripts and highlight a previously unappreciated role for RNA binding proteins and post-transcriptional control mechanisms in regulating macrophage gene expression.

RESULTS

SRSF and hnRNP proteins control expression of distinct sets of transcripts in resting and *Salmonella*-infected macrophages

To understand how splicing regulatory proteins shape global innate immune gene expression, we first needed to identify factors most likely to play a privileged role in the macrophage innate immune response. Two recent publications identified a number of splicing factors that were differentially phosphorylated during bacterial infection (specifically during infection with the intracellular bacterial pathogen *Mycobacterium tuberculosis*) in a RAW 264.7 macrophage cell line [32] or in primary mouse macrophages [33]. We manually annotated these lists and prioritized a set of SRSF and hnRNPs for transcriptomics analysis. Based on these proteomics data, SRSF1, SRSF2, SRSF6, SRSF7, SRSF9, hnRNP C, hnRNP F, hnRNP K, hnRNP M, and hnRNP U, were predicted to be phosphorylated or dephosphorylated at one or more amino acid residues over the first 24h of *M. tuberculosis* infection (Fig. S1A). To begin to determine how each of these factors contribute to gene expression in macrophages, we generated murine macrophage cell lines (RAW 264.7) in which each factor was stably knocked down via expression of an shRNA construct targeting the 3'UTR for each factor. Hairpin targets were carefully chosen to ensure that all protein coding isoforms of each factor could be targeted by the shRNA. Each SR knockdown cell line was selected and derived at the same time, alongside a scramble (SCR) control hairpin-expressing cell line; hnRNP KD cell lines were derived the same way. Knockdown efficiency of each factor was validated by RT-qPCR using exonic primer sets and the most efficient knockdown cell line (from 6 different knockdown constructs) is shown (Fig. 1A and 1B). A notable caveat of these stable knockdown cell lines is that overall knockdown efficiency varied between factors, with only about 50% knockdown efficiency achieved for hnRNP C, hnRNP K, SRSF1 and SRSF7 and 70-90% knockdown achieved for SRSF2, SRSF6, SRSF9, hnRNP F and hnRNP U. We predict this variation in knockdown efficiency is indicative of the macrophage's ability to tolerate loss of each of these factors and likely correlates with the cell's reliance on each of these factors for proper RNA processing of essential housekeeping genes.



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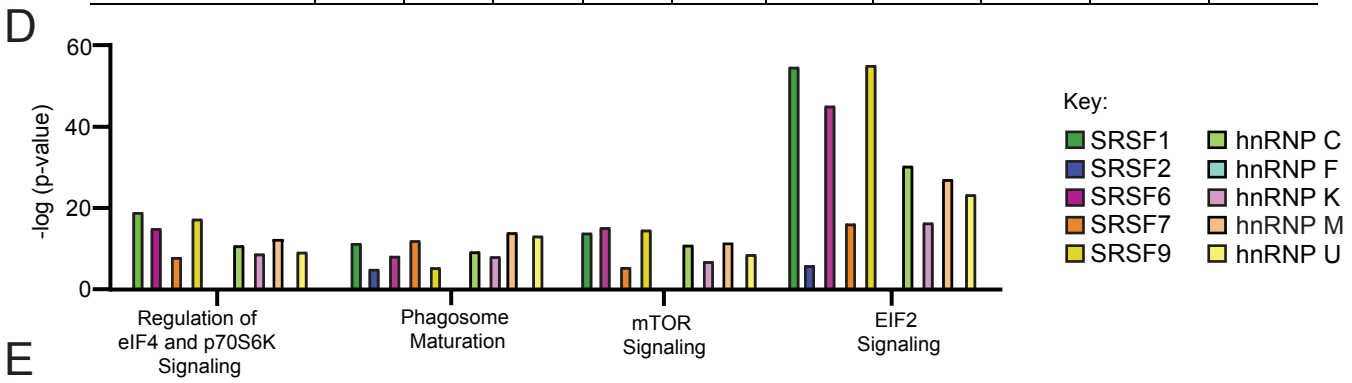
	SRSF1	SRSF2	SRSF6	SRSF7	SRSF9	hnRNP C	hnRNP F	hnRNP K	hnRNP M	hnRNP U
% DEGs downregulated upon *Salmonella*-infection	1.3	3.5	1.8	3.2	2.8	4.3	4.7	5.8	4.6	6.3



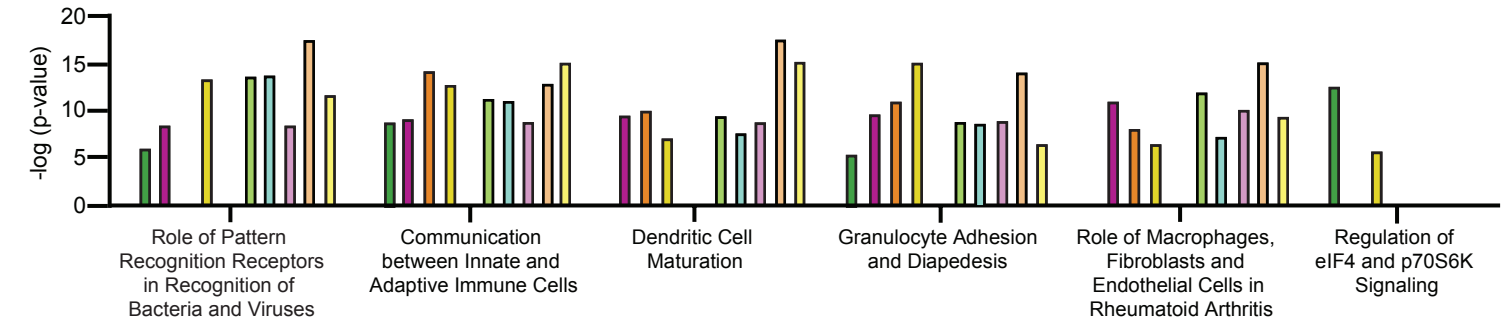


Figure 1

<u>Figure 1:</u> RNA-Seq Reveals Hundreds of hnRNP and SRSF-dependent Gene Expression In Resting and *Salmonella*-Infected macrophages.

(A) For each SR knockdown RAW 264.7 macrophage cell line, overlap between uninfected and *Salmonella*-infected macrophages is shown via Venn Diagram. Heatmaps illustrate genes whose expression is up or downregulated in uninfected macrophages; the same genes in *Salmonella* infected cells are shown alongside. Orange indicates genes downregulated in respective KD; purple indidcates genes upregulated in respective knockdown (colorbar shown to the right). All genes represented as differentially expressed had a statistically significant difference between knockdown and SCR control cell lines, $p<0.05$. Knockdown efficiency for each SR factor as measured by RT-qPCR, shown as expression relative to *Actb* and relative to SCR expression. Ratios are the mean of 3 biological replicates, error bars indicate standard deviation. (B) as in A but with hnRNP knockdown macrophages. (C) Percentage of DEGs in each SR/hnRNP knockdown cell line that are downregulated >2.0 fold in *Salmonella*-infected vs. uninfected SCR macrophages. (D) Ingenuity pathway analysis (-log (p-value)) of differentially expressed genes in uninfected SR and hnRNP knockdown macrophages. Pathways enriched in eight or more knockdown cell lines are shown. (E) As in (C) but in *Salmonella*-infected macrophages. Pathways enriched in eight or more knockdown cell lines are shown except for “Regulation of eIF4 and p70S6K Signaling.”

To induce macrophage innate immune gene expression, we infected each of the RAW 264.7 knockdown cell lines alongside two SCR control cell lines with *Salmonella enterica* serovar Typhimurium at an MOI of 10. *Salmonella* was chosen because it elicits a widely-studied innate immune response wherein TLR-4 sensing of *Salmonella* lipopolysaccharide (LPS) triggers two distinct transcription factor regulons—NFκB downstream of via the MyD88 adapter protein and IRF3 downstream of the adapter TRIF [36]. *Salmonella* Typhimurium infection of RAW 264.7 macrophages has previously been shown to elicit similar gene expression responses as LPS treatment at 4h [37]. We collected total RNA from resting and *Salmonella*-infected macrophages at 4h post-infection and performed bulk RNA sequencing via an Illumina HiSeq 4000 sequencer (150bp; paired-end reads). An average of ~60.2 million raw sequencing reads were generated from three biological replicates of each knockdown (for uninfected and *Salmonella*-infected samples). Reads were aligned, quantified, and analyzed using CLC Genomics Workbench (Qiagen).

Having generated measurements of DEGs in SR/hnRNP knockdown macrophages compared to SCR controls, we first wanted to know whether SR and hnRNP knockdown impacted expression of different transcripts in uninfected (resting) vs. *Salmonella*-infected macrophages. We identified transcripts whose expression was significantly altered ($p<0.05$; up- or down-regulated) in the knockdown cell lines relative to SCR controls and generated Venn diagrams to visualize overlap between affected genes in uninfected and *Salmonella*-infected macrophages. Each of the SRSF/hnRNPs queried regulate a distinct set of transcripts in resting vs. *Salmonella*-infected macrophages, with only about 1/3 or less overlap between the two conditions for most of the SRSF (Fig. 1A) and hnRNP (Fig. 1B) knockdown macrophage cell lines. Knockdown of most factors affected steady state RNA levels of approximately 200-400 genes in either resting or *Salmonella* infected macrophages, with knockdown of hnRNP F KD, which altered expression of 1943 genes in resting macrophages and 732 genes in *Salmonella*-infected macrophages, being a notable exception (Table 1 contains all gene expression changes $p<0.05$).

Upon *Salmonella* infection of macrophages, myriad innate immune transcripts including cytokines, chemokines, and antimicrobial mediators are dramatically upregulated. Because the vast majority of these genes are either expressed at very low levels or not at all in resting macrophages, it was not surprising that many of the DEGs in *Salmonella*-infected cells are not represented amongst the uninfected DEGs. However, we did find it curious that uninfected DEGs, presumably representing genes with housekeeping functions, are seemingly no longer sensitive to loss of SR or hnRNPs in the context of *Salmonella* infection (Fig. 1A and 1B Venn Diagrams and heatmaps). Previous studies demonstrate that global downregulation of ribosomal protein genes occurs rapidly upon stresses like starvation [38, 39] and in yeast, this repression occurs in

large part by shifting the splicing machinery away from such transcripts [40-42]. Therefore, we hypothesized that DEGs identified in uninfected macrophages were downregulated during *Salmonella* infection.

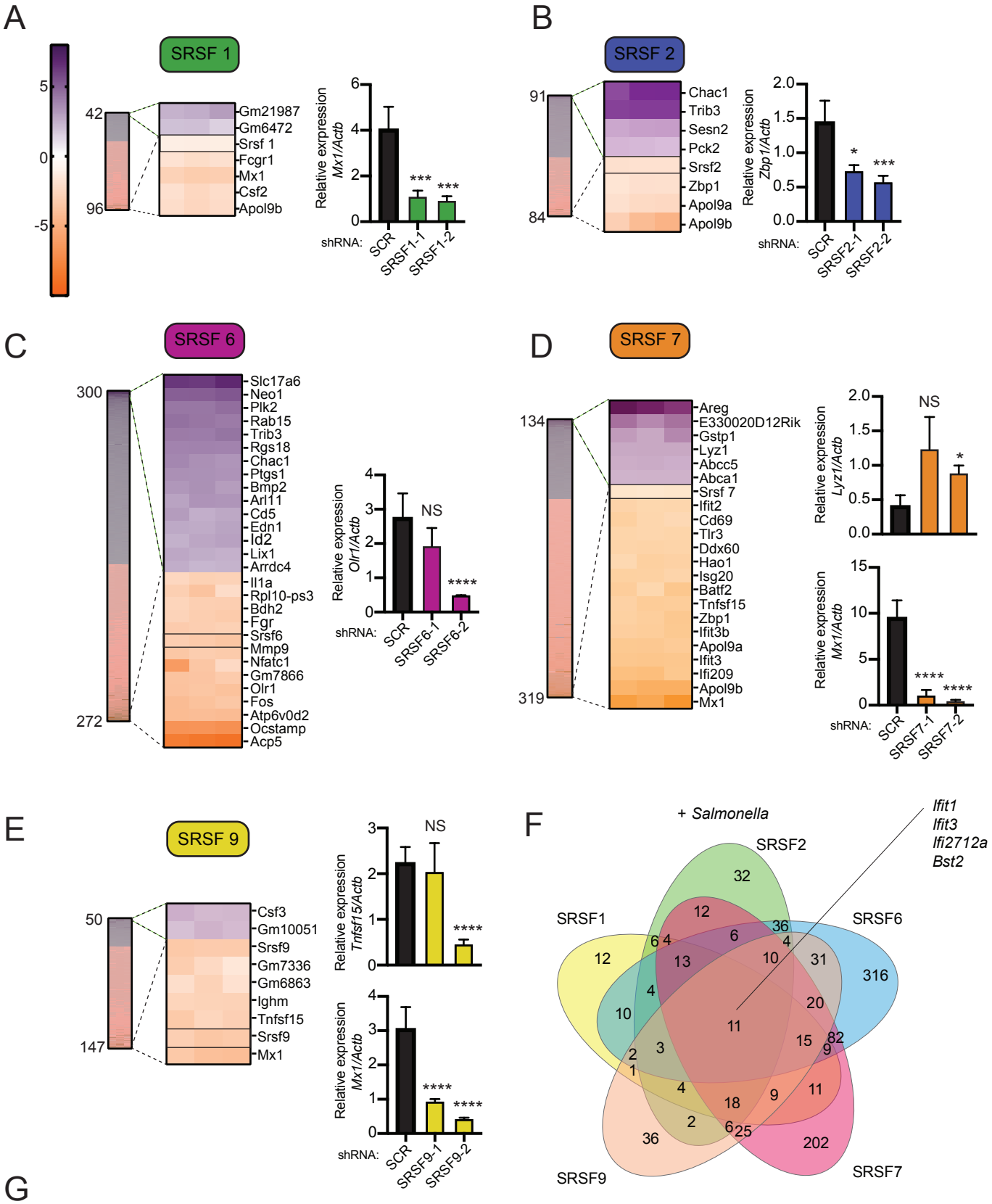
To test whether the lack of overlap between uninfected and *Salmonella*-infected macrophages was due to lower expression of uninfected "hits" in *Salmonella*-infected cells, we manually cross-referenced SR/hnRNP-sensitive genes against genes whose expression was downregulated 2-fold or more in *Salmonella*-infected SCR control macrophages (Table 1). Although 365 genes were downregulated 2-fold or more in *Salmonella*-infected SR/hnRNP knockdown macrophages compared to SCR controls at 4h post-infection, surprisingly only a handful of these genes were among the uninfected DGEs (Fig. 1C). In fact, in no case were more than 6.3% (hnRNP U) of uninfected DEGs downregulated upon *Salmonella* infection, arguing that genes that rely on particular SR/hnRNPs for proper expression in resting cells are no longer sensitive to loss of that factor when macrophages are infected, despite still being expressed. We confirmed lack of downregulation for several representative genes (e.g. *Bnip3*, *Id2*, *Hpgd*) in SRSF and hnRNP KD cells by RT-qPCR (Fig. S1B-E). This finding begins to suggest that SR proteins and hnRNPs are functionalized such that the nature of their target transcripts changes in the context of macrophage infection.

We next performed Ingenuity Pathway Analysis (Qiagen) to identify pathways enriched for DEGs in in uninfected vs. *Salmonella*-infected macrophages. We observed significant enrichment in SR/hnRNP-sensitive DEGs in pathways related to translation initiation, mTOR signaling, and phagosomal maturation (Fig. 1D, and Table 1 for full list), which paralleled previous analyses in hnRNP M knockdown macrophages [35]. IPA of the set of 365 downregulated genes from above revealed no overlap between pathways enriched for SR/hnRNP-sensitive DEGs and those enriched for downregulated genes (Fig. S1F), supporting our earlier conclusion that SR/hnRNP targets are not globally downregulated upon infection. Major pathways enriched for SR/hnRNP DEGs in *Salmonella*-infected macrophages were generally related to innate immune responses, namely "Recognition of Bacteria and Viruses by Pattern Recognition Receptors," "Communication between Innate and Adaptive Immune Cells," and "Granulocyte Adhesion and Diapedesis" (Fig. 1E). Consistent with our observation that uninfected DEGs are no longer dependent on SR/hnRNPs for proper expression upon *Salmonella*-infection, the only pathway that was significantly enriched for DEGs in both conditions was the translation/mTOR related pathway "Regulation of eIF4 p70S6K." This finding suggests that SR and hnRNP targets differ dramatically between uninfected and *Salmonella*-infected macrophages.

SRSF and hnRNP proteins can both repress and activate innate immune gene expression during *Salmonella* infection

Having observed distinct sets of transcripts whose expression was altered by loss of splicing regulatory factors in uninfected and *Salmonella*-infected macrophages, we set out to identify functional trends in these data. Specifically, we wanted to know whether loss of SR/hnRNPs generally caused up- or down-regulation of gene expression compared to SCR controls and whether SR and hnRNP family proteins impacted expression of the same or different regulons. For each *Salmonella*-infected knockdown cell line, we quantified the number of transcripts whose expression was up- or down-regulated in the absence of each SR or hnRNP, compared to SCR controls ($p<0.05$). As visualized in Fig. 2A and 3A, most splicing factors queried are able to act as both activators and repressors of gene expression in *Salmonella*-infected macrophages, as roughly equal numbers of genes had positive or negative expression changes in most knockdowns. hnRNP F was a notable exception to this rule, as almost all genes affected by loss of hnRNP F were significantly downregulated (680 genes down, 52 genes up), suggesting a previously unappreciated role for hnRNP F in activating innate immune gene expression. Similar trends of up and down-regulation were observed in uninfected macrophages as well (Table 1). As part of the same analysis, we wanted to identify specific transcripts that were up or down-regulated by SR/hnRNP KD in *Salmonella*-infected macrophages and whether DEGs were shared between multiple SR/hnRNP knockdown cell lines. To these ends, we generated heatmaps that show the top 5% of genes ($p<0.05$) both up- and down-regulated in *Salmonella*-infected SR/hnRNP knockdown macrophages compared to SCR. The comparative expression level of each SR/hnRNP gene itself was manually included as a reference in each of the heatmaps (Fig. 2A-E and Fig. 3A-E). These heatmaps show clear hyper- or hypo-induction of many critical innate immune genes in splicing factor knockdowns, including genes encoding nitric oxide synthase (*Nos2*), the influenza restriction factor *Mx1*, and many other interferon stimulated genes including Apolipoprotein 9 (*Apol9a/b*), and members of the IFIT (Interferon-induced protein with tetratricopeptide repeats) family of antiviral proteins (i.e. *Ifit2*, *Ifit3*, *Ifit1bl1*). We validated differential expression of several genes by RT-qPCR (Fig. 2A-E and Fig. 3A-E). Knockdown efficiency was measured by RT-qPCR for two knockdown cell lines for each factor and protein levels were monitored in cases where antibodies specific for endogenous proteins were available (SRSF6 and SRSF7 (Fig. S2) and hnRNP F and hnRNP M (Fig. S3)).

We also asked whether DEGs were shared between multiple SR or hnRNP knockdowns. Interestingly, we observed a great deal more diversity between genes whose expression was sensitive to loss of SRSF proteins vs. hnRNPs, with only 11 genes common to all the SRs vs. 104



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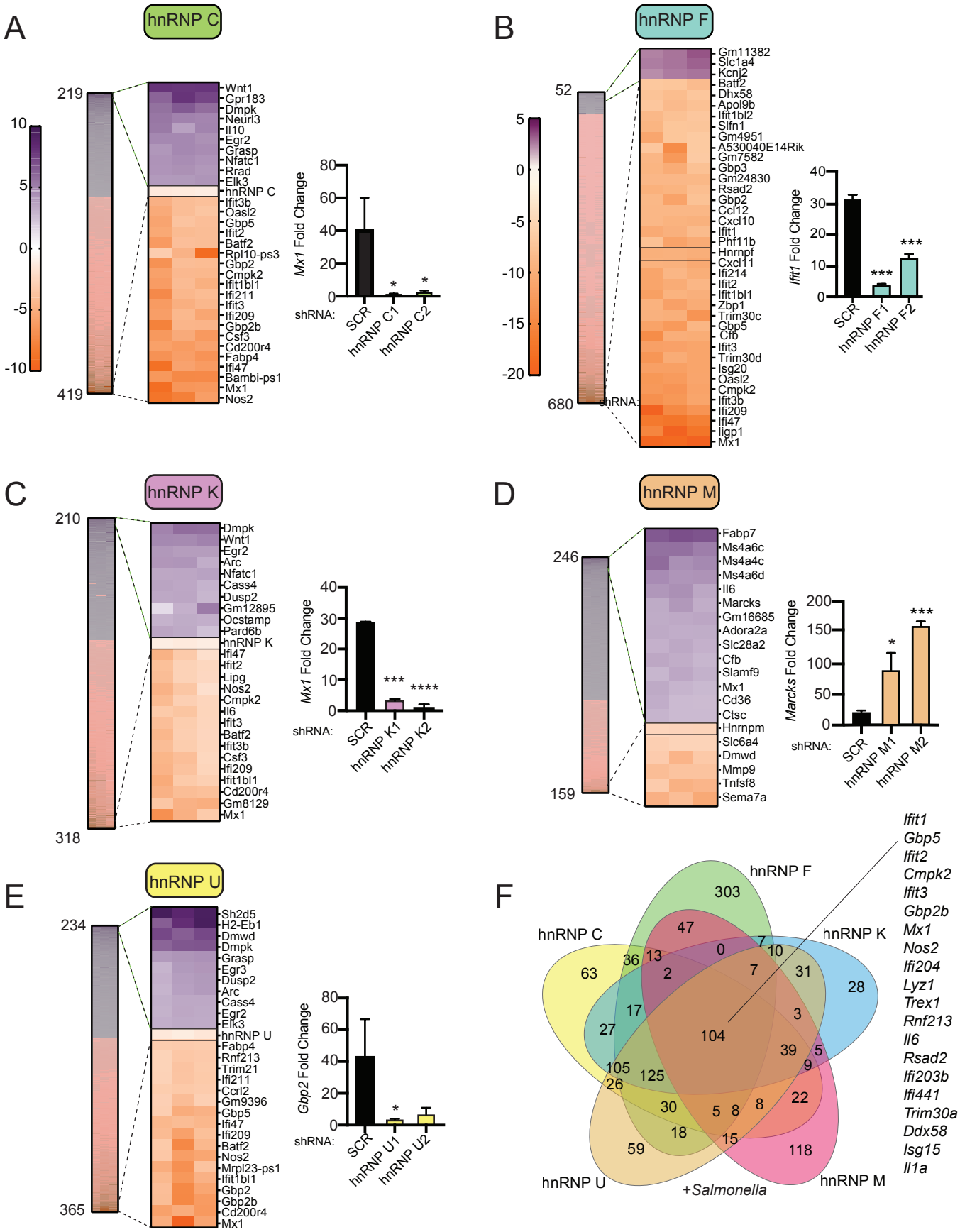
SRSF knockdown cell line	% induced innate immune genes whose expression changes in KD
SRSF1	9% (59/655)
SRSF2	9.5% (62/655)
SRSF6	27% (179/655)
SRSF7	28% (185/655)
SRSF9	9.5% (62/655)

Figure 2

<u>Figure 2:</u> SR protein knockdown causes up and downregulation of gene expression in Salmonella-infected macrophages.

(A) On left, heatmap represents all up- and down-regulated genes in SRSF1 knockdown macrophages relative to SCR control at 4h post-*Salmonella* infection ($p<0.05$). Zoom in represents the top 5% up- and down-regulated genes (fold change). On right, RT-qPCR validation of expression of a representative DEG (*Mx1*) in two knockdown cell lines vs. a SCR control. (B) As in (A) but for SRSF2; RT-qPCR of Zbp1. (C) As in (A) but for SRSF6; RT-qPCR of *Olr1*; (D) As in (A) but for SRSF7; RT-qPCR of *Lyz1* and *Mx1*; (E) As in (A) but for SRSF; RT-qPCR of *Tnfsf15* and *Mx1*. (F) Venn diagram representing DEGs common to one or more SR knockdown cell line ($p<0.05$). Interferon stimulated genes (ISGs) whose expression is impacted by loss of all five SRSF proteins are highlighted. (G) Percentage of all genes induced at 4h post-*Salmonella* infection (>2.0-fold) that are differentially expressed in each SRSF knockdown macrophage cell line ($p>0.05$). For all RT-qPCRs values are the mean of 3 biological replicates and error bars indicate standard deviation. * = $p<0.05$; ** = $p<0.01$; *** = $p<0.005$; **** = $p<0.001$.



hnRNP knockdown cell line	% induced innate immune genes whose expression changes in KD
hnRNP C	40.5% (266/655)
hnRNP F	43.5% (286/655)
hnRNP K	33.5% (219/655)
hnRNP M	25% (164/655)
hnRNP U	37% (242/655)

Figure 3

<u>Figure 3:</u> hnRNP protein knockdown causes up and downregulation of gene expression in *Salmonella*-infected macrophages.

(A) On left, heatmap represents all up- and down-regulated genes in hnRNP C knockdown macrophages relative to SCR control at 4h post-*Salmonella* infection ($p<0.05$). Zoom in represents the top 5% up- and down-regulated genes (fold change). On right, RT-qPCR validation of expression of a representative DEG (*Mx1*) in two knockdown cell lines vs. a SCR control. (B) As in (A) but for hnRNP F; RT-qPCR of *Ifit*. (C) As in (A) but for hnRNP K; RT-qPCR of *Mx1*. (D) As in (A) but for hnRNP M; RT-qPCR of *Marcks1*; (E) As in (A) but for hnRNP U; RT-qPCR of *Gbp2*; (F) Venn diagram representing DEGs common to one or more SR knockdown cell line ($p<0.05$). Interferon stimulated genes (ISGs) whose expression is impacted by loss of all five hnRNP proteins are highlighted. . (G) Percentage of all genes induced at 4h post-*Salmonella* infection (>2.0-fold) that are differentially expressed in each SRSF knockdown macrophage cell line ($p>0.05$). For all RT-qPCRs values are the mean of 3 biological replicates and error bars indicate standard deviation. * = $p<0.05$; ** = $p<0.01$. *** = $p<0.005$.

shared between all five of the hnRNPs (and another 125 shared between hnRNP C, K, and U, in particular) (Fig. 2F and 3F). Interestingly, most of the shared DEGs in SR protein knockdown macrophages and in hnRNP knockdown macrophages are categorized as ISGs (Fig. 2F and 3F), suggesting that genes in this category, expressed by transcription factors in the IRF and STAT family of transcription factors, are particularly reliant on SR and hnRNP proteins to regulate their induction. Our finding that hnRNP knockdown macrophages share many of the same DEG targets is consistent with previous global analysis of hnRNP target RNAs in human 293T cells that described cooperation between these factors [43]. We also looked at what percentage of genes that are induced as part of the innate immune response to *Salmonella* infection are dependent on each SR/hnRNP for proper induction levels. We found that between 25-43% of all genes induced >2-fold at 4h post-*Salmonella* infection had altered gene expression in one or more hnRNP knockdown cell line and 9-25% in one or more SRSF knockdown cell line (Fig. 2G and 3G), providing further support that splicing regulatory factors modulate innate immune gene expression outcomes and suggesting a privileged role for hnRNP proteins in this response.

Gene expression profiles for several SR/hnRNPs hint at possible mechanisms through which these factors influence innate immune gene expression. For example, hnRNP F knockdown RAW 264.7 cells are characterized by a dramatic decrease in virtually every ISG, suggesting these cells can not properly activate transcription factors like IRF3 or IRF7 (Fig. 3B). SRSF6 knockdown macrophages also had an interesting phenotype, whereby they express higher than normal levels of ISGs in uninfected cells, suggesting misregulation of cytosolic nucleic acid sensing or loss of negative regulation of these pathways (Table 1). Together, these data implicate SR and hnRNP splicing regulatory factors in making important contributions to the overall levels of innate immune transcript expression and point to considerable levels of specificity regarding the transcripts regulated by each factor.

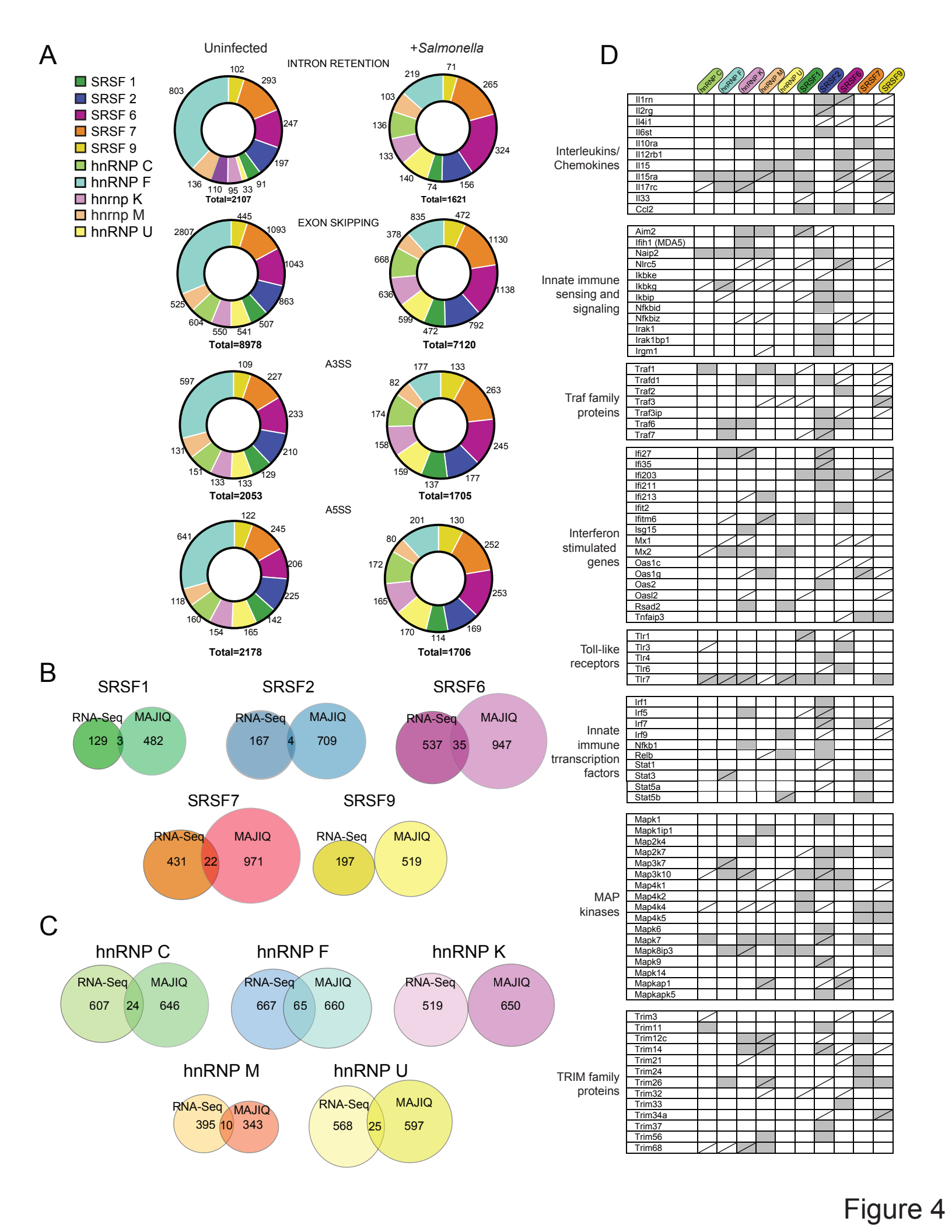
SR/hnRNPs-mediated alternative splicing events are not common in DEGs

Data presented so far generally argue against a global up- or down-regulation of pre-mRNA splicing in *Salmonella*-infected macrophages and instead support a model whereby individual SRs and hnRNPs dictate RNA processing decisions for particular transcripts. Both SR and hnRNP proteins have been implicated in many steps of gene expression and RNA processing, from chromatin remodeling and transcription, to constitutive and alternative splicing, and mRNA export and stability [44-47]. One obvious way in which all of these proteins can shape the steady state transcriptome, and likely innate immune outcomes, is via alternative splicing. For example, is it possible that loss of SR/hnRNPs increases or decreases inclusion of so-called poison exons

that introduce premature stop codons and target transcripts to nonsense mediated decay, thus impacting transcript levels. Recent work from the Baltimore lab has shown that innate immune transcripts are frequently regulated by alternative splicing events that introduce premature stop codons [16]. To begin to investigate potential mechanisms through which SR/hnRNPs control induction/repression of innate immune transcripts, we employed an algorithm to identify and quantify local splicing variations (LSV) called Modeling Alternative Junction Inclusion Quantification (MAJIQ) [48]. MAJIQ allows identification, quantification, and visualization of diverse LSVs, including alternative 5′ or 3′ splice site usage, exon skipping, and intron retention across different experimental conditions.

MAJIQ successfully identified hundreds of statistically significant alternative splicing changes (probability [|delta PSI|, ≥10%] >95%) in all SR and hnRNP KD macrophages, both in resting cells and during *Salmonella* infection. We observed all types of alternative splicing changes, with the majority of changes being categorized as exon skipping events, consistent with the canonical roles of SR and hnRNPs in enhancing or repressing exon inclusion, respectively (Fig. 4A) [49-51]. There were no dramatic differences between the overall number of LSVs between different SR/hnRNPs nor did we detect major differences in the number of LSVs in uninfected compared to *Salmonella*-infected macrophages (Fig. 4A). One exception to this trend was that loss of hnRNP F (teal) impacted more LSVs of all varieties (intron retention, exon skipping, alternative 3'SS, alternative 5'SS) in uninfected vs. *Salmonella*-infected cells. In uninfected cells, loss of hnRNP F impacted 2807 exon skipping events (vs. 835 in *Salmonella*-infected), 803 intron retention events (vs. 219), 597 alternative 3'SS events, (vs. 177) and 641 alternative 5'SS events (vs. 201) compared to 835, further supporting our model that macrophage activation and differential phosphorylation alters the function of regulatory splicing factors, perhaps by changing their protein binding partners, RNA targets, or subcellular localization.

To investigate a role for alternative splicing in regulating innate immune gene expression in *Salmonella*-infected macrophages, we calculated the overlap between DEGs and genes with one or more LSV. Surprisingly, we saw almost virtually no overlap between genes whose expression was changed in SR/hnRNP KDs vs. those whose alternative splicing was changed. This was the case for both uninfected (Fig. S4A) and *Salmonella*-infected conditions (Fig. 4C and 4D). Likewise, we observed little to no enrichment for SR/hnRNP-dependent alternative splicing events in genes related to innate immune pathways via IPA. In fact, no pathway was enriched for MAJIQ hits more than -log(p-value) = 5 (Table 1), suggesting that SR/hnRNP-dependent alternative splicing changes in macrophages do not occur in functionally related genes from any particular pathway.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Uninfected
+Salmonella
SRSF 1
SRSF 2
SRSF 6
SRSF 7
SRSF 9
hnRNP C
hnRNP F
hnrnp K
hnrnp M
hnRNP U
INTRON RETENTION
Total=2107
Total=1621
EXON SKIPPING
Total=8978
Total=7120
A3SS
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RNA-Seq 537 35 MAJIQ 947
SRSF7
RNA-Seq 431 22 MAJIQ 971
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RNA-Seq 395 10 MAJIQ 343
hnRNP U
RNA-Seq 568 25 MAJIQ 597
D
hnRNP C
hnRNP F
hnRNP K
hnRNP M
hnRNP U
SRSF1
SRSF2
SRSF6
SRSF7
SRSF9
Interleukins/ Chemokines
Il1rn
Il2rg
Il4i1
Il6st
Il10ra
Il12rb1
Il15
Il15ra
Il17rc
Il33
Ccl2
Innate immune sensing and signaling
Aim2
Ifih1 (MDA5)
Naip2
Nlrc5
Ikbke
Ikbkg
Ikbip
Nfkbid
Nfkbiz
Irak1
Irak1bp1
Irgm1
Traf family proteins
Traf1
Trafd1
Traf2
Traf3
Traf3ip
Traf6
Traf7
Interferon stimulated genes
Ifi27
Ifi35
Ifi203
Ifi211
Ifi213
Ifit2
Ifitm6
Isg15
Mx1
Mx2
Oas1c
Oas1g
Oas2
Oasl2
Rsad2
Tnfaip3
Toll-like receptors
Tlr1
Tlr3
Tlr4
Tlr6
Tlr7
Innate immune trranscription factors
Irf1
Irf5
Irf7
Irf9
Nfkb1
Relb
Stat1
Stat3
Stat5a
Stat5b
MAP kinases
Mapk1
Mapk1ip1
Map2k4
Map2k7
Map3k7
Map3k10
Map4k1
Map4k2
Map4k4
Map4k5
Mapk6
Mapk7
Mapk8ip3
Mapk9
Mapk14
Mapkap1
Mapkapk5
TRIM family proteins
Trim3
Trim11
Trim12c
Trim14
Trim21
Trim24
Trim26
Trim32
Trim33
Trim34a
Trim37
Trim56
Trim68

Figure 4

<u>Figure 4:</u> Local splicing variations are abundant in SR/hnRNP knockdown macrophages, but they are not enriched in differentially expressed genes.
(A) Quantitation of intron retention, exon skipping, alternative 3' and 5' splice site usage, events in uninfected and *Salmonella*-infected SR and hnRNP knockdown macrophages (probability [|delta PSI|, ≥10%], >95%). (B) Overlap between DEGs ($p<0.05$) and genes with MAJIQ LSVs (PSI ≥10%) in each SRSF knockdown macrophage cell line +*Salmonella* infection. (C) As in (B) but for hnRNP knockdown cell lines. (D) Identification of genes with differential isoform usage (DIU) in each SR/hnRNP knockdown macrophage cell line vs. SCR control. Genes included in chart have one or more significant LSV by MAJIQ analysis. Shaded boxes indicate an LSV occurs preferentially in resting knockdown macrophages; slashes indicate an LSV occurs preferentially in *Salmonella*-infected macrophages. Genes were manually annotated into different innate immune categories.

Earlier studies also found a lack of overlap between transcriptome changes and alternative splicing events in *Salmonella*-infected human monocytes [15] and influenza virus-infected A549 cells [52]. Together these data suggest that changes to constitutive intron removal and/or alternative splicing of an upstream master regulator are more likely explanations for how loss of SR/hnRNPs influences steady state RNA expression levels in cells.

To address the latter possibility that innate immune gene expression is globally impacted by an alternatively spliced isoform of an innate immune sensor or signaling factor, we manually cataloged genes with one or more instance of DIU in SR/hnRNP knockdown cell lines into categories related to immune function and asked whether SR/hnRNP knockdown generated DIU in resting and/or *Salmonella*-infected macrophages (Fig. 4D). While differential expression of innate immune genes was generally unique to *Salmonella*-infected macrophages (with SRSF6 being a notable exception), we detected LSVs in a number of immune-related transcripts in resting and infected SR/hnRNP knockdown RAW 264.7 cells. These immune-related genes represent only a subset of total genes with LSVs in SR/hnRNP knockdown macrophages, as IPA did not demonstrate significant enrichment for alternatively spliced genes in any functional pathway (Fig. S4). Some genes showed alternative splicing changes only in resting cells (e.g. *Naip2*, *Traf6*, *Il12rb1*, *Nfkb1*, and *Trim56*), others were alternatively spliced in both conditions (e.g. *Tlr7*, *Il15ra*, *Trim14*), and others were mostly impacted by loss of SR/hnRNPs in the context of *Salmonella* infection (e.g. *Trim3*, *Mx1*, *Traf3*, *Ikbkg*). These results again argue against a general regulatory network of alternative splicing “activation” or “repression” upon macrophage activation. Likewise, they argue against a global “reallocation” of SR/hnRNPs to focus solely on innate immune-specific splicing decisions following pathogen sensing.

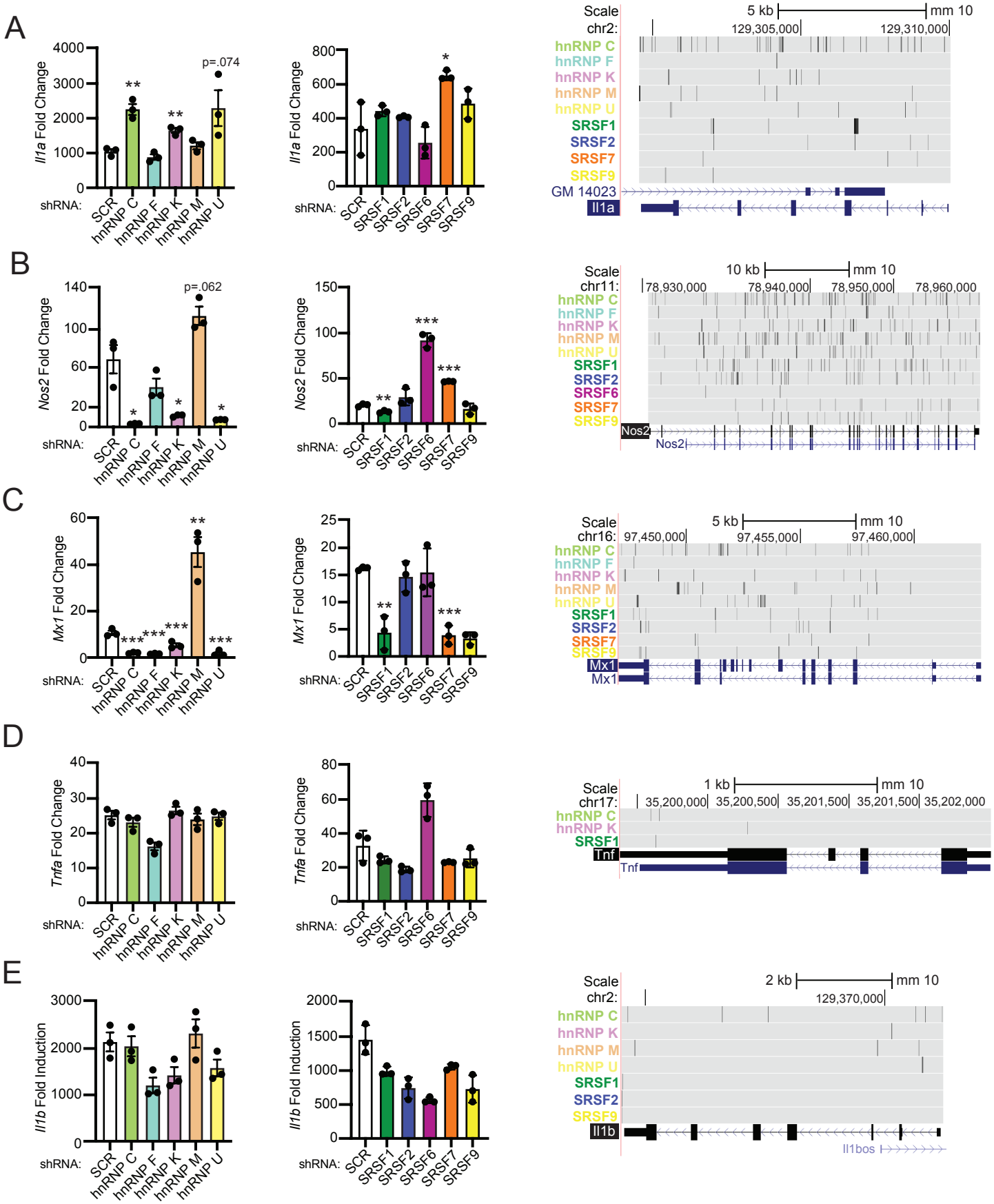
These findings do, however, suggest that a complex regulatory network of SR and hnRNPs controls alternative splicing decisions of many critical innate immune transcripts. Previous high-throughput sequencing studies have annotated alternatively spliced isoforms of virtually all of these transcripts and in some cases, isoforms have been functionally characterized. For example, alternative splicing of TRAFs, which mediate TNF signaling and MAPK activation, is thought to play an important role in inflammation and cancer development [53] and alternative splicing of TRIM family genes has been shown to generate dominant negative isoforms that regulate nucleic acid sensing pathways [54, 55].

Earlier, we hypothesized that one mechanism through which SR/hRNPs could regulate steady innate immune gene expression is through changing alternative splicing of a master regulator, like a transcription factor. There is certainly evidence for this possibility in our MAJIQ analysis, with LSVs measured in transcripts encoding several transcription factors in the IRF, STAT,

and NFκB families. While changes to *Irf1*, *Irf5*, *Irf7*, *Irf9*, and *Stat1* alternative splicing are consistent with the dramatic lack of ISG induction in *Salmonella*-infected hnRNP F knockdown macrophages (Fig. 3B), other SR/hnRNP knockdown macrophages did not demonstrate phenotypes consistent with transcription factor-dependence. While we cannot rule it out, it seems unlikely that alternatively spliced transcription factors or signaling molecules are solely responsible for SR/hnRNP-dependent DEGs, as genes impacted in most of the knockdown cell lines are not part of a single, shared transcriptional regulon.

Certain innate immune genes are especially reliant on SR/hRNP proteins for maintaining proper expression levels.

Altogether, our transcriptomics data suggest that each of the SR/hnRNPs queried impact its own cohort of transcripts. However, there are many innate immune genes whose induction level varied dramatically in multiple SR/hRNP knockdown cell lines including *Il1a*, (a pro-inflammatory cytokine and stimulator of thymocyte proliferation [56]), *Nos2* (nitric oxide synthase, an important antimicrobial effector molecule [57]) and *Mx1* (a viral restriction factor [58]). We validated hyper- and hypo-induction of these genes by measuring their expression by RT-qPCR in *Salmonella*-infected samples from all ten of our knockdown macrophage cell lines and our two SCR controls. We observed that *Il1a* induction was higher in the absence of hnRNP C, hnRNP K, SRSF7, and to limited extent, hnRNP U (Fig. 5A). Likewise, *Nos2* expression varied dramatically in different SR/hnRNP knockdown macrophages, with hnRNP C, K, U and SRSF1 being required for maximal induction of *Nos2* and factors like hnRNP M, SRSF6 and SRSF7 contributing to *Nos2* repression (Fig. 5B). Curiously, upregulation of *Nos2* in SRSF6 knockdown cells was seen both at rest and following *Salmonella* infection, suggesting that loss of SRSF6 somehow stimulates basal innate immune activation (Table 1). *Mx1* expression was lower in all of the hnRNP as well as in SRSF1 and 7 knockdowns compared to scramble controls, except for hnRNP M (Fig. 5C), consistent with our previously characterized role for this factor in repressing *Mx1* expression [35]. However, reliance on SR/hnRNP for proper induction was not observed for all innate immune genes as *Tnfa* and *Il1b* showed little to no differential gene expression in any of the ten SR/hnRNP knockdown cells (Fig. 5D-E). To begin to understand why loss of SR/hnRNPs impacts expression of some innate immune genes and not others, we turned to a computational prediction method called RBPFinder to identify potential binding sites for the SR and hnRNPs of interest (Paz Kosti Ares 2014). RBPFinder detected hundreds of potential binding sites for these RNA binding proteins in *Il1a*, *Nos2*, and *Mx1* but remarkably, almost none for *Tnf* or *Il1b* (Fig. 5F). This trend held even when we normalized for gene length (Fig. 5F). While this analysis on its own is merely



F

Gene	Length in nt	RBP binding sites/nt	% Coverage
Il1a	10463	0.01290	1.29
Nos2	39568	0.01218	1.22
Mx1	15869	0.00907	0.91
Tnfa	2727	0.00147	0.15
Il1b	6670	0.00270	0.27

Figure 5

<u>Figure 5</u>: Innate immune genes vary in their reliance of SR/hnRNP family members for proper induction in *Salmonella*-infected RAW 264.7 macrophages. (A) (left) RT-qPCR of relative gene expression of *Il1a* relative to *Actb* in *Salmonella*-infected SRSF and hnRNP knockdown macrophages (shown as fold change relative to uninfected). (right) Gene diagram of Il1a from UCSC genome browser showing RBPFinder tracks for each SR/hnRNP with one or more predicted binding sites. Mouse genome (GRCm38/mm10); high stringency; conservation filter applied). (B) As in (A) but for *Nos2*. (C) As in (A) but for *Mx1*. (D) As in (A) but for *Tnfa*. (E) As in (A) but for *Il1b*. (F) Calculation of RBP binding sites per nucleotide and percent RBP site coverage for each gene analyzed by RBPFinder. * = $p<0.05$; ** = $p<0.01$; *** = $p<0.005$

correlative, it does begin to suggest that exonic and intronic splicing enhancers/silencers are enriched in select innate immune transcripts that consequently rely on SR/hnRNPs for regulation.

Reliance on SR/hnRNPs to maintain proper steady state RNA levels of innate immune transcripts is independent of level of induction, gene length, and/or number of introns/exons

Having observed so much diversity in genes whose expression and alternative splicing changed in our transcriptomics analyses, we were eager to see if we could identify any trends related to SR/hnRNP-sensitive innate immune genes. We hypothesized that genes whose expression is the most upregulated in response to *Salmonella* infection could be more sensitive to loss of SR/hnRNPs, perhaps via a need to "suck up" rate limiting spliceosome components. To address this possibility, we ranked all top induced genes from *Salmonella*-infected SCR control macrophages and looked at those top 100 innate immune transcripts for differential expression in SR/hnRNP knockdown cell lines (Fig. 6A). Consistent with previous reports, we observed dramatic upregulation of hundreds of macrophages genes at 4h post-*Salmonella* infection, with some genes like *Il1a* and *Il1b* being upregulated approximately 1500-fold. We observed no clear correlation between level of induction/expression level and whether or not a transcript was differentially regulated by loss of an SR/hnRNP: DEGs induced 1000-fold were impacted by the loss of a similar number of SR/hnRNPs and to a similar magnitude as DEGs induced 5-fold (Fig. 6A; top vs. bottom of heatmap). To examine if other attributes of a gene influenced whether its expression was altered by loss of an SR/hnRNP, we conducted Pearson's correlation tests to determine the relationship between differential expression ($p<0.05$) and gene length (Fig. 6B), exon length (total exonic sequence, or sum of all exon nucleotides) (Fig. 6C), intron length (total intronic sequence, or sum of all intron nucleotides) (Fig. 6D), and quantity of exons (Fig. 6E). We observed little to no correlation between any of these gene attributes and the degree to which a gene's expression was altered in any of the hnRNP and SR knockdown cell lines, with all tests generating Pearson correlation coefficients close to zero. Together, these analyses further support our model whereby certain innate immune genes rely more on SR/hnRNPs than others by virtue of transcript intrinsic features like enrichment for SR and hnRNP binding sites.

Rapidly induced innate immune genes are subject to repression from SR and hnRNPs

With no apparent correlation between various gene architecture attributes and SR/hnRNP reliance, we looked to see if we could identify trends between SR/hnRNP reliance and dynamics of transcriptional activation as previously described by other studies. One commonly used categorization of innate immune genes is into primary and secondary response genes. Of the 53 LPS-

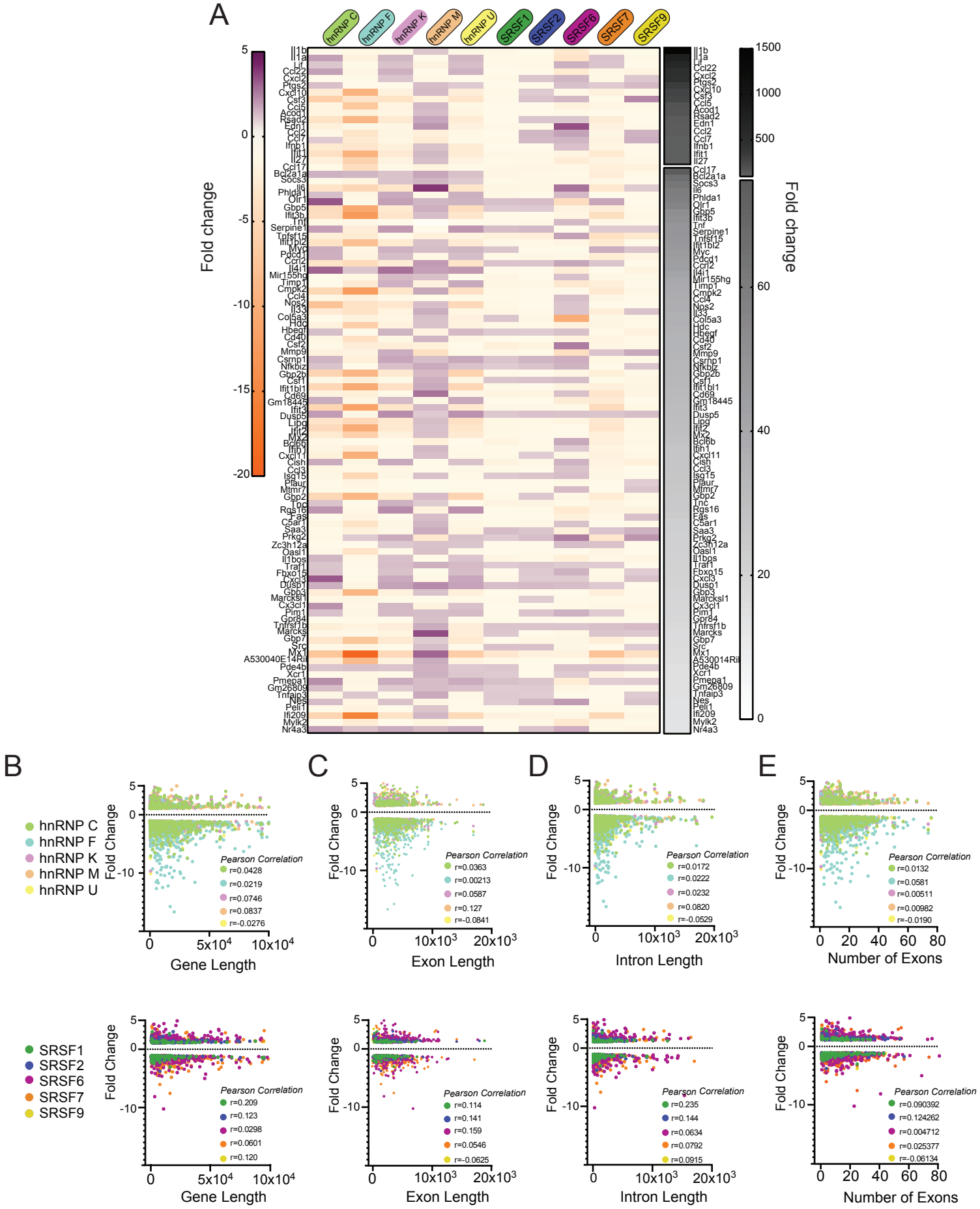

A
hnRNP C
hnRNP F
hnRNP K
hnRNP M
hnRNP U
SRSF1
SRSF2
SRSF6
SRSF7
SRSF9
Fold change
B
C
D
E
hnRNP C
hnRNP F
hnRNP K
hnRNP M
hnRNP U
Pearson Correlation
r=0.0428
r=0.0219
r=0.0746
r=0.0837
r=-0.0276
Gene Length
r=0.0363
r=0.00213
r=0.0587
r=0.127
r=-0.0841
Exon Length
r=0.0172
r=0.0222
r=0.0232
r=0.0820
r=-0.0529
Intron Length
r=0.0132
r=0.0581
r=0.00511
r=0.00982
r=-0.0190
Number of Exons
SRSF1
SRSF2
SRSF6
SRSF7
SRSF9
r=0.209
r=0.123
r=0.0298
r=0.0601
r=0.120
r=0.114
r=0.141
r=0.159
r=0.0546
r=-0.0625
r=0.235
r=0.144
r=0.0634
r=0.0792
r=0.0915
r=0.090392
r=0.124262
r=0.004712
r=0.025377
r=-0.06134
Fold Change

Figure 6

<u>Figure 6</u>: Level of induction, gene length, and number of introns/exons do not positively correlate with a gene's reliance of SR/hnRNPs for proper expression. (A) (right, black and white) Heatmap of the top 100 genes induced at 4h post-*Salmonella* infection in SCR control RAW 264.7 macrophages. (left) Heatmap of up- or down-regulation (fold change) of each induced gene conferred by hnRNP or SRSF knockdown. Purple, genes upregulated in SR/hnRNP knockdowns; Orange, genes downregulated in SR/hnRNP knockdowns. (B) Scatter plot depicting correlation of the fold change of each DEGs vs. coding sequence (CDS) length in *Salmonella*-infected hnRNP (top) and SRSF (bottom) knockdown macrophages. (C) as in (B) but comparison of DEG fold change vs. exon length. (D) As in (B) but comparison of DEG fold change vs. intron length. (E) As in (B) but comparison of DEG fold change vs. number of exons in a DEG. Genes represented were significantly differentially expressed ($p<0.05$) in each knockdown compared to SCR controls. Y-axes were made all the same to facilitate comparison between multiple knockdown cell lines.

driven primary response genes annotated by [10], 35 of them were upregulated in the absence of one or more SR/hnRNP, suggesting a role for factors like hnRNP C, K, M, U and SRSF6 in repressing the expression of primary response genes (Fig. 7A). A repressive role for the same factors was less evident for secondary response genes, which were mostly downregulated in the absence of the SR/hnRNPs (except for hnRNP M and SRSF6). This analysis begins to suggest that primary response genes may be more reliant on pre-mRNA splicing to control the proper timing and magnitude of their induction than secondary response genes, which rely on multiple other layers of regulation.

We next looked at innate immune gene categories as defined by Bhatt et al. [6], who divided RefSeq genes exceeding 400bp in length into 12 clusters based on their pattern of transcript levels in three cellular compartments (chromatin, nucleoplasm, cytoplasm) in primary macrophages following Lipid A treatment. The vast majority of genes we identified as SR/hnRNP-sensitive in *Salmonella*-infected RAW 264.7 macrophages fell into Groups 1-3 (Fig. S7A), which are comprised mostly of Lipid-A-induced genes. As was the case with the primary response genes, most of the Group 1 genes showed increased expression in various SR/hnRNP knockdowns. We then looked specifically at another categorization by Bhatt et al., whereby they grouped only Lipid A-responsive genes based on their expression patterns. Genes in Groups A1, A2, and B were characterized by a “burst” of induction, with maximal chromatin-associated reads measured at 15 min. post-Lipid A for Group A1 genes and slightly slower kinetics seen for Groups A2 and B, respectively. The general dynamics of each of the Groups’ chromatin-reads is represented schematically in Fig. 7C. Almost all the genes in Group A1 were differentially expressed in the absence of one or more SR/hRNP (70%, $p<0.05$) (Fig. 7B). About 40% of genes in other Classes were affected, save for Class C in which 67.3% of genes were differentially expressed in one or more SR/hnRNP knockdown macrophage cell line. Class C genes are induced early and usually peak at 30 min post-Lipid A treatment but show more sustained expression past peak induction. Overall, many more Class A1, A2, B, and C were upregulated than downregulated in the absence of several of SR/hnRNPs (in particular, hnRNP C, K, M, U, SRSF2, 6, and 7) suggesting these factors may contribute to repression of rapidly induced innate immune genes (Fig. 7B).

As earlier work implicated high CpG promoters with rapid, transiently induced primary response genes and low CpG promoters with secondary response genes with delayed, sustained transcription kinetics, we looked to see if there was any correlation between our SR/hnRNP DEGs and CpG promoter architecture. Generally, DEGs in SR/hnRNP knockdown *Salmonella*-infected macrophages were about evenly split between high CpG and low CpG promoters (Fig. S7B),

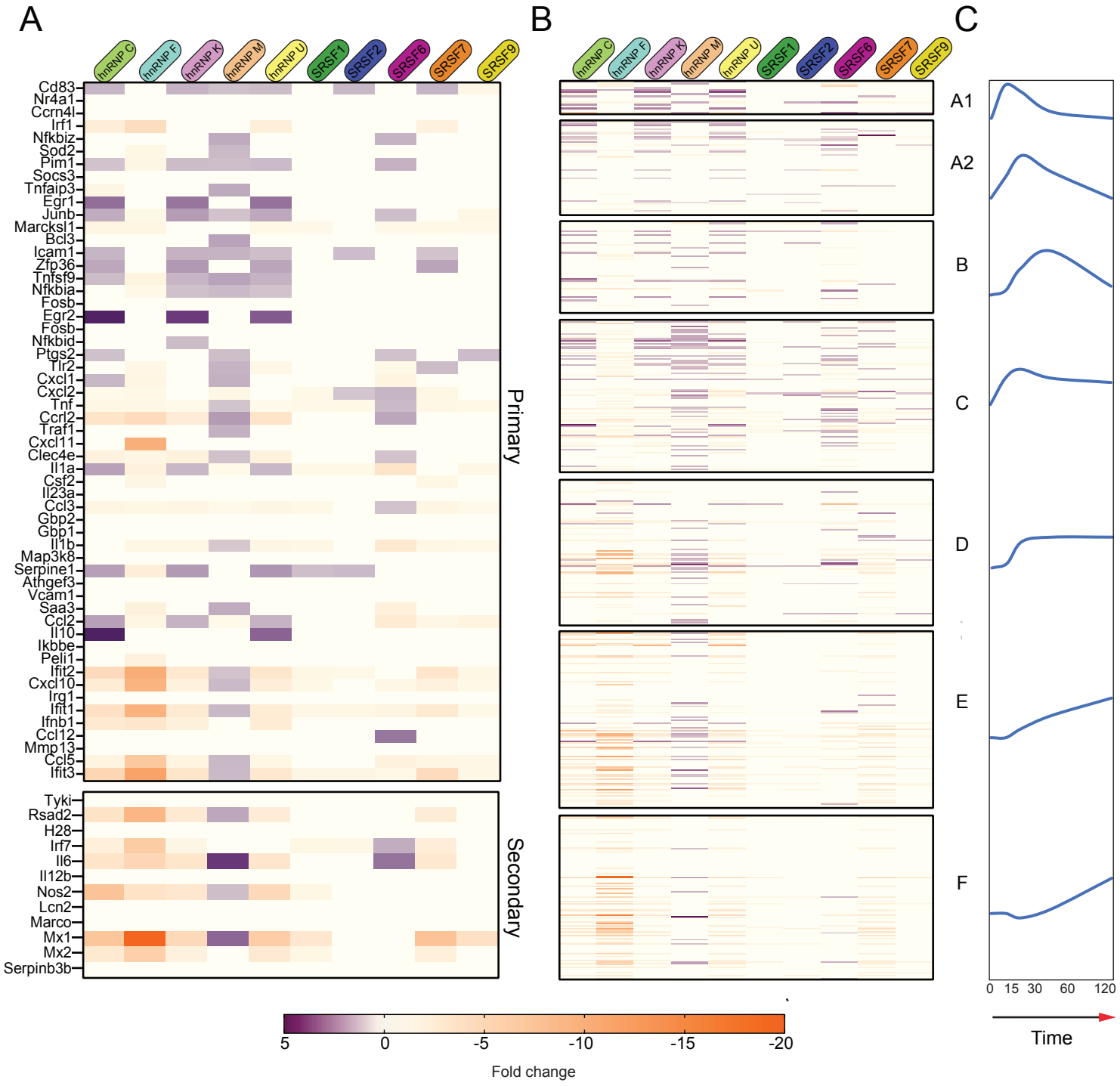

A
B
C
hnRNP C
hnRNP F
hnRNP K
hnRNP M
hnRNP U
SRSF1
SRSF2
SRSF6
SRSF7
SRSF9
Cd83
Nr4a1
Ccrn4l
Irf1
Nfkbiz
Sod2
Pim1
Socs3
Tnfaip3
Egr1
Junb
Marcksl1
Bcl3
Icam1
Zfp36
Tnfsf9
Nfkbia
Fosb
Egr2
Fosb
Nfkbid
Ptgs2
Tlr2
Cxcl1
Cxcl2
Tnf
Ccrl2
Traf1
Cxcl11
Clec4e
Il1a
Csf2
Il23a
Ccl3
Gbp2
Gbp1
Il1b
Map3k8
Serpine1
Athgef3
Vcam1
Saa3
Ccl2
Il10
Ikbbe
Peli1
Ifit2
Cxcl10
Irg1
Ifit1
Ifnb1
Ccl12
Mmp13
Ccl5
Ifit3
Primary
Tyki
Rsad2
H28
Irf7
Il6
Il12b
Nos2
Lcn2
Marco
Mx1
Mx2
Serpinb3b
Secondary
A1
A2
B
C
D
E
F
0 15 30 60 120
Time
5 0 -5 -10 -15 -20
Fold change

Figure 7

<u>Figure 7</u>: Primary response and early induced innate immune genes are generally repressed by SR/hnRNPs. (A) Fold change of SR/hnRNP DEGs defined in *Salmonella*-infected RAW 264.7 macrophages compared to SCR controls with genes categorized as primary and secondary response genes by Ramirez-Carrozzi et. al. 2009. (B) Fold change of SR/hnRNP DEGs in *Salmonella*-infected RAW 264.7 macrophages compared to SCR controls with Lipid A-induced genes categorized on basis of induction kinetics as defined by Bhatt et al., 2012. (C) Schematic representation of induction kinetics over a 120-minute time course following Lipid A treatment. Adapted from Bhatt et al., 2012.

although Class C DEGs were particularly enriched for CpG promoters (75%) and Class F were notably depleted (23%). Altogether, these data suggest the presence of CpG islands is not dictating whether an innate immune gene relies on SR/hnRNPs for proper induction, although they do support a general role for these regulatory factors in repressing expression of primary response genes.

hnRNP C and hnRNP K have similar effects on gene expression in uninfected and *Salmonella*-infected macrophages and loss of hnRNP K and U enhances restriction of viral replication

Lastly, we set out to determine whether we could use DEG and/or DUI profiles of SR/hnRNP knockdown macrophages to predict whether cells would be better or worse at controlling infection with an actual pathogen. We began by applying a simple hierarchical clustering algorithm to calculate similarities in DEG profiles between knockdowns. Remarkably, we found significant similarity between genes affected by loss of hnRNP K and hnRNP U (and to a lesser extent, hnRNP C) in uninfected cells (correlation between K/U: 0.78; correlation between C/K/U: 0.69) as well as in *Salmonella*-infected cells (correlation between K/U: 0.79; correlation between C/K/U: 0.76). To begin to understand how these knockdown cell lines may be phenotypically similar, we identified several clusters of upregulated and dowregulated genes common to hnRNP K and hnRNP U. Two clusters of upregulated genes are highlighted in Figure 8A. Interestingly, Cluster 1 contains mostly ISGs while Cluster 2 is enriched for a diverse group of kinases (Dmpk, Ripk3), regulators of GTPase activity (Gng10, Rgs16, Fgd2), and mitochondrial related factors (Pmaip1, Ucp2). Upon *Salmonella* infection, several of the upregulated ISGs from Cluster 1 are expressed at lower levels in hnRNP K and U relative to SCR controls (*Oasl2*, *Ddx58*, *Isg15*, *Usp18*), suggesting components of this antiviral pathway are dysregulated in the absence of hnRNP K and U. Conversely, Cluster 2 DEGs remain overexpressed in *Salmonella*-infected hnRNP K and U knockdown macrophages (see Clusters 4 and 5)

Consistent with hnRNP K and U sharing target transcripts, overlap between DEGs and DUIs in hnRNP K and U knockdowns is also highlighted in Venn Diagrams that show 1/3 shared DEGs and 3/4 shared DUIs in hnRNP K and U knockdown macrophage cell lines. Having discovered that loss of hnRNP K and U impacted antiviral gene expression in both resting and *Salmonella*-infected macrophages, we asked whether viral replication was impacted at early infection time points in hnRNP K and U knockdown cell lines compared to SCR controls. We infected SCR, hnRNP K and hnRNP U knockdown RAW 264.7 macrophages with vesicular stomatitis virus (VSV) at a MOI of 1. VSV is enveloped RNA virus that can replicate and elicit robust gene

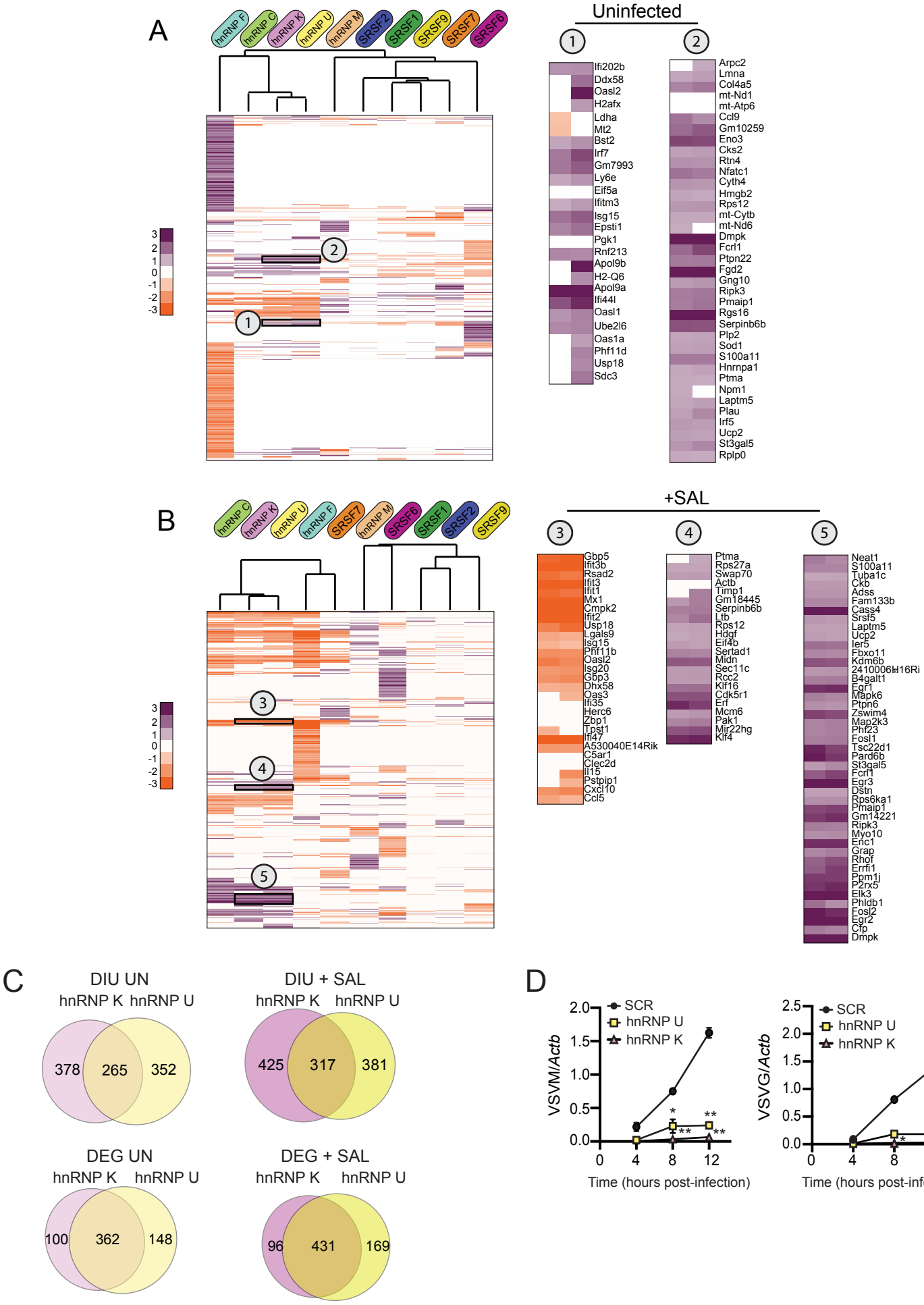

A
hnRNP F
hnRNP C
hnRNP K
hnRNP U
hnRNP M
SRSF2
SRSF1
SRSF9
SRSF7
SRSF6
Uninfected
1
2
Ifi202b
Ddx58
Oasl2
H2afx
Ldha
Mt2
Bst2
Irf7
Gm7993
Ly6e
Eif5a
Ifitm3
Isg15
Epsti1
Pgk1
Rnf213
Apol9b
H2-Q6
Apol9a
Ifi44l
Oasl1
Ube2l6
Oas1a
Phf11d
Usp18
Sdc3
Arpc2
Lmna
Col4a5
mt-Nd1
mt-Atp6
Ccl9
Gm10259
Eno3
Cks2
Rtn4
Nfatc1
Cyth4
Hmgb2
Rps12
mt-Cytb
mt-Nd6
Dmpk
Fcrl1
Ptpn22
Fgd2
Gng10
Ripk3
Pmaip1
Rgs16
Serpinb6b
Plp2
Sod1
S100a11
Hnrnpa1
Ptma
Npm1
Laptm5
Plau
Irf5
Ucp2
St3gal5
Rplp0
3
2
1
0
-1
-2
-3
B
hnRNP C
hnRNP K
hnRNP U
hnRNP F
SRSF7
hnRNP M
SRSF6
SRSF1
SRSF2
SRSF9
+SAL
3
4
5
Gbp5
Ifit3b
Rsad2
Ifit3
Ifit1
Mx1
Cmpk2
Ifit2
Usp18
Lgals9
Isg15
Pfif11b
Oasl2
Isg20
Gbp3
Dhx58
Oas3
Ifi35
Herc6
Zbp1
Tpst1
Ifi47
A530040E14Rik
C5ar1
Clec2d
Il15
Pstpip1
Cxcl10
Ccl5
Ptma
Rps27a
Swap70
Actb
Timp1
Gm18445
Serpinb6b
Ltb
Rps12
Hdgf
Eif4b
Sertad1
Midn
Sec11c
Rcc2
Klf16
Cdk5r1
Erf
Mcm6
Pak1
Mir22hg
Klf4
Neat1
S100a11
Tuba1c
Ckb
Adss
Fam133b
Cass4
Srsf5
Laptm5
Ucp2
Ier5
Fbxo11
Kdm6b
2410006H16Ri
B4galt1
Egr1
Mapk6
Ptpn6
Zswim4
Map2k3
Phf23
Fosl1
Tsc22d1
Pard6b
St3gal5
Fcrl1
Egr3
Dstn
Rps6ka1
Pmaip1
Gm14221
Ripk3
Myo10
Efnc1
Grap
Rhof
Errfi1
Ppm1j
P2rx5
Elk3
Phldb1
Fosl2
Egr2
Cfp
Dmpk
C
DIU UN
hnRNP K
hnRNP U
378
265
352
DIU + SAL
hnRNP K
hnRNP U
425
317
381
DEG UN
hnRNP K
hnRNP U
100
362
148
DEG + SAL
hnRNP K
hnRNP U
96
431
169
D
SCR
hnRNP U
hnRNP K
VSVM/Actb
VSVG/Actb
Time (hours post-infection)
Time (hours post-infection)

Figure 8

<u>Figure 8</u>: Loss of hnRNP K and hnRNP U controls similar DEGs and DIU and enhances the ability of RAW 264.7 macrophages to control viral replication (A) Hierarchical clustering of up- and down-regulated DEGs in uninfected macrophages (SR/hnRNP knockdown vs. SCR). 1 and 2 indicate clusters (right: zoom-ins of each cluster). Correlation of Cluster 1 is 0.84; Cluster 2 is 0.76. To allow for better visualization of DEGs, the scale is set at -3 to +3. (B) As in (A) but in Salmonella-infected macrophages. Correlation of Cluster 3 is 0.75; Cluster 4 is 0.78; Cluster 5 is 0.72. (C) Overlap of genes with DIU in uninfected hnRNP K and U knockdown macrophages. (D) As in (C) but comparing DIUs in *Salmonella*-infected macrophages. (E) Overlap of DEGs in uninfected hnRNP K and U knockdown macrophages. (F) As in (E) but comparing DEGs in *Salmonella*-infected macrophages. (G) Viral replication in hnRNP K and hnRNP U knockdown macrophages compared to SCR controls infected with VSV (MOI = 1) at 4, 8, and 12h post-infection. * = $p<0.05$; ** = $p<0.01$.

expression changes in RAW 264.7 cells [59]. Viral replication was measured over a 12h time course by following expression of two viral genes, VSV-G and VSV-M by RT-qPCR. Remarkably, we observed almost no replication of VSV in either hnRNP knockdown cell line at any time point. A similar hyper-restriction phenotype was recently reported for hnRNP K knockdown A549 cell lines infected with influenza virus by Thompson et al. [52]. These results argue strongly for splicing factors contributing to innate immune and infection outcomes in important ways and suggest that some of these factors may work in a cooperative fashion.

DISCUSSION

Our global transcriptomics analysis of SR and hnRNP knockdown macrophages highlights an unappreciated role for splicing regulatory proteins in modulating gene expression levels and alternative splicing decisions in *Salmonella*-infected macrophages. While it is easy to predict that splicing factors would regulate steady state gene expression in both resting and activated macrophages, our findings regarding the diversity of genes and gene regulons impacted by individual splicing factors and the dramatic reliance of some innate immune genes versus others on SR/hnRNPs for establishing or maintaining proper steady state levels, is surprising. Indeed, our observation that over 70% of innate immune transcripts induced more than 2-fold at 4h post-infection with Salmonella are differentially induced in the absence of one or more SR/hnRNPs queried here, demands a rethinking of how the innate immune response is post-transcriptionally regulated.

At the onset of this study, we hypothesized that we might see evidence for splicing factor reallotment, whereby downregulation of certain genes “frees up” splicing factors to promote processing of induced innate immune genes. While our data clearly show that the transcriptomes of uninfected and *Salmonella*-infected macrophages are differentially impacted by loss of splicing factors, we did not detect correlation between genes that are downregulated in *Salmonella*-infected macrophages and those that have/lose reliance on SR/hnRNPs. This finding is at seemingly at odds with earlier studies that examined splicing in cells upon various environmental triggers (e.g. starvation [38, 39, 60], heat shock [61-64], and T cell activation [65]) and found global repression of splicing. Generally, these studies conclude that inhibiting splicing is one way a cell can “reassign” the spliceosome to different target genes and/or shut maturation of pre-mRNAs down entirely. However, unlike stresses that trigger downregulation of gene expression so that cells can conserve energy, macrophage activation requires *de novo* biogenesis of hundreds of new proteins that are either secreted to notify other immune cells of a detected threat or increase antimicrobial capacity of the macrophage itself. Our data suggests that macrophages may

achieve this feat not by massively downregulating gene expression to relieve reliance on splicing factors but instead by functionalizing splicing factors such that they have different preferred targets in infected (i.e. activated) vs. uninfected macrophages. While this study focused mostly on how genes that are induced as part of the innate immune response are differentially expressed in SR/hnRNP knockdown macrophages, it will be interesting for future studies to investigate what happens to genes that are differentially expressed at rest upon macrophage activation. Does their pre-mRNA accumulate? Do other splicing factors take over as certain regulatory splicing factors are repurposed?

One lingering question from this analysis relates to the nature of the mechanism(s) through which genes—both in resting and *Salmonella*-infected macrophages—are up or down regulated in SR/hnRNP knockdown RAW 264.7 macrophages relative to SCR controls. Since our data does not generally support models wherein (1) an upstream master regulatory is mis-spliced or functionally altered (save for hnRNP F) or (2) alternative splicing is targeting transcripts to NMD, how are splicing factors activating and/or repressing induction of innate immune genes? Previous work from our lab, which found hnRNP knockdown leads to hyper-induction of a number of innate immune transcripts following inflammatory triggers, suggests that slowing/inhibiting pre-mRNA maturation may be involved. Specifically, we found that overexpression of hnRNP M promotes accumulation of intron-containing *Il6* pre-mRNAs, while loss of hnRNP M increases removal of *Il6* intron 3. From these findings we concluded that by repressing constitutive intron removal in *Il6*, hnRNP M acts as a hand break to slow early or spurious activation of this pro-inflammatory cytokine. It is possible that SR and hnRNPs here work in the same way, by contributing to specific constitutive intron removal events that can fine-tune the kinetics of transcript maturation and influence steady state RNA levels. Such a model would predict increased levels of reads from particular introns in DEGs. Although we do not see evidence for this in our RNA-seq data, this is not particularly surprising given the low abundance of intron-containing pre-mRNAs relative to mRNAs. Indeed, an important caveat of these studies is that they were carried out at a single, rather late, time point following *Salmonella* infection. While we chose this time-point to maximize mRNA transcript accumulation, we may have inadvertently minimized our ability to detect transient accumulation of unprocessed transcript intermediates. Kinetic transcriptome analysis from the Black and Smale labs demonstrates that for most transcripts, pre-mRNA splicing of innate immune transcripts occurs co-transcriptionally but accumulation of nascent pre-mRNA at the chromatin level is generally not evident at time points following 15-30 minutes, except in select cases where splicing catalysis on especially long transcripts is delayed [6, 7]. Thus, it is possible that for many of our transcripts of interest, the most important contribution of SR/hnRNP

proteins to constitutive and/or alternative splicing could occur during that early transcriptional burst. Indeed, our previous experiments looking at hnRNP M support this type of mechanism whereby loss of hnRNP M depressed Il6 and Mx1 expression at early time points (2 and 4h) but by 6 and 8h, hnRNP M was dispensable [35].

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MATERIALS AND METHODS

Cell lines and bacterial strains

RAW 264.7 macrophages (ATCC) (originally isolated from male BALB/c mice) were cultured at 37°C with a humidified atmosphere of 5% CO2 in DMEM (Thermo Fisher) with 10% FBS (Sigma Aldrich) 0.5% HEPES (Thermo Fisher). For RAW 264.7 macrophages stably expressing scramble knockdown and respective hnRNP or SRSF knockdown, cells were transfected with scramble non-targeting shRNA constructs and respective shRNA constructs targeted toward the 3′ UTR of the specific hnRNP or SRSF. After 48 hours, media was supplemented with hygromycin (Invitrogen) to select for cells containing the shRNA plasmid. Salmonella enterica serovar Typhimurium (SL1344) was obtained from Helene Andrews-Polymenis, TAMHSC.

S. Typhimurium Infection

Infections with S. Typhimurium were conducted by plating RAW 264.7 macrophages on tissue-cultured treated 12-well dishes at 7.5 x105 and incubated overnight. Overnight cultures of S. Typhimurium were diluted 1:20 in LB broth containing 0.3M NaCl and grown until they reached an OD600 of 0.9. Unless specified, cell lines at a confluency of 80% were infected with the S. Typhimurium strains at an MOI of 5 (hnRNP and SRSF RNA-Seq) for 30 minutes in Hank's buffered salt solution (HBSS), and subsequently cells were spun for 10 minutes at 1,000rpm, washed twice in HBSS containing 100µg/ml of gentamycin, and refilled with media plus gentamicin (10 µg/ml). After removal of supernatant, cells were lysed in Trizol (Thermo Fisher) for RNA collection and analyzed using RT-qPCR.

RNA-Seq

The RNA-Seq experiment was made up of 60 samples: biological triplicate of SCR uninfected, SCR Salmonella-infected, each knockdown uninfected, and each Salmonella-infected knockdown. RNA-Seq and library prep was performed by Texas A&M AgriLife Genomics and Bioinformatics Service. Samples were sequenced on Illumina 4000 using 2 × 150-bp paired-end reads. Raw reads were filtered and trimmed and Fastq data was mapped to the Mus musculus Reference genome (RefSeq) using CLC Genomics Workbench 8.0.1. Differential expression analyses were performed using CLC Genomics Workbench. Relative transcript expression was calculated by counting Reads Per Kilobase of exon model per Million mapped reads (RPKM). statistical significance was determined by the EDGE test via CLC Genomics Workbench. The differentially expressed genes were selected as those with p value threshold < 0.05 to include in the heatmaps represented.

Gene Ontology (GO) Canonical Pathway and Hierarchical Analysis

To determine the most affected pathways in control versus knockdown RAW 264.7 macrophages, canonical pathway analysis was conducted using Ingenuity Pathway Analysis software from QIAGEN Bioinformatics. Genes that were differentially expressed wth a p value < 0.05 from our RNA-SEQ analysis were used as input from uninfected and Salmonella Typhimurium infected cells. Hierarchical clustering was done in cluster3 (3.0) with the parameters of “complete linkage”, “absolute correlation (centered)”, and visualized using Treeview. Gene clusters were then analyzed using IPA Bio Functions to identify their relative function.

Scatter Plots and Correlation Analysis

For (p<0.05) differentially expressed genes, fold change was plotted to compare to coding sequence length which is identified by CLC Genomics Workbench to be equal to the total length of all exons (not all transcripts). Exon number and intron number were identified by CLC Genomics Workbench to be the number of exons/introns based on the mRNA annotations of the reference genome. Total gene length was calculated using “chromosome region start” and “chromosome region end” which are determined by CLC Genomics Workbench and the reference sequence to be the start position and end position of the annotated gene. Pearson Correlation was calculated using the values described above.

RNA isolation and qRT-PCR analysis

For transcript analysis, cells were harvested in Trizol and RNA was isolated using Direct-zol RNA Miniprep kits (Zymo Research) with 1 hr DNase treatment. cDNA was synthesized with

iScript cDNA Synthesis Kit (Bio-Rad). CDNA was diluted to 1:20 for each sample. A pool of cDNA from each treated or infected sample was used to make a 1:10 standard curve with each standard sample diluted 1:5 to produce a linear curve. RT-qPCR was performed using Power-Up SYBR Green Master Mix (Thermo Fisher) using a Quant Studio Flex 6 (Applied Biosystems). Samples were run in triplicate wells in a 96-well plate. Averages of the raw values were normalized to average values for the same sample with the control gene, beta-actin. To analyze fold induction, the average of the treated sample was divided by the untreated control sample, which was set at 1.

Alternative Splicing Analysis

Alternative splicing events were analyzed using MAJIQ and VOILA with the default parameters (Vaquero-Garcia et al., 2016). Briefly, uniquely mapped, junction-spanning reads were used by MAJIQ to construct splice graphs for transcripts by using the RefSeq annotation supplemented with de-novo detected junctions. Here, de-novo refers to junctions that were not in the RefSeq transcriptome database but had sufficient evidence in the RNA-Seq data. The resulting gene splice graphs were analyzed for all identified local splice variations (LSVs). For every junction in each LSV, MAJIQ then quantified expected percent spliced in (PSI) value in control and knockdown samples and expected change in PSI (dPSI) between control and knockdown samples. Results from VOILA were then filtered for high confidence changing LSVs (whereby one or more junctions had at least a 95% probability of expected dPSI of at least an absolute value of 10 PSI units between control and KD) and candidate changing LSVs (95% probability, 10% dPSI). For the high confidence results (dPSI > = 10%), the events were further categorized as single exon cassette, multi-exon cassette, alternative 5′ and/or 3′ splice site, or intron-retention.

VSV infection

7x105 RAW cells were seeded in 12-well plates 16h before infection. Cells were infected with VSV-GFP virus (Dalton and Rose, 2001) at multiplicity of infection (MOI) of 1 in serum-free DMEM (HyClone SH30022.01). After 1h of incubation with media containing virus, supernatant was removed, and fresh DMEM plus 10% FBS was added to each well. At indicated times post infection, cells were harvested with Trizol and prepared for RNA isolation.

Quantitation and Statistical Analysis

Statistical analysis of data was performed using GraphPad Prism software. Two-tailed unpaired Student's t tests were used for statistical analyses, and unless otherwise noted, all results are representative of at least three biological experiments (mean ± SEM (n = 3 per group)).

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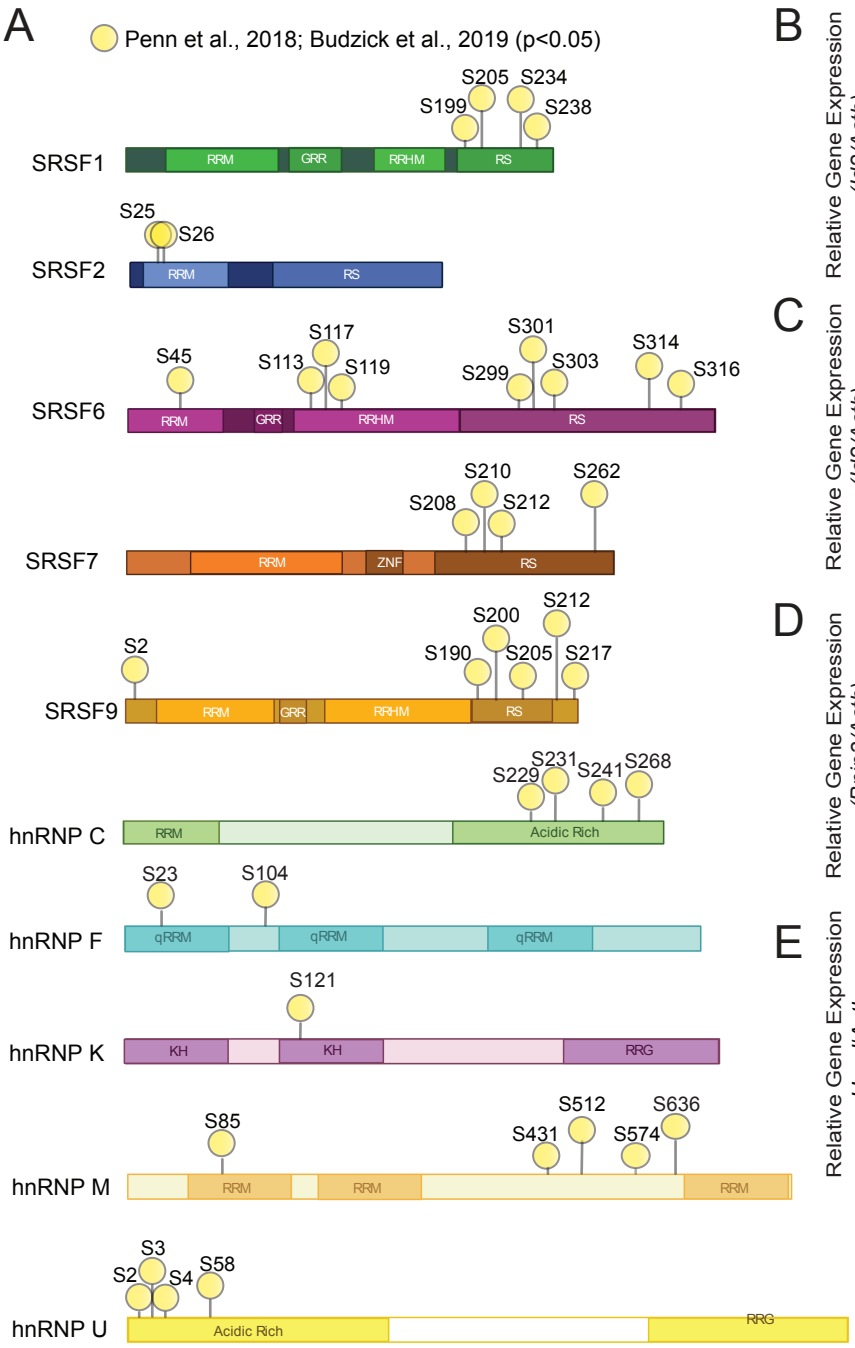


RRM = RNA Recognition Motif

KH = K Homology Domain

qRRM = Quasi RMM

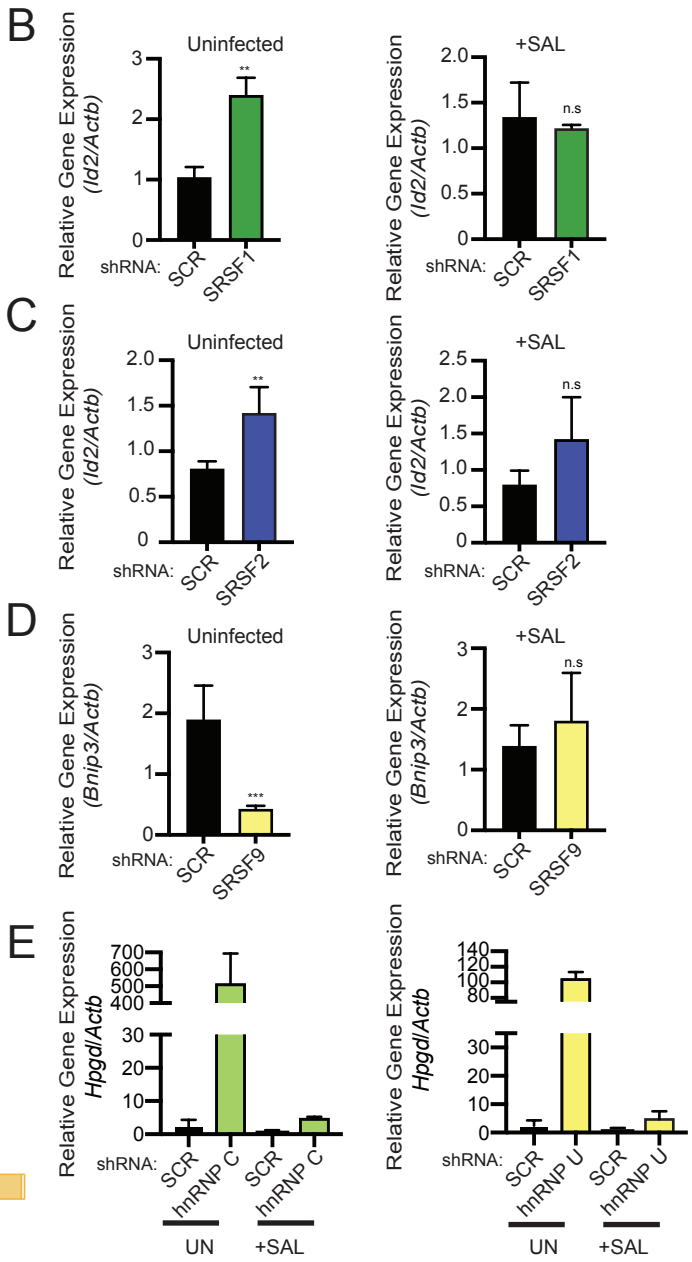
GRR = Glycine Rich Region

RS = Arginine-Serine Rich

RRG = Arginine-Glycine Repeat Region

RRHM = RRM Homology

ZNF = Zinc-Binding Domain



F

Ingenuity Canonical Pathways	-log(p-value)
Senescence Pathway	5.46
Cell Cycle: G1/S Checkpoint Regulation	5.21
Chronic Myeloid Leukemia Signaling	4.67
Role of BRCA1 in DNA Damage Response	4.64
Cyclins and Cell Cycle Regulation	4.6
Pancreatic Adenocarcinoma Signaling	3.68
Molecular Mechanisms of Cancer	3.51
Colorectal Cancer Metastasis Signaling	3.41
Estrogen-mediated S-phase Entry	3.28

Figure S1

Figure S1:

(A) Schematic representation of sites of differential phosphorylation reported by Penn et al., 2019 and Budzik et al., 2020 for SRSF and hnRNPs queried.

(B) Relative gene expression of *Bnip3* over *Actb* in uninfected DEGs in SRSF1 knockdown macrophages comparing uninfected (KD vs. SCR) and *Salmonella*-infected (KD vs. SCR). ** = $p<0.01$

(C) As in (B) but *Id2* expression in SRSF2 knockdown macrophages. ** = $p<0.01$

(D) As in (B) but *Bnip3* in SRSF9 knockdown macrophages. *** = $p<0.005$.

(E) As in (B) but *Hpgd* in hnRNP C and hnRNP U knockdown macrophages.

(F) Ingenuity pathway analysis (-log (p-value)) of genes downregulated in *Salmonella* vs. uninfected SCR macrophages (fold-change of -2 or more)

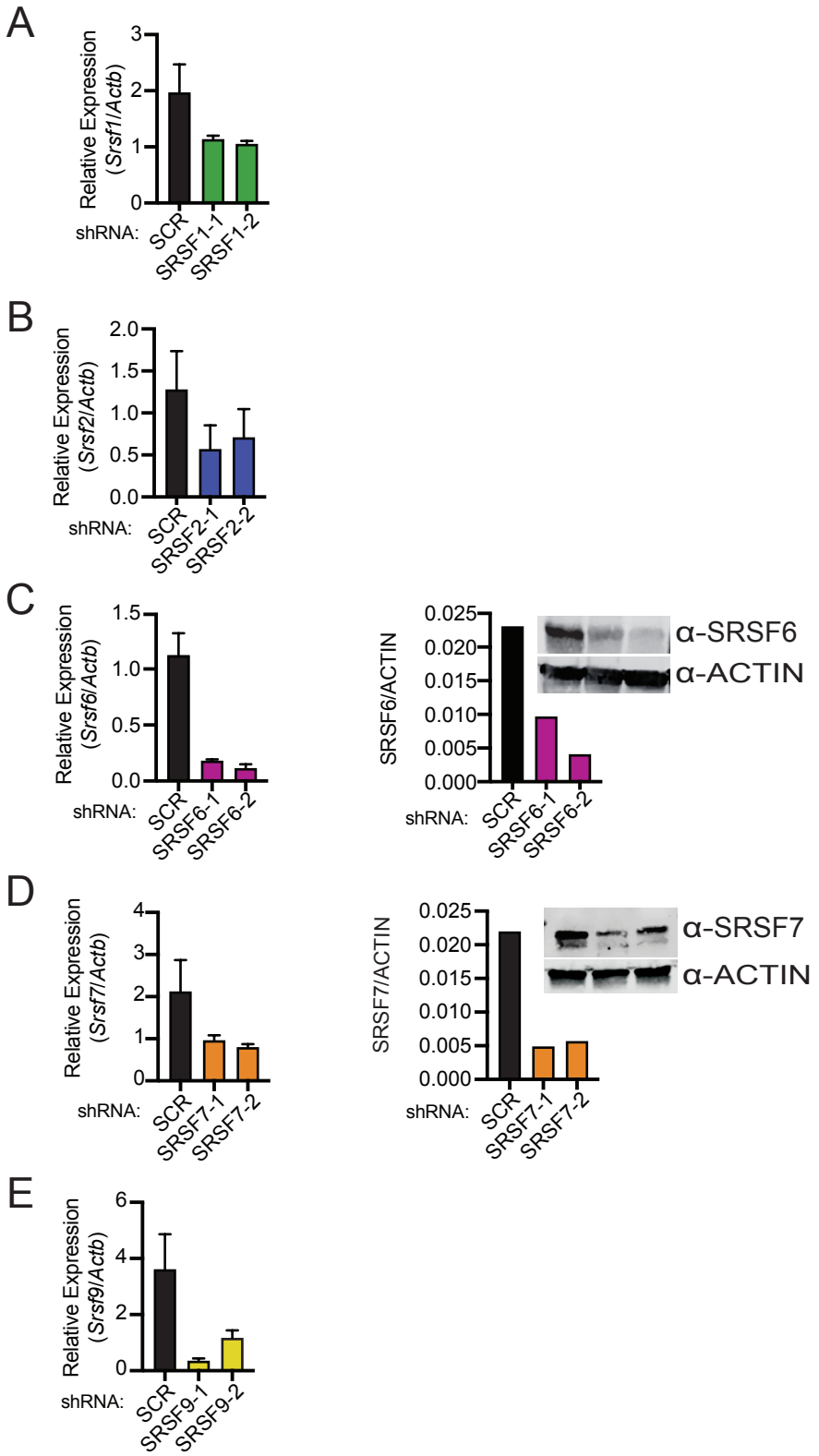
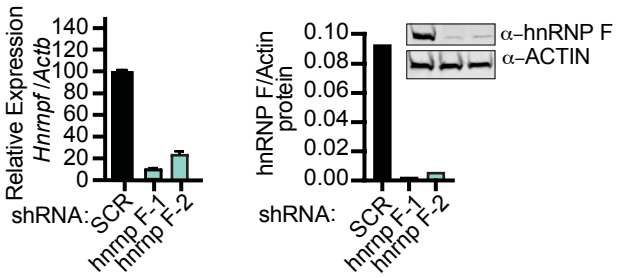

A
Relative Expression (Srsf1/Actb)
3
2
1
0
shRNA:
SCR
SRSF1-1
SRSF1-2
B
Relative Expression (Srsf2/Actb)
2.0
1.5
1.0
0.5
0.0
shRNA:
SCR
SRSF2-1
SRSF2-2
C
Relative Expression (Srsf6/Actb)
1.5
1.0
0.5
0.0
shRNA:
SCR
SRSF6-1
SRSF6-2
SRSF6/ACTIN
0.025
0.020
0.015
0.010
0.005
0.000
α-SRSF6
α-ACTIN
D
Relative Expression (Srsf7/Actb)
4
3
2
1
0
shRNA:
SCR
SRSF7-1
SRSF7-2
SRSF7/ACTIN
0.025
0.020
0.015
0.010
0.005
0.000
α-SRSF7
α-ACTIN
E
Relative Expression (Srsf9/Actb)
6
4
2
0
shRNA:
SCR
SRSF9-1
SRSF9-2

Figure S2

Figure S2:

(A-E) Knockdown efficiency of SRSF RAW 264.7 macrophages by RT-qPCR (SRSF1, 2, 6 7, 9) and immunoblot (SRSF6, 7). For all RT-qPCRs, values are the mean of 3 biological replicates, error bars represent standard deviation, and immunoblots are representative of 2 or more independent experiments.

A


Relative Expression
Hnrnpf/Actb
140
120
100
80
60
40
20
0
shRNA:
SCR
hnrnp F-1
hnrnp F-2
hnRNP F/Actin protein
0.10
0.08
0.06
0.04
0.02
0.00
α–hnRNP F
α–ACTIN

B

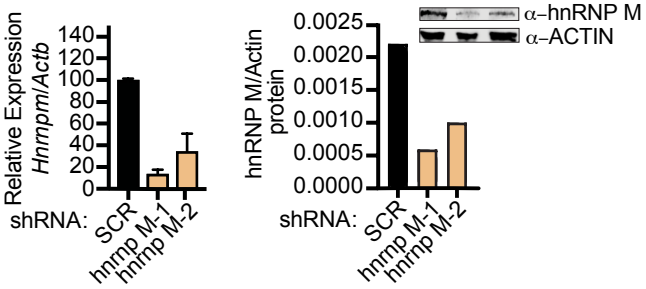

Relative Expression
Hnrnpm/Actb
140
120
100
80
60
40
20
0
shRNA:
SCR
hnrnp M-1
hnrnp M-2
hnRNP M/Actin protein
0.0025
0.0020
0.0015
0.0010
0.0005
0.0000
α–hnRNP M
α–ACTIN

Figure S3

Figure S3:

(A-E) Knockdown efficiency of hnRNP RAW 264.7 macrophages by RT-qPCR (hnRNP C, F, K, M, U) and immunoblot (hnRNP F, hnRNP M). For all RT-qPCRs, values are the mean of 3 biological replicates, error bars represent standard deviation, and immunoblots are representative of 2 or more independent experiments.

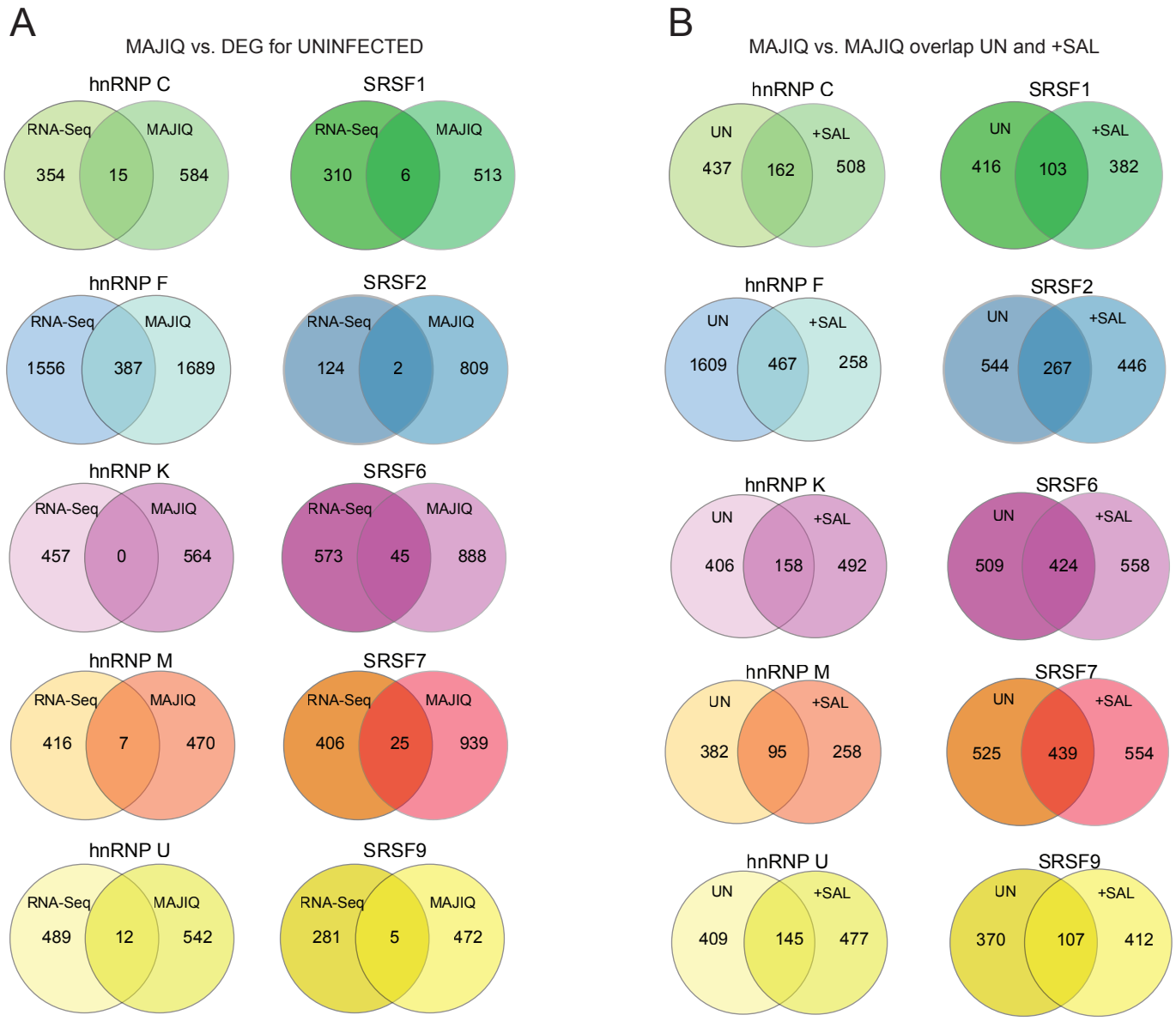
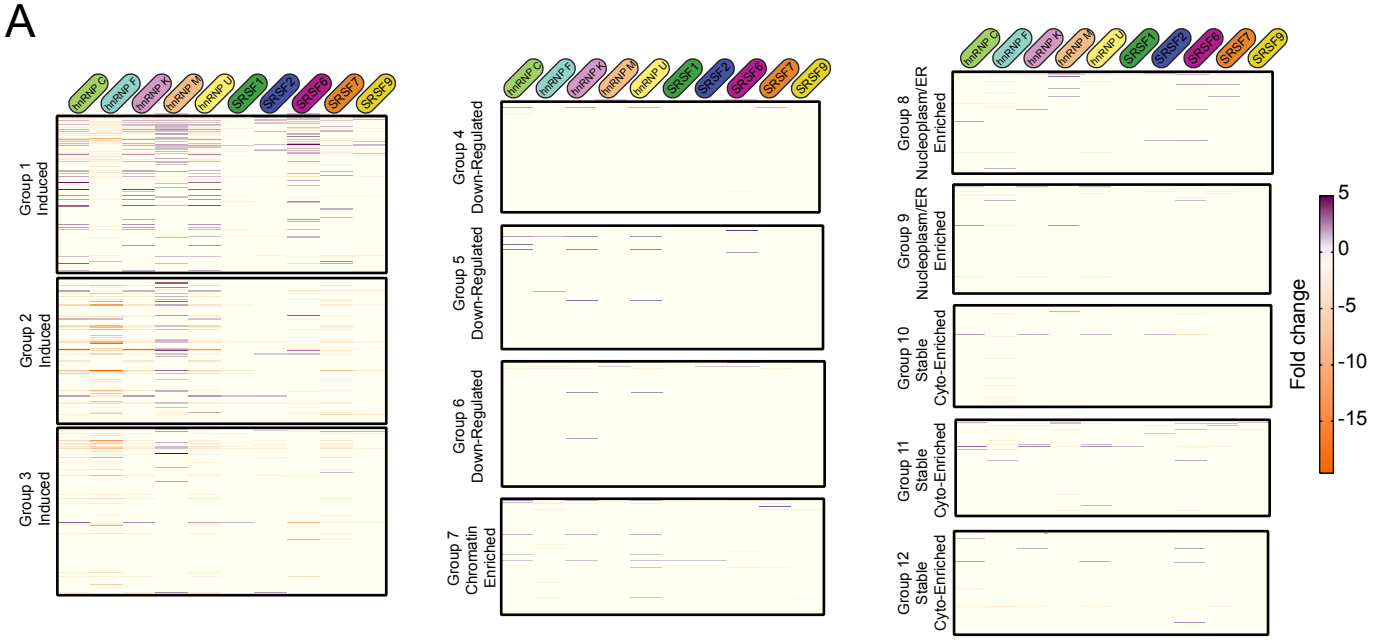

A
MAJIQ vs. DEG for UNINFECTED
hnRNP C
RNA-Seq
MAJIQ
354
15
584
SRSF1
RNA-Seq
MAJIQ
310
6
513
hnRNP F
RNA-Seq
MAJIQ
1556
387
1689
SRSF2
RNA-Seq
MAJIQ
124
2
809
hnRNP K
RNA-Seq
MAJIQ
457
0
564
SRSF6
RNA-Seq
MAJIQ
573
45
888
hnRNP M
RNA-Seq
MAJIQ
416
7
470
SRSF7
RNA-Seq
MAJIQ
406
25
939
hnRNP U
RNA-Seq
MAJIQ
489
12
542
SRSF9
RNA-Seq
MAJIQ
281
5
472
B
MAJIQ vs. MAJIQ overlap UN and +SAL
hnRNP C
UN
+SAL
437
162
508
SRSF1
UN
+SAL
416
103
382
hnRNP F
UN
+SAL
1609
467
258
SRSF2
UN
+SAL
544
267
446
hnRNP K
UN
+SAL
406
158
492
SRSF6
UN
+SAL
509
424
558
hnRNP M
UN
+SAL
382
95
258
SRSF7
UN
+SAL
525
439
554
hnRNP U
UN
+SAL
409
145
477
SRSF9
UN
+SAL
370
107
412

Figure S4

Figure S4:
(A) Overlap of DEGs with genes that have significant MAJIQ LSVs for hnRNP (left) and SRSF (right) knockdown RAW 264.7 macrophages vs. SCR controls. DEG $p<0.05$; LSV PSI (PSI ≥10%).
(B) Overlap of genes with LSVs in uninfected vs. *Salmonella*-infected hnRNP (left) and SRSF (right) knockdown RAW 264.7 macrophages vs. SCR controls. LSV PSI (PSI ≥10%).

A



B

Group (Bhatt et al., 2012)	% DEGs with a CpG promoter	% DEGs with a LPG promoter
A1	79% (11/14)	50% (1/2)
A2	45% (22/49)	55% (5/9)
B	48% (19/40)	48% (6/16)
C	75% (43/57)	40% (24/40)
D	49% (23/47)	48% (24/50)
E	40% (24/60)	55% (31/56)
F	23% (10/43)	54% (40/74)

Figure S7

Figure S7:
(A) Fold change of SR/hnRNP DEGs in *Salmonella*-infected RAW 264.7 macrophages compared to SCR controls with all genes (RefSeq genes exceeding 400-bp in length with an RPKM of at least 1 in at least one sample) categorized on basis of expression kinetics and subcellular accumulation in chromatin, nucleoplasm, and cytoplasm fractions as defined by Bhatt et al., 2012.
(B) Calculation of genes with a high (CpG) and low CpG (LPG) promoter islands as defined by Bhatt et al., 2012 that were differentially expressed in one or more SR/hnRNP knockdown macrophage cell line compared to a SCR control.