Core splicing architecture and early spliceosomal recognition determine microexon sensitivity to SRRM3/4

Sophie Bonnal[1,*], Simon Bajew[1], Rosa Martinez Corral[1] and Manuel Irimia[1,2,3,4,*]

1. Centre for Genomic Regulation (CRG), The Barcelona Institute of Science and Technology, Barcelona, Spain
2. Universitat Pompeu Fabra (UPF), Barcelona, Spain
3. ICREA, Barcelona, Spain
4. Lead contact

* corresponding authors

Sophie Bonnal
Centre for Genomic Regulation
Dr. Aiguader, 88, 08003 Barcelona, Spain
e-mail: sophie.bonnal@crg.eu
Phone: +34933160215 Fax: +34933160099

Manuel Irimia
Universitat Pompeu Fabra
Dr. Aiguader, 88, 08003 Barcelona, Spain
e-mail: mirimia@gmail.com
Phone: +34933160189 Fax: +34933160099

ABSTRACT

Microexons are essential for proper functioning of neurons and pancreatic endocrine cells, where their inclusion depends on the splicing factors SRRM3/4. However, in pancreatic cells, lower expression of these regulators limits inclusion to only the most sensitive subset among all neuronal microexons. Although various *cis*-acting elements can contribute to microexon regulation, how they determine this differential dose response and high or low sensitivity to SRRM3/4 remains unknown. Here, Massively Parallel Splicing Assays probing 28,535 variants show that sensitivity to SRRM4 is conserved across vertebrates and support a regulatory model whereby high or low microexon sensitivity is largely determined by an interplay between core splicing architecture and length constraints. This conclusion is further supported by distinct spliceosome activities in the absence of SRRM3/4 and by a mathematical model that assumes that the two types of microexons differ only in their efficiency to recruit early spliceosomal components.

Keywords

Microexon, core spliceosome, Massively Parallel Splicing assays

INTRODUCTION

Tissue specific alternative splicing plays a central role in transcriptome and proteome specialization. A set of RNA binding proteins exhibits tissue specific expression patterns and regulates diverse programs of alternative splicing that are instrumental for the development and functioning of specific cell and tissue types[1–3]. One prominent example is that of the paralogous proteins SRRM3 and SRRM4, which are highly expressed in neurons and, in the case of SRRM3, also but to a lesser extent in pancreatic endocrine cells[4]. Both proteins trigger the tissue-specific and dose-dependent inclusion of a highly conserved class of short exons, known as microexons, ranging from 3 to 27 nucleotides (nts)[4–6]. By expressing increasing levels of either SRRM3 or SRRM4, we have previously identified two types of microexons based on their sensitivity to SRRM3/4 regulation. Interestingly, because of their different sensitivity, the low expression of SRRM3 in pancreatic islets is sufficient to drive the inclusion of a subset of highly sensitive (HS) microexons in that tissue, while a higher expression is required to trigger the inclusion of the lowly sensitive (LS), neuronal-specific microexons[4,7]. Despite the functional relevance of the differential sensitivity of microexons to SRRM3/4, the *cis*-regulatory determinants of this sensitivity have not been investigated.

Splice sites are core splicing elements involved in recruiting the spliceosome in a stepwise and dynamic manner. In the early stages of spliceosome assembly, specifically during E complex formation, the 5′ splice site (5′ss) recruits the U1 snRNP through base-pairing interactions between the 5′ end of the U1 snRNA and the 5′ss. Simultaneously, the 3′ splice site (3′ss) mediates the recruitment of the heterodimer U2AF (composed of U2AF1 and U2AF2, associated to the dinucleotide AG and polypyrimidine tract (Py), respectively) and SF1, bound to the branch point (BP), another key core splicing element. The U2 snRNP is subsequently recruited to the BP through base-pairing interactions between the U2 snRNA and the BP, resulting in the formation of the A complex. Later in the process, the tri-snRNP (U4/U6/U5) is recruited. Multiple RNA-RNA, RNA-protein and protein-protein interactions undergo dynamic rearrangements leading to the formation of the catalytic spliceosome, which ultimately orchestrates the removal of introns and the ligation of exons[8].

Compared to other exons, SRRM3/4-dependent microexons exhibit distinct features in relation to their adjacent splice sites. They have stronger 5′ss, i.e. more complementary to the 5′ end of U1 snRNA and more similar to the species' consensus sequence. They also have stronger Py tract and BP signals, but these are located farther away from the AG dinucleotide, which results in a relatively weak 3′ss context[9,10]. This provides space for the accommodation of an intronic splicing enhancer (ISE) composed of a bipartite motif UCU_CUC[N1-50]_UGC capable of binding either positive or negative *trans*-acting regulators of microexon splicing, including SRRM3/4 (associated to UGC), SRSF11, U2AF2 and PTBP1, among others[9,11–18]. The contribution of those *cis*-regulatory elements to

SRRM3/4-mediated regulation has only been investigated in detail for a few microexons[9,17,19,20], and never in the context of their differential sensitivity to SRRM3/4.

Two contrasting yet non-mutually exclusive models taking into account core and SRRM3/4-specific splicing signals may explain the differences in SRRM3/4 sensitivity (Figure 1A). On the one hand, both classes of microexons (LS and HS) could have similar core splicing architectures, but differ in their ability and/or affinity to recruit SRRM3/4 to the pre-mRNA (Figure 1A, Model I). This model would predict differences in the contribution of SRRM3/4-related features between the two classes of microexons (e.g. UGC presence and/or location). On the other hand, both microexon classes could differ in the composition and strength of core splicing motifs involved in scaffolding the spliceosome, while the recruitment and effect of SRRM3/4 could be similar (Figure 1A, Model II). This model would predict different effects on each type of microexons if the core splicing architecture is modified in the same manner.

In this study, we have used Massively Parallel Splicing Assays (MaPSy) to test predictions made by these two models by assessing the impact of thousands of mutations on microexon sensitivity to SRRM3/4. This type of libraries, consisting of pools of sequences with either random mutations or rationally designed variants, have previously been used by other groups to investigate splicing regulation. These studies focused on investigating the role of *cis*-acting elements in 3′ss selection and regulation upon splicing factor mutation[21,22], in 5′ss selection and regulation upon perturbations[22–24], as well as of exonic sequences[25–38]. Here, we generated and analyzed 28,535 unique variants to assess the evolutionary conservation and regulatory contribution of both intronic (3′ss / 5′ss) and exonic *cis*-acting elements to SRRM3/4 regulation and sensitivity. We report the results of the quantitative dose response of those variants to different levels of SRRM4 activity by titrating its expression in HEK 293 Flp-In T-REx cells. While LS and HS microexons may also exhibit individual differences in their response to changes in SRRM3/4-related motifs, our results are overall highly consistent with the regulatory Model II (Figure 1A), whereby the main determinants of microexon sensitivity are related to differences in core splicing architecture. This conclusion is further supported by a mathematical model that assumes that the two types of microexons differ in their efficiency of recruitment of early spliceosomal components and the activity of the spliceosome in the absence of SRRM3/4.

RESULTS

MaPSy applied to tissue specific microexons recapitulates SRRM4 splicing regulation

To categorize microexons based on their response to SRRM3/4, we utilized RNA sequencing (RNA-seq) data from two human cell lines (HeLa and HEK 293) expressing either SRRM3 or SRRM4 at four levels and their corresponding control cells. Using Percent Splice-In (PSI) thresholds at each expression level (see Methods), we defined 71 LS and 73 HS SRRM3/4-dependent microexons (Figure S1A, Table S1). These microexons were respectively associated with either the neuronal-specific (N) or shared neuronal and pancreatic endocrine cell (NP) specific programs reported previously[4] (Figure 1B).

We next developed a MaPSy approach in HEK 293 Flp-In T-REx cell lines to investigate the determinants of the differential sensitivity of LS and HS microexons to SRRM4. Since the main *cis*-acting elements involved in microexon regulation are expected to be upstream and proximal to the dinucleotide AG and at the 5′ ss[9,10,17,19,20], we designed and cloned each sequence to contain the microexon flanked by the adjacent intronic 93 nts of the 3′ ss and 25 nts of the 5′ ss (Figure 1C). We then assessed how each class of microexons responded to different levels of SRRM4 expression in the context of the MaPSy libraries. For comparison, we also included 28 non-SRRM3/4-responding (NR) microexons, 20 cryptic (CR) microexons, and 21 constitutively spliced (CS) 42-nt long exons (Figure S1A, Table S1; see Methods). The inclusion profiles of exons with sufficient read coverage across all conditions showed that each (micro)exon group exhibited the expected relative behavior compared to their endogenous counterparts, despite an average lower inclusion level across all exon groups (Figure 1D).

Next, to generate the subsequent variant libraries, we selected the subset of exons that most faithfully recapitulated the regulation observed at the endogenous levels, assuming that these constructs carried all the essential *cis*-regulatory elements required for endogenous SRRM4 regulation. We selected 15 HS, 15 LS, 3 CR microexons and 9 CS exons (Figure S1B, Table S2), and used them to conduct a targeted high throughput mutagenesis within intronic and exonic sequences. The wild type sequences (WT) and their resulting variants (VAR) were then transfected in HEK 293 cells expressing four levels (none [=GFP], LOW, MID and HIGH) of SRRM4 expression, and RNA-seq was used to quantify the effects of each sequence variant on inclusion levels (Figure 1C; see Methods).

Sequence determinants of SRRM4 sensitivity are widely conserved across vertebrates

We first investigated the evolutionary conservation of the *cis*-regulatory architecture underlying the sensitivity to SRRM4. For this purpose, we retrieved the sequences of LS and HS microexons and surrounding splice sites from 47 species covering the main groups of jawed vertebrates across ~450 million years of evolution (Table S3), and generated equivalent minigenes to those of the human counterparts. In line with their high intronic and exonic sequence conservation[5], relative PSI levels

were remarkably conserved from shark to mammals for both LS and HS microexons under different SRRM4 conditions in human cells (Figure 1E, S1C). Teleosts showed more dissimilar inclusion patterns relative to their corresponding human sequences (Figure S1C), even though their dose response was also conserved. Consistent with this *cis*-regulatory conservation, endogenous orthologous HS microexons exhibited significantly higher overall inclusion levels in neural and endocrine pancreas than LS ones in five studied vertebrates (Figure S1D). Next, to directly test the conservation of the *cis*-regulatory elements in their intronic sequences, we swapped both flanking intronic sequences between each species variant and its human ortholog (Figure 1F, swap (iii)), or the human event and its ortholog from the other species (Figure 1F, swap (ii)). As a control, we swapped all flanking intronic sequences between human microexons with different sensitivity (Figure 1F, swap (iv)). We observed relatively little variation in PSI across those chimeric evolutionary variants compared to the WT human minigene, and this variation was much lower in all cases and conditions compared to intronic swaps among non-homologous human microexons (Figure 1F, S1E). These results showed that the *cis*-regulatory elements involved in splicing regulation by – and sensitivity to – SRRM4 are largely evolutionarily conserved and capable of recapitulating the expected sensitivity when introduced into human cells.

Exon length contributes to exon recognition and sensitivity to SRRM4

To start investigating the contribution of various features to SRRM4 sensitivity, and given that microexons are characterized by their short length, we first assessed the impact of increasing or decreasing their length. To increase it, we designed sixteen 36-nt long sequences devoid of potential splicing regulatory motifs such as Exonic Splicing Enhancers (ESE) or Silencers (ESS) according to either the Human Splicing Finder tool[39] or data from[40] ("designed sequences"; Methods). Then, for each microexon, we increased the length of the microexon through the stepwise addition of 3 nts to its center for each of the designed sequences, up to a maximal exon length of 42 nts (Figure S2A,B, Table S4). As expected, when added to LS, HS and CR events, those sequences led to minimal variation of Exonic Splicing Regulatory sequences (ESRseq) scores throughout those exons[40] (Figure S2C-F). In parallel, we also decreased the length of each microexon and CS exon by stepwise removal of 3 nts from the middle of the exon until its length reached 6 nts.

This analysis revealed that lengthening generally enhanced exon recognition, as evidenced by the increase in PSI of the longer variants with respect to the WT ones observed across all conditions (Figure 2A,B; $rho \geq 0.65$ and $P < 0.05$ for all conditions, Spearman Correlation), and despite the unexpected effects of some added sequences (Figure S2G). Conversely, shortening the length had a negative impact on the degree to which the exons responded to SRRM4, as evidenced under conditions of HIGH expression of SRRM4 (Figure 2A,B). Therefore, this analysis uncovered an overall

positive association between length and microexon inclusion in line with previous work[41]. However, we found important differences between the behavior of LS and HS microexons. First, while both have PSI ~ 0 in the absence of SRRM4 (GFP condition) (insets in Figure 2A,B), only HS events showed a strong PSI increase upon lengthening in that condition (Figure 2B). This indicates that HS microexons can be included in the absence of SRRM4 simply by lengthening, unlike most of LS microexons, whose variants still require SRRM4 to achieve a substantial inclusion level. Second, whereas elongated LS microexons respond to increased SRRM4 levels more strongly than their shorter versions, lengthening and shortening of HS microexons did not generally change their relative response to the different levels of SRRM4 or did so in a non-monotonic way (Figure 2C,D). In other words, the change in PSI between GFP and the different SRRM4 expression levels progressively increased with length for LS microexons for MID and HIGH conditions (*rho* ≥ 0.54 and $P \leq 0.01$; Spearman Correlation), but remained similar across lengths for HS (*rho* < 0.28 and $P > 0.05$; Spearman Correlation).

We next examined the consequence of reducing the size of the 42 nt-long CS exons. In general, all studied events displayed progressively reduced inclusion under the GFP condition as the length decreased (Figure 2E,F). However, the specific length at which inclusion started to decrease and when it reached nearly zero varied for each event (Figure S2H,I). Strikingly, we also noticed that some of the CS events became responsive to SRRM4 at a certain length (ΔPSI (HIGH-GFP) ≥ 20; Figure S2H). The gain of SRRM4 regulation by shortened CS variants was independently validated for four of these events through individual transfection in HEK 293 cells expressing either GFP or SRRM4 (Figure 2G,H). Finally, CR microexons also exhibited a positive response to lengthening while the shortening had a more dispersed pattern (Figure S2J,K**).**

Altogether, these results show that microexon length is one of the determinants of exon recognition and contributes to sensitivity to SRRM4, and it does so differently for HS and LS microexons. For HS microexons, the sensitivity is largely maximized while increases in length often lead to substantial microexon variant inclusion even in the absence of SRRM4. For LS microexons, longer variants have increased sensitivity to SRRM4, but they do not usually achieve high inclusion levels in the GFP condition.

Exonic elements contribute to microexon regulation in a context dependent manner

Cis-acting elements located within exons are known to contribute to splicing regulation[30,34,40]. As we observed distinct effects for some sequences used for microexons lengthening (Figure S2G), we next asked whether exon-specific regulatory motifs were present. For that purpose, we performed a systematic mutagenesis of three successive nucleotides using a sliding window approach from the 5′ end to the 3′ end of each microexon (Figure S3A). Interestingly, similar to the pattern observed for

lengthening, only a few mutations in HS microexons showed substantial positive responses (ΔPSI > 10) in the absence of SRRM4 (Figure S3B,C,E, *P* = 1.2e-4 two-sided Fisher's Exact test). Upon different levels of SRRM4 expression, mutations with strong effects were still a minority, but these were balanced between those with a positive and a negative effect on SRRM4 regulation for both LS and HS microexons (Figure S3B,C,E,F). In the case of CS exons and their corresponding shorter variants, we noticed exon length-dependent effects for some *cis*-acting elements, which only contributed substantially to splicing regulation in the shortened variants (Figure S3D,E). Moreover, we also detected specific mutations that enabled regulation by SRRM4, even for the 42 nt-long exon variants (Figure S3F).

To complement this mutational scan, we created another set of variants by inserting previously described ESE or ESS hexamers[40,42,43] at two exonic positions (Figure S3G). As expected, ESE and ESS insertions usually led to increased and decreased (micro)exon inclusion, respectively, with some context dependent effects observed across all exon types and conditions. However, we again observed that ESE insertion led to substantial SRRM4-independent inclusion only for HS but not LS microexons (Figure S3G). Interestingly, this pattern was also observed for shortened SRRM4-dependent CS events in the GFP condition (Figure S3G).

Altogether, these results indicate that, while it is likely that microexon regulation mainly relies on elements in the flanking intronic regions, *cis*-regulatory elements within the microexon may also impact its inclusion and response to SRRM4.

The sensitivity to SRRM4 is to a large extent encoded in the flanking intronic sequences

To assess to what extent the different sensitivities to SRRM4 are encoded in the flanking intronic sequences, we systematically swapped the 3′, the 5′ or both flanking intronic sequences among all 15 HS and 15 LS studied microexons (3,480 total combinations) (Figure 3A). These swappings showed that sensitivity information was encoded in both the 3′ and, less expectedly, the 5′ neighboring intronic sequences (Figure 3). Replacing the intronic sequences from HS events with those from LS events negatively impacted the response to SRRM4 (Figure 3B), while the opposite was observed when replacing LS with HS intronic sequences (Figure 3C). Surprisingly, the effect was more pronounced when swapping the 5′ss as compared to the 3′ss and even more pronounced when both flanking intronic regions were replaced at given concentrations of SRRM4 (Figure 3A-C). These results thus point towards a more important role of the 5′ss than previously anticipated[5,9,17]. Swapping sequences within HS events or within LS events yielded more moderate impacts (Figure 3A-C), suggesting the presence of more compatible intronic architectures for a given sensitivity.

Given the importance of length on microexon inclusion (Figure 2), we next focused on swappings between events of comparable length (both ≤15 nts, short; or both > 15 nts long) or between events of different length groups (short with long and vice versa). Remarkably, this analysis revealed that the *cis*-regulatory architecture of HS and LS is adapted to the length context of the microexons. Therefore, when HS intronic sequences from short microexons (predicted to be the most sensitive) were placed around long LS microexons (predicted to be the least sensitive), we observed an overall increase in PSI across all conditions, including a substantial inclusion even in the absence of SRRM4 (Figure 3D). On the contrary, when LS intronic sequences from long microexons were placed around short HS microexons, we found an overall decrease in inclusion, particularly noticeable under HIGH SRRM4 levels (Figure 3E).

These experiments show that the sensitivity to SRRM4 is to a large extent encoded in the intronic sequences surrounding the microexons, which prompted us to investigate the relevance of specific *cis*-acting elements globally or individually within these intronic sequences.

Context and position requirements for the UGC motif are similar between sensitivity groups

Next, we focused on the other highly characteristic feature of SRRM3/4-regulated microexons, the UGC motif near the 3′ss. A UGC motif between the BP and the AG was found in the majority, but not all, SRRM3/4-dependent microexons. However, its presence is not associated with their sensitivity, since a UGC motif was found in 79% (57/73) HS and 74% (51/71) LS microexons (P = 0.444 two-sided Fisher's Exact test). To systematically explore the role of this motif in SRRM3/4-dependent splicing regulation, we mutated either one or multiple UGCs within the upstream intronic sequences of each human microexon used in our mutagenesis study. Consistent with previous reports, most single or multiple mutations of UGC motifs disrupted the response to SRRM4 for both LS and HS events (Figure 4A). Moreover, when multiple UGC motifs were present, not all of them had an impact when disrupted (Figure 4A).

Surprisingly, however, the presence of a UGC motif was not strictly necessary for SRRM4-dependent inclusion of microexons, in contrast to previous reports[6,9,10,19,20]. In particular, HIGH levels of SRRM4 could, to varying extents, overcome the disruption of UGC motifs for most microexons. In such cases, HS microexons displayed higher propensity for inclusion despite the mutation of UGC motifs (Figure 4A). Moreover, a similar pattern was observed for orthologous microexons from 23 other species, for which all present UGC motifs were mutated (Figure S4A). The patterns of splicing were independently validated by RT-PCR assays for three microexons after co-expression of either human SRRM4 or a more efficient regulator obtained from amphioxus[10] (*Srrm234* isoform AB) (Figure 4B). These results suggest that, while the UGC motif seems to strongly facilitate the action of SRRM3/4, likely favoring

its binding, it is not strictly necessary for it. This points towards the presence of additional interactions triggering its recruitment to microexons.

Next, we assessed the relative position of the most relevant UGC, as identified by mutagenesis (Figure 4A), with respect to the AG, best predicted BP and 3′ end of the Py tract. We found that the relative position of the UGC motif was similar between LS and HS events (Figure 4C), although the UGC was closer to the BP in HS microexons, suggesting a more compact architecture. In line with this, when all endogenous microexons were considered, the distance between the closest UGC and the AG was also significantly shorter for HS events (Figure 4D; P = 1.1e-04, Mann-Whitney-Wilcoxon test). To assess whether the most relevant UGC was located at the most optimal position in both types of microexons, we performed a "UGC-addition walk", i.e., we systematically inserted a UGC motif at all possible positions between the 3′ end of the Py tract and the dinucleotide AG after mutating all natural UGC motifs, and assessed the inclusion level of the resulting microexon variants (Figure 4E, Methods). This analysis showed that for both types of microexons, the most relevant natural UGC is generally at the position where a UGC motif has its maximal effect, even if UGCs at neighboring positions can have similarly strong effects (Figure 4E,F). Importantly, this result also indicates the UGC motifs are not placed in more optimal configurations in HS than in LS microexons with regard to the general architecture of each exon, i.e., their core splicing elements, as potentially predicted by Model I (Figure 1A).

Finally, we assessed how additional UGC motifs impacted microexon inclusion and the degree of response to SRRM4. For this purpose, we added one, two or three UGCs downstream to the best predicted BP alone or in combinations with upstream shifts of the BP and its associated Py tract (Figure S4C,D and Methods). Somewhat surprisingly, most of these mutations had a detrimental effect on the response to SRRM4 for both HS and LS events (Figure S4C,D), likely because they led to either decreased Py tract strength or increased distance from BP to AG.

Overall, these findings show that a UGC motif at a specific location is of high relevance for SRRM4 response and this location is largely equivalent for HS and LS microexons. However, the presence of this motif is not strictly necessary for the inclusion of either HS or LS microexons. Therefore, additional *cis*-regulatory elements likely contribute to the splicing pattern and the regulation by SRRM4.

Sequences in proximity to splice sites are key for SRRM4 splicing regulation

To investigate the parts of the pre-mRNA that are most relevant for microexon splicing and their regulation by SRRM4, we performed a semi-deep mutagenesis scan of both intronic and exonic sequences (Figure 5A, S3A-C). Overall, nearly all mutations had a neutral or negative impact on

splicing. As expected, intronic mutations in proximity to the 3′ss (i.e., likely within the BP, Py tract and intronic splicing regulatory elements) or the 5′ss more negatively impacted the regulation of LS and HS events by SRRM4 (Figure 5A). Similar trends were observed for CS events and their shortened variants (Figure S5A), although strong negative effects on inclusion were also observed for WT CS exons in the GFP condition, given their high starting PSI. Moreover, impactful mutations at the 3′ss of CS events tended to be located closer to the dinucleotide AG in comparison with SRRM4-dependent microexons, consistent with the peculiar architecture of the latter, with more distant BPs (see Introduction, Figure S5B).

To assess the role of the strength of the BP and the Py tract in microexon splicing and regulation by SRRM4, we then replaced the BP of LS and HS events by either two different strong or two weak BPs. As expected, this revealed a general increased or decreased response upon strengthening or weakening, respectively (Figure 5B). However, the strengthening effect of the most consensus BP (UACUAAC) was particularly remarkable for HS microexons in the GFP condition, and it revealed that modulating core splicing signals is sufficient to generate SRRM4-independent inclusion specifically for HS microexons. In the case of LS microexons, the strengthening of the BP resulted in an overall increase in sensitivity, with very little impact on the PSI in the GFP condition (Figure 5B). Hence, solely by modulating this core splicing signal, we could convert LS into HS-like microexons, as predicted by Model II (Figure 1A). Conversely, weakening of the BP signal was sufficient to reduce the sensitivity to SRRM4 of HS and LS microexons (Figure 5B).

Strengthening or weakening the Py tract[44] (Table S5), alone or in combination with the above mentioned BP mutations, also positively or negatively impacted splicing regulation, respectively. Again, this resulted in SRRM4-independent inclusion, specially for HS microexons, and to the expected changes in sensitivity for both HS and LS microexons (Figure 5C,D). The joint contribution of the BP and Py tract to regulation by SRRM4 was further confirmed by the full replacement of both elements by the one from Adenovirus Major Late (AdML)[45] or from immunoglobulin mu (IgM)[46], two well established splicing substrates with strong or weak 3′ss features, respectively (Figure 5E). Interestingly, a similar behavior to HS microexons was observed for the SRRM4-dependent microexons obtained from reducing the length of CS events, suggesting that their initial constitutive architecture may be closer to that of HS than of LS microexons (Figure S5C,D). Finally, other probed elements, including UCUC motifs involved in SRSF11 / PTBP1 mediated regulation of microexons[9,17], did not have a major consistent impact on the tested microexons (Figure S5E).

Altogether, these results show that core elements involved in spliceosomal recruitment contribute to the splicing pattern of microexons and to their sensitivity to SRRM4.

Unappreciated contribution of the 5′ splice site to splicing regulation by SRRM4

We next assessed more specifically the contribution of the 3′ss and 5′ss sequences. We first focused on the 3′ss, which is recognized by U2AF1 based on positions -3 to +1 (YAG|R[47–49]). Interestingly, microexons have similar features to AG-independent exons[50] and have been shown to be generally repressed by U2AF1[13]. Thus, we performed a deep mutagenesis of positions -3, +1, +2 and +3 to globally examine the contribution of the nucleotides surrounding the AG to the regulation by SRRM4. Overall, we noticed microexon-specific dependencies on certain nucleotides (Figure S5G). Also, somewhat unexpectedly, the shortest variants of the CS events were more affected than their longer counterpart, and, to a similar extent as LS and HS events (Figure S5G). These results suggest a complex and microexon-specific relationship between AG recognition and SRRM4-dependent regulation that would require further investigation.

Next, we explored the 5′ss. While it was previously noted that the 5′ss of SRRM3/4-dependent microexons are generally strong[10], their role in shaping splicing patterns and their contribution to SRRM4 regulation have not been experimentally investigated. As shown above, swaps of intronic regions including the 5′ flanking intronic region had stronger effects than swapping only the upstream intron (Figure 1F, 3B,C), and mutations in the 5′ss had highly detrimental effects (Figure 3, 5A). Thus, we sought to determine whether strengthening the 5′ss alone could influence SRRM3/4-dependent splicing regulation. For this purpose, we increased 5′ss strength by introducing mutations in the last three nts of the exon and up to the eight first nts of the downstream intron, enhancing its complementarity to U1 snRNA (U1cons) (Figure 6A). Additionally, we performed mutations from positions +5 to +9 in the intron to enhance the complementarity to U6 snRNA (U6cons)[51] (Figure S6A). Each of those mutations led to a significant increase in the maximal entropy (MAXENT) score of the 5′ss[52] (Figure 6B, S6B,C). Strengthening of the 5′ss had a very strong positive effect on inclusion for both types of microexons across most SRRM4 conditions (Figure 6C,D). In the case of LS microexons, their inclusion reached near saturation at HIGH SRRM4 levels, similar to HS events, and both LS and HS events increase their inclusion levels at low levels of SRRM4, and thus their sensitivity to the regulator. Moreover, we observed a substantial degree of inclusion in the GFP condition for both LS and HS microexons, showing that modulating 5′ss signals is sufficient to drive inclusion of microexons even in the absence of SRRM4. These findings were independently validated by RT-PCR assays for two LS microexons (Figure 6E, S6D,E). To rule out a potential impact of the specific minigene design on these findings, we validated the impact of strengthening the 5′ss using three additional minigenes for which the endogenous upstream exon/intron, microexon and downstream intron/exon belonged to the same event (Figure S6F).

The reverse pattern was observed upon weakening the strength of the 5′ss by GU to GC mutation or by using a weak 5′ss sequence (Figure S6A,G,H). To exemplify these effects, we focused on WT orthologous sequences for the LS microexon in *ITSN1* (HsaEX0032608). We noticed that the orthologous microexon for pig, cow and dolphin (belonging to the monophyletic group of Artiodactyls) had the lowest response to SRRM4 among twenty probed species (Figure S6I, right panel). A multiple sequence alignment revealed an A-to-G substitution at position +3 of the 5′ss in this clade (Figure S6I, left panel), which decreased its maximal entropy score from 10.24 to 8.73 (Figure 6E). To test the effect of this variant in the response to SRRM4, we generated an A-to-G single point mutation at position +3 of the human sequence, which led to a complete loss of response to SRRM4 expression (Figure 6E). The contribution of the 5′ss in early spliceosomal recruitment was further examined *in vitro* for this event. The A complex formation efficiency on *ITSN1* microexon substrates in the presence of the minimal domain of SRRM3 necessary and sufficient for regulation (eMIC) was higher when a stronger 5′ss was present (Figure 6F), suggesting a potential contribution of exon definition in the regulation. Another set of examples was provided by two likely pathogenic SNPs, according to ClinVar, which weaken the 5′ss and led to decreased regulation of a HS microexon in *CASK* (HsaEX0012545) and a CS exon in *SNAP25* (HsaEX6077184) (Figure S6J). These patterns are consistent with the global results obtained by performing deep mutagenesis of every position involved in base pairing to U1 snRNA (from -3 to +6) (Figure S6K). Interestingly, while mutations at positions -1 and +3 were detrimental for all types of (micro)exons, only HS and shortened CS events were highly sensitive to mutations at position +5 (Figure S6K).

Next, to systematically evaluate the contribution of the 5′ss to the regulation of SRRM4-dependent microexons, we calculated the difference in scores measuring either the 5′ss strength (MAXENT5), the length of the AG exclusion zone (agez) or the 3′ss strength (MAXENT3) between a given variant and its corresponding WT, and correlated it with the change in exon inclusion at HIGH SRRM4 expression between that variant and the corresponding WT. For both LS and HS events, we observed a strong positive correlation only between the variation in MAXENT5 and the change in inclusion, while the correlations with the agez and, specially, MAXENT3 were much weaker (Figure 6G).

Altogether, these results highlight a previously unappreciated relevance of 5′ss strength in inclusion of the microexon and response to SRRM4 expression.

Early recognition of splice sites in the absence of SRRM4 correlates with microexon sensitivity

A number of findings presented thus far collectively support a model by which HS and LS microexons differ in their recognition capacities by the spliceosome under conditions where SRRM4 is not expressed. Specifically, HS microexons were more often included in the absence of SRRM4 (GFP

condition) upon different types of mutations. This occurred when the microexon length was increased (Figure 2), an ESE was introduced (Figure S3), splice sites between sensitivity groups were swapped (Figure 3), a stronger 3′ss arrangement was employed (Figure 5) or the strength of the 5′ss was increased (Figure 6). On the other hand, key features specifically associated with SRRM4 regulation, such as the presence and optimal location of UGC motifs, did not show differential behavior for HS and LS microexons (Figure 4). Thus, our results are consistent with the model in which the sensitivity to SRRM3/4 is, at least to large extent, related to the background core splicing configuration of each microexon type (Figure 1A, Model II), in which (i) HS microexons are much closer than LS microexons to forming productive splicing assemblies in the absence of SRRM4, and (ii) SRRM4's function on both types of exons is similar.

To gather further support to this hypothesis, we next developed a minimal mathematical model that could explain our data. Our model considers a pre-mRNA that is transcribed and can be bound by the spliceosome complex (considered at a coarse level as a single enzymatic entity) as well as the regulator SRRM4 ((S) and (R), respectively, Figure 7A; see Methods). The pre-mRNA can undergo splicing decisions in favor of skipping or inclusion, with different rates depending on whether or not the spliceosome and/or the regulator are bound. In order to account for the inclusion-enhancing effect of SRRM4, we assume that it enhances spliceosome recruitment and activity. We consider the model at steady-state, so that the PSI measure corresponds to the ratio of the steady-state concentration of the mature mRNA with the microexon included (I*) over the sum of both the steady state concentrations of included and excluded mature mRNA species (I*+E*). As data to fit the model to, we selected the inclusion profiles of WT LS and HS events, both at the endogenous level and in the MaPSy context, as well as mutations in the 3′ss (BP strong or weak) or in the 5′ss (U1cons). We fitted the model parameters to the median of the inclusion profiles of the various groups of sequences, assuming that they differ only in parameters determining the basal spliceosomal assembly and activity (c_2, k_{is}). In fact, it was enough to only assume differences in c_2 to recapitulate the data, without the need to invoke additional differences in the action of the regulator over LS and HS microexons (c_a, c_d, $c_{3\text{-}6}$, c_i) (Figure 7B-D). In line with this, publicly available SRRM4 PAR-iCLIP data[9] show that, while the distribution of SRRM4 binding to HS microexons at the 3′ss is narrower (Figure S7B), HS and LS microexons do not differ in their capacity to recruit SRRM4 (Figure S7C,D). These results thus offer orthogonal support to the model where differences in core spliceosomal architecture could be sufficient to produce microexons with different sensitivities to their master regulator.

Additional experimental support to this hypothesis is provided by assays of A complex formation for HS and LS microexons in basal conditions, i.e., with no SRRM4 expression. Specifically, we *in vitro* transcribed the variants bearing the 3′ flanking intronic, microexon and 5′ flanking intronic sequences

together with the adaptor sequences, and incubated these RNAs in HeLa cell nuclear extracts under splicing conditions (Figure S7E; see Methods). After separation by electrophoresis, the variants incorporated within the A complex were isolated from the gel and subjected to RNA-seq. The proportion of variants detected in A complex as compared to the proportion in the pool of RNAs used for the reaction was defined as the enrichment score (ES), which is a proxy of the capacity of each variant to recruit early spliceosomal components (Methods). Focusing on the WT human microexons and their orthologs in 23 species, we observed that HS events had significantly higher ESs in basal condition than LS events (Figure 7E). Similarly, despite being very low in both groups, the levels of inclusion in the absence of SRRM4 (GFP condition) were also significantly higher for HS than for LS exons (Figure 7E).

Next, we took advantage of publicly available data from a recent study mapping the binding of U2 snRNP to 3′ss in the high molecular weight fraction associated with chromatin in HEK 293 cells[53]. Since these cells do not express SRRM3/4, they can be used as a proxy for basal spliceosomal recognition of microexons. Therefore, we asked whether we could detect differential recruitment of U2 snRNP on endogenous HS and LS microexons. For SF3A3, RBM5 and RBM10 IP, a significantly higher proportion of HS microexons presented U2 snRNP peaks in the last 93 nts of the intron upstream to the microexons as compared to LS events (Figure 7F,G), and these were of higher intensity (Figure 7G, S7F-H). Importantly, this did not correlate with differences in mRNA expression of the host genes (Figure S7I) or differences in intensity for other peaks within the same genes (Figure S7J). These data further support the hypothesis that HS and LS microexons are different in their initial basal state, with HS being more primed to enter into the splicing reaction. In line with this, experimental depletion of various splicing factors involved in 3′ss recognition or repression in human cell lines with no SRRM3/4 expression was sufficient to induce mild but significant upregulation of HS microexons but not of LS ones (Figure 7H).

DISCUSSION

Since their discovery a decade ago, neural microexons were considered a paradigmatic example of switch-like splicing regulation: expression of their master regulators SRRM3/4, endogenously or ectopically, was sufficient to activate microexon inclusion in any tested cell type[4,6,9,10,12,13,16,17,54–57]. However, recent work has shown that this switch-like model is incomplete. Two types of SRRM3/4-dependent microexons were identified, one type included in transcripts in endocrine pancreatic cells and neurons, and another expressed only in neurons[4]. Remarkably, this tissue-specific pattern was not driven by co-factors but mainly by the differential sensitivity of each type of microexon to SRRM3/4

activity. While pancreatic microexons were highly sensitive (HS) and needed only low levels of the regulator, neural-specific microexons were lowly sensitive (LS) and required much higher levels of SRRM3/4[4]. Therefore, microexon activation follows a dim-switch like model rather than an on/off one[16]. Interestingly, this opened up the possibility that differences in microexon sensitivity corresponded to different cell type-specific needs. However, it left a major unanswered question: how is microexon sensitivity to SRRM3/4 encoded?

In this study, we aimed at answering this question and understanding the *cis*-regulatory exonic and intronic determinants governing the pattern of microexon splicing and their response to SRRM3/4. We developed a microexon-focused MaPSy approach that recapitulated their regulation by using 93 nts of the 3′ flanking intron - the microexon - 25 nts of the 5′ flanking intron in a minigene reporter and HEK 293 Flp-In T-REx cells expressing either GFP or various SRRM4 levels in an inducible manner. This approach demonstrated the complex contribution of elements in the 3′ss, the 5′ss and the microexons themselves, as well as the conservation of their regulation over ~450 MYA of vertebrate evolution. Moreover, it reemphasized that exonic length is a major determinant of the regulatory logic of SRRM3/4-dependent microexons. By elongating and shortening individual microexons, we have found that, as a general rule, longer microexons are more efficiently recognized, often to a point where SRRM4 was no longer necessary for their inclusion. This aligns with pioneering work on nSRC[41] and suggests that the short length of microexons imposes idiosyncratic constraints, ensuring a fine balance between repression in the absence of SRRM3/4 and avoiding excessive repression that would hinder the regulator's function. We propose that, by adjusting these constraints to the core splicing architecture of each microexon, the optimal dose response level, or sensitivity, to SRRM3/4 is achieved.

To thoroughly assess this possibility, we explicitly confronted two contrasting (yet not mutually exclusive) models that could explain the difference in sensitivity between HS and LS microexons (Figure 1A). According to the first model (Model I), HS and LS microexons would have a similar "core splicing" architecture (a long BP-AG distance, strong 5′ss, etc.[5,9,11]), but they would differ in their specific SRRM3/4-responding architecture, e.g., by varying in number of UGC motifs, with stronger binding affinities and/or more accessible in the pre-mRNA. This would translate into a stronger response at a given level of SRRM3/4 expression for HS than for LS microexons. In the second model (Model II), the absolute effect of SRRM3/4 regulation would be similar for both HS and LS microexons (i.e., they would be in fact equally sensitive to it), but the "core splicing" architecture of each type would be distinct, with HS microexons being basally more primed for exon inclusion. In both models, the effect of SRRM3/4 at a given concentration can be represented as a enhancing effect towards higher inclusion in a response curve (Figure 1A). In the first model, such a “push” would be of different

magnitude for HS than for LS microexons. In the second model, however, the magnitude of the push would be similar for both types and the difference in resulting PSI would be due to the distinct "starting position" along the inclusion response curve, which is in turn related to the general or core splicing architecture of the microexon. It is crucial to note that for both HS and LS microexons, the default configuration is not sufficient for proper exon recognition and substantial inclusion without SRRM3/4, which is needed to further recruit early spliceosomal components[10].

Importantly, each model makes specific predictions that we could test. First, in contrast to Model I, Model II predicts that recruitment of SRRM4 should not differ much between HS and LS exons, as we found. Moreover, we find that for both types of microexons, UGC architecture and relative position, when present, seem to be optimized. A second key prediction of Model II is that small changes in core splicing elements (e.g. the addition of ESE/ESSs, strengthening/weakening of the 5′ or 3′ ss, etc.) would be sufficient to cause increases or decreases in microexon sensitivity to SRRM4, both for HS and LS, as observed across all analyses. Third, when such changes in core splicing architecture increase spliceosomal recruitment, i.e., result in a forward shift in the inclusion response curve, they should cause SRRM3/4-independent inclusion for HS but not LS microexons, as we indeed observed. Forth, similarly, changes in expression in splicing factors involved in splice site recognition or repression in non-SRRM3/4 expressing cells would more often lead to preferential inclusion of HS microexons compared to LS ones. Fifth, early steps of spliceosome assembly should be more readily observed in HS than in LS microexons in the absence of SRRM3/4, as we observed for *in vitro* A complex formation as well as for U2 snRNP recruitment on nascent RNA isolated from chromatin. Moreover, somehow unexpectedly, we also reported the conversion of several CS events into SRRM4-dependent microexons upon exon shortening, whose behavior was usually closer to that of HS microexon. This suggested that the core architecture required for a high sensitivity response to SRRM4 is, to some extent, closer to that of CS exons. Finally, the integration of these concepts into a mathematical model further supported that a different initial state between HS and LS microexons, attributed solely to their basal association with the spliceosome, could be sufficient to explain the patterns of sensitivity we observed in endogenous microexons as well as in our minigene libraries. Therefore, and although we have not formally ruled out Model I and, in fact, it is very possible that it also contributes to some extent to microexon sensitivity to SRRM3/4, our data collectively provides strong support for Model II in determining microexon sensitivity. This model is also consistent with the model of global scaling of splicing regulation[26], by which seemingly small differences in the initial inclusion levels may highly affect the magnitude of the response of exons to splicing perturbations.

Subsequent work should examine the effects of different types of mutations and on a more extended set of microexons, as well as integrate the effects of modulating the activity of other *trans*-acting

factors. Moreover, with the exponential growth of machine learning and Artificial Intelligence approaches, complementary analyses of these data should bring us closer to define a microexon code that accurately predicts splicing outcomes under multiple conditions. Finally, whether or not this model for differential sensitivity also applies to the exon targets of other tissue-specific splicing regulators (e.g. RBFOX, NOVA, etc.) needs to be investigated.

Limitations of the study

First, the experiments presented here were performed in HEK 293 cells. Even though these cells recapitulate the dose response inclusion of the microexons to different levels of SRRM4, they may not fully reconstitute the tissue specific context from neural or pancreatic endocrine cells that could contribute to their finely tuned splicing regulation. Second, the analyses were performed using a limited subset of microexons from the nested programs (15 LS vs 15 HS), which may not fully represent the diversity of *cis*-acting elements arrangements, particularly for LS microexons with large BP-UGC-AG distances. Additionally, the pre-mRNA sequences were restricted to 93 nts in the upstream intron and 25 nts in the downstream intron surrounding the microexons and inserted in an ectopic context, which may have led to the loss of longer distance molecular determinants and co-transcriptional splicing regulation. Finally, while the proposed mathematical model shed light into the mechanistic differences between HS and LS microexons and particular relevance of early spliceosomal recruitment, additional experiments are needed to investigate the microexon specific contribution of SRRM4 in assembly and splicing catalysis.

ACKNOWLEDGEMENTS

The authors would like to thank Juan Valcárcel, all members of the Irimia Lab, Lawrence Chasin for providing a comprehensive list of ESRseq scores [40], the authors of [53] for providing the HMW peak intensities and discussion on their data, Antonio Torres-Mendez for insightful discussion on microexons regulation, Luis Iñiguez-Rabago and Miquel Anglada Girotto for assistance with bioinformatics, David Gray for his insights into pharmacological models, the Genomics Unit at the CRG for assistance with the sequencing and the Protein Technologies Unit at the CRG.

Funding: The research has been funded by the European Research Council (ERC) under the European Union's Horizon 2020 research and innovation program (ERCCoG-LS2-101002275 to MI) and Spanish Ministry of Science and Innovation (PID2020-115040GB-I00 to MI). CRG acknowledges support of the Spanish Ministry of Science and Innovation through the Centro de Excelencia Severo Ochoa (CEX2020-001049-S, MCIN/AEI/10.13039/501100011033), and the Generalitat de Catalunya through the CERCA program.

AUTHORS CONTRIBUTION

Conceptualization, SoB, SB, RMC, MI; Methodology, SoB, SB, RMC, MI; Software, SB, RMC; Validation, SoB; Formal Analysis, SoB, SB; Investigation, SoB, SB, RMC, MI; Resources, SoB, MI; Writing - Original Draft, SoB, MI; Writing - Review & Editing, SoB, MI; Visualization, SoB, SB, RMC; Supervision, SoB, MI; Project Administration, MI; Funding Acquisition, MI.

DECLARATION OF INTERESTS

The authors declare no conflict of interest.

DATA AVAILABILITY

The datasets supporting the conclusions of this article have been deposited to the Gene Expression Omnibus (GSE276143) repository. All original codes used to generate the results in this manuscript are deposited at the GitHub repositories: https://github.com/simon-bt/lme-mapsy and https://github.com/theobiolab/SRRM4_MapSy_2024.git

SUPPLEMENTAL INFORMATION

Document S1. Figures S1-S7 legends, Minigene reporter sequence

Tables S1-S20: All tables are provided as standalone excel documents.

METHODS

Definition of microexon sensitivities

We used RNA-seq data corresponding to (i) the ectopic expression in HEK Flp-In T-REx cells of either GFP (PRJNA474911[10]) or human SRRM4 upon titration after induction using 0 (uninduced, CTR), 0.3 (LOW), 1.2 (MID), 4.8 (HIGH) ng/ml doxycycline (generated in this paper, see below, Table S9), and (ii) and the ectopic expression in HeLa FLIp IN cells of either GFP or mouse SRRM3 (PRJNA817274[4]). Microexon inclusion levels (PSI) were quantified for each condition with *vast-tools* v2.5.1[60]. Deprecated events in the final output of *vast-tools* were retrieved from the intermediate raw inclusion tables (option *--keep_raw_incl*). We then defined each event as follows based on various PSI thresholds, irrespectively of the read coverage (Figure S1A, Tables S10, S11):

- Non-Responding events: $(PSI_{HIGH_SRRM4}) - (PSI_{GFP}) < 10$ AND $(PSI_{HIGH_SRRM3}) - (PSI_{GFP}) < 10$.
- Highly Sensitive (HS) events: $(PSI_{LOW_SRRM4}) - (PSI_{GFP}) >= 25$ OR $(PSI_{LOW_SRRM3}) - (PSI_{GFP}) >= 25$.
- Lowly Sensitive (LS) events: $[(PS_{LOW_SRRM4}) - (PSI_{GFP}) < 25$ OR $(PSI_{MID_SRRM3}) - (PSI_{GFP}) < 25]$ AND $[(PSI_{CTR_SRRM4}) - (PSI_{GFP}) < 5$ OR $(PSI_{CTR_SRRM3}) - (PSI_{GFP}) < 5]$.

Thirty cryptic (CR) microexons (PSI < 10 in > 90% of the samples with coverage and a PSI range < 25) were manually selected from VastDB to represent different cases with and without UGC and UCUC motifs. Finally, the output was manually curated to assign the sensitivity when NAs were present in any condition.

mRNA sequencing

RNA was isolated using the RNeasy Mini Qiagen kit and the RNA quality was checked using Bioanalyzer (Agilent). Libraries were prepared at the CRG Genomics Unit using the TruSeq stranded mRNA Library Prep (ref. 20020595, Illumina) according to the manufacturer's protocol. Each library was sequenced on a HiSeq2500 Illumina machine, to produce an average of 79.6 million 125-nt long paired end reads. This data was submitted to GEO (GSE276143).

Retrieval and curation of vertebrate microexon orthologs

To generate the evolutionary variants, we first identified the orthologous microexon events in 47 vertebrate species (Table S3). For this purpose, we searched LASTZ alignments (available from Ensembl REST API) for available amniote species using as queries each tested human HS and LS microexon and their 93 nts upstream and 25 nts downstream coordinates, -500 and +500 nts respectively. Alignments were reverse complemented if needed and automatically searched for the presence of microexons with maximum 3 nts substitutions and no more than of 40% deletion with respect to the human orthologous event, and AG and GT at 3′ and 5′ ends of the identified exons.

Orthologous sequences were then manually curated and checked for completion in the original genome assemblies. For frogs, teleosts and the elephant shark, as well as for additional ammonite species with available "UCSC Chain Files" (https://hgdownload.cse.ucsc.edu/goldenpath/*/liftOver/), we manually searched for each microexon through UCSC liftover and Ensembl Blast searches. The information related to the divergence time per species (million years ago; MYA) and tree were recovered from http://timetree.org/[58] (Table S3). The tree was adapted using *ete3* at http://etetoolkit.org/[61]. Sequences' alignment provided in Figure S6I was generated using Jalview v2.11.3.3.

Microexon inclusion and *Srrm3/4* gene expression levels in other species

Orthologous events in *Mus musculus (mm10)*, *Rattus norvegicus (rn6)*, *Gallus gallus (galGal4)*, *Bos taurus* (*bosTau6*) and *Danio rerio* (*danRer10*) were obtained from the "EVENT CONSERVATION" table from *VastDB* (https://vastdb.crg.eu/wiki/Downloads). PSI and gene expression (cRPKM) in tissues from those five species were collected from the "MAIN PSI TABLE" and "MAIN EXPRESSION TABLE" from *VastDB*. Tissues were classified either as Neural, Pancreatic beta cells (when available), Others or discarded (Table S12). The PSIs supported by sufficient read coverage (*vast-tools* quality scores VLOW, LOW, OK, SOK) were plotted for all biological samples. The cRPKMs are represented only for tissues in which the PSI of LS and HS events were quantified. Animal silhouettes in Figure S1D were obtained from https://www.phylopic.org/.

Generation of the barcoded minigene backbone for library cloning

First, a DNM1 minigene encompassing 'upstream exon (116 nts) - intron (433 nts) - microexon (12 nts) - intron (647 nts) - downstream exon (171 nts) - first 25 nts of the downstream intron' was constructed for the *DNM1* human microexon (HsaEX0020439). The corresponding genomic region was amplified by PCR from genomic DNA extracted from HEK 293 cells using genElute mammalian genomic DNA miniprep kit (Sigma) and cloned using EcoRV and NotI restriction sites under a *Cytomegalovirus* promoter. Furthermore, PT1 and PT2[62] sequences were added at the 5′ and 3′ ends of the minigene, respectively, for detection by reverse transcription PCR after transfection (Figure 1C). Second, for accurate PSI quantification, both the inclusion and exclusion isoforms should be readily identifiable in the output RNA-seq. Therefore, barcodes were added between PT1 and the first exon in the DNM1 minigene backbone prior to the cloning of the variants. For this purpose, the plasmid bearing the DNM1 minigene was amplified by PCR using the Taq Precision Plus enzyme (Agilent Technologies) with primers in PT1 and at the 5′ end of the first exon. An oligonucleotide pool containing the 34-nt long barcode NNNNAGCTNNNNTCAGNNNNTAGCNNNCAGTNNN and flanking sequences was ordered to IDT as 'hand mix' ssDNA and amplified through 10 cycles PCR using Taq Precision Plus enzyme (Agilent Technologies). The barcodes were cloned using the Gibson Assembly

cloning strategy with a mix of enzymes provided by the CRG Protein Technologies Unit. The plasmids were transformed into Stellar cells (Takara Bio). A pool of an estimate of 209,000 barcoded vectors was obtained. This minigene pool was used as backbone for the cloning of all the libraries used in this study.

Library design and cloning

A total of 234 events were chosen to build the MaPSy LME (Library of Micro(&)Exons) T0, including 207 microexons of lengths from 6 to 27 nts and 27 42-nt long constitutive exons. The microexons included 71 lowly sensitive (LS), 73 highly sensitive (HS) and 63 cryptic/control events. HS and LS microexons encompassed different proportions of pancreatic endocrine cell- and neuronal- specific microexons (Figure 1C, Table S1). The events used in LME T1-4 are listed in Table S2 and they were selected based on the similarity between their endogenous and MaPSy-derived inclusion profiles.

Each of the five libraries were designed to contain the WT sequences and a subset of the full set of variants (up to 6,000 sequences per library). These variants included various types of mutations and modifications (e.g. UGC mutations, strengthening/weakening of splice sites and other splice signals, semi-deep mutagenesis, inclusion of additional motifs, etc.), as well as swappings between events. All variants and the rationale behind their design are described in detail in Tables S5, S13, S14. These designed variants were ordered as ssDNA (from 164 to 200 nt in length) pools to Twist Bioscience (Table S15). Each sequence contained 93 nts of the upstream 3′ss, microexon/exon, and 25 nts of the downstream 5′ss, surrounded by two 20-nt long artificial sequences (Mega and Moe). The libraries were cloned into the barcoded DNM1 minigene backbone replacing the central exon and surrounding splice sites (93 nts of the upstream intron and 25 nts of the downstream intron) (Figure 1C). For this purpose, the plasmid was amplified by PCR using the Taq Precision Plus enzyme (Agilent Technologies) with primers designed to anneal intronic sequences and bear Mega / Moe overhang sequences, and further treated with DpnI (New England Biolabs). The ssDNA libraries were amplified with the Taq Precision Plus polymerase (Agilent Technologies) through 10 cycles PCR and cloned using the Gibson Assembly cloning strategy with a mix of enzymes provided by the CRG Protein Technologies Unit. The resulting plasmids were transformed into Stellar cells (Takara Bio). The total number of clones (barcode-variant pairs) was estimated after transformation to be: LME T0 76,000 clones; LME T1 145,700 clones; LME T2 104,084 clones; LME T3 198,400 clones; LME T4 163,680 clones. The plasmids were amplified and purified (Plasmid Maxiprep Kit, Qiagen).

The final pools of plasmids bearing the variants have the following structure: PT1 BC (34-nt long) exon 1 intron 1 with <u>Mega</u> **<u>VARIANT</u>** (*93 nt- EXON - 25 nt*) <u>Moe</u> intron 2 exon 3 25 nts downstream intron PT2, whose sequences are provided in SI.

Transfection of libraries, SRRM4 induction and RNA extraction and sequencing

Tetracycline inducible HEK 293 Flp-In T-REx cells expressing either GFP or SRRM4 generated in[10] were used. Each condition was performed in 6 replicates. Each replicate consisted in a transfection of 6 wells from a 6 wells plate. For each well, 400,000 cells were seeded in 1 ml of culture media the day before transfection and 80 ng of plasmids bearing the barcoded variants were transfected using Lipofectamine 2000 (Invitrogen) following the manufacturer's instructions. At the time of transfection, doxycycline at various concentrations was added to induce the expression of the protein of interest.

- LME T0: Non-induced GFP line (GFP) and SRRM4 expressing lines treated with 4.8 (LOW); 19.2 (MID) and 50 (HIGH) ng/ml doxycycline were used.
- LME T1/T2: Non-induced GFP line (GFP) and SRRM4 expressing lines treated with 0 (LOW); 1.2 (MID) and 50 (HIGH) ng/ml doxycycline were used.
- LME T3/T4: Non-induced GFP line (GFP) and SRRM4 expressing lines treated with 0 (LOW); 0.032 (MID) and 50 (HIGH) ng/ml doxycycline were used.

For all libraries, 24 hours after transfection, the cells from 6 wells of each condition were pooled and the RNA extracted using the Illustra RNAspin Mini RNA Isolation Kit (GE Healthcare). The consistency of regulation from T1/T2 and T3/T4 is represented as a scatter plot of GFP (VAR-WT) and HIGH (VAR-WT) for the 277 duplicated sequences in Figure S1G.

Amplification of the inputs and outputs of the libraries for RNA deep-sequencing

In order to associate each barcode with a variant in each plasmid, the pool of plasmids for each library (inputs) was amplified using primers bearing sequences from Illumina (Read 1 and Read 2) and complementary to PT1 and down to Moe (Figure 1C), represented as green amplicon). Each input was amplified and sequenced from three independent PCRs [7 cycles each using Taq Precision Plus (Agilent Technologies)]. The pattern of alternative splicing (outputs) was assessed by RT (with oligo-d(T) (IDT) / random hexamer (Roche Diagnostics GmbH) with AMV Reverse Transcriptase (Promega) and 10 cycles PCR with Taq Precision Plus (Agilent Technologies) using six primers (three forward and three reverse) bearing sequences from Illumina for sequencing 5′-Read 1 – (N/NB/NBK) – PT1-3′ and 5′-Read 2 – (N/NB/NBK) – part of last exon of DNM1-3′ reporter (Figure 1C, represented as red amplicon). All primers used are reported in Table S16.

For both inputs and outputs, PCR elongation times of 5 minutes were used to decrease the likelihood of generating truncated products that could serve as "mega-primer" in the next PCR cycles. The products of amplification were purified on AMPure XP beads (Beckman Coulter). Amplicons were checked using Bioanalyzer (Agilent) and the final steps amplification (consisting in the addition of the Illumina adaptors) were performed at CRG Genomics Unit. Both the input and output PCR products

were deep sequenced. After quantification by qubit, amplification was performed by PCR using NEBNext Q5 Hot Start HiFi PCR Master Mix (ref. M0543L) and Index 1 (i7) and Index 2 (i5) primers containing Illumina's P5 and P7 sequences. Final libraries were analyzed using Agilent Bioanalyzer or Fragment analyzer High Sensitivity assay (ref. 5067-4626 or ref. DNF-474) to estimate the quantity and check size distribution, and were then quantified by qPCR using the KAPA Library Quantification Kit KK4835 (Ref. 07960204001, Roche). Library LME T0 was sequenced in an Illumina HiSeq2000 machine using a 125+125 nt paired end read format; Libraries LME T1/T2 were sequenced in a NextSeq500 machine as 100+200 nt paired end reads; Libraries LME T3/T4 were sequenced in a NextSeq2000 machine as 100+200 nt paired end reads. An average of 13.4 and 37.6 million reads were generated for the inputs and outputs, respectively (Table S17). This data was submitted to GEO (GSE276143). For MaPSy T0, each of the six replicates was analyzed individually and the overlap of the six independent replicates is presented in Figure S1F. Based on the high correlation observed, the libraries T1, T2, T3, T4 were also performed in six independent replicates but pooled prior to sequencing [i.e., post amplification and purification on AMPure XP beads (Beckman Coulter)].

Identification of barcode-variant associations from MaPSy inputs

To identify barcode-variant associations we used the *identify_associations.py*, available at https://github.com/simon-bt/lme-mapsy. Each forward read contains the information of the barcode, whose sequence was retrieved using the regular expression *".*(CTTGCTCAAC){{s<=5}}(.{{{barcode_length}}})(GAATGTCTAC){{s<=5}}.*"* (PT1 34 nts BC exon 1). Each reverse read contains the information of the associated variant, whose identity was retrieved using the regular expression *'.*(GGGATAAGACGGTAGGC){s<=5}(.{93})'+(.{{{length}}})'+(.{25})(TCGTAGCACGTCACGGTTGG){s<=5}.*')* for reads of 200 nts (LME T1-4) and *'(.{{{lenupint-2}}}AG)'+ '(.{{{length}}})'+'(GT.{23})(TCGTAGCACGTCACGGTTGGAGCTCCAGCCAGGTTTTCAAGC){s<=7}.*')'* for reads of different lengths (LME T0), where *lenupint = len(read_sequence) - 42 - 25 - length*, and *length* corresponds to the length of the exon of each variant, and *s* to the number of allowed mismatches in each substring. The sequence identity of each variant was evaluated by calculating the Hamming distance between the retrieved sequence and the reference variant sequence.

Since MaPSy libraries were generated using a pre-barcoded minigene reporter, it is conceivable that the same barcode gets associated with one or multiple variants. Also, it is possible that template switching during PCR amplification leads to the detection of reads supporting spurious barcode-variant associations. To ensure an accurate quantification of variant inclusion, each barcode should be associated with a unique variant, or otherwise filtered out. Filtering of barcode-variant associations was performed using *resolve_associations.py*, available at https://github.com/simon-bt/lme-mapsy. In

brief, the identified barcode-variant associations were filtered for maximum percent of the mismatched sequence based on the Hamming distance (*max_mismatch_pct = 50,* for multiple matching variants associated with a barcode we considered the closest matching variant), a minimum read support of 5 for the main association (*min_nreads = 5*), presence of the main barcode-variant association in at least 2 input sequencing replicates (*min_rep = 2*), a maximum of 1 read supporting the second most represented variant associated to a given barcode (*nreads_second_highest = 1*), and maximum percentage (10%) of reads supporting the second most supported variant (*pct_second_best* <= 10). The barcodes that did not meet these requirements to be considered uniquely associated to a variant were considered ambiguous and discarded from further analyses. The set of filtered unique barcode-variant associations for each LME library was used to quantify inclusion of each variant from MaPSy output data.

Quantification and correction of variant inclusion levels from MaPSy outputs

The analysis of the output was performed using *quantify_inclusion.py* and *process_inclusion_data.py*, available at https://github.com/simon-bt/lme-mapsy. For each valid barcode supported by at least 3 reads (*min_nreads = 3*), exclusion and inclusion reads in the different experimental conditions were identified using the regular expression *'TGAGCGTGTTGGG(.*?)GCCAGCGAGACCG'*, corresponding to the last and first 13 nts of constant exons 1 and 3, and potentially a sequence in between. Exclusions read counts (EXC) corresponded to those for which no internal sequence was detected (exon1-exon3 junction with no sequence in between). Potential inclusion reads were then further investigated to assess if the sequence between the two flanking exons corresponded to the variant uniquely associated with the barcode. The inclusion reads (INC) were directly accepted and counted if the included sequence matched the reference sequence. We further added a read correction to account for potential INC sequences that do not match the expected variant sequence, and could result from various experimental (template switching) and splicing errors. For this, we calculated a proportion of reads derived from such reads to the sum of total potential inclusion reads (i.e. with any sequence in between exons 1-3). This proportion was used to calculate corrected inclusion level values, such that $INC_{corrected}$ = INC * ((100 + proportion) / 100).

Next, we selected barcode-variant associations supported by at least 3 reads after error correction (min_reads = 3), for which proportion of erroneous inclusion reads was smaller than 75% (max_proportion < 75). For every variant represented by at least 2 barcodes (*min_bc* = 2) in at least 2 samples (min_samples = 2), we derived a single PSI per variant (and condition). For this purpose, we first calculated a corrected PSI value for each barcode-variant association using corrected inclusion reads, such that $PSI_{corrected}$ = * 100 / ($INC_{corrected}$ + EXC). Next, we calculated the median PSI per variant (and condition) of all $PSI_{corrected}$ values (*median_psi*), and an absolute difference between that value

and each barcode-variant $PSI_{corrected}$ value (*psi_diff* = *median_psi* - $PSI_{corrected}$) to assess the trend in per-barcode values. We accepted barcode-variant association under the following conditions: (i) if (*median_psi* >= 90 OR *median_psi* < 10) AND *psi_diff* < 10, (ii) elsif (*median_psi* >= 70 OR *median_psi* < 30) AND *psi_diff* < 15, (iii) elsif (30 <= *median_psi* < 70) AND *psi_diff* < 20. For each variant, we then aggregated error-corrected inclusion reads ($INC_{corrected}$) and exclusion reads for filtered barcoded data per variant (and condition) and calculated a single PSI value per variant and condition. Manual inspection of multiple WT constructs show that the PSI values after these two correction steps better recapitulated the endogenous microexon inclusion levels.

Finally, to obtain information for as many designed variants as possible, for those variants for which no PSI was calculated due to the applied thresholds (generally a single barcode associated to the variant), we retrieved the inclusion information, when possible, from intermediate files (CORRECTED_COUNTS, see README.md) and similar steps as above were applied. While these PSIs are less robust, they were included in the analyses to increase the proportion of variants covered and they were nonetheless only a minority of cases. Specifically, for each library and condition, 88.3-96.4% of variants were covered by the main quantification strategy and 3.4-8.7% corresponded to these "rescued" PSIs. The percent of variants per library without an assigned PSI ranged from 0.2% (T4 library) to 3.3% (T1 library).

The level of correlation of the PSI between the initial calculated PSI and the retrieved ones in LME_T0 in condition of HIGH level of SRRM4 expression and six biological replicates are presented in Figure S1F.

Specific sequence feature analyses

In Figure 4A, the most relevant UGC was defined as the UGC mutation having the strongest impact on inclusion levels (negative ΔPSI) in the condition of HIGH level of expression of SRRM4. The most optimal UGC in the “UGC-addition walk” was defined as the single UGC addition having the strongest impact on inclusion levels (positive ΔPSI) in HIGH level of expression of SRRM4. The relative distance between the positions of these two UGCs was calculated for each microexon and the counts plotted as a density. The distances from UGC to AG, Py 3′ end and BP were calculated considering the distances defined for the best predicted BP[63]. In Figure 6G, features for each variant were recovered using either SVM-BP or MAXENT tools (Table S9) and the “variation in feature” was calculated as the score of a feature for a given variant with respect to its corresponding value in the WT sequence. All LS or HS events in LME T1-4 for which the delta feature (VAR-WT) for MAXENT5 (Figure 6G, left), MAXENT3 (Figure 6G, right) or agez (Figure 6G, middle) was not zero were considered for the corresponding analysis after removing the length variants (either elongated or shortened). ESRseq

scores[40] were used to generate the line plots in Figure S2B. The score of each successive hexamer in each of the 15 sequences described in Figure S2A is represented as a line from the 5′ end to the 3′ end of each sequence. The same approach was used to calculate the ESRseq scores for LS, HS, CS and CSrem events upon shortening or lengthening with 15 sequences in LME T1, whose mean values are represented in Figure S2C-F.

In Figure 7H, publicly available datasets were collected and analyzed using *vast-tools* v2.5.1 (Table S11). The change in splicing in the different experimental conditions of LS and HS microexons was calculated as ΔPSI (KD-CTR), irrespectively of read coverage to increase the sample size. For Figure 7F,G and S7F-H, hg19 coordinates of the LS and HS events described in Table S1 were retrieved using liftover (https://genome.ucsc.edu/cgi-bin/hgLiftOver). The intensities of the peaks corresponding to the 3′ss occupancy by U2 snRNP in the high molecular weight chromatin fraction in the last 93 nts of the upstream intron upon immunoprecipitation of RBM5, RBM10 and SF3A3 were retrieved from[53] (supplementary materials kindly provided by the authors) and used to generate Figures 7F,G and S7F-H. In addition, to ensure that the results were not biased by differences in gene expression, (i) peak intensities over the entire transcripts with microexons for which U2 snRNP binding was detected, and (ii) their gene expression (*vast-tools* option *--expr*) were analyzed (Figure S7I,J). In Figure S7B-D, PAR-iCLIP tag densities and positions were retrieved from[9] from the *GSE57278_293TnSR100_PariCLIP_pool2.bedgraph* strand agnostic processed file and matched against hg19 microexon coordinates. Events with tags in the last 200 nts of the upstream intron were retrieved to generate the barplot (Figure S7C) and a distance of 150 nts was chosen to generate the Figure S7B,D.

Experimental validations with single minigene transfection

DAAM1 minigene amplified from human genomic DNA, surrounded by PT1/PT2[62], was cloned under a *Cytomegalovirus* promoter and mutated using primers described in Table S16. *apbb1* and *asap1* minigenes from *Danio rerio* were previously generated for[10]. Stronger 5′ss mutations were performed using primers described in Table S16. Other variants from the MaPSy were cloned using the same strategy as for the library cloning. Inserts were either ordered as a unique ssDNA encompassing Mega, 93 nts of the 3′ss, microexon, 25 nts of the 5′ss, and Moe, or two overlapping sequences (Table S16). The inserts were amplified by PCR using either Mega Fwd and Moe Rev primers or Fwd and Rev primers from the overlapping sequences, respectively. The fragment was cloned using the Gibson cloning strategy into the DNM1 minigene backbone. Specific mutations were generated by PCR around the vector using Taq Precision Plus polymerase (Agilent Technologies). The amplified vector was treated with DpnI (New England Biolabs), T4 DNA polymerase (New England Biolabs) and ligated using T4 DNA ligase (New England Biolabs) following the manufacturer's instructions. The clones

were transformed in *in-house* prepared XL1-Blue heat shock competent cells, single clones were amplified in LB supplemented with ampicillin, purified using Qiaprep miniprep kit (Qiagen) and sequenced (Eurofins).

To investigate the pattern of alternative splicing, the minigenes were transfected. For this purpose, 400,000 cells (HEK 293 or HEK 293 Flp-In T-REx cells expressing SRRM4 in a doxycycline inducible manner) were seeded in a 6 wells plate the day prior the transfection. 24 hours later, HEK 293 Flp-In T-REx cells were transfected with 10ng of minigene and the expression of SRRM4 was induced with 50 ng/ml of doxycycline. For transient expression, HEK 293 cells were transfected with 10 ng minigene and 1 ug of the plasmid allowing the expression of the protein of interest (beta galactosidase as negative control and SRRM4 human / SRRM234 Amphioxus[10]). The transfection was performed using Lipofectamine 2000, following the instructions of the provider. Cells were collected 24 hours post-transfection and the RNA was isolated using the Illustra RNAspin Mini RNA Isolation Kit (GE Healthcare). The pattern of alternative splicing of individual minigenes was detected by RT-PCR assays. Reverse transcription was performed using oligo-d(T) (IDT) and random hexamer (Roche Diagnostics GmbH) using an enzyme provided by the CRG Protein Technologies Unit in 5X First-Strand Buffer (250 mM Tris-HCl pH 8.3; 375 mM KCl; 15 mM $MgCl_2$). The PCR was performed using primers PT1 and in the last exon of the minigene with the enzyme GoTaq (Promega). The amplicons were separated on 7% acrylamide 37,5:1 (PanReac Applichem) run in TBE 1X (10.8 g Trizma base; 5.5 g Boric Acid and 4 ml of 0.5M EDTA pH 8.0 per liter) and detected by Gel Red staining (Biotum) using the gel doc software Quantity One 4.6.9. Fiji (version 1) was used for the quantification of the amplicons' intensities. The PSI for each biological replicate is calculated as 100 * [inclusion / (inclusion + skipping)] and the quantifications are provided in Table S19.

In vitro **spliceosomal A complex formation analyses**

The ssDNA libraries were converted into dsRNA by PCR using primers in T7 promoter _ Mega Fwd and Moe Rev sequences and the Taq Precision Plus enzyme (Agilent Technologies). Cy5-CTP/Cy5-UTP (Cytiva) labeled RNA were transcribed from the PCR templates using the Megascript T7 Transcription kit (Ambion) according to the manufacturer's instructions. Each library was assembled in four independent reactions. Per reaction, 15 ng/ul fluorescently labeled RNA were incubated with 3 ul of HeLa cell nuclear extracts (CILBIOTECH, CC-01-20-50) supplemented with 3 mM $MgCl_2$, 24.9 mM KCl, 3.33% PVA, 13.3 mM HEPES pH 8, 0.13 mM EDTA,13.3 % glycerol, 0.03 % NP-40, 0.66 mM DTT, 2 mM ATP and 22 mM creatine phosphate in a final volume of 9 microliters. The mixture was incubated for 18 minutes at 30ºC. 1 microliter of heparin (50 ug/ul stock) was added and incubated for 10 minutes at room temperature. 3 microliters of 50% glycerol were added and 9 microliters loaded on a composite gel (4% acrylamide, 0.05% bis-acrylamide, 0.5% agarose, 50 mM Tris, 50 mM

Glycine). The gel was run for ~ 4 hours at 200 Volts in 50 mM Tris / 50 mM Glycine buffer. After electrophoresis, the fluorescence was detected using a Typhoon PhosphorImager (Amersham and corresponding Cytiva software). The pieces of gel containing the products corresponding to twice two reactions of A complex formation were cut (Figure S7B) and the RNA extracted in an RNA elution buffer (0.5 M NH_4Ac, 10 mM $MgCl_2$, 1 mM EDTA, 0.1 % SDS) for 6 hours under shaking at 50 degrees. The supernatant was extracted with phenol / chloroform and precipitated with three volumes of ethanol absolute. The RNA was resuspended in water and used for one RT reaction further used in 2 PCR. The reverse transcription was performed using the Moe Rev primer using the AMV Reverse Transcriptase (Promega). The PCR was performed using the Taq Precision Plus (Agilent Technologies) and primers bearing Illumina compatible reads, the corresponding amplicons are represented in green (Figure S7B). The PCRs were pooled prior to purification on AMPure XP beads (Beckman Coulter). In parallel, the RNA used to perform the assay were amplified by RT-PCR to determine the enrichment scores of each variant with respect to its initial expression. The enrichment scores (ES) for libraries T1, T2 are provided in Table S20. Each library was sequenced on an Illumina NextSeq500 machine, to produce an average of 1.95 and 1.56 million 100+200 long paired end reads for inputs and outputs, respectively. This data was submitted to GEO (GSE276143). Spliceosomal A complex formation on ITSN1 was performed as described in[10] on substrates bearing 93 nts of the flanking 3′ss _ microexon _ 25 nts of the flanking 5′ss surrounded by Mega and Moe sequences. Quantification of the biological replicates presented in Figure 6F is provided in Table S8.

Mathematical model of microexon regulation by SRRM4

We modeled microexon inclusion regulation by SRRM4 with a system of coupled Ordinary Differential Equations (ODEs). See Figure 7A for a cartoon of the model with the species and rates. The model considers an unbound pre-mRNA species P which is transcribed at rate α. The spliceosome, considered at a coarse level as a single enzyme, assembles around the microexon on the pre-mRNA at rate c_1 to yield species S, and disassembles at rate c_2. On the other hand, SRRM4 can bind to the pre-mRNA at rate c_3x, with x the concentration of SRRM4, to yield species R, and unbinding occurs at rate c_4. We assume that bound SRRM4 can favor spliceosomal recruitment to the microexon, by enhancing its assembly by a factor $c_a>1$, and reducing disassembly by a factor $0<c_d<1$. We also assume that SRRM4 binding can follow spliceosomal assembly, and for simplicity we assume that this happens at the same rates as when there is no spliceosome bound ($c_5= c_3$ and $c_6= c_4$).

In addition to binding and unbinding, the pre-mRNA can undergo microexon skipping or inclusion. We assume that there is a basal skipping rate k_e, which changes to k_{es} when the spliceosome is on the microexon. Furthermore, the inclusion rate when the spliceosome is present alone is k_{is}, and we

assume that SRRM4 can modulate this rate by a factor c_i. Finally, the mature mRNA products are degraded at rate δ. This yields the following system of coupled ODEs:

$$\frac{dP}{dt} = \alpha_0 + c_2 S + c_4 R - (k_e + c_1 + c_3 x)P$$
$$\frac{dS}{dt} = c_1 P + c_6 C - (c_2 + c_5 x + k_{es} + k_{is})S$$
$$\frac{dR}{dt} = c_3 x P + c_2 c_d C - (c_4 + c_1 c_a + k_e)R$$
$$\frac{dC}{dt} = c_5 x S + c_1 c_a R - (c_6 + c_2 c_d + k_{es} + k_{is} c_i)C$$
$$\frac{dE}{dt} = k_e P + k_{es} S + k_e R + k_{es} C - \delta E$$
$$\frac{dI}{dt} = k_{is} S + k_{is} c_i C - \delta I$$

PSI is quantified as: $PSI = \frac{I^*}{(I^* + S^*)}$

where Y^* denotes steady-state species Y.

We set δ=1 (time unit)$^{-1}$, so that the rest of the rates are in units of the mRNA degradation rate. In order to find the steady state, we solve for the solution of the corresponding linear system using the linalg.solve function from the Python numpy package. We double checked that the same solutions are obtained by integrating the ODE system until steady-state using the *odeint* function of Scipy. The corresponding code is provided at https://github.com/theobiolab/SRRM4_MapSy_2024.git.

Model fitting

In order to test whether the model could account for the diversity of the responses to SRRM4 observed in the data, we selected the responses of the WT LS and HS for endogenous microexons and the minigene constructs, as well as the mutants that make the interaction with the U1 snRNA stronger (U1cons_b), and the mutants that make the BP stronger (BP_stg1) or weaker (BP_weak3). We took the median of the PSI values across the sequences for the various groups at each of the four regulator concentrations (GFP, LOW, MID, HIGH). We fixed the concentration of SRRM4 to 0 for the GFP condition, and to 1 for the LOW condition, so that the concentrations of the MID and HIGH conditions are in units of SRRM4 concentration in the LOW condition. We then searched for parameter sets that would best fit the data allowing the groups to share the MID and HIGH SRRM4 concentration, as well as all binding, unbinding, skipping and inclusion rates except for c_2, or c_2 and k_{is}. These are parameters related to basal spliceosome recruitment (c_2) or activity (k_{is}), and by allowing them to be the only differences across groups we could test whether the data could be explained by just allowing changes at this basal spliceosome recruitment and or activity level, without involving differences in recruitment and or effects of SRRM4. For the groups shown in Figure 7D, we found a good fit even if only

considering variations in c_2 alone, so we show the results corresponding to this scenario. We observed that other groups of sequences were better fitted by varying the two parameters (not shown).

Given the coarse level of our models and the lack of measurements for the parameters, we allowed the parameters to be within a broad range, spanning from 10^{-4} to 10^{4}. We searched for optimum parameter sets by using the genetic algorithm implementation of the eaSimple function of the Python Deap package for evolutionary algorithms. We used the following settings and hyperparameters: two-point crossover, tournament selection with size 3, gaussian mutation, a population size of 400, 500 generations, probability of crossover 0.25, probability of mutating an individual 0.75, the standard deviation of the gaussian mutation 0.5, and the probability of mutating an individual parameter 0.3. After this, the best solution was refined by refining c_2 by local minimization, using the minimize function of scipy optimize, starting from the identified c_2 as well as 10 additional random conditions from within the bounds.

Parameter name	Parameter value	Units
x (MID)	11.1	c.u.
x (HIGH)	378.3	c.u.
α	0.0009	$t.u.^{-1}$
c_1	0.119	$t.u.^{-1}$
c_2 (WTendo-LS)	167	$t.u.^{-1}$
c_2 (WTendo-HS)	0.029	$t.u.^{-1}$
c_2 (WTminigene-LS)	92.7	$t.u.^{-1}$
c_2 (WTminigene-HS)	0.092	$t.u.^{-1}$
c_2 (U1cons_b-LS)	0.0513	$t.u.^{-1}$
c_2 (U1cons_b-HS)	0.004	$t.u.^{-1}$
c_2 (BP_stg1-LS)	15.9	$t.u.^{-1}$
c_2 (BP_stg1-HS)	0.0014	$t.u.^{-1}$
c_2 (BP_weak3-LS)	10000	$t.u.^{-1}$
c_2 (BP_weak3-HS)	129.4	$t.u.^{-1}$
c_3	0.0026	$(c.u. \times t.u.)^{-1}$
c_4	0.221	$t.u.^{-1}$
c_a	10.1	-
c_d	0.033	-
k_e	0.030	$t.u.^{-1}$

k_{es}	0.00085	$t.u.^{-1}$
k_{is}	0.00032	$t.u.^{-1}$
c_i	464.5	-

Parameter values for the modeling plots in Figure 7D.
c.u: Concentration of SRRM4 in the LOW condition (x (LOW)=1 c.u.)
t.u: time units (mRNA degradation rate = 1 t.u.).

Data visualization

In all panels with boxplots, the boxes represent the interquartile range of the PSI or ΔPSI from the 25th percentile to the 75th percentile, the median is represented by the line in the box, the whiskers represent the minimal and maximal values. Outliers are not displayed. In all panels with line plots, the lines represent an estimate of the central tendency and the corresponding 95% confidence interval of the represented values.

REFERENCES

1. Ule, J. & Blencowe, B. J. Alternative Splicing Regulatory Networks: Functions, Mechanisms, and Evolution. *Mol Cell* **76**, 329–345 (2019).
2. Baralle, F. E. & Giudice, J. Alternative splicing as a regulator of development and tissue identity. *Nat Rev Mol Cell Biol* **18**, 437–451 (2017).
3. Mantica, F. & Irimia, M. The 3D-Evo Space: Evolution of Gene Expression and Alternative Splicing Regulation. *Annu Rev Genet* **56**, 315–337 (2022).
4. Juan-Mateu, J. *et al.* Pancreatic microexons regulate islet function and glucose homeostasis. *Nat Metab* **5**, 219–236 (2023).
5. Irimia, M. *et al.* A highly conserved program of neuronal microexons is misregulated in autistic brains. *Cell* **159**, 1511–1523 (2014).
6. Nakano, Y., Wiechert, S. & Bánfi, B. Overlapping Activities of Two Neuronal Splicing Factors Switch the GABA Effect from Excitatory to Inhibitory by Regulating REST. *Cell Rep* **27**, 860-871.e8 (2019).
7. Ciampi, L., Serrano, L. & Irimia, M. Unique transcriptomes of sensory and non-sensory neurons: insights from Splicing Regulatory States. *Mol Syst Biol* **20**, 296–310 (2024).
8. Wan, R., Bai, R., Zhan, X. & Shi, Y. How Is Precursor Messenger RNA Spliced by the Spliceosome? *Annu Rev Biochem* **89**, 333–358 (2020).

9. Raj, B. *et al.* A global regulatory mechanism for activating an exon network required for neurogenesis. *Mol Cell* **56**, 90–103 (2014).
10. Torres-Méndez, A. *et al.* A novel protein domain in an ancestral splicing factor drove the evolution of neural microexons. *Nat Ecol Evol* **3**, 691–701 (2019).
11. Gonatopoulos-Pournatzis, T. & Blencowe, B. J. Microexons: at the nexus of nervous system development, behaviour and autism spectrum disorder. *Curr Opin Genet Dev* **65**, 22–33 (2020).
12. Nakano, Y. *et al.* A mutation in the Srrm4 gene causes alternative splicing defects and deafness in the Bronx waltzer mouse. *PLoS Genet* **8**, e1002966 (2012).
13. Torres-Méndez, A. *et al.* Parallel evolution of a splicing program controlling neuronal excitability in flies and mammals. *Sci Adv* **8**, eabk0445 (2022).
14. Li, Y. I., Sanchez-Pulido, L., Haerty, W. & Ponting, C. P. RBFOX and PTBP1 proteins regulate the alternative splicing of micro-exons in human brain transcripts. *Genome Res* **25**, 1–13 (2015).
15. Matalkah, F. *et al.* The Musashi proteins direct post-transcriptional control of protein expression and alternate exon splicing in vertebrate photoreceptors. *Commun Biol* **5**, 1011 (2022).
16. Ciampi, L., Serrano, L. & Irimia, M. Unique transcriptomes of sensory and non-sensory neurons: insights from Splicing Regulatory States. *Mol Syst Biol* **20**, 296–310 (2024).
17. Gonatopoulos-Pournatzis, T. *et al.* Genome-wide CRISPR-Cas9 Interrogation of Splicing Networks Reveals a Mechanism for Recognition of Autism-Misregulated Neuronal Microexons. *Mol Cell* **72**, 510-524.e12 (2018).
18. Lee, J.-S., Lamarche-Vane, N. & Richard, S. Microexon alternative splicing of small GTPase regulators: Implication in central nervous system diseases. *Wiley Interdiscip Rev RNA* **13**, e1678 (2022).
19. Ohnishi, T., Shirane, M. & Nakayama, K. I. SRRM4-dependent neuron-specific alternative splicing of protrudin transcripts regulates neurite outgrowth. *Sci Rep* **7**, 41130 (2017).
20. Rusconi, F. *et al.* LSD1 Neurospecific Alternative Splicing Controls Neuronal Excitability in Mouse Models of Epilepsy. *Cereb Cortex* **25**, 2729–2740 (2015).
21. Gupta, A. K. *et al.* Degenerate minigene library analysis enables identification of altered branch point utilization by mutant splicing factor 3B1 (SF3B1). *Nucleic Acids Res* **47**, 970–980 (2019).
22. Mikl, M., Hamburg, A., Pilpel, Y. & Segal, E. Dissecting splicing decisions and cell-to-cell variability with designed sequence libraries. *Nat Commun* **10**, 4572 (2019).
23. Wong, M. S., Kinney, J. B. & Krainer, A. R. Quantitative Activity Profile and Context Dependence of All Human 5' Splice Sites. *Mol Cell* **71**, 1012-1026.e3 (2018).
24. Ishigami, Y. *et al.* Specificity, synergy, and mechanisms of splice-modifying drugs. *Nat Commun* **15**, 1880 (2024).

25. Julien, P., Miñana, B., Baeza-Centurion, P., Valcárcel, J. & Lehner, B. The complete local genotype-phenotype landscape for the alternative splicing of a human exon. *Nat Commun* **7**, 11558 (2016).
26. Baeza-Centurion, P., Miñana, B., Schmiedel, J. M., Valcárcel, J. & Lehner, B. Combinatorial Genetics Reveals a Scaling Law for the Effects of Mutations on Splicing. *Cell* **176**, 549-563.e23 (2019).
27. Baeza-Centurion, P., Miñana, B., Valcárcel, J. & Lehner, B. Mutations primarily alter the inclusion of alternatively spliced exons. *Elife* **9**, e59959 (2020).
28. Rhine, C. L. *et al.* Massively parallel reporter assays discover de novo exonic splicing mutants in paralogs of Autism genes. *PLoS Genet* **18**, e1009884 (2022).
29. Rong, S. *et al.* Large-scale functional screen identifies genetic variants with splicing effects in modern and archaic humans. *Proc Natl Acad Sci U S A* **120**, e2218308120 (2023).
30. Soemedi, R. *et al.* Pathogenic variants that alter protein code often disrupt splicing. *Nat Genet* **49**, 848–855 (2017).
31. Wang, Z. *et al.* Systematic identification and analysis of exonic splicing silencers. *Cell* **119**, 831–845 (2004).
32. Enculescu, M. *et al.* Exon Definition Facilitates Reliable Control of Alternative Splicing in the RON Proto-Oncogene. *Biophys J* **118**, 2027–2041 (2020).
33. Findlay, G. M., Boyle, E. A., Hause, R. J., Klein, J. C. & Shendure, J. Saturation editing of genomic regions by multiplex homology-directed repair. *Nature* **513**, 120–123 (2014).
34. Fairbrother, W. G. & Chasin, L. A. Human genomic sequences that inhibit splicing. *Mol Cell Biol* **20**, 6816–6825 (2000).
35. Rosenberg, A. B., Patwardhan, R. P., Shendure, J. & Seelig, G. Learning the sequence determinants of alternative splicing from millions of random sequences. *Cell* **163**, 698–711 (2015).
36. Adamson, S. I., Zhan, L. & Graveley, B. R. Vex-seq: high-throughput identification of the impact of genetic variation on pre-mRNA splicing efficiency. *Genome Biol* **19**, 71 (2018).
37. Ke, S. *et al.* Saturation mutagenesis reveals manifold determinants of exon definition. *Genome Res* **28**, 11–24 (2018).
38. Baeza-Centurion, P. *et al.* Deep indel mutagenesis reveals the regulatory and modulatory architecture of alternative exon splicing. Preprint at https://doi.org/10.1101/2024.04.21.590414 (2024).
39. Desmet, F.-O. *et al.* Human Splicing Finder: an online bioinformatics tool to predict splicing signals. *Nucleic Acids Res* **37**, e67 (2009).
40. Ke, S. *et al.* Quantitative evaluation of all hexamers as exonic splicing elements. *Genome Res* **21**, 1360–1374 (2011).

41. Black, D. L. Does steric interference between splice sites block the splicing of a short c-src neuron-specific exon in non-neuronal cells? *Genes Dev* **5**, 389–402 (1991).
42. Cáceres, E. F. & Hurst, L. D. The evolution, impact and properties of exonic splice enhancers. *Genome Biol* **14**, R143 (2013).
43. Paronetto, M. P. *et al.* Regulation of FAS exon definition and apoptosis by the Ewing sarcoma protein. *Cell Rep* **7**, 1211–1226 (2014).
44. Mackereth, C. D. *et al.* Multi-domain conformational selection underlies pre-mRNA splicing regulation by U2AF. *Nature* **475**, 408–411 (2011).
45. Zillmann, M., Zapp, M. L. & Berget, S. M. Gel electrophoretic isolation of splicing complexes containing U1 small nuclear ribonucleoprotein particles. *Mol Cell Biol* **8**, 814–821 (1988).
46. Watakabe, A., Tanaka, K. & Shimura, Y. The role of exon sequences in splice site selection. *Genes Dev* **7**, 407–418 (1993).
47. Wu, S., Romfo, C. M., Nilsen, T. W. & Green, M. R. Functional recognition of the 3' splice site AG by the splicing factor U2AF35. *Nature* **402**, 832–835 (1999).
48. Zorio, D. A. & Blumenthal, T. Both subunits of U2AF recognize the 3' splice site in Caenorhabditis elegans. *Nature* **402**, 835–838 (1999).
49. Merendino, L., Guth, S., Bilbao, D., Martínez, C. & Valcárcel, J. Inhibition of msl-2 splicing by Sex-lethal reveals interaction between U2AF35 and the 3' splice site AG. *Nature* **402**, 838–841 (1999).
50. Joudaki, A. *et al.* FexSplice: A LightGBM-Based Model for Predicting the Splicing Effect of a Single Nucleotide Variant Affecting the First Nucleotide G of an Exon. *Genes (Basel)* **14**, 1765 (2023).
51. Rhode, B. M., Hartmuth, K., Westhof, E. & Lührmann, R. Proximity of conserved U6 and U2 snRNA elements to the 5' splice site region in activated spliceosomes. *EMBO J* **25**, 2475–2486 (2006).
52. Yeo, G. & Burge, C. B. Maximum entropy modeling of short sequence motifs with applications to RNA splicing signals. *J Comput Biol* **11**, 377–394 (2004).
53. Damianov, A. *et al.* The splicing regulators RBM5 and RBM10 are subunits of the U2 snRNP engaged with intron branch sites on chromatin. *Mol Cell* **84**, 1496-1511.e7 (2024).
54. Head, S. A. *et al.* Silencing of SRRM4 suppresses microexon inclusion and promotes tumor growth across cancers. *PLoS Biol* **19**, e3001138 (2021).
55. Quesnel-Vallières, M., Irimia, M., Cordes, S. P. & Blencowe, B. J. Essential roles for the splicing regulator nSR100/SRRM4 during nervous system development. *Genes Dev* **29**, 746–759 (2015).
56. Quesnel-Vallières, M. *et al.* Misregulation of an Activity-Dependent Splicing Network as a Common Mechanism Underlying Autism Spectrum Disorders. *Mol Cell* **64**, 1023–1034 (2016).
57. Capponi, S. *et al.* Neuronal-specific microexon splicing of TAF1 mRNA is directly regulated by SRRM4/nSR100. *RNA Biol* **17**, 62–74 (2020).

58. Kumar, S. *et al.* TimeTree 5: An Expanded Resource for Species Divergence Times. *Mol Biol Evol* **39**, msac174 (2022).
59. Freund, M. *et al.* Extended base pair complementarity between U1 snRNA and the 5' splice site does not inhibit splicing in higher eukaryotes, but rather increases 5' splice site recognition. *Nucleic Acids Res* **33**, 5112–5119 (2005).
60. Tapial, J. *et al.* An atlas of alternative splicing profiles and functional associations reveals new regulatory programs and genes that simultaneously express multiple major isoforms. *Genome Res* **27**, 1759–1768 (2017).
61. Huerta-Cepas, J., Serra, F. & Bork, P. ETE 3: Reconstruction, Analysis, and Visualization of Phylogenomic Data. *Mol Biol Evol* **33**, 1635–1638 (2016).
62. Sakamoto, H., Inoue, K., Higuchi, I., Ono, Y. & Shimura, Y. Control of Drosophila Sex-lethal pre-mRNA splicing by its own female-specific product. *Nucleic Acids Res* **20**, 5533–5540 (1992).
63. Corvelo, A., Hallegger, M., Smith, C. W. J. & Eyras, E. Genome-wide association between branch point properties and alternative splicing. *PLoS Comput Biol* **6**, e1001016 (2010).

MAIN FIGURES

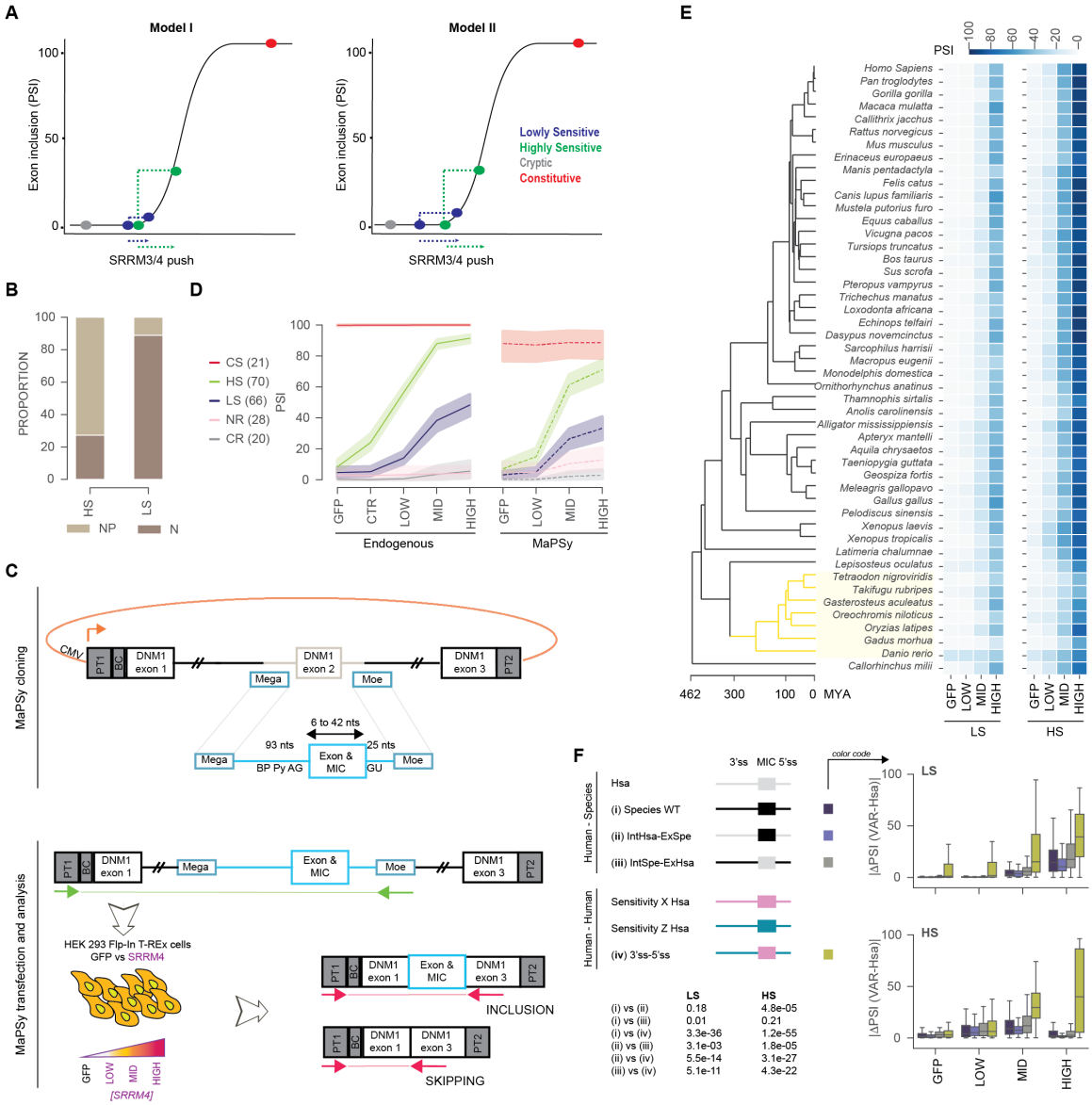

Figure 1: MaPSy strategy recapitulates endogenous microexon sensitivity to SRRM3/4

(**A**) Proposed models of dose-dependent response of microexons with different sensitivities to SRRM3/4. For each exon type, its inclusion level (PSI) in the absence of SRRM3/4 is represented in a PSI continuum, from 0 to 100, which follows a sigmoid curve, as described by[26]. The effect of having a given amount of SRRM3/4 is represented for LS and HS microexons as an enhancing effect, or push, towards higher PSI levels. The difference between the two models resides on the magnitude of this effect and the starting position within this curve for the two types of microexons. According to Model I, the starting position is similar, but the enhancing effect is stronger for HS microexons. In

contrast, according to Model II, the effect is similar for both types but HS microexons are shifted towards the right in the PSI curve, i.e. they are more primed for recognition and inclusion under basal conditions. (**B**) Proportion of LS and HS microexons previously reported as neuronal-specific (N) or neuronal + pancreatic endocrine-specific (NP)[4]. HS = 40 NP + 15 N, LS = 5 NP + 40 N. The remaining microexons were not assigned to either category. (**C**) Overview of the MaPSy strategy for investigating the patterns of microexon splicing *ex vivo*. Pre-designed variants were cloned into a barcoded minigene reporter (top panel) and transfected into HEK 293 Flp-In T-REx cells expressing either GFP (black) or SRRM4 at different levels upon doxycycline titration (purple) (bottom panel). Primers used for sequencing are depicted in green and red (Methods). (**D**) Inclusion levels upon SRRM4 titration in the endogenous (left) or in the MaPSy (right) context for the subset of CS, HS, LS, NR (Non Responding), CR events presented in Figure S1A with sufficient read coverage across all nine experimental conditions (x-axis, Methods). (**E**) The heatmaps represent the mean of the PSI for orthologous WT sequences in four experimental conditions (x-axis; GFP and LOW, MID, HIGH expression of SRRM4) for LS (left) or HS (right) events across a phylogeny of 47 jawed vertebrates (y-axis). Time scale in million years ago (MYA) from TimeTree[58]. The teleost branch is marked in yellow. (**F**) Left: representation of the different intron swappings performed. Variants include: (i) WT orthologous non-human sequences (Species WT), (ii) swapping both flanking intronic sequences from human (Hsa) to another species, (iii) swapping both flanking intronic sequences from another species to human, and (iv) swapping both flanking intronic sequences from one sensitivity group X (LS or HS) to a different sensitivity group Z (HS or LS). The color codes used in the boxplots on the right side are indicated. Right: the boxplots show the absolute change in PSI of each variant type respect to the WT human sequence (|ΔPSI (VAR-Hsa)|). Teleosts were removed from the analysis. Results of Mann-Whitney-Wilcoxon test in HIGH expression level of SRRM4 are indicated for LS and HS events for the indicated comparisons (bottom-left panel). See also Figure S1.

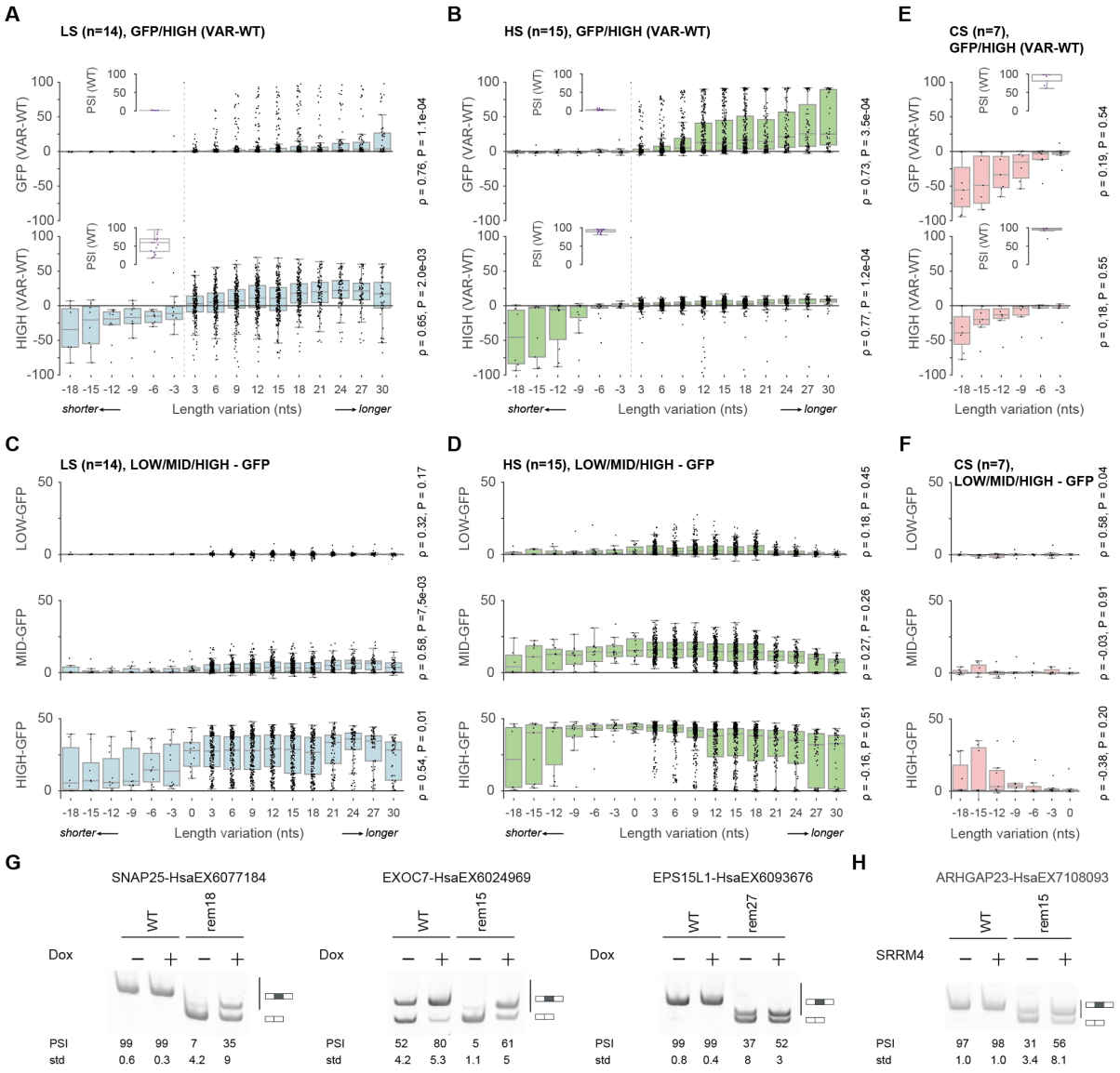

Figure 2: Exonic length contributes to splicing regulation in response to SRRM3/4

(**A,B,E**) Impact of exon length on the pattern of splicing and regulation by SRRM4 for LS (A), HS (B) and CS (E) events. Change in exon inclusion upon shortening or lengthening was quantified as ΔPSI (VAR-WT) (i.e. difference in PSI between the different length VARiants and their WT counterpart) in either the GFP condition or under HIGH level of expression of SRRM4. Each data point corresponds to the effect of adding a given number of nucleotides from one of the fifteen designed sequences (positive number on the x-axis), or removal of a given number of nucleotides from the exonic sequence (negative number on the x-axis). The boxplots in the insets represent the distribution of PSIs of the WT constructs in each experimental condition. In (A,B), the reference length of the WT is indicated by the dashed vertical line. (**C,D,F**) Impact of length variation on the pattern of splicing

and regulation under different levels of expression of SRRM4 with respect to GFP condition for each sequence, expressed as ΔPSI (HIGH/MID/LOW - GFP), for LS (C), HS (D) and CS (F) events. In (A-F), the numbers at the right side of each subpanel indicate the Spearman correlation (ρ) and corresponding p-value (*P*) of the effects in PSI according to length variation. (**G,H**) RT-PCR assays showing the splicing pattern under control condition (-) or expression of SRRM4 (+) of four CS events (WT length of 42 nt) and their length variants, in which the indicated number of nucleotides, N, has been removed (remN). PSI and standard deviations (std) from five biological replicates are provided. In (G), HEK 293 Flp-In T-REx cells non treated or treated with doxycycline were used. In (H), transient expression of a control vector (-) or SRRM4 expression vector (+) in HEK 293 cells was performed. See also Figures S2,S3.

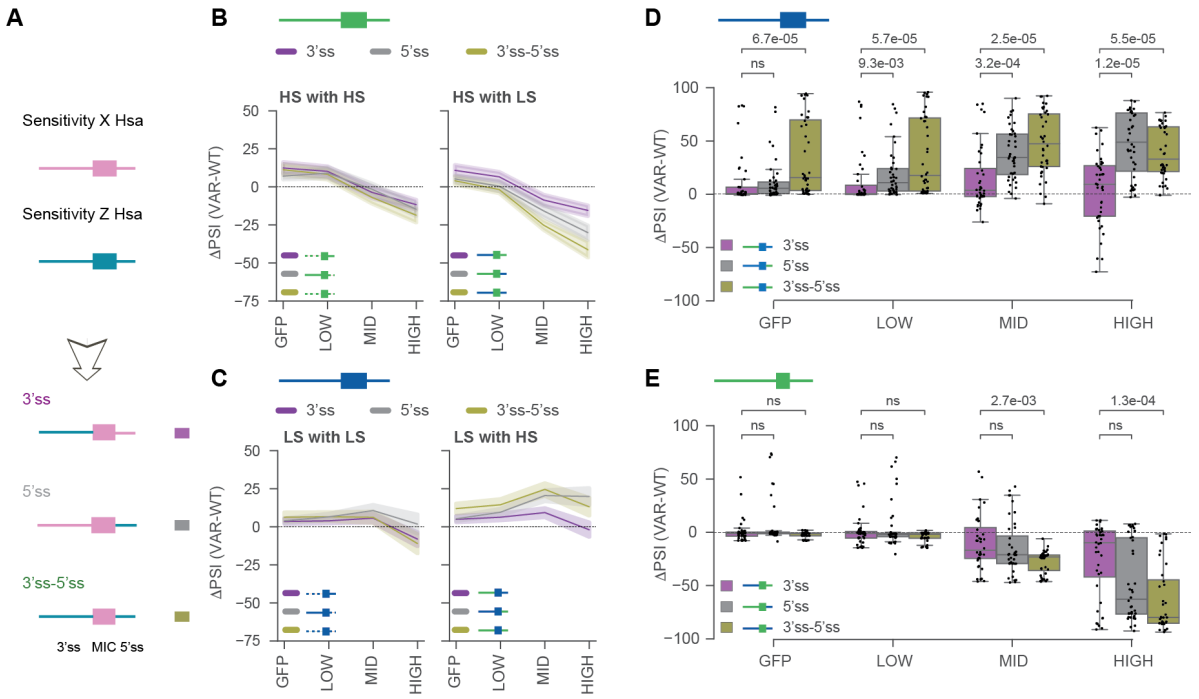

Figure 3: Intronic elements contribute to the different microexon sensitivities

(**A**) Representation of the different intron swappings performed in (B-E). Flanking introns from events in Sensitivity group Z are used to replace the corresponding regions in events from Sensitivity group X. Color codes used in (B-E) are indicated. (**B-E**) Effect of swappings intronic sequences between events from Sensitivity group X and Sensitivity group Z. (B) group X: HS, group Z: HS, LS. (C) group X: LS, group Z: LS, HS. (D) group X: LS long (i.e., exon length > 15 nt), group Z: HS short (i.e., exon length < 16 nt). (E) group X: HS short, group Z: LS long. LS and HS pre-mRNA regions are represented in blue, and green, respectively. For each subpanel, the WT sequence is depicted at the top and a scheme of the part(s) of the pre-mRNA involved in the swapping is represented along with the color legend of the lines or boxes. Statistics in (B,C): results of one sample t-tests are presented in Table S6, in (D,E): Mann-Whitney-Wilcoxon test two-sided with Bonferroni correction (ns: non significant).

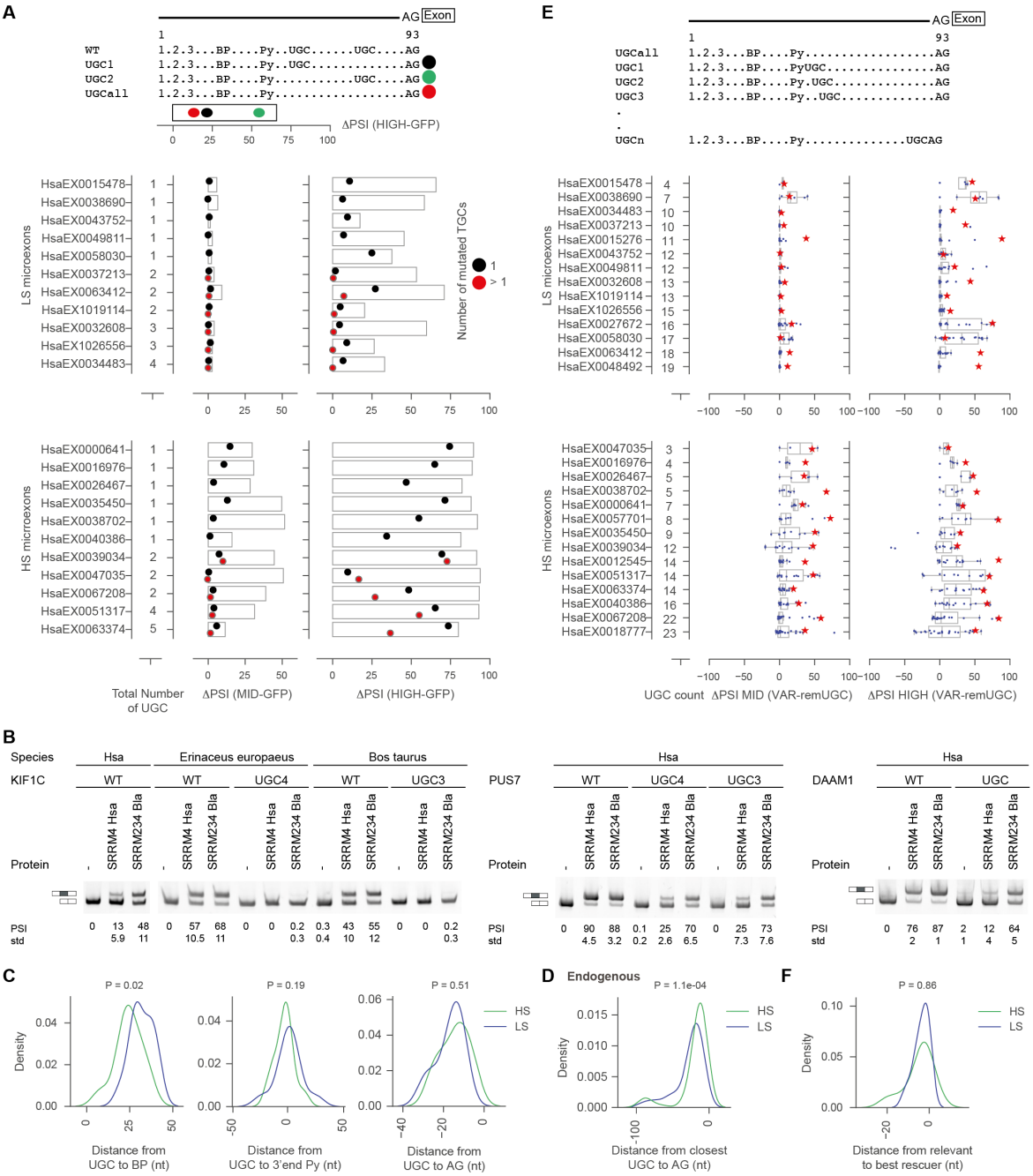

Figure 4: The UGC motif contributes to splicing regulation by SRRM4 in a context and position dependent manner

(**A**) Top: Scheme of the generation and quantification of UGC variants. UGC motifs in the 3'ss of the WT events were mutated either individually (black and green dots) or in combinations (red dot). Bottom: Quantification of the impact of UGC mutations for LS and HS microexons. The barplots represent the ΔPSI of the WT sequences in MID (left) and HIGH (right) expression of SRRM4 with respect to GFP. The effect of mutating either the UGC with the strongest impact (black dots) or all

UGCs (red dots, total number considered for mutagenesis is provided as “Total number of UGCs”) is shown as the ΔPSI between MID or HIGH expression of SRRM4 with respect to GFP for each variant. The closer to x = 0, the stronger the effect of the UGC mutation(s). (**B**) RT-PCR assays showing the splicing pattern of three events (WT and UGC variant) from different species under control condition (-) or expression of human SRRM4 (Hsa) or amphioxus SRRM234 (Bla). PSI and standard deviations (std) from three biological replicates are provided. The PSI response of KIF1C-HsaEX0034483 and PUS7-HsaEX0051317 variants in MaPSy is presented in Figure S4B. (**C**) Distribution of the distances between the most relevant UGC motif (as identified in A, black dot) and the dinucleotide AG, the 3′ end of the polypyrimidine (Py) tract or the best predicted branch point (BP). The UGC-AG distance is expressed in negative values to account for the reverse arrangement of the two elements with respect to BP-UGC and Py-UGC pairs. (**D**) Distribution of the distances between the dinucleotide AG and the closest UGC for all endogenous LS and HS events with UGC in the 93-nt long flanking 3′ss. (**E**) The UGC-addition walk (top panel) examines the effect of adding a single UGC between the 3′ end of the Py tract and the dinucleotide AG. This effect is quantified as ΔPSI with respect to the variant in which all UGCs are mutated (UGCall in panel (A)) in MID or HIGH expression levels of SRRM4. The red stars represent the WT and the blue dots the variants of the UGC-addition walk, whose distribution is shown as a boxplot. (**F**) Distribution of the distance between the most relevant UGC identified in (A) (black dots) and the UGC with the strongest effect identified in (C). Statistics in (C,D,F): Mann-Whitney-Wilcoxon test. See also Figure S4.

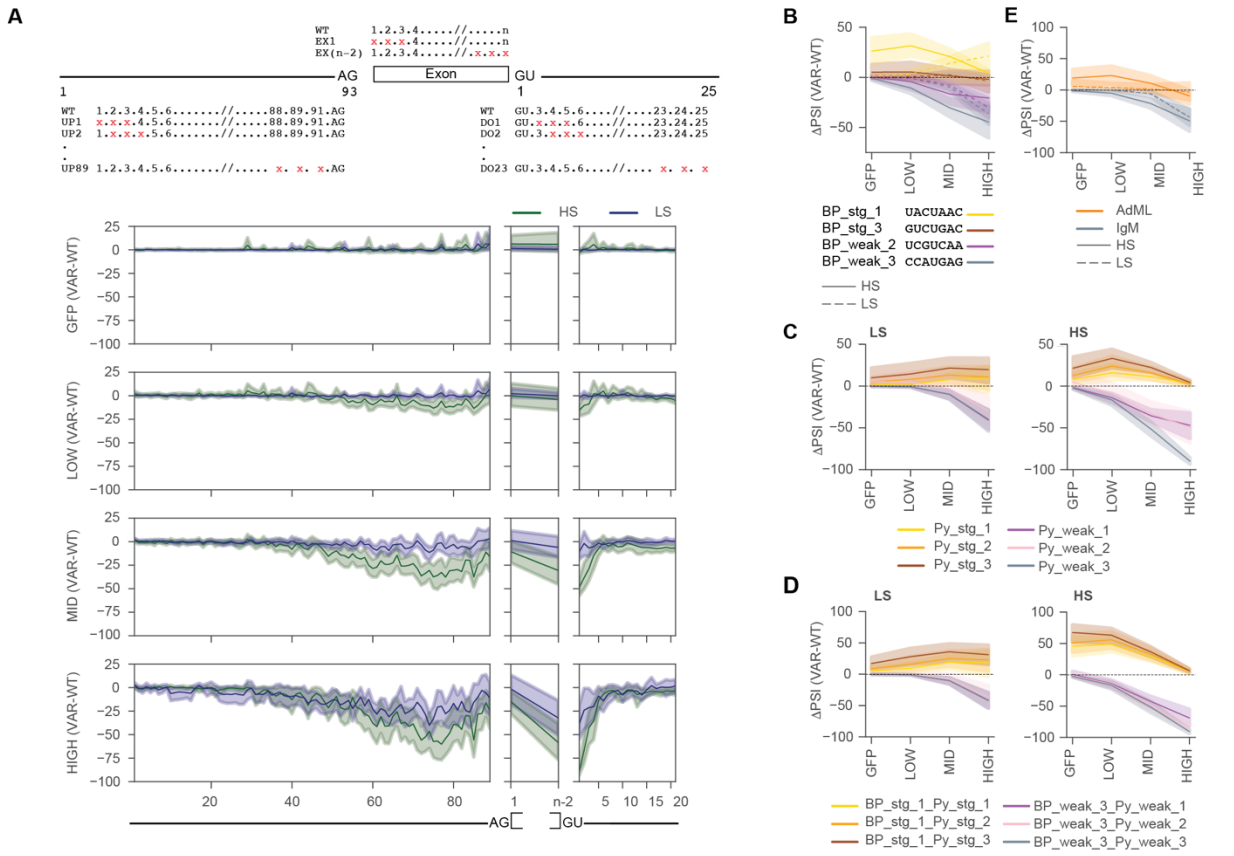

Figure 5: Intronic core splicing elements close to splice sites are relevant for regulation by SRRM4

(**A-E**) Effects of (A) mutating three consecutive nucleotides following the strategy depicted at the top, (B) strengthening (stg) or weakening (weak) the BP (Table S7), (C) strengthening (stg) or weakening (weak) the Py tract, (D) combining the mutations described in (B) and (C), or (E) replacing the endogenous BP-Py tract sequence by the ones from AdML or IgM. The effects are shown for LS and HS events for the four experimental conditions quantified as ΔPSI (VAR-WT) (i.e., PSI of the VARiant with respect to the WT sequence). In (A), the positions of the intronic mutations are indicated along the x-axis according to the nomenclature described at the top and only the impact of mutating the first (1) or last 3 (n-2) exonic nucleotides is reported here. The impact of other exonic mutations is reported in Figure S3A-C. See also Figure S5. In (B), the BP sequences used are indicated. For other mutations, see Table S5. Statistics in (B-E): results of one sample t-tests are presented in Table S6.

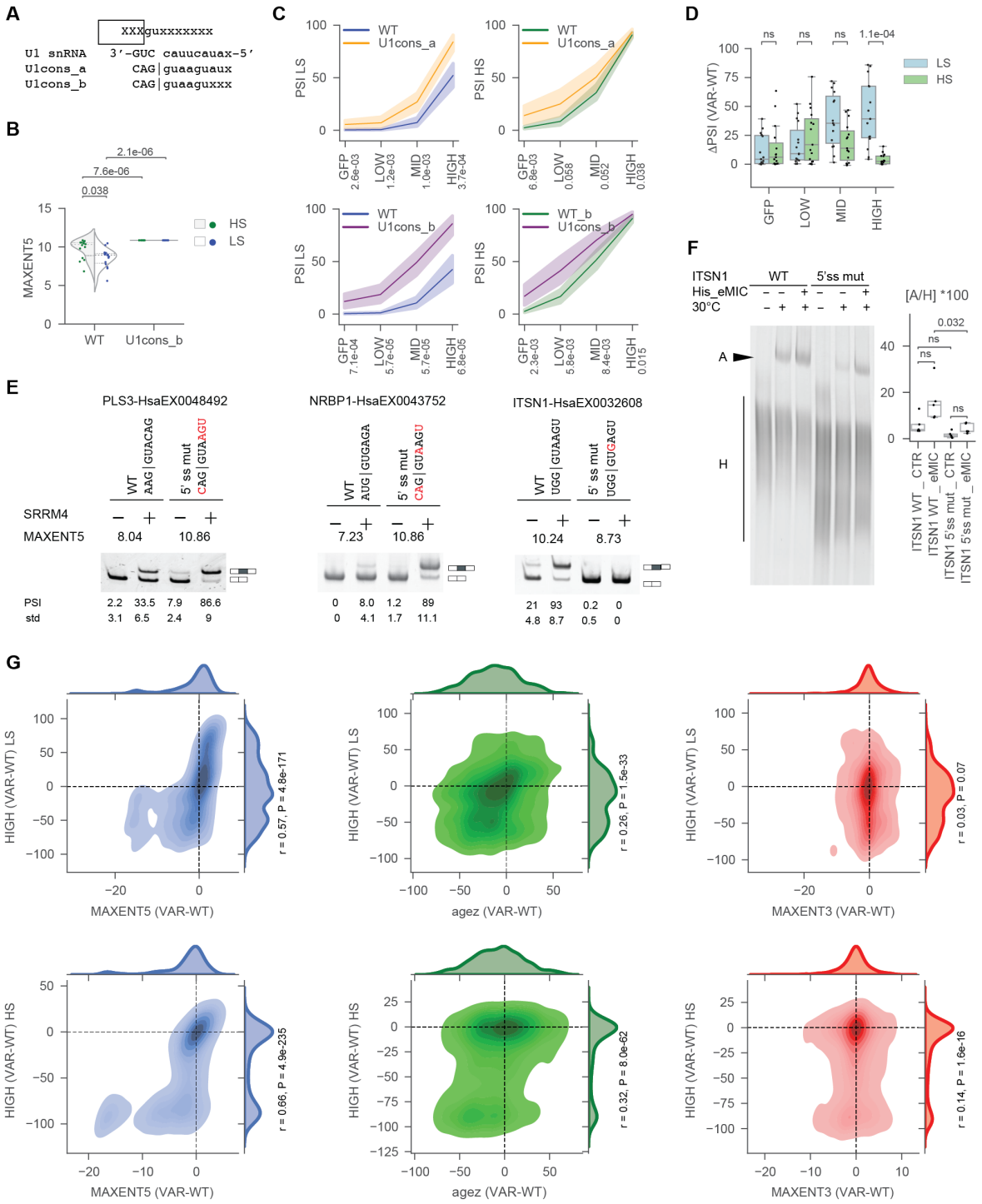

Figure 6: The strength of the 5′ss contributes to sensitivity to SRRM4 expression

(**A**) Mutations performed to enhance base-pairing between the 5′ss and U1 snRNA. U1cons_a corresponds to 9 nts complementarity to U1 snRNA[52], while U1cons_b corresponds to an extended based pairing involving MAG|GURAGUAU[59]. (**B**) Maximum entropy scores of the 5′ss (MAXENT5) for

WT and U1 variants for LS and HS events. (**C**) Splicing profiles quantified as PSI in four experimental conditions for WT and U1 variants depicted in (A) for LS and HS events. Statistics: Mann-Whitney-Wilcoxon test. (**D**) The effects of U1cons_b mutations represented in (A, C) quantified as ΔPSI (VAR-WT) in four experimental conditions for LS and HS events. Each dot represents an event. (**E**) RT-PCR assays showing the splicing pattern of the WT and corresponding U1 variants for three events under control condition (-) or expression of SRRM4 (+). Sequences of the 5′ss are indicated at the top and the mutated nucleotides are highlighted in red. PSI and standard deviations (std) from at least three biological replicates are provided. PSI in the MaPSy are provided in Figure S6D,E. (**F**) *In vitro* spliceosomal A complex formation on ITSN1 pre-mRNA, either WT or the 5′ss variant in (E), under control condition or expression of the recombinant eMIC domain of SRRM3[10]. Quantification of the ratio A/H complexes from five biological replicates is provided (Table S8). (**G**) Distribution of ΔPSI (VAR-WT) values upon HIGH level of expression of SRRM4 with respect to ΔMAXENT5 (VAR-WT), Δagez (VAR-WT), ΔMAXENT3 (VAR-WT) values for the LS and HS variants with WT exon length and that modify each corresponding feature (see Methods). MAXENT, maximum entropy score; agez, AG exclusion zone. Statistics in B,D,F: Mann-Whitney-Wilcoxon test two-sided with Bonferroni correction (ns: non significant). Statistics in G: Pearson correlation coefficients and p-values. See also Figure S6.

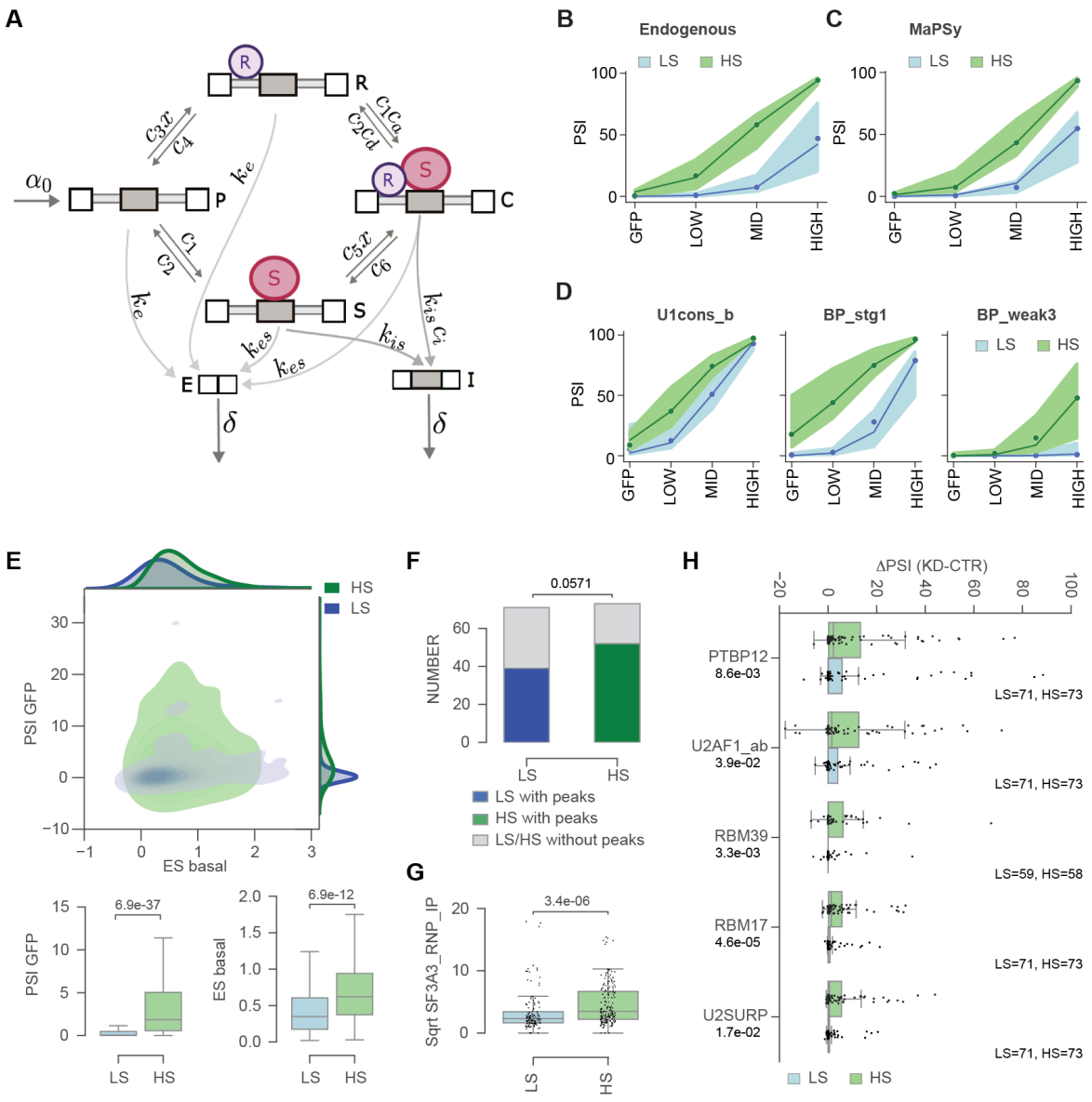

Figure 7: Early recognition of splice sites in the absence of SRRM4 correlates with microexon regulation

(**A**) Integration of the splicing regulation into a mathematical model. (**B-C**) Mathematical model applied to WT LS (in blue) and HS (in green) events at the endogenous level (B) or in the context of MaPSY (C) in four experimental conditions (GFP or LOW, MID, HIGH level of SRRM4 expression). The points for each condition represent the medians of the experimental data, the filled area the interquartile range, and the line depicts the model fit. (**D**) Model applied to mutations in 5′ss (U1cons_b, Figure 6C, S7A) or the 3′ss (BP_stg1, BP_weak3, Figure 5B, S7A). (**E**) Distribution of PSI in control condition (GFP) (y-axis) versus enrichment score (ES) for *in vitro* spliceosomal A complex formation in basal condition (x-axis) for WT sequences for LS and HS events from human and 23 species. The

corresponding PSI and ES distributions are separately represented as boxplots below. (**F**) Number of events with or without U2 snRNP related peaks in the 93 nts upstream of the microexons in[53]. Statistics: two-sided Fisher's Exact test. (**G**) *Ex vivo* U2 snRNP association, represented as square root of SF3A3_RNP_IP peak intensities in[53], to LS and HS events identified in (F). (**H**) Effects of knocking down individual RNA binding proteins on the splicing pattern of LS and HS events quantified as ΔPSI with respect to control conditions (ΔPSI = KD - CTR). Number of LS and HS events quantified in each condition is reported and each dot represents an event. Statistics in (E,G,H): Mann-Whitney-Wilcoxon test. See also Figure S7.