A Cytoplasmic RNA Virus Alters the Function of the Cell Splicing Protein SRSF2

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ABSTRACT To replicate efficiently, viruses must create favorable cell conditions and overcome cell antiviral responses. We previously reported that the reovirus protein μ2 from strain T1L, but not strain T3D, represses one antiviral response: alpha/beta interferon signaling. We report here that T1L, but not T3D, μ2 localizes to nuclear speckles, where it forms a complex with the mRNA splicing factor SRSF2 and alters its subnuclear localization. Reovirus replicates in cytoplasmic viral factories, and there is no evidence that reovirus genomic or messenger RNAs are spliced, suggesting that T1L μ2 might target splicing of cell RNAs. Indeed, RNA sequencing revealed that reovirus T1L, but not T3D, infection alters the splicing of transcripts for host genes involved in mRNA posttranscriptional modifications. Moreover, depletion of SRSF2 enhanced reovirus replication and cytopathic effect, suggesting that T1L μ2 modulation of splicing benefits the virus. This provides the first report of viral antagonism of the splicing factor SRSF2 and identifies the viral protein that determines strain-specific differences in cell RNA splicing.

IMPORTANCE Efficient viral replication requires that the virus create favorable cell conditions. Many viruses accomplish this by repressing specific antiviral responses. We demonstrate here that some mammalian reoviruses, RNA viruses that replicate strictly in the cytoplasm, express a protein variant that localizes to nuclear speckles, where it targets a cell mRNA splicing factor. Infection with a reovirus strain that targets this splicing factor alters splicing of cell mRNAs involved in the maturation of many other cell mRNAs. Depletion of this cell splicing factor enhances reovirus replication and cytopathic effect. Our results provide the first evidence of viral antagonism of this splicing factor and suggest that downstream consequences to the cell are global and benefit the virus.

KEYWORDS RNA processing, SRSF, reovirus, splicing
difference is determined by the reovirus \( \mu_2 \) protein (6, 7) encoded by the M1 gene. \( \mu_2 \) is a minor capsid protein but is expressed abundantly in infected cells and has RNA binding (8) and NTPase activities (9, 10). It also determines virus strain-specific differences in the morphology of VFs through its capacity to bind and stabilize microtubules (10, 11). Strain-specific differences in repression of IFN-\( \beta \) signaling and stabilization of microtubules are determined by a polymorphism in amino acid 208 of the \( \mu_2 \) protein (6, 7, 11). This may reflect the impact of amino acid 208 on \( \mu_2 \) stability (12) or on \( \mu_2 \) function. During infection, \( \mu_2 \) localizes predominantly to VFs (10-13) but can also be visualized diffusely in the cytoplasm and nucleus (10, 11, 14). However, the role of \( \mu_2 \) in the nucleus remains to be elucidated.

Here, we show that \( \mu_2 \) from T1L and reovirus recombinants that encode the T1L \( \mu_2 \) amino acid polymorphism form a complex with the pre-mRNA splicing factor SRSF2 (previously known as SC35 [15]) in nuclear speckles. Moreover, T1L but not T3D alters the splicing of transcripts for genes involved in RNA processing and maturation. Finally, depletion of SRSF2 enhances reovirus replication and cytopathic effect, suggesting that T1L \( \mu_2 \) modulation of splicing benefits the virus. We provide here the first report of viral antagonism of the splicing factor SRSF2 and suggest that the consequences to the cell are global.

RESULTS

\( \mu_2 \) undergoes constant nuclear shuttling, but the predominant intracellular localization is strain specific. Reovirus replication is exclusively cytoplasmic (4), and yet the reovirus protein \( \mu_2 \) can translocate to the nucleus (10, 11, 14). Given that the capacity for reovirus \( \mu_2 \) to repress IFN-\( \beta \) signaling is virus strain specific (6, 7), we probed \( \mu_2 \) intracellular localization further. Notably, we found that the predominant intracellular localization of \( \mu_2 \) is also strain specific. T1L-\( \mu_2 \)-HA localized primarily to the nucleus, whereas T3D-\( \mu_2 \)-HA localized to the cytoplasm (Fig. 1A). Interestingly, nuclear T1L \( \mu_2 \) localized to intranuclear filamentous structures reminiscent of microtubules and also to discrete aggregates (Fig. 1A and B). T1L \( \mu_2 \) nuclear localization is dependent on the nuclear export signal receptor protein CRM1/exportin 1 (10). To determine whether
T3D μ2 has the ability to transiently shuttle to the nucleus and associate with similar subnuclear structures, we inhibited CRM1 with the irreversible inhibitor leptomycin B (LMB) (16) for 5 h prior to immunostaining (Fig. 1A and C). Treatment with LMB revealed that T3D μ2 does indeed shuttle into the nucleus, but the predominant localization is cytoplasmic under normal nuclear protein export conditions. To further probe nuclear-cytoplasmic shuttling, eGFP-CRM1 or an eGFP empty vector control was coexpressed with μ2 (Fig. 1D and E). As expected, T1L μ2 was found mainly in the nucleus when coexpressed with eGFP but was found in the cytoplasm in cells overexpressing CRM1. Together, the results indicate that μ2 undergoes constitutive CRM1-dependent nuclear-cytoplasmic shuttling, but the predominant localization is reovirus strain specific. Interestingly, even when T3D μ2 accumulated at high levels in the nucleus upon CRM1 inhibition (Fig. 1A), it never associated with subnuclear structures, as seen for T1L μ2. T1L μ2 is known to bind and stabilize microtubules in the cytoplasm (11), but association with subnuclear aggregates has not been previously described. Although the role of intranuclear bodies in the life cycle of several viruses is of increasing interest (17), a role for μ2 in the nucleus has not been previously identified.

**Intranuclear μ2 localizes to nuclear speckles.** To determine the nature of the intranuclear T1L μ2 structures, AD-293 cells were transfected with μ2-HA and immunostained for markers of candidate nuclear bodies. Although viruses are known to modulate promyelocytic leukemia (PML) body function, particularly relating to the IFN system (reviewed in references 18 and 19), T1L μ2 did not colocalize with PML bodies (Fig. 2A). Similarly, μ2 did not colocalize with coillin (Fig. 2B), a marker of Cajal bodies (20). T1L μ2, however, colocalized with the serine/arginine-rich splicing factor 2 (SRSF2; previously known as SC35 [15]), a marker of nuclear speckles (Fig. 2C). In metazoans, nuclear speckles are enriched in mature snRNPs, non-snRNP pre-mRNA splicing factors, and polyadenylated RNA (21–23). Association of μ2 with nuclear speckles was specific to T1L μ2, since T3D μ2 failed to associate with SRSF2 even after treatment with LMB to induce its nuclear localization (Fig. 2D).

**Amino acid 208 in μ2 determines μ2 localization to nuclear speckles during infection.** Amino acid 208 in the μ2 protein determines several strain-specific differences in μ2 function (7, 11, 12). To determine whether this amino acid is also a determinant of μ2 localization to nuclear speckles, AD-293 cells were infected with either the parental reovirus strains T1L or T3D or recombinant mutant viruses and then immunostained (Fig. 3). As expected (11), μ2 localized to cytoplasmic viral factories that displayed either a filamentous or globular morphology during T1L or T3D infection, respectively. In addition, T1L but not T3D μ2 colocalized with SRSF2 in nuclear speckles (in 61.5% ± 3.3% versus 0.0% of infected cells, respectively), a finding consistent with our results using plasmid-derived μ2 (Fig. 2). Remarkably, substitution of T3D μ2 amino acid 208 with that of T1L (T3D-S208P) induced μ2 localization to nuclear speckles (in 50.9% ± 2.7% of infected cells), whereas this localization was lost in a T3D virus expressing T1L μ2 with amino acid 208 reverted to that of T3D (T3D T1L-M1 P208S; 0.0% of infected cells). Together, results demonstrate that strain-specific differences in μ2 amino acid 208 are both required and sufficient for μ2 localization to nuclear speckles during reovirus infection.

**μ2 alters the localization of SRSF2 in a microtubule-dependent manner.** Interestingly, ectopic expression of T1L μ2 resulted in the mislocalization of SRSF2 to filamentous structures in approximately half of transfected L929 or AD293 cells (Fig. 4A, E, and F). This effect was specific for SRSF2, since T1L μ2 did not affect localization of the nuclear speckle matrix protein Son (Fig. 4B). T1L μ2 binds to and stabilizes cytoplasmic microtubules (11) and, not unexpectedly, T1L μ2 colocalized with nuclear alpha-tubulin in nuclear filaments (Fig. 4C). To determine whether T1L μ2-induced changes in SRSF2 localization were a consequence of μ2 association with nuclear microtubules, cells were treated with nocodazole and paclitaxel to inhibit polymerization of or to stabilize microtubules, respectively (24), and the cytoskeletal effects of these drugs were confirmed (Fig. 4D). Depolymerization of microtubules with nocoda-
FIG 2 Intranuclear T1L μ2 specifically localizes to nuclear speckles. AD-293 cells were transfected with reovirus T1L- or T3D-M1-HA for 20 h, fixed, and immunostained with antibodies against HA and markers of PML bodies (PML) (A), Cajal bodies (collin) (B), or nuclear speckles (SRSF2) (C). (D) AD-293 cells were transfected with T3D M1-HA for 18 h, treated with LMB for 5 h, and immunostained as in panel C. Nuclei were counterstained with DAPI. Histograms display measured fluorescence intensity along the drawn line in the overlay inset panels. Scale bar, 10 μm.
zole reduced the fraction of T1L \( \mu 2 \)-expressing cells that displayed mislocalized SRSF2, demonstrating that this TL \( \mu 2 \) effect requires microtubules (Fig. 4E and F). However, stabilization of microtubules with paclitaxel did not alter SRSF2 localization in T3D \( \mu 2 \)-expressing cells demonstrating that stabilized microtubules are not sufficient to support \( \mu 2 \) effects on SRSF2 localization (Fig. 4E and F). Together, results demonstrate that T1L \( \mu 2 \), but not T3D \( \mu 2 \), alters the localization of SRSF2 to filamentous nuclear structures, likely by interactions with both microtubules and SRSF2.

\( \mu 2 \) forms a complex with the pre-mRNA splicing factor SRSF2. To determine whether T1L \( \mu 2 \) interacts with SRSF2, we first expressed T1L- and T3D-\( \mu 2 \)-HA in AD-293 cells and performed an in situ proximity ligation assay (PLA [25]) (Fig. 5A). Antibody against tubulin was used as a control for the approach and, as expected, T1L \( \mu 2 \)-HA generated much greater PLA signals than T3D \( \mu 2 \)-HA did (Kruskal-Wallis nonparametric test; \( P < 0.001 \)). Importantly, T1L \( \mu 2 \)-HA also generated much greater PLA signals than T3D \( \mu 2 \)-HA did with endogenous SRSF2 in the nucleus (Kruskal-Wallis nonparametric test; \( P < 0.001 \)). Consistent with the lack of \( \mu 2 \)-mediated changes in Son localization (Fig. 4B), PLA experiments demonstrated that T1L \( \mu 2 \)-HA does not interact with Son (data not shown). Next, we confirmed formation of a complex between \( \mu 2 \) and SRSF2 biochemically by coimmunoprecipitation of cell lysates. AD-293 cells were cotrans-
fected with T1L-M1-HA and myc-tagged SRSF2 (because endogenous SRSF2 is not expressed at sufficient levels) or myc-tagged IRF9 as a negative control (Fig. 5B). Myc-tagged SRSF2, but not myc-tagged IRF9, coimmunoprecipitated with T1L/H92622-HA. Together, results demonstrate that T1L/H92622 but not T3D/H92622 forms a complex with both microtubules and SRSF2 in the nucleus.

**μ2 nuclear localization requires SRSF2 expression.** Given the impact of T1L μ2 on SRSF2 localization, we next sought to determine whether SRSF2 affects μ2 localization. SRSF2 was depleted by small interfering RNA (siRNA) in L929 cells (Fig. 6A). T1L- or T3D-M1-HA were transfected, and μ2 localization was assessed by immunofluorescence (Fig. 6B). As expected, in control siRNA-transfected cells, T1L μ2 and T3D μ2 localized primarily to the nucleus and the cytoplasm, respectively. However, T1L μ2 localization became primarily cytoplasmic in cells with reduced SRSF2 expression, mimicking T3D μ2. Thus, SRSF2 function is required for T1L μ2 nuclear localization where, given the exclusively nuclear location of SRSF2 (26), μ2 likely subsequently forms a complex with SRSF2.
Depletion of SRSF2 increases reovirus replication and cytopathic effect and impact is strain specific. The interaction of T1L and SRSF2 suggested the possibility that reovirus modulates SRSF2 function to generate a more favorable cell environment. To determine whether SRSF2 affects viral replication and cytopathic effect,
L929 cells were depleted of SRSF2 and then infected with T1L or T3D. SRSF2 depletion increased T1L but not T3D replication and cytopathic effect in both primary (Fig. 7A and B) and secondary infections (Fig. 7C and D). The absence of an effect of SRSF2 depletion on T3D infection suggests that SRSF2-independent events are the predominant determinants of T3D replication in L929 cells. The increased T1L replication and cytopathic effect suggest that T1L/2 does not inhibit SRSF2 activity entirely and that further depletion of SRSF2 is beneficial to the virus.

Reovirus T1L alters cellular mRNA splicing. SRSF2 is a pre-mRNA splicing factor, but reovirus replication is exclusively cytoplasmic, and there is no evidence that any reovirus RNAs are spliced (4). We therefore used RNA-seq to determined whether reovirus T1L but not T3D alters cellular mRNA splicing. Because T3D induces significantly more IFN-β than T1L does (6, 7, 27) and because IFN-β alone alters mRNA splicing of some IFN-stimulated genes (28), we compared mock-infected, untreated cells to mock-infected or reovirus-infected cells stimulated with IFN-β. This identifies differences in T1L and T3D effects on splicing separate from differences in their induction of IFN-β and subsequent IFN-β effects on splicing. The results from RNA sequencing (RNA-seq) were analyzed by the mixture-of-isomers (MISO) statistical model (29) to assess changes in mRNA alternative splicing (Fig. 8A; see also Tables S1 to S3 in the supplemental material). IFN-β stimulation generated 42 novel splicing variants in 41 different genes relative to untreated cells. Excluding those IFN-β-induced variants, T3D infection generated 142 novel splicing variants while, remarkably, T1L generated 369. Only 35 of these variants were found to overlap between the two reovirus strains, resulting in a total of 297 and 97 genes by T1L and T3D, respectively. Splicing events that were induced only in T1L were confirmed by quantitative reverse transcription-PCR (qRT-PCR) for two cases (RanBP3 and SRSF3) identified by MISO analysis (Fig. 8B). Whereas T3D also altered RanBP3 and SRSF3 splicing to some extent (2.4- and 2.5-fold, relative to the Mock group), T1L altered their splicing much more dramatically (10.4- and 10.8-fold, respectively). Moreover, these qRT-PCR results confirmed that IFN-β has minimal or no impact on viral effects on splicing and that the same μ2 amino acid that determines μ2 association with SRSF2 (Fig. 3) determines reovirus effects on splicing (S208P altered RanBP3 and SRSF3 splicing 7.0- and 11.5-fold, respectively, Fig. 8B). Thus, while both reovirus strains induce unique splicing variants, T1L induces more than three times more than T3D does (RNA-seq results: chi square, \( P < 0.001 \)), and a single μ2 amino acid can determine reovirus effects on splicing.

To gain further insights into reovirus-induced global changes in host mRNA splicing, we used Ingenuity pathway analysis (IPA) and gene ontology (GO) analysis. Specifically, we performed IPA on the 297 and 97 genes whose splicing was altered uniquely by T1L.
or by T3D, respectively. The pathways most affected by T1L involved RNA posttranscriptional modifications (Fig. 8C). Indeed, T1L (but not T3D) altered the splicing of SRSF3, SRSF6, SRSF7, and SRSF11 (see Table S3 in the supplemental material), all of which have specialized roles in posttranscriptional regulation (30). Moreover, the top five predicted upstream regulators for altered splicing events induced by T1L (but not T3D) included SRSF1 (data not shown), another SR family member (31). Importantly, the minimal changes in splicing that occurred during T3D did not cluster in any of these categories, and there was no overlap with those obtained for T1L. Lastly, we used the network of genes affected by T1L with the highest statistical significance to create a web of known and predicted interactions. One-third of the genes in the network related to mRNA splicing or mRNA processing (data not shown). These IPA results were supported by GO analysis of the same data sets. Specifically, the top two processes for T1L were “mRNA processing” and “ribonucleoprotein complex assembly,” whereas those were 47th and 8th in priority for T3D, respectively (T1L different from T3D at $P < 0.001$ and $P < 0.05$, respectively).

FIG 8 Reovirus infection alters cellular mRNA splicing. (A) Venn diagram indicating differences in splicing based on MISO analysis of RNA-seq results. (B) Confirmation of differential expression of novel splicing variants using qRT-PCR. L929 cells were infected and stimulated with IFN-β using conditions identical to those used for RNA-seq. Transcripts with a specific exon skipped or spliced-in (as identified by RNA-seq) were quantitated by qRT-PCR for two representative genes. The results are expressed as a ratio of the two splicing events to capture the events comprising MISO analysis. The results of statistical analyses ($P < 0.05$), indicated by lowercase letters, were as follows: a, mock different from T1L and S208P; b, mock or mock/IFN different from all viruses or viruses/IFN; c, T1L or T1L/IFN different from T3D or T3D/IFN; d, T3D or T3D/IFN different from S208P or S208P+IFN; and e, S208P or S208P+IFN not different from T1L or T1L+IFN. (C) IPA results for genes whose splicing was altered uniquely by either T1L (297 genes) or T3D (97 genes) and IFN-β stimulation, excluding genes whose splicing was altered by IFN-β stimulation alone.
In sum, results suggest that SRSF2 is required for T1L/H9262 nuclear localization whereupon T1L/H9262 forms a complex with SRSF2 and antagonizes the organization of nuclear speckles, resulting in dysregulated splicing of other splicing factors and regulators of mRNA at the posttranscriptional level to benefit viral replication (Fig. 9).

DISCUSSION

To replicate efficiently, viruses must create favorable cell conditions and overcome cell antiviral responses. Here, we report that the μ2 protein from strain T1L localizes to nuclear speckles, where it forms a complex with and alters the localization of the pre-mRNA splicing factor SRSF2. Moreover, infection with reovirus T1L alters mRNA splicing of genes involved in mRNA processing and maturation, likely as a consequence of T1L/μ2 antagonistic effects on SRSF2. Finally, depletion of SRSF2 enhances reovirus replication and cytopathic effect, suggesting that T1L/μ2 modulation of splicing benefits the virus. This provides the first report of viral antagonism of the splicing factor SRSF2 and suggests that by altering mRNA processing and maturation, the virus induces cell consequences that are global rather than in a single pathway.

Reovirus μ2 stability, repression of IFN-β signaling, and stabilization of microtubules are all determined by a single amino acid polymorphism in the μ2 protein (6, 7, 11). Here, we demonstrate that this amino acid similarly determines μ2 localization to nuclear speckles (Fig. 3). The common dependence on a single amino acid could reflect its impact on μ2 stability or its impact on function. The N terminus of μ2 includes a polybasic region that can function as a nuclear localization signal (10) and, interestingly, domains that are rich in basic amino acids have been identified as nuclear speckle-targeting signals (32). However, this μ2 polybasic region is conserved between T1L and T3D strains, indicating that the primary protein sequence alone does not predict its localization and that other factors contribute to the differential localization of μ2. This complexity in determinants of μ2 localization to intracellular compartments is highlighted by the observation that SRSF2 is required for μ2 nuclear localization (Fig. 6) and that T3D μ2 does not associate with nuclear speckles even when concentrated in the nucleus after inhibition of nuclear export (Fig. 2D). The requirement for SRSF2 could reflect trapping of μ2 in a complex, an impact on nuclear import or export processes, or an impact on cell proteins that affect μ2 itself (Fig. 9). Posttranslational modifications of μ2, including acetylation (33) and phosphorylation (34), have been reported, and yet the impact(s) of these modifications on μ2 function has not been fully elucidated. Future studies will address the roles of SRSF2 and posttranslational modifications on μ2 subcellular localization.
Multiple viruses regulate the cell splicing machinery to generate alternative viral RNA products. Several RNA viruses (35–38) and DNA viruses (39) use extensive alternative splicing of their mRNAs. The influenza A virus NS1 protein disorganizes nuclear speckles and Cajal bodies and binds members of the snRNP family of splicing factors and proteins required for 3’-terminal polyadenylation of host transcripts to facilitate splicing of viral RNAs (35). Retroviruses regulate cell splicing machinery to balance generation of viral mRNAs with maintenance of unspliced RNA for production of viral progeny (40, 41). There is no evidence for splicing of viral RNA for the cytoplasmically replicating reovirus (4). Reovirus RNAs also lack modifications at their 3’ termini (4); another RNA modification function associated with nuclear speckles (42–44). Finally, we did not detect any redistribution of splicing factors to cytoplasmic VFs during reovirus infection (data not shown). Thus, there is no evidence that reovirus modulation of SRSF2 affects viral RNAs. Instead, reovirus likely modulates the cell transcriptome to its benefit.

Alternative splicing of cell mRNAs can affect cell function and disease (45–47), and several viruses modulate cell splicing machinery to alter the cell transcriptome. The adenovirus E4-ORF4 antagonizes the function of SRSF1 and SRSF9 by promoting their dephosphorylation (48, 49). The herpes simplex virus ICP27 protein binds the SR protein kinase 1 (SRPK1), resulting in its relocalization from the cytoplasm to the nucleus to dephosphorylate SR proteins and regulate host mRNA splicing events (50–52). The vaccinia virus VH1 phosphatase appears to directly dephosphorylate SR proteins (53). Epstein-Barr virus (EBV) itself encodes a splicing factor, SM, that influences splicing and processing of host pre-mRNAs (54, 55). Splice variants, including those that encode dominant negative proteins, have been described for several genes involved in the IFN-α/β response (56–63). Notably, the EBV SM protein skews the mRNA splicing patterns of STAT1, enhancing production of the dominant-negative STAT1β isoform (55). Very recently, it has been shown that the N55 protein of dengue virus binds to and interferes with components of the U5 snRNP particle and alters mRNA splicing of several antiviral factors (64). In contrast to our results for reovirus, dengue N55 does not appear to affect the intranuclear localization of its splicing targets (64). Finally, in a recent report reovirus T3D was shown to alter cell mRNA splicing, but the impact on viral replication and the underlying mechanism were not investigated (65). It is interesting that a single reovirus protein, μ2, can modulate both IFN signaling (7) and SRSF2 function to benefit the virus. The results suggest that this protein has evolved to target both a specific antiviral pathway and mRNA maturation for more global effects.

Several SR proteins play roles in addition to those in splicing that affect mature mRNA expression and nuclear export (30). For example, SRSF1 participates in the organization of nuclear speckles and in the promotion of decay of aberrantly spliced transcripts (31, 66, 67), whereas SRSF2 influences transcriptional elongation of some mRNAs (68). With the exception of SRSF2, mammalian SR proteins constitutively shuttle between the nucleus and cytoplasm (26). Once in the cytoplasm, SRSF1 impacts translation by modulating mRNA entrance into polyribosomes (69, 70). Lastly and perhaps most intriguingly, SRSF1 has been associated with RIG-I and RNA polymerase III-dependent sensing of transfected non-self cytosolic DNA to facilitate IFN-β production (71); however, its role during viral infection remains unclear. Similarly, the spliceosomal protein SNRNP200 can also translocate to the cytoplasm, where it modulates the viral induction of IFN-β (72). Although T1L did not affect splicing of SRSF1 or SNRNP200 mRNA, the loss of SRSF2 can affect SRSF1 function (73), and the regulation of snRNPs is complex, leaving open the possibility that T1L alters the cytoplasmic function of spliceosomal proteins to benefit the virus.

T1L infection alters SRSF3 splicing (see Table S3 in the supplemental material), suggesting another possible mechanism by which T1L μ2 interactions with SRSF2 could impact cell RNA function. SRSF3 (previously known as SRp20 [15]) is involved in 5’-cap-independent internal ribosome entry site (IRES)-mediated translation of picornavirus transcripts (74, 75). The 2A proteinase of several picornaviruses induce a change in localization of SRSF3 from the nucleus to the cytoplasm and stress granules (76), and
depletion of SRSF3 impacts the efficiency of IRES-mediated translation (74). Reovirus transcripts are 5’ capped and undergo 5’-cap-dependent translation in ribosomes within membrane-associated VFs (4, 5, 77). Thus, it is unlikely that reovirus modulation of SRSF3 splicing is related to IRES-dependent translation. However, T1L infection induces novel splice variants of SRSF3, SRSF6, SRSF7, and SRSF11 (see Table S3 in the supplemental material), all of which have specialized roles in posttranscriptional regulation (30). Thus, reovirus modulation of the cell response may be indirectly mediated through a dysregulation of mRNA nuclear export, processing, nonsense-mediated decay, and/or efficient translation through the targeting of SRSF2 and changes in the isoforms of other SR proteins.

Lastly, reovirus μ2 repression of the IFN-β response is a determinant of reovirus induction of myocarditis (7, 27), and the same μ2 amino acid polymorphism that determines this repression and severity of myocarditis (7) determines μ2 association with SRSF2 (Fig. 3). Together, these results suggest that SRSF2 could participate in protecting the heart against viral myocarditis. While this could reflect SRSF2 effects on the IFN response, it has been shown that mutations and/or decreased expression of splicing factors results in cardiac development defects (45), and cardiac tissue-specific ablation of SRSF2 results in dilated cardiomyopathy (78). The fact that viruses, such as adenoviruses, herpesviruses, and reoviruses, known to induce myocarditis (79) can modulate splicing factors raises the intriguing possibility that altered splicing represents yet another mechanism by which viruses induce cardiac damage.

MATERIALS AND METHODS

Cells. Mouse L929 cells were maintained in minimal essential medium (MEM; SAFC Biosciences) as a suspension culture supplemented to contain 5% fetal calf serum and 2 mM l-glutamine. AD-293 cells were maintained in high glucose Dulbecco MEM (DMEM; Gibco) supplemented to contain 10% fetal calf serum and 1% of sodium pyruvate. Exponentially growing L cells or trypsinized AD-293 cells were plated and incubated for at least 3 h prior to transfection or infection.

Viruses. All viruses were CsCl-purified, low-passage-number stocks originating from plaques of either cultured virus or virus generated by reverse genetics (80) as previously described (6, 7). The results were the same regardless of virus source.

Plasmids and plasmid transfections. A plasmid expressing C-terminus hemagglutinin (HA)-tagged T1L μ2 in a pCAGGs backbone was described previously (34). The C-terminus HA-tagged T3D μ2 was generated from pCAGGs-M1-T3D (6) using a similar strategy and the primer 5’-CCCGGTCACGTAGCCGT AATCTGGAACATCGTATGGGTACGCCAAGTCAGATCGGAAAGCTAGTC-3’. The eGFP-N1 and eGFP-HsCRM1 plasmids were obtained from Clontech Laboratories (catalog no. 6085-1) and GeneCopoeia (catalog no. EX-T0446-M29), respectively. The Myc-DDK-IRF9 plasmid was custom-cloned by OriGene Technologies, Inc., using the murine IRF9 cDNA (NM_001159417) in a pCMV6 expression vector. The HisSRSF2-c-Myc in a pcDNA3.1 backbone plasmid (81) was a gift from Kathleen Scotto (Addgene; plasmid 44721). Plasmids were transfected using Lipofectamine 3000 (Thermo Fisher Scientific) for 20 to 24 h.

siRNAs and siRNA transfections. L929 cells were transfected for a final 60 nM concentration of control nontargeting siRNA or siRNA against murine SRSF2 (GE Dharmacon; catalog no. D-001810-10 or L-044306) using the transfection reagent RNAiMAX (Thermo Fisher Scientific). At 24 h posttransfection, the IFN response was assayed using a 1,000 U/ml diluted in supplemented DMEM. Recombinant mouse interferon-β (PBL Assay Science, catalog no. 12410-1) was used at 1,000 U/ml diluted in supplemented MEM.

Antibodies and chemical treatments. The following antibodies and dilutions were used for immunoblotting: anti-μ2 (generated against two μ2 peptides by Open Biosystems; 1:1,000), anti-β-actin (Santa Cruz Biotech, sc-1615-hrp; 1:3,000), anti-SRSF2 (Millipore, 04-1550; 1:1,000), anti-SRSF1 (Abcam, ab129108; 1:350), anti-c-Myc (catalog no. sc-40, 1:3,000; Santa Cruz Biotech), goat horseradish peroxidase (HRP)-conjugated anti-rabbit immunoglobulin (Ig; Millipore, 12-348; 1:2,000), and goat HRP-conjugated anti-mouse Ig (Millipore, 12-349; 1:2,000). For immunoprecipitation experiments, secondary antibodies were either TrueBlot Ultra anti-rabbit IgG-HRP (Rockland Immunocohemeis, 18-8816-33 and 18-8817-33; 1:1,000). Primary antibodies used for immunofluorescence and PLA were anti-collin (Cell Signaling Technology, 14168; 1:800), anti-SRSF2/SC35 (Abcam, ab11826; 1:1,000), anti-Son (Abcam, ab109472; 1:200), anti-HA epitope tag (GeneTex, 18181; 1:1200; or Sigma, H9098; 1:1,000 or 1:1,200 for PLA), rabbit anti-μ2 antisera (82) (1:1,000), and a mix of anti-reovirus T1L and anti-reo virus 88 mouse antisera (B. Sherry, unpublished; 1:5,000 each). The secondary antibodies were Alexa Fluor 488- or Alexa Fluor 594-conjugated goat anti-mouse or anti-rabbit IgG (Thermo Fisher Scientific; 1:1,000). The CRM1-inhibitor leptomycin B (LMB; Sigma, L2913) was used at a final concentration of 20 nM in supplemented DMEM. Nocodazole (Sigma, M1404) and paclitaxel (Sigma, T7191) in dimethyl sulfoxide were used at a final concentration of 10 μM in supplemented DMEM. Recombinant mouse interferon-β (PBL Assay Science, catalog no. 12410-1) was used at 1,000 U/ml diluted in supplemented MEM.

SDS-PAGE and immunoblotting. Whole-cell protein extracts were obtained 2 days postplating using radiolabeled protein assay (RIPA) lysis buffer (50 mM Tris HCl [pH 7.4], 1% NP-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA) supplemented to contain 1% sodium dodecyl sulfate

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Contaminating DNA was removed, and an aliquot of the RNA was converted to cDNA for qRT-PCR. After 5 h of incubation, the overlays were removed, and the total RNA was harvested, treated with RNase-free DNase I, and subjected to SDS-PAGE and immunoblotting as described above.

In situ proximity ligation assay. Transfected cells in poly-L-lysine-coated chamber slides were fixed with 4% paraformaldehyde, permeabilized in 0.25% Triton X-100, blocked with Duolink blocking solution, and probed using a Duolink PLA kit (Sigma, catalog no. DUO92101). Dried slides were mounted on Duolink mounting medium with DAPI and imaged as z-stacks with confocal microscopy.

Quantitative (real-time) reverse transcription-PCR. For confirmation of the MISO results, oligonucleotide primers were designed to amplify transcripts that either skipped or spliced-in a specific exon, identified by RNA-seq. The primers were as follows: RanBP3 skipped exon (forward, 5'-CGCGTGTCTCTCTCCGTGAGTA-3'), RanBP3 spliced-in exon (forward, 5'-CGACCCGTGTCTTTGGTTT-3'), reverse, 5'-AAAAATGACCCTCATCAAA-3'), SRSF3 spliced-in exon (forward, 5'-TGAACGTACGAGGAGGATC-3'), reverse, 5'-GACCACCCGACCGGCTGATT-3'), and SRSF3 spliced-in exon (forward, 5'-GATTACCCGACGGAGGATC-3'), reverse, 5'-TGACGCTGAAAGGGCTAGTT-3'). The total RNA was harvested using an RNAeasy kit (Qiagen, Inc.), treated with RNase-free DNase I (Qiagen, Inc.), and then probed using a Duolink PLA kit (Sigma, catalog no. DUO92101). Dried slides were mounted on Duolink mounting medium with DAPI and imaged as z-stacks with confocal microscopy.

Viral replication and cytopathic effect in siRNA-transfected cells. L929 cells were transfected with siRNA as described above and infected 2 days later at the indicated multiplicity of infection (MOI). The siRNA-mediated depletion of SRSF2 was maintained throughout the experiment (data not shown). Viral replication and cytopathic effect were assessed by plaque assay (84) and MTT assay (27).

Confocal microscopy and image analysis. A Zeiss LSM 710 confocal microscope equipped with a 40× C-Apochromat/1.1 NA water immersion objective from the Cellular and Molecular Facility at NC State University was used for all experiments. The pinhole diameter was maintained at 1 Airy unit, and all images were obtained using multitrack sequential scanning for each fluorophore to prevent bleed-through. The excitation/emission wavelengths during micrograph acquisition were as follows: 488 nm/492 to 554 nm for Alexa Fluor 488, 561 nm/584 to 666 nm for Alexa Fluor 594 and PLA Duolink Red, and 405 nm/407 to 507 nm for DAPI. The images were processed for presentation using Photoshop CS4. Intensity plot profiles were generated using ImageJ software (82). Quantification of in situ PLA signals per cell was performed using the particle analysis tool of ImageJ.

RNA-seq. L929 cells were infected at an MOI of 100 PFU per cell, and at 20 h postinfection overlays were replaced with supplemented media or mouse IFN-β diluted in supplemented medium to 1,000 U/ml. After 5 h of incubation, the overlays were removed, and the total RNA was harvested, the contaminating DNA was removed, and an aliquot of the RNA was converted to cDNA for qRT-PCR. Total RNA samples were submitted to the North Carolina State University Genomic Sciences Laboratory (Raleigh, NC) for Illumina RNA library construction and sequencing. mRNA was purified using oligo(dT) beads (NEBNext poly(A) mRNA magnetic isolation module [New England BioLabs, USA]), chemically fragmented, and primed with random oligonucleotides for first-strand cDNA synthesis (NEBNext Ultra Directional RNA library prep kit [NEB] and NEBNext Multiplex Oligos for Illumina [NEB]). Second-strand cDNA synthesis was carried out with dUTPs to preserve strand orientation information. Double-stranded cDNA was“A-tailed” for adapter ligation and selected for a final size of 250 to 400 bp, including adapters (AMPure XP bead isolation; Beckman Coulter, USA). Library enrichment was performed, and indexes were added during PCR amplification. Libraries were pooled in equimolar amounts and sequenced on an Illumina NextSeq 500 DNA sequencer, and real-time analysis was used to generate raw base call files, which were then demultiplexed by sample into fastq files.

Annotation and clustering of changes in alternative splicing during reovirus infection. Only those reads with a minimum of 50 bp were used, and all reads were trimmed to 50 bases (fastx_trimmer tool [http://hannonlab.cshl.edu/fastx_toolkit/]). Reads were mapped to the mm10 mouse reference genome using TopHat2 (85), and the results merged to create a single bam file for each sample. Picard
Supplemental Material

Supplemental material for this article may be found at https://doi.org/10.1128/JVI.02488-16.

DATA SET S1, xlsx file, 0.01 MB.
DATA SET S2, xlsx file, 0.01 MB.
DATA SET S3, xlsx file, 0.01 MB.

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