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## Advances in analyzing viral-induced alterations of host-cell splicing

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### Keywords

Alternative splicing

Virus-host interaction

Genome-wide transcriptomics

Systems biology

35 **Abstract**

36 Alteration of host cell splicing is a common feature of many viral infections which is  
37 underappreciated because of the complexity and technical difficulty of studying alternative  
38 splicing regulation. Recent advances in RNA sequencing technologies revealed that up to  
39 several hundreds of host genes can show altered mRNA splicing upon viral infection. The  
40 observed changes in alternative splicing events can either be a direct consequence of viral  
41 manipulation of the host splicing machinery or result indirectly from the viral-induced innate  
42 immune response or cellular damages. Analysis at a higher resolution with single-cell  
43 RNAseq and a higher scale with the integration of multiple omics datasets in a systems  
44 biology perspective will be needed to further comprehend this complex facet of virus-host  
45 interactions.

46

47

## 48 **Alteration of cellular splicing: a complex facet of virus-host interactions**

49 In higher eukaryotic cells most genes are transcribed as precursor messenger RNAs (pre-  
50 mRNAs) that undergo splicing, a maturation process through which RNA sequences (introns)  
51 are removed and the remaining sequences (exons) are ligated together. Splicing occurs in  
52 the nucleus and is catalyzed by the spliceosome, a large and highly dynamic  
53 ribonucleoprotein complex [1, 2]. Most mammalian pre-mRNAs are subject to alternative  
54 splicing (AS), and human genes contain on average 8.8 exons and 7.8 introns per gene, giving  
55 rise to an average of 3.4 alternatively spliced isoforms [3, 4]. The most common types of AS  
56 events are the use of alternative donor and acceptor splice sites, exon skipping, alternative  
57 use of mutually exclusive exons, and intron retention. Alternative splicing expands the  
58 diversity of proteins that can be expressed from a given gene, and can also modify cis-  
59 regulatory elements that govern the stability and translation of mRNAs. In recent years,  
60 head-to-tail back-splicing events that result in the formation of non-coding circular RNAs  
61 (circRNAs) have also been observed to play key regulatory roles in a variety of biological  
62 processes, including antiviral immunity [5, 6]. Splicing is tightly coupled to transcription, and  
63 is controlled by cis- and trans-acting elements as well as through chromatin structure and  
64 signalling pathways [3, 5, 7]. The advent of high-throughput RNA sequencing technologies  
65 (RNAseq) has opened up a new era in studying how AS is regulated and shapes the cellular  
66 proteome in response to changes in environmental conditions (e.g. [8]).

67 Viruses modulate host gene expression in order to favor viral replication and evade antiviral  
68 responses. They have evolved mechanisms to affect cellular transcription, mRNA processing  
69 and nuclear export, mRNA decay, and translation [9-11]. RNAseq, proteomic and  
70 interactomic studies are now beginning to provide a global view of viral-induced alterations  
71 of cellular splicing and insights into how they may impact viral pathogenesis. Herein, we  
72 review the recent developments in the field, we discuss how current limitations could be  
73 overcome in the future, and what advances may be expected from the integration of splicing  
74 isoforms datasets into a systems biology perspective.

75

## 76 **Viral manipulation of the host splicing machinery**

77 Viruses that replicate in the nucleus of infected cells and gain access to the splicing  
78 machinery (e.g. adenoviruses, herpesviruses, and influenza viruses) have evolved an

79 expansion of their coding capacity by producing spliced viral mRNAs. However, manipulation  
80 of the host splicing machinery is not exclusive to nuclear viruses and has also been observed  
81 with viruses that replicate in the cytoplasm such as picornaviruses or flaviviruses (Table 1).  
82 This can be accounted for by the nucleo-cytoplasmic shuttling of some viral proteins (e.g. the  
83 dengue virus NS5 protein [12]) and splicing factors (e.g. SR proteins [13]), increased nuclear  
84 permeability upon viral infection [14] or signaling pathways triggered by viral infection [15].  
85 Components of the splicing machinery commonly targeted across different virus families are  
86 the small nuclear ribonucleoproteins (snRNPs), serine/arginine-rich (SR) proteins, and  
87 heterogeneous nuclear ribonucleoproteins (hnRNPs). The U1 to U6 snRNPs are core  
88 components of the spliceosome, whereas SR and hnRNP proteins are involved in the  
89 regulation of constitutive and alternative splicing. Different viruses appear to induce similar  
90 types of alterations, *i.e.* changes in the level of expression, protein-protein or protein-RNA  
91 interaction pattern, localization, phosphorylation and/or intrinsic activity of splicing factors  
92 (Table 1).

93 Among the genes that appear differentially expressed upon viral infections in transcriptomic  
94 and proteomic datasets, the overall enrichment of splicing-related genes is generally not  
95 reported. However, particular splicing factors can undergo significant changes in expression  
96 upon viral infection. As an example, expression of EFTUD2, a U5-snRNP associated factor,  
97 was found to be decreased upon HCV infection of cultured cells and in liver samples from  
98 HCV-infected patients; downregulation of EFTUD2 impairs the splicing of RIG-I and MDA5  
99 pre-mRNAs and therefore enables the virus to circumvent the innate antiviral response [16].  
100 Phosphoproteomic profiling provides an additional layer of information by revealing viral-  
101 induced changes in the phosphorylation status of host proteins. Dynamic  
102 phosphorylation/dephosphorylation is known to regulate the function of splicing factors,  
103 most notably SR proteins [17]. HIV-1 entry triggers early changes in the phosphorylation of  
104 five SR proteins, including SRRM2 which regulates the splicing of HIV-1 transcripts [18]. Later  
105 in infection there is evidence that the HIV-1 protein Vpr modulates the activity of SR protein-  
106 specific kinases and the phosphorylation of SRRM2 [19]. In the pre-omics era, the  
107 herpesvirus ICP27, adenovirus E4-ORF4 and papillomavirus E1<sup>^</sup>E4 proteins have also been  
108 reported to regulate SR proteins phosphorylation in order to facilitate their replication [20-

109 22] (Table 1). Altogether these findings point to changes in SR phosphorylation as a  
110 mechanism commonly triggered by multiple viruses to co-opt the splicing machinery.  
111 Interrogation of the VirHostNet 2.0 public database dedicated to virus-host protein-protein  
112 interactions (PPIs) [23] reveals that a large proportion of known splicing factors have at least  
113 one reported interaction with a viral protein, pointing to the spliceosome as a frequent viral  
114 target (Figure 1A and 1B). Most of the data derive from a yeast two-hybrid or affinity-  
115 purification mass spectrometry screen with no systematic experimental validation of the  
116 interactions and therefore cannot be assumed *a priori* as high confidence data [14]. However  
117 data integration helps reveal splicing factors which are found to be associated with multiple  
118 viral species and represent potentially relevant targets for onward studies, e.g. DDX5 and  
119 FUS (Figure 1C). Some PPIs have been identified in low throughput studies and are well  
120 documented such as the recently identified interaction between the reovirus protein  $\mu 2$  and  
121 the SR protein SRSF2, which alters SRSF2 function and determines the virus ability to  
122 counteract the interferon response [24]. Additional PPIs and associated effects on the host  
123 splicing machinery are listed in Table 1. Furthermore, viral RNAs can act on the splicing  
124 machinery, as exemplified by the changes in AS events observed upon sequestration of the  
125 HuR protein by the 3'UTR of Sindbis virus [25], (Table 1).

126

### 127 **Peering into viral-induced alterations of host alternative splicing events**

128 To date there are only a few studies that performed transcriptome-wide micro-array or  
129 RNAseq analysis of cellular AS events in virus-infected cells (Table 2). They concern  
130 herpesviruses [26-28], reoviruses [24, 29], dengue viruses (DENV) [12, 30], Zika virus [31]  
131 and influenza viruses [32], and reveal several hundreds of host genes that show altered  
132 mRNA splicing upon infection. When examined, no correlation was found between changes  
133 in AS events and changes in mRNA expression levels. Validation by RT-PCR or RT-qPCR of a  
134 selected subset of the predicted differential AS events is compulsory and usually performed  
135 to sort out and confirm RNAseq findings. Validation rates can only be determined if a large  
136 enough number of AS events is assayed, and are likely to depend on the metrics and  
137 statistical analysis that are used. In the few studies which provide GO terms enrichment  
138 analysis on the list of differentially spliced genes in virus-infected cells, an enrichment in  
139 genes related to cell cycle, gene expression and/or RNA metabolism was reported [24, 27,

140 30-32]. Consistently with previous reports that the AS landscape varies between human  
141 tissues [33], neural progenitor cells infected with the human cytomegalovirus (HCMV)  
142 exhibited fewer AS changes compared to fibroblasts and only half of altered exon-skipping  
143 overlapped in both cell types [26]. Exon-skipping, which is most frequently detected in the  
144 human transcriptome [33] accounts for the largest share of infection-altered AS events in all  
145 except the DENV5 study [12]. Interestingly intron retention (IR) which, although identified  
146 early on in spliced viral mRNAs, was until recently considered a rare event in mammalian  
147 cells, was found to represent a substantial proportion (> 20%) of infection-altered AS events  
148 in herpes simplex virus-1 (HSV-1) and DENV5-infected cells [12, 27]. These observations align  
149 with recent findings that IR is actually a common AS event in mammalian cells, although the  
150 fate of intron-retaining mRNAs regarding nucleo-cytoplasmic export or nonsense mediated  
151 decay is not fully understood [34]. Taken together with a recent report that influenza virus  
152 NS1 protein primarily binds intronic sequences [35], they suggest that some viruses may  
153 have evolved specific mechanisms to alter host cell expression through increased IR. Finally,  
154 within the last couple of years the first reports of viral-induced alterations of cirRNAs were  
155 published. Notably, cirRNAs were found to be expressed and associated with the  
156 NF90/NF110 factor at lower levels upon Vesicular Stomatitis Virus (VSV) infection, which  
157 results in increased NF90/NF110 binding to viral mRNAs in the cytoplasm and thereby likely  
158 contributes to the antiviral immune response [36]. A global dysregulation of circRNAs was  
159 observed upon infection with HSV-1, which could potentially modulate the cellular  
160 transcriptional responses through the miRNA sponge function of circRNAs [37].

161 Splicing alterations observed upon infection potentially result from the combination of two  
162 types of mechanisms: i) they can be caused by a viral manipulation of the splicing machinery,  
163 as described above; or ii) they can be related to viral-induced cellular damages or innate  
164 immune responses (Figure 2). Indeed, there is increasing evidence that AS is a mechanism to  
165 regulate the immune responses to pathogens [38-40] (Box 1), as well as apoptosis [41], DNA  
166 damage response [42] and endoplasmic reticulum stress response [43]. Integration of multi-  
167 omics data will probably be key to distinguishing between direct and indirect effects of viral  
168 infection on the cellular splicing machinery and uncovering of the complex mechanisms by  
169 which the host-cell splicing landscape is modified (see below).

170 In some instances, differences in viral-induced splicing changes were reported when viral  
171 variants were compared. Reovirus strain T1L, compared to strain T3D which differs in that its  
172  $\mu 2$  protein does not localize to nuclear speckles, triggered more splicing changes *i.e.* 369  
173 compared to 142, with an overlap of only 35 changes. The cellular processes which were  
174 most affected by T1L infection, *i.e.* gene expression and RNA post-transcriptional  
175 modification, were not strongly affected by T3D infection [24]. Comparison of a subset of AS  
176 events upon infection with DENV2 and DENV4 revealed differences, suggesting a possible  
177 serotype specificity in AS alteration [12]. However the molecular mechanisms that lead to  
178 specific alterations of the splicing landscape remain largely unknown. The only evidence for  
179 a sequence recognition mechanism comes from Tang et al. who showed that the ICP27  
180 protein of HSV-1 mediates splicing alterations in genes that are GC-rich, with suboptimal  
181 splicing sites and cytosine-rich sequences close to the 5' splice site [44]. The presence of a  
182 conserved 41 nucleotide motif was observed in 93 out of 240 AS events that were  
183 dysregulated upon infection with the reovirus strain T3D [29], however this motif does not  
184 correspond to a predicted RNA regulatory motif and the significance of this observation  
185 remains to be demonstrated.

186 Importantly, transcriptomics studies revealed that not only splicing but also other features of  
187 cellular mRNAs can be altered upon viral infection. For instance, DENV1 was found to induce  
188 alterations in the relative usage of transcriptional start sites, in addition to splicing changes  
189 [30]. Infection with herpesviruses was shown to trigger widespread disruption of  
190 transcription termination of cellular mRNAs [26-28], which in the case of HCMV infection  
191 was attributed to a strong induction of the host RNA-binding protein CPEB1 [26].  
192 Transcription extends over thousands of nucleotides beyond poly(A) sites and into  
193 downstream genes, it interferes with the analysis of transcriptional and splicing regulation of  
194 the downstream gene and can generate novel intergenic splicing between exons of two  
195 neighbouring genes [28]. Defective transcription termination of cellular mRNAs was also very  
196 recently reported in influenza virus-infected cells [45-47]. While most studies provide  
197 information about the steady-state levels of mRNA isoforms, a few of them rely on the NET-  
198 Seq method [45] or on 4sU tagging of mRNAs [28] to provide a real-time view of viral-  
199 induced changes in co-transcriptional RNA processing. As these methods allow to detect  
200 actively or newly transcribed mRNAs, they can also help avoid biases due to isoform-specific



201 differences in the stability of mRNA. Transcriptomic analysis and mechanistic understanding  
202 of how viral infection impacts cellular mRNA co- and post-transcriptional processing is an  
203 expanding field, constantly evolving in response to progress in cellular biology (e.g. the  
204 recent findings on the biogenesis and function of circRNAs) and in technologies (as discussed  
205 below).

206

### 207 **Towards a more accurate view: methodological challenges**

208 RNAseq transcriptomic analysis of AS is a challenging issue, and even more so when  
209 performed on a virus-host system. Accurate quantification of isoform abundance requires a  
210 high read number (about 50 millions paired-end reads of at least 75 bp are recommended  
211 for the human transcriptome), which to date can only be provided by the Illumina  
212 technology. However a serious limitation of Illumina RNAseq is that it relies on short reads,  
213 so that the resolution of exon connectivity and full-length isoform structure cannot be  
214 achieved (Box 2). Upon viral infection the reads mapping to viral mRNAs may represent 5-  
215 75% of the total number of reads (Table 2), therefore the sequencing depth of virus- and  
216 mock-infected samples must be adjusted accordingly. The degree of variability of AS viral-  
217 induced perturbations also need to be taken into account when setting the sequencing  
218 depth and the number of biological replicates, in order to differentiate biologically relevant  
219 changes from transcriptional noise.

220 Many viruses induce host-cell transcription shut-off [10] although specific genes (e.g. IFN-  
221 stimulated genes, cytokines) can escape this shut-off and be up-regulated. Marked  
222 differences between the gene expression profiles of mock- and virus-infected cells poses a  
223 challenge for accurate comparison of isoform abundance. Commonly used RNAseq  
224 normalization methods [48] make the assumption that a core set of genes is not  
225 differentially expressed and mainly correct for sequencing depth. Normalization methods  
226 taking into account other variability factors (e.g. the average amount of cellular RNA per cell)  
227 besides sequencing depth for host-pathogen dual RNAseq experiments need to be  
228 developed [49].

229 Another methodological challenge of viral-host RNAseq lies in the ability to deal with cell-to-  
230 cell heterogeneity during infection. Usually high multiplicities of infection are used and it is  
231 verified or assumed that almost all cells are infected. However, single-cell RNAseq

232 (scRNAseq) experiments revealed extensive variability from one individual cell to another in  
233 terms of production of intracellular viral RNAs and cellular responses [50-52]. RNAseq  
234 experiments on polyadenylated mRNAs or circRNAs extracted from bulk cell cultures or  
235 tissues are providing an average measure of isoform abundance; they are potentially  
236 masking heterogeneity that occurs in the dynamics of infection and/or specific  
237 transcriptomic profiles in a subset of cells which could be relevant to the viral phenotype.  
238 Single cell RNAseq can be used for transcriptome-wide differential splicing isoform  
239 quantification, however this requires specific bioinformatics tools and normalization  
240 procedures to be developed, to cope with the low reads counts, the heterogeneity and noise  
241 in the datasets [53-55].

242 So far, most RNAseq analyses of splicing events in virus-infected cells have been conducted  
243 with cancer cell lines and/or viral laboratory strains. Such experimental conditions have  
244 practical advantages (ready availability, ability to infect almost 100% of the cells, and higher  
245 reproducibility), however they may not accurately reflect physiological infection. The  
246 development of scRNAseq, which allows discrimination between infected and uninfected  
247 cells, will probably encourage the use of field viral isolates and more relevant cellular  
248 systems such as primary cells or tissue explants. Novel perspectives will be opened by the  
249 use of induced human pluripotent cells (iPS) which upon differentiation can serve as  
250 convenient surrogates for primary cells that are difficult to isolate and culture [56], and 3D  
251 organoid cultures which can provide an accurate model for the micro-anatomy of an organ  
252 [57].

253

#### 254 **Integration of isoforms datasets in a systems biology perspective**

255 The rapid advancement of high-throughput technologies has led to the development of the  
256 systems biology field, which aims at modeling the properties of complex biological systems,  
257 and predicting their response to biological or chemical perturbations. The commonly used  
258 “top down” systems biology approach turns RNAseq measurement of expression levels into  
259 a variable which can be included in a mathematical model, such as a generalized linear  
260 model or a multivariate analysis (Principal Component Analysis, Singular Value  
261 Decomposition, Partial Least Square). The variance across genes or isoforms, samples and  
262 conditions is computed to identify statistically significant transcriptomics signatures, *i.e.*

263 particular combinations of thousands of genes or isoform expression levels. The mechanistic  
264 interpretation of these signatures and the prioritization of candidate genes for downstream  
265 experimental validation remain challenging tasks. However, the use of transcriptomic  
266 signatures has already allowed genetically close viral strains to be robustly distinguished  
267 [58], species-specific responses to infection outlined [59] and the outcome of viral infections  
268 predicted [60]. It can also pave the way towards the discovery of relevant biomarkers [61].  
269 Transcriptomic signature analysis has become a routine at the gene level, but little has been  
270 done so far at the isoform-level. Recent methodological developments - such as splicing  
271 signature comparison workflows for the discovery of candidate splicing regulatory elements  
272 - have been applied to psoriasis studies [62, 63] and could be transferred to the analysis of  
273 infected-cells in the future. Further progress in the field will also be enhanced by the  
274 accessibility and the integration of published RNAseq datasets within open access  
275 knowledge bases (e.g. ArrayExpress, Gene Expression Omnibus), which allows meta-analyses  
276 to be conducted.

277 The integration of RNAseq datasets with other omics datasets, so far mostly proteomics,  
278 interactomics and metabolomics datasets, has become a new way to rationalize the  
279 deconvolution of the transcriptomics signal, and has been applied to virus-infected cells [64-  
280 66] or patients [67]. To facilitate this integration step, network-based methods such as  
281 correlation network (e.g. WGCNA) or probabilistic models (e.g. MERLIN) are currently being  
282 investigated (reviewed in [68]). The main objective is to infer the transcriptional gene  
283 regulatory network and to prioritize a set of candidate genes, transcriptional factors or  
284 functional modules that are involved in viral infection. A growing number of studies combine  
285 newly generated experimental data with published datasets available in open access  
286 databases to identify the most relevant molecular pathways. For example, by combining  
287 proteomics and RNA-seq experimental data along with available gene-regulatory and  
288 protein-protein interaction networks Sychev *et al.* successfully implemented a  
289 computational model based on the Prize-Collecting Steiner Forest algorithm, which  
290 highlighted peroxisome lipid metabolism as an important function involved in KSHV (Kaposi's  
291 sarcoma associated herpesvirus) latent infection [65].

292 One should stress that integration is for now only performed at the gene-level. Gene  
293 annotation knowledge bases (GO, Interpro, and KEGG) are exploited to investigate

294 enrichment of specific biological or molecular functions (GSEA). Although they are  
295 continuously being improved, gene annotations are still far from being complete [69], and  
296 even more so at the isoform level and for non-model organisms. Gene annotations often  
297 relate to a “reference isoform” which is assumed to be the predominant one, whereas the  
298 relative proportion of splicing isoforms may differ from one tissue to another. Alternative  
299 splicing may lead to the gain or loss of functional domains, catalytic sites and/or protein-  
300 protein interfaces. However, the full set of alternative isoforms that effectively contribute to  
301 proteomic diversity and represent “functional alloforms” remains to be characterized [70,  
302 71]. Available Exon Ontology resources can be used to readily identify enriched functions of  
303 gene isoform subsets but they restrict the analysis at the exon-level [72]. The systematic  
304 characterization of AS isoforms function is a challenging task and might be accelerated  
305 through bioinformatics prediction [72-74] or experimentally by using interactomics  
306 approaches [75, 76].

307 As molecular approaches to study virus-host interactions at a high level of mechanistic detail  
308 are also making steady progress, the “bottom up” systems biology approach also seem  
309 promising. In this approach the knowledge generated by the molecular and biochemical  
310 characterization of a subsystem and its response to perturbations is used to generate  
311 specific sub-networks, which can subsequently be integrated together with high-throughput  
312 data into larger molecular interaction networks [77-79]. In the case of the spliceosome  
313 machinery, subnetwork modeling is making progress through Bayesian probability modeling  
314 [80], food-web modeling [81], or deep-learning approaches [82], which offers advanced  
315 tools for studying its involvement and vulnerability upon viral infection. In return, research  
316 on the virus-spliceosome interplay will likely contribute to a better definition of the complex  
317 set of rules that can predict the splicing pattern of each isoform based on a comprehensive  
318 catalogue of cis-regulatory elements and their functional molecular interactions in various  
319 physiological and pathological conditions.

320

### 321 **Concluding remarks**

322 The importance of characterizing the transcriptome landscape of virus-infected cells at the  
323 splicing level is highlighted by recent studies, which reveal significant AS alterations in  
324 response to viral infections. The observed changes in AS events, which are regulated through

325 a very complex protein-RNA interaction network, can either be a direct consequence of viral  
326 manipulation of the host splicing machinery or result indirectly from viral-induced immune  
327 responses or cellular damages. Integration of multiple omics datasets in a systems biology  
328 perspective will be needed to comprehend this complex facet of virus-host interactions.  
329 Beyond proteomics and interactomics, which have been most commonly combined with  
330 RNAseq analysis so far, epigenomics and epitranscriptomics would also be relevant. Indeed,  
331 viral infections may induce epigenetic [83, 84] or epitranscriptomic [85] modifications, and  
332 both types of modifications may in turn affect splicing [86, 87]. Genetic mutations that affect  
333 AS, and consequently may determine phenotypic variability and individual susceptibility to  
334 viral diseases, is also an interesting direction for future research [88]. Species-dependent AS  
335 patterns of cellular genes could possibly be determinant for viral host-range, as suggested  
336 recently for influenza viruses [89]. As technological advances in RNA sequencing and RNA  
337 genomics will allow to study the interplay between cellular AS and viral infections at an  
338 increasing scale and resolution, major challenges in terms of computational analysis and  
339 storage of the corresponding datasets will need to be addressed, and the building of  
340 pluridisciplinary research teams along the lines of the European Virus Bioinformatics Center  
341 (<http://evbc.uni-jena.de>) [90] will be paramount. Integration of splicing isoform datasets  
342 with other omics data may well contribute to the development of personalized prognosis  
343 and management of infectious diseases and lead to therapeutic innovations.  
344

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352

353 **Box 1 : Role of alternative splicing in shaping immune responses.**

354 In the recent years there has been growing evidence for a role of AS in shaping both innate  
355 and adaptative immune responses [38-40]. Different isoforms have been found for key  
356 players of the antiviral innate immunity, including pattern-recognition receptors (e.g. TLRs,  
357 RIG-I, and MDA5), downstream signaling proteins (e.g. MyD88, MAVS, STING, TBK1, and  
358 IRF3), and effectors (IFN Type I, IFNAR, cytokines and chemokines). The splicing factors  
359 involved are known in a few cases : EFTUD2 regulates the splicing of RIG-I, MDA5 and  
360 MyD88 [91], while SF3A1 affects the splicing of several genes of the TLR signaling pathway  
361 including MyD88 [92]. In several instances, splice variants exert a negative feedback loop on  
362 the signaling pathway, thereby probably controlling the intensity and duration of the  
363 antiviral and inflammatory responses. Notably, RIG-I and STING splice variants whose  
364 expression are up-regulated upon viral infection strongly inhibit RIG-I and STING signaling  
365 pathways, respectively [93, 94]. A viral-induced, alternatively spliced isoform of TPK-1  
366 disrupts the interaction between RIG-I and MAVS and inhibits IFN-beta signaling [95]. Short  
367 isoforms of MAVS negatively regulate TLR3-mediated nucleic acid sensing [96], and limit self-  
368 aggregation of the full-length MAVS protein thereby preventing accidental antiviral innate  
369 immune signaling [97]. The contribution of AS to the regulation of humoral and cellular  
370 adaptative immune responses is also clearly recognized, however the mechanisms involved  
371 remain largely unknown. In B cells two mechanisms were recently uncovered : the HuR  
372 protein, by regulating mRNA splicing upon B cell activation, is essential for antibody  
373 response to a variety of antigens [98], while the ZFP318 factor regulates the AS-dependent  
374 balance between IgM and IgD immunoglobulins [99]. In T cells, one of the best documented  
375 examples is the AS of CD45 in response to antigen receptor-mediated signaling, which is  
376 differentially regulated depending on the T cell lineage and the stage of activation (reviewed  
377 in [39, 40]).

378

379

380

381 **Box 2 : Resolving alternative splicing by short- or long-reads sequencing technologies.**

382 Currently Illumina is the most commonly used RNAseq technology for transcriptome-wide  
383 analysis of AS. However Third Generation Sequencing (TGS) technologies, such as the Pacific  
384 Biosciences (PacBio) and Oxford Nanopore (ON) technologies, are emerging as alternative  
385 platforms for AS analysis [100].

386 The major advantage of Illumina over the TGS technologies is its higher sequencing depth  
387 (up to 400 million reads instead of 1 million for TGS), even more so in the case of dual  
388 RNAseq when both the viral and host transcriptome need to be sequenced. High depth is  
389 needed for the detection of minor isoforms and the robust quantification and statistical  
390 analysis of AS events [101]. Another advantage of Illumina resides in its higher sequencing  
391 accuracy (about 0.1% error rates instead of 10-15% for TGS), which is particularly  
392 advantageous when working on poorly annotated genomes of non-model organisms.  
393 However, a major limitation of Illumina is that it generates short reads (75-300 bp) which  
394 provides only local information about AS events and entails a challenging computational  
395 reconstruction of full-length isoforms. The strength of TGS is their read length (1-100 kb),  
396 which allows the direct characterisation of full-length transcripts with information not only  
397 on alternative splicing and the coordination of distant exons [102] but also on alternative  
398 transcription start and termination sites [103]. Moreover long reads allow to resolve  
399 repetitive sequences, that are posing a major challenge for sequence assembly or alignment  
400 from short-read datasets.

401 Future advances may lie in the Hybrid-Seq approach which combines short and long-read  
402 technologies [101], the Synthetic Long-Read technology which exploits the assets of Illumina  
403 with a short-read barcoding system to reconstruct full-length transcripts [104], or the very  
404 swift evolution of long-read technologies. Particularly promising is the perspective of direct  
405 RNA sequencing proposed by ON, which would avoid reverse-transcription- and  
406 amplification-related biases in isoforms quantification [105].

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410 **Table 1. Viral targeting of the splicing machinery.**

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<b>Virus</b>	<b>Viral factor</b>	<b>Cellular Target</b>	<b>Associated cellular changes<sup>a</sup></b>	<b>References</b>
<b>HSV-1</b>	ICP27	Binding to, relocalisation and inhibition of SRPK1	Altered phosphorylation of SR proteins	[22]
<b>HSV-1</b>	ICP27	Binding to SF3B2*	ND	[106]
<b>HIV-1</b>	Vpr	Binding to SF3B2*	Altered splicing of pre-mRNAs	[107]
<b>EBV</b>	SM	Binding to SRSF3	Altered splicing of STAT1 pre-mRNA	[108]
<b>HPV1</b>	E1 <sup>^</sup> E4	Binding to and inhibition of SRPK1	Altered phosphorylation of SR proteins	[21]
<b>HPV16</b>	E2	Transactivation of SRSF1-3 promoters	ND	[109]
<b>Adenovirus</b>	E4-ORF4	Binding to SRSF1, SRSF9	Modulation of pre-mRNA splicing	[20]
<b>Influenza V</b>	NS1	Binding to U6snRNA	Inhibition of pre-mRNA splicing	[110]
<b>Influenza V</b>	NS1	Relocalisation of SRSF2	ND	[111]
<b>Influenza V</b>	NS1	Binding to and relocalisation of NS1-BP	Altered splicing of some pre-mRNAs regulated by NS1-BP	[112, 113]
<b>Poliovirus</b>	2A	Relocalisation of HuR, TIA-1, TIAR	Modulation of Fas6 pre-mRNA splicing	[114]
<b>Poliovirus</b>	2A	Relocalisation of SRSF3	ND	[115]
<b>EV-71</b>	3D (Pol)	Binding to and inhibition of PRPF8**	Blockage of pre-mRNA splicing and mRNA synthesis	[116]
<b>FMDV</b>	3C (Pro)	Cleavage and relocalisation of Sam68	ND	[117]
<b>Reovirus</b>	μ2	Binding to and relocalisation of SRSF2	Altered splicing of pre-mRNAs	[24]
<b>Rotavirus</b>	NSP2, NSP5	Association with and relocalisation of hnRNPs and HuR	ND	[118]
<b>Sindbis V</b>	nsP2	Binding to hnRNPK	ND	[119]
<b>Alphaviruses</b>	3' UTR	Binding to and relocalisation of HuR	Altered stability, splicing and polyadenylation of mRNAs	[25, 120]
<b>HCV</b>	3'UTR	Binding to and relocalisation of HuR	ND	[121]
<b>DENV-1</b>	NS5 protein	Binding to CD2BP2 DDX23**	Altered pre-mRNA splicing in vitro	[12]
<b>VSV</b>	M	Relocalisation of hnRNPH	ND	[122]

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<sup>a</sup>ND: not determined, \* component of the U2 snRNP, \*\* component of the U5 snRNP,

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**Table 2. Transcriptome-wide analyses of AS in virus-infected cells.**

	Rutkowski et al. 2015 [28]	Hu et al. 2016 [27]	Batra et al. 2016 [26]	Boudreault et al. 2016 [29]	Rivera-S et al. 2017 [24]	Sessions et al. 2013 [30]	De Maio et al. 2016 [12]	Hu et al. 2017 [31]	Fabozzi et al. 2018 [32]
<b>Virus</b>	HSV-1	HSV-1	HCMV	Reovirus	Reovirus	Dengue V	Dengue V	Zika V	Influenza V
<b>Host-cell model<sup>a</sup></b>	Human Fibroblasts	Human Fibroblasts	Human Fibroblasts & NPC	Murine L929 cells	Murine L929 cells	Human HuH7 cells	Human A549 cells	Human NPC	Human BEAS-2B cells
<b>Number of replicates</b>	n=2	n=3 pooled	n=1	n=3	n>1 pooled	n=3	n=3	n=2	n=3
<b>Sequencing library</b>	ribo-depleted	polyA+	polyA+	polyA+	polyA+	polyA+	polyA+	polyA+	ribo-depleted
<b>Sequencing platform</b>	HiSeq 2500	HiSeq 2000	HiSeq	HiSeq 2000	NextSeq 500	HiSeq 2000/GA II x	HiSeq 4000	NextSeq 500	HiSeq 2000
<b>Reads features<sup>a</sup></b>	2 x 101 nt	2 x 90 nt	1 x 101 nt	2 x 100 nt	2 x 50 nt	2 x 75 nt	2 x 90 nt	2 x 75 nt	2 x 50 nt
<b>Sequencing depth</b>	> 25 x 10 <sup>6</sup> reads	~ 26 x 10 <sup>6</sup> reads*	~ 130-230 x 10 <sup>6</sup> reads	> 40 x 10 <sup>6</sup> reads	NA	~ 20-100 x 10 <sup>6</sup> reads	> 20 x 10 <sup>6</sup> reads	~ 7 x 10 <sup>6</sup> reads	~ 30 x 10 <sup>6</sup> reads
<b>Fraction of viral reads<sup>b</sup></b>	27%	30%	22-68%	NA	NA	NA	30-40%	NA	~5-75%**
<b>Mapping</b>	Context Map	TopHat2	GSNAP	Bowtie2	TopHat	TopHat	TopHat2	TopHat2	TopHat2
<b>Gene expression analysis</b>	RPKM	Cufflinks	RPKM	RSEM	NA	Cufflinks	NA	Cufflinks	DESeq2
<b>Alternative splicing analysis</b>	Home-made scripts	Cufflinks ASD DaPars	Olego Quantas MISO	RSEM	MISO	Cufflinks MISO	ASpli edgeR	Cufflinks ASD	MISO
<b>Data availability<sup>b</sup></b>	GSE59717	NA	GSE74250	GSE81017	NA	NA	GSE84285	GSE78711	GSE61517

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<sup>a</sup> NPC: Neural Progenitor Cells

<sup>b</sup> nt: nucleotide

<sup>c</sup> NA: not available

\* number of reads for the pooled samples, \*\* depending on the viral strain and time-point

426 **Figure legends.**

427

428 **Figure 1. Virus-spliceosome protein-protein interaction network.**

429 **A.** Summary of virus-spliceosome protein-protein interactions, as recorded in the VirHostNet  
430 database, January release 2018 [23]. For each viral family, the number of viral species (as  
431 defined by the International Committee on Taxonomy of Viruses and the National Center for  
432 Biological Information or NCBI) and viral proteins (as defined by the NCBI Reference  
433 Sequence and the UniProt databases) reported to interact with at least one cellular  
434 spliceosomal factor (among the list of 244 factors defined in [123]) is indicated. The number  
435 of viral proteins obtained upon merging of homologous proteins derived from different  
436 strains or isolates of a single viral species, grouped thereafter under the name “viral proteins  
437 types” and represented as a single node in B, are indicated into brackets.

438 **B and C.** Interaction network between viral proteins (B) or viral species (C) and cellular  
439 spliceosomal proteins. The network was built with Cytoscape (version 3.2.1). Nodes and  
440 edges between nodes represent protein and protein-protein interactions, respectively. Core  
441 and regulatory spliceosomal factors are represented by square and triangular black nodes,  
442 respectively. **B.** Viral proteins types, as defined in A, are represented by colored nodes (the  
443 color code is according to the Table in A). The size of the viral protein nodes is proportional  
444 to their degree of connectivity (i.e. the number of interacting partners of a protein) and the  
445 layout is done according to their centrality in the network. **C.** Viral species are represented  
446 by colored nodes (the color code is according to the Table in A). The size of the spliceosomal  
447 factor nodes is proportional to their degree of connectivity and the layout is done according  
448 to their centrality. The five spliceosomal factors showing the highest degree of connectivity  
449 (interaction with 11 to 15 distinct viral species) are indicated with a white star and edges  
450 representing their protein-protein interactions with viral species are highlighted in red.

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452 **Figure 2. Direct and indirect mechanisms for viral-induced splicing alterations.**

453 Red arrows represent viral manipulation of the splicing machinery and the resulting AS  
454 changes, which have in turn the potential to modulate innate immunity [16, 95] or apoptosis  
455 [124]. Blue arrows represent viral-induced cell damages and innate immune responses and  
456 the subsequent AS changes in the infected cell [40-43]. Grey arrows represent viral-induced

457 B- and T-cell immune responses, which are subject to AS-mediated regulation [38-40]. UPR:  
458 Unfolded Protein Response. DDR : DNA Damage Response.  
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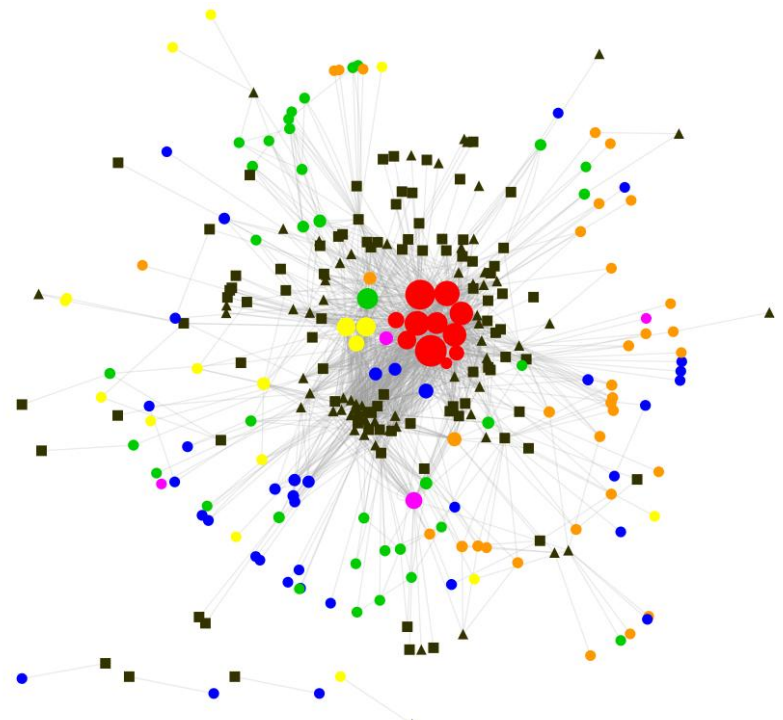
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905

**A**

Viral family	Viral species	Viral proteins (non-redundant)
Papillomaviridae	9	34(34)
Retroviridae	8	17(17)
Herpesviridae	5	49(36)
Flaviviridae	4	9(4)
Togaviridae	3	3(3)
Paramyxoviridae	2	4(3)
Polyomaviridae	2	3(3)
Coronaviridae	2	2(2)
Parvoviridae	2	2(2)
Rhabdoviridae	2	2(2)
Orthomyxoviridae	1	27(11)
Poxviridae	1	13(12)
Filoviridae	1	2(2)
Pneumoviridae	1	2(2)
Adenoviridae	1	1(1)
Circoviridae	1	1(1)
Hepadnaviridae	1	1(1)
Peribunyaviridae	1	1(1)
Phenuiviridae	1	1(1)
Reoviridae	1	1(1)
<b>Total</b>	<b>49</b>	<b>175(139)</b>

**B**



**C**

