

Deep indel mutagenesis reveals the regulatory and modulatory architecture of alternative exon splicing

Corresponding Author: Professor Juan Valcárcel Juárez

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

The authors use the apoptosis-related FAS exon 6, which they have previously studied in the context of trans-regulation as well as saturation mutagenesis, to characterize and compare all possible substitutions, deletions and insertions (up to 3-4 nt) in PCR/sequencing-based reporter screen. This allows them to dissect the interplay of cis-regulatory features responsible for intermediate inclusion of this alternative exon. Among other novel findings, they find that insertions more often cause increased exon inclusion compared to deletions or substitutions, and they uncover novel ways to generate microexons by insertion of splice sites near a natural exonic poly-pyrimidine tract – a feature associated with exons encoding transmembrane helices, which they show are regulated by known microexon regulators. They further demonstrate that assembly of spliceosomal A complexes within the exon competes with the endogenous 3'-splice site, dependent on exonic poly-pyrimidine tract and branch points. Importantly, they demonstrate that the effect of deletions is well-predicted by the SpliceAI and correlates with the effect of ant-sense oligos (ASOs). They go on to predict ASO effects across 18K exons and validate predictions for the model exon.

This manuscript is of interest from a fundamental (splicing-regulatory) perspective as well as for the study and development of ASOs. The claims are generally well-supported, and the figures refreshingly accessible. The authors are requested to clarify the following minor points:

1. For the transcriptome-wide analysis of SRE density (Fig. 6, S14-15), the authors present figures based on the density of state transitions in an HMM, but it is not clear if they intend to demonstrate that it is density of SREs (as opposed to neutral sequence) or rather the frequent succession of enhancer and silencer elements that is relevant. If the former, a high density of highly-sorted elements (i.e., little neutral sequence but few E/S transitions because of consecutive enhancer or silencer elements) could be just as meaningful for regulation as frequent state transitions. For example, in line 489 ff, the authors purport to test the hypothesis that short, SRE-dense exons have similar fractions of sequence within enhancer and silencer elements, which would be more straightforwardly done by showing the actual percentages of nt within each state rather than comparing the frequency of state transitions. Similar line 470 ff.
2. SVM-BP does not strictly predict 3'-splice sites, as the authors state (line 378), but specifically branch point features. To put the SVM-BP scores in perspective and support the notion that some exons have scores similar to 3'-splice sites (Fig. 4C), the authors could show the scores for the bona fide 3'-splice sites. Furthermore, the model provides a separate score for the poly-pyrimidine tract. Does this explain the SVM-BP score differences? Are one-pass transmembrane helices more often encoded in short, alternative exons than expected (e.g. compared to other exons within the same proteins, or multi-pass transmembrane domains)?
3. There are some relatively strong discrepancies between the effects of point mutations report in this manuscript compared to Julien et al. (2016), e.g. at A2, T10, T52, despite the same cell and reporter construct being used. Can the authors point to possible sources for these differences?
4. Line 204: Reference for the pyrimidine-rich PTB silencer at positions 28-39 missing.
5. Fig. S10: x-axis label should probably read 'Exon length bin' – are bins the same as in Fig. 3B?

6. Fig. 3F-G: Describe (in the main text or figure legend) how the endogenous poly-pyrimidine tract was strengthened.

7. Line 374: The sentence, 'To investigate whether alternative exons, and particularly those coding for transmembrane domains, are generally regulated by this type of mechanism, we first used SVM-BPfinder to scan exons throughout the genome harbouring branchpoint motif followed by a polypyrimidine tract.' is ambiguous. Were all alternative exons scanned, or only exons containing a branch point followed by a polypyrimidine tract, and/or coding for transmembrane domains?

8. Fig. 6F: It is difficult to see from the ternary plot whether exons < 100 nt indeed tend to have less than 20% N state due to overplotting.

9. Line 487: Not all 50/50 E/S exons are shorter than 100 nts; qualify this statement.

10. The estimated PSI of a variant is given as $PSI^{wt} * \exp(ES^x) / \exp(ES^{wt})$ in Fig. 5B but $PSI^{wt} * ES^x / ES^{wt}$ in the Methods. For consistency, clarify if ES is the ln of ratios.

11. Is the pathway for microexon generation the authors describe here, i.e. through shortening of existing exons containing a poly-pyrimidine tract, a natural source for microexons during evolution?

Reviewer #2

(Remarks to the Author)

The submitted manuscript uses mutagenesis of FAS exon 6 to understand existing and introduced splicing regulatory elements (SREs). The authors characterize in detail the SREs for this specific exon and validate examples of splicing regulation impacting cryptic exons, and propose this as a broader mechanism of splicing regulation in transmembrane helices. The authors apply what they've learned using this approach to build a model to identify SREs in other exons and characterize the exons in terms of their abundance of different types of SREs as well as exon length. The authors benchmarked computational tools to predict the impact of deletions on splicing on FAS exon 6 and used the output of these models to aid the design of antisense oligonucleotides (AONs).

Generally speaking, the conclusions drawn are well supported by the experiments and analyses performed in the paper; the statistical methods seem sound and many of the findings are novel. This is a rich paper with a lot of data and creative and informative analyses. The SRE characterization exome-wide and development of an AON design software for altering splicing I especially find potentially appealing to a broad audience and novel (albeit see important caveats below).

There are however revisions that the authors should pursue to identify experimental biases in their approach and understand the scope of their conclusions. Additionally, the clarity and completeness of the supplemental figures needs to be improved generally. Specifically my major concerns are as follows:

1. Nonsense-mediated decay (NMD) is a potential source of bias in the acquired data, especially when so many of the experimental interventions are insertions and deletions. The FAS exon 6 is 63 nucleotides long, so NMD would be predicted to act upon a subset of these transcripts where a stop codon is introduced within 50 nucleotides upstream of the last exon junction either by frameshift or inserting nucleotides that code for a stop codon. It is possible that NMD has a modest or negligible effect on the reporter, but this should be demonstrated by comparing the PSI of NMD predicted variants to nearby non-NMD predicted variants in similar locations in the exon. If these are not broadly different, then it's likely that NMD can be ignored, but if not this is a caveat that needs to be taken into consideration when drawing conclusions from these data. If the reporter isn't translated or should not be subject to NMD for other experimental design reasons, this should be explicitly addressed by the authors. Another possible way to address this would be to perform the experiments in NMD deficient cells, but this is likely a more difficult alternative.

2. In the proposal of pyrimidine rich regions being a potential mechanism driving protein localization via being soluble vs. membrane bound, there is no broad analysis of this in proteins with annotated transmembrane domains; this limits the generalizability of this proposed mechanism. Non-polar amino acids and hydrophobic domains are not exclusive to transmembrane domains, so what proportion of the exons analyzed which are transmembrane domains is unclear. One way to address this is to examine exons which overlap with annotated transmembrane domains (annotated by UniProt, Pfam, or another database) and ask whether these have either better pyrimidine tracts for splicing or are observed splice sites compared to exons encoding other protein domains (including non-transmembrane hydrophobic domains). The examples presented in Figure 4F are convincing examples of this, but how broad this may be as a mechanism of transmembrane domain regulation is still unclear given the evidence presented.

3. The accuracy of deletion effect predictions (Fig 5A) - if I understand correctly, this includes all deletions, short and longer. Would be prediction improve if limiting to short deletions? Add this analysis for an apples to apples comparison to substitutions and insertions would seem useful. Understanding the quality of predictions as a function of deletion length more broadly would also be informative regarding DANGO.

4. The DANGO model and the AON effect analysis are interesting, but not sufficiently supported by the data. The empirical data in Fig 7B is sparse - 9 deletions in a single exon of a single gene - and the validation of the DANGO score in Fig 7C is based on these 9 data points. In Fig 7B the correlation is quite high, but the actual PSI values don't match, which should be pointed out in the text. Additionally, it would demonstrate the robustness of both Dango and the SRE HMM if the

directionality of Dango matched the splicing enhancer or silencer states from their HMM. Overall, the current data is not sufficient to support the conclusions and framing, including the highlighting of AONs in the abstract and statements like “DANGO is therefore a genome-wide resource that accurately predicts the effects of 21-nt deletions that can be used for AON selection” and “we have shown that deletion scanning accurately predicts the effects of AONs”. I’m not against presenting these analyses, but it should be either toned down or supported with more (validation) data.

5. There are multiple issues with the supplemental material, which make some claims difficult to interpret not matching the quality of the rest of the figures. These include:

- a) Low resolution images, which are difficult to read even when zoomed in (S5-S10)
- b) Missing figure labels (S13)
- c) Unclear legends (i.e. what is the x-axis of figure S10? Length deciles?, if so please say explicitly)
- d) In the absence of a ladder, I’m unconvinced there aren’t microexons in figure S11B. I am unsure as to whether the boxed-in parts of the gel (see attachment) are shadows of overloaded bands or microexons. A picture gel with a ladder showing the scale of separation, adjacent microexon positive controls of the expected size, or junction specific qPCR would be useful in clearing this up. To be clear, I don’t believe this to be an issue with the other microexons detected in S11C, or figure 3, as these appear to be well separated.
- e) Figure S16 is referenced in the text, but not present in the supplement. It could be that S14 is missing and S15 and S16 are labeled as S14 and S15, as this seems consistent with their references in the text (line 470 doesn’t match E/S state transitions in S14).

While most of the paper is clear, there are additional minor alterations that I believe would improve clarity and reader comprehension.

- Figure 1D; horizontal line should be labeled at reference sequence PSI somewhere (either on figure or figure legend).
- A reference to Figure 2A would be useful in the paragraph beginning at line 144 to orient readers visually to the statistics mentioned in that paragraph.
- Line 360-361: “Collectively, our results reveal a novel mechanism of exon skipping based upon assembly of U2 snRNP on exonic sequences that resemble (but cannot be active as) 3’ splice sites.” Authors should clarify how this is novel because they go on to say how U2AF2 binds pyrimidine tracts in the exon of IL7R to promote exon skipping in a separate paper (Line 366-367). Is it novel for FAS exon 6, is the proposed mechanism different from the IL7R mechanism?
- Figure 5B-C being referenced in the text before Figures 3 and 4 could be very confusing for a reader depending on how the final paper is formatted.
- HMM identified SREs in 6C would be easier to evaluate for readers if they were aligned with FAS exon 6 experimental data (as a sub panel on top or something). This would also visually support the statement on lines 445-448: “Our model captured the regulatory architecture of FAS exon 6 as uncovered by our deep indel mutagenesis experiment: regions of the exon corresponding to inferred enhancers were predicted to be in state E, and regions corresponding to inferred silencers in state S (figure 6C).”
- Figure 7C (which has Dango score on the y-axis) is referenced in the text before Dango is introduced and is a bit confusing for the reader.
- Figure S12 should be labeled “UAG” instead of “TAG”.
- Line 641 typo: “Quiagen”

Separately, there are a couple of other improvements that could be made regarding code, data, and reagent availability.

- It would be useful to the community if the HMM predictions for SREs genome wide were available in a usable format for researchers (like a ucsc track or bedgraph).
- The code availability on github is nice, but it would be useful for people trying to reproduce analysis if there was a README file explaining the repository contents and dependencies to reproduce the analysis.
- The authors should comment on the availability of the plasmid used in their experiments for other researchers.

Aside from the recommended changes mentioned, I think the experimental approach is sound, the interpretation for the data is appropriate, and exceptions are well addressed in separate experiments. I find that the statistical analysis in this manuscript is appropriate and well-described in methods and in figures. The references provided seem appropriate for the content of the paper. The abstract, introduction, and conclusion are accessible and appropriate.

Reviewer #3

(Remarks to the Author)

I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Reviewer #4

(Remarks to the Author)

This work by Baeza-Centurion et al describes a systematic exploration of the regulatory architecture of human exons. The authors built a minigene based on a specific exon of interest, FAS exon 6, and used a synthetic genetic library to introduce into it all possible deletions, all possible 1-3 nucleotide insertions plus a collection of randomly chosen 4 nucleotide insertions, together with all single nucleotide substitutions. Then they measure the exon inclusion rate using deep

sequencing.

First, this high throughput approach reveals the full regulatory architecture of the exon of interest, exposing that splicing enhancers and silencers are found on the exon in an alternating pattern. They also demonstrate a strong correlation between k-mers insertion effects in the library and the effects of having these k-mers in exons in the genome, demonstrating the power of the synthetic library to predict genomic features.

Second, the authors show that some library variants lead to an alternatively spliced micro-exon, which leads them to suggest an alternative splicing regulatory mechanism through exonic cryptic splice sites. The authors also predict using bioinformatic analysis that other short exons that code for transmembrane domains (similar to the exon of interest) should have this mechanism, and demonstrate this mechanism on 3 other such exons.

Next, the authors examine the performances of 5 different computational models on predicting indels effects on splicing. After demonstrating that one of the models performs well on their indel library they use this model to generalize their observations on exonic regulatory architecture for the entire human exome.

Finally, the authors claim that the method presented in this work can be very beneficial for screening for effective antisense oligonucleotides (AONs), to treat splicing-associated genetic syndromes. And they provide a new computational model to predict AON efficiency.

We think that the parts of the paper where the authors analyze the regulatory architecture of the exon of interest and then generalize their observations over the entire human exome contribute significantly to our understanding of splicing regulation. Moreover, this work demonstrates the power of deep indel mutagenesis in revealing splicing regulatory elements, and their mechanism.

On the other hand, in the last part of the paper where they claim this method contributes to discovering new AONs with therapeutic potential, the contribution and the novelty are less clear to us. First, it's not clear to us from the text how deletion scanning is more accessible than directly performing AON scanning as was done for Figure 7B. If there are significant benefits for deletion scanning over AON scanning it should be better described in the text. Second, we think that the last section in the results which describes a new computational model named DANGO is not novel, since they merely use specific inputs (21-nt deletions) as input to an existing computational model (spliceAI). We think that naming this as "a new model" is misleading.

Since the abstract and introduction of the paper put a lot of focus on the AON discovery part we suggest this paper be revised. Either put more focus on the first parts describing the regulatory architecture, or better describe the advantage of deletion scanning as an AON discovery method over AON scanning. As for the computational model, we think it shouldn't be presented as a new model, only as a way to use an existing model and how it can help with AON discovery.

Below are specific comments:

Major points:

Calculation of PSI values per variant is based on counting the frequency of mature mRNA molecules per variant, divided by the frequency of the variant in the input DNA library. There is a caveat in the experimental design since only mRNAs with fully included exons can be assigned to a library variant. When the exon is skipped, all mRNA products from the library have identical sequences. Therefore, there is no way to measure the total mRNA per variant. So it's impossible to differentiate between the mutant's effect on PSI and the mutant's effect on total mRNA expression level. The authors should address this caveat and come with means to mitigate it. The experimental validation with RT-PCR (Figure 1C) does measure both skipped and included transcripts per variant, so the authors can use this validation experiment to check for effects on total mRNA levels per variant in this validation subset. If they don't observe significant variation in total mRNA between mutants it can justify the library's experimental design.

In line 530 the authors write "We reasoned that deletion mutagenesis might provide a rapid method to predict the effects of AONs binding...". However, the authors don't describe what are the benefits of deletion scanning over AON scanning. Is it faster? cheaper? easier to perform? The authors should elaborate on that.

Minor points:

The experimental design in Figure 1B is not clear enough. From the figure, it can be seen as if mRNA levels for all library variants were measured through RT-PCR as described in line 705, which is not the case.

The calculation of PSI value as described in Figure 1B and equations in lines 696-704 is not clear enough. According to the equations in Figure 1B, the ES value for WT equals 0 by definition, and then normalizing the PSI value by $\exp(\text{ES}[\text{WT}])$ is redundant. In addition, we suggest adding the ES calculation as an equation in the method section, and not only in Figure 1B.

In lines 374-387 the authors describe how due to the nature of the genetic code, exons that code for transmembrane domains are rich in pyrimidines and therefore have a high probability of including cryptic 3' splice sites that can lead to exon skipping. Since we assume some transmembrane domains in the genome are essential, we think it would be interesting to check bioinformatically if essential domains have a way to avoid the effect of such cryptic splice sites.

In line 40 there is a typo, "that that".

Reviewer #5

(Remarks to the Author)

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

The authors have addressed all points this reviewer has raised.

Reviewer #2

(Remarks to the Author)

The authors have done careful and comprehensive revisions that have improved the manuscript. All my concerns have been addressed.

Reviewer #3

(Remarks to the Author)

I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Reviewer #5

(Remarks to the Author)

The authors have addressed sufficiently most of my comments.

However, I am still not convinced by the benefit of deletion mutagenesis over AON walk.

First, in the rebuttal letter, the authors refer to lines 585-595 in the revised manuscript. I assume this is a mistake, as these lines describe the DANGO genome browser track. In the section above titled "Deletion scans accurately predict the effects of antisense oligonucleotides on exon inclusion" the only change I notice from the previous version relates to analyses of two previously published AONs. Could it be that a part of the revised text is missing?

Second, in the rebuttal letter, the authors provide two benefits of deletion scanning over AON scanning.

1) "higher resolution in the definition of regulatory elements"

I see how deletion scanning can provide this higher resolution, because deleted elements can be shorter than the 18-21nt AONs, hence, deletion scanning should be a better tool to explore the regulatory landscape as beautifully demonstrated in this work. However, for clinical applications, I still struggle to understand how deletion scanning is better than AON scanning for predicting AONs effect. If one performs an empirical scanning, wouldn't it be better to perform the scanning as close as possible to the treatment itself? If there are other benefits for deletion scanning (like cost or simplicity), the authors should elaborate on them.

2) "that the good correlation between experimental and computational predictions of the effects of extensive deletion analyses opens up the possibility of predicting in silico AONs for any desired exon, as well as transcriptome-wide."

In my opinion, the added analysis provided by the authors in Figure S19 doesn't support this claim. As noted by the authors in the figure legend, in the case of Mnk2b spliceAI predicts the opposite effect than the AON scan, and in the case of SMN2 there is a good correlation only for 3/9 AONs.

So, eventually, taking all three analyses together (Figure 7C, and Figure S19 A-B), only one of them demonstrates good correlation between in-silico predictions and AON effect.

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Response to reviewers

Reviewer #1 (Remarks to the Author):

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This manuscript is of interest from a fundamental (splicing-regulatory) perspective as well as for the study and development of ASOs. The claims are generally well-supported, and the figures refreshingly accessible. The authors are requested to clarify the following minor points:

We appreciate the reviewer's positive overall evaluation of our work and the detailed helpful feedback.

1. For the transcriptome-wide analysis of SRE density (Fig. 6, S14-15), the authors present figures based on the density of state transitions in an HMM, but it is not clear if they intend to demonstrate that it is density of SREs (as opposed to neutral sequence) or rather the frequent succession of enhancer and silencer elements that is relevant. If the former, a high density of highly-sorted elements (i.e., little neutral sequence but few E/S transitions because of consecutive enhancer or silencer elements) could be just as meaningful for regulation as frequent state transitions. For example, in line 489 ff, the authors purport to test the hypothesis that short, SRE-dense exons have similar fractions of sequence within enhancer and silencer elements, which would be more straightforwardly done by showing the actual percentages of nt within each state rather than comparing the frequency of state transitions. Similar line 470 ff.

We very much appreciate the reviewer's suggestion because this exercise has helped to further clarify some important messages of our manuscript. Representing the actual percentage of nucleotides within each state for different levels of exon inclusion and exon length (presented in the manuscript as new Figure S16, also shown below for the reviewer's convenience as **Figure R1**), the following conclusions have been strengthened:

- 1) Highly included exons display lower density of regulatory elements, in all likelihood related to stronger flanking splice sites characteristic of highly included/constitutive exons. This is in line with multiple previous reports, including our previous publication focusing on this issue (PMID: 33112234).
- 2) The density of nucleotides within splicing enhancer elements generally decreases with exon length, suggesting that splicing enhancers are required to sustain the inclusion of alternative exons flanked by weaker splice sites, but the density of enhancer sequences does not determine the extent of exon inclusion.
- 3) In contrast, the density of splicing silencers generally correlates inversely with the levels of exon inclusion, suggesting that splicing silencers, while generally less frequent than enhancers, are key determinants of exon inclusion.

We incorporate these analyses and further discuss these points in the text (lines 475-493 and 622-625).

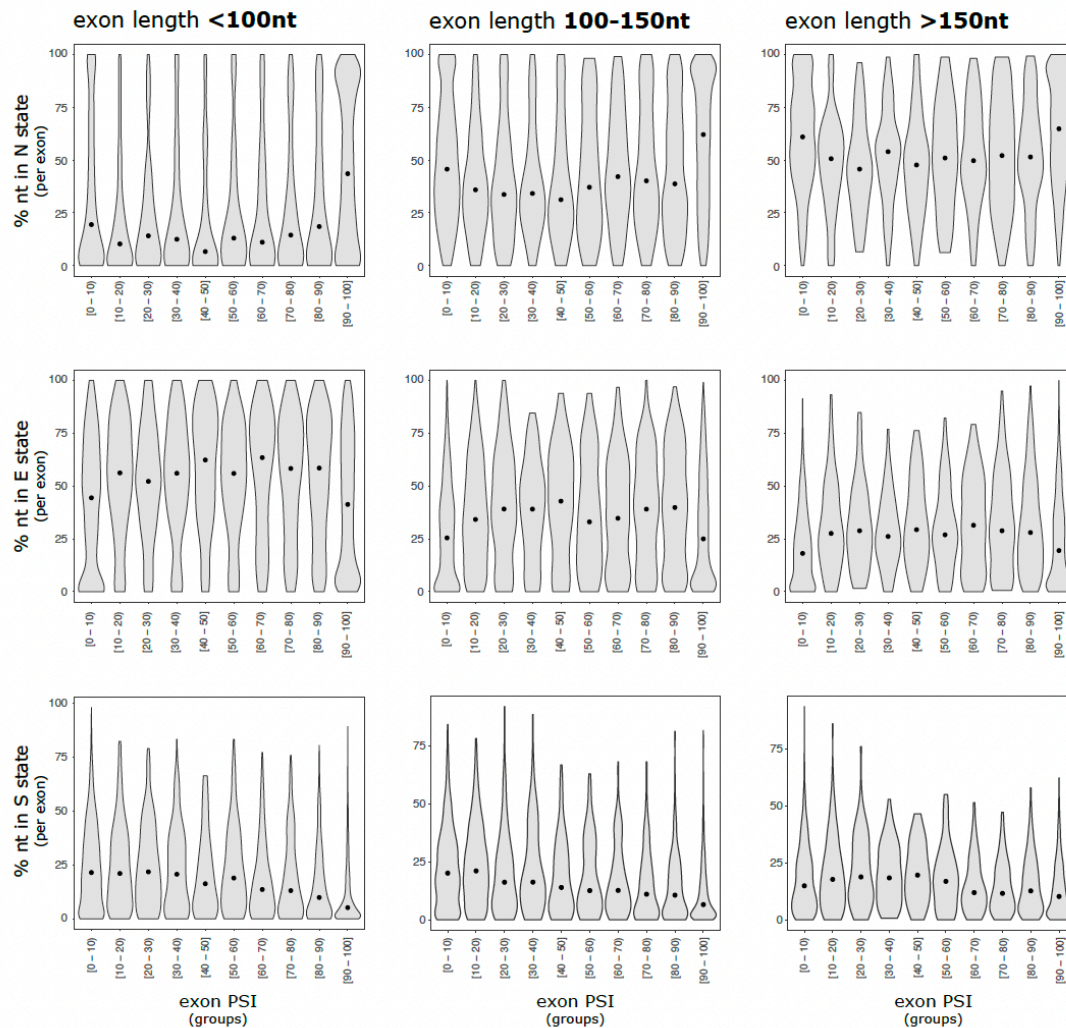


Figure R1 (incorporated in the manuscript as **new Figure S16**). **Percentage of nucleotides in N, E or S states for exons of different lengths and inclusion levels.** The distribution of the fraction of nucleotides in Neutral (N), Enhancer (E) or Silencer (S) states is shown for the indicated exon lengths and PSI values. Data source as for Figures 6 and S13-S15.

2. SVM-BP does not strictly predict 3'-splice sites, as the authors state (line 378), but specifically branch point features. To put the SVM-BP scores in perspective and support the notion that some exons have scores similar to 3'-splice sites (Fig. 4C), the authors could show the scores for the bona fide 3'-splice sites. Furthermore, the model provides a separate score for the poly-pyrimidine tract. Does this explain the SVM-BP score differences? Are one-pass transmembrane helices more often encoded in short, alternative exons than expected (e.g. compared to other exons within the same proteins, or multi-pass transmembrane domains)?

Thank you for the suggestion. We now show, in addition to the predictions of the full SVM-BP model in main Figure 4C, the separate effects of the polypyrimidine tract and of the branch point sequence (Figure S13, also shown as **Figure R2** below for the reviewer's convenience) and conclude that the polypyrimidine tract is the main contributor to the effects observed (lines 394-396).

Finally, one-pass transmembrane helices are often (84%, PMID: 22708632) encoded in distinct alternative exons (and are not interrupted by intron junctions PMID: 14992489), which fits with the observation that they are frequently involved in regulated expression of membrane-bound versus soluble isoforms which typically display antagonistic functions in processes spanning from programmed cell death (e.g. Fas/CD95 exon 6), cell growth signaling (e.g. FGFR3 exon 9) to proteolytic ectodomain shedding (e.g. syndecan-4 exon V-a) (e.g. PMID: 14675776, PMID: 22708632) and whose deregulation has been linked to various pathologies (PMID: 22708632).

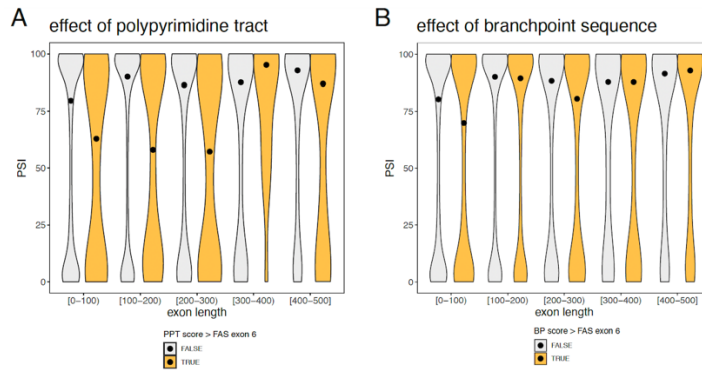


Figure R2. Inclusion of exons categorized into different length groups, divided by whether they have a polypyrimidine tract (A) or a branch point (B) sequence stronger to that in the 3' splice site of FAS exon 6. Analyses as in Figure 4C following Corvelo et al (2010) (Ref. 48).

3. There are some relatively strong discrepancies between the effects of point mutations report in this manuscript compared to Julien et al. (2016), e.g. at A2, T10, T52, despite the same cell and reporter construct being used. Can the authors point to possible sources for these differences?

Thank you for the detailed analyses. To address this issue, we have compared directly the PSI measurements in Julien et al (2016) and in our new study. The results in **Figures R3** and **R4** below show, reassuringly, a high level of concordance between the two measurements and in addition they document lower variability between replicates in our new study, likely related to more accurate estimates due to streamlined procedures and higher number of replicates.

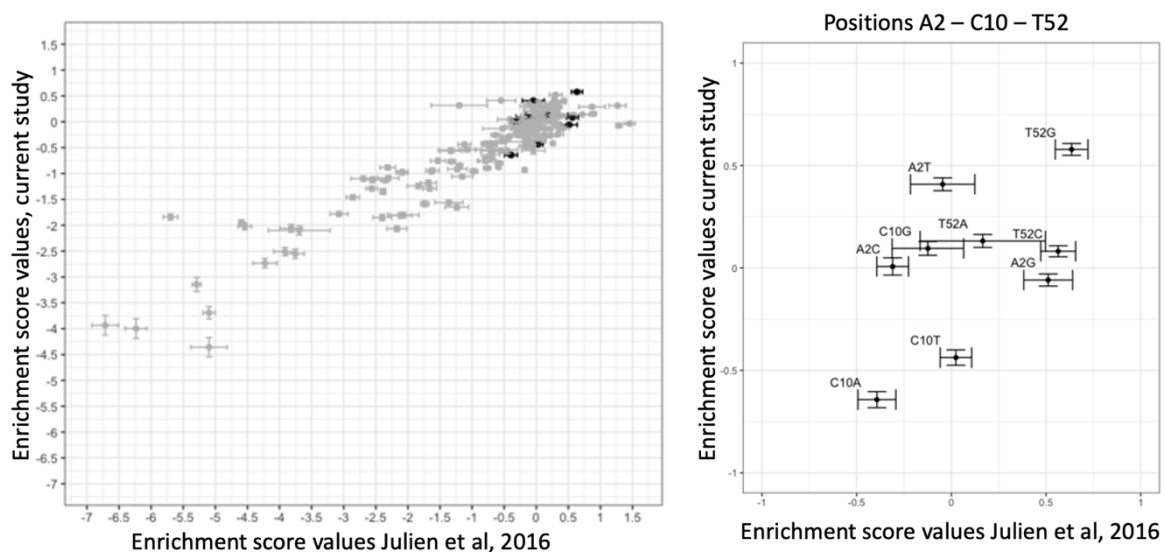


Figure R3. The diagram plots PSI estimates based upon enrichment score values reported by Julien et al, 2016 and this study for all single nucleotide substitutions (left panel) and specifically for substitutions in positions 2, 10 and 52. Please notice the significant wider range of enrichment score values obtained in replicates of the Julien et al, 2016 study than in the current study.

Thank you. We state now (line 503-505) that 50/50 E/S exons tend to be shorter than 100 nts (trend illustrated in new Figure 6F, circled area).

10. The estimated PSI of a variant is given as $PSI^{WT} \cdot \exp(ES^X) / \exp(ES^{WT})$ in Fig. 5B but $PSI^{WT} \cdot ES^X / ES^{WT}$ in the Methods. For consistency, clarify if ES is the ln of ratios.

Thank you for pointing out this inconsistency. Enrichment scores are in log space and therefore the equations in the methods section (lines 727-735) now read:

$$\frac{PSI_{variant}}{PSI_{WT}} = \frac{\exp(ES_{variant})}{\exp(ES_{WT})}$$
$$PSI_{variant} = PSI_{WT} \cdot \frac{\exp(ES_{variant})}{\exp(ES_{WT})}$$
$$PSI_{variant} = 49.1 \cdot \frac{\exp(ES_{variant})}{\exp(ES_{WT})}$$

Because ES_{WT} is 0, this is equivalent to:

$$PSI_{variant} = 49.1 \cdot \exp(ES_{variant})$$

11. Is the pathway for microexon generation the authors describe here, i.e. through shortening of existing exons containing a poly-pyrimidine tract, a natural source for microexons during evolution?

This is an interesting question but we feel that a rigorous assessment of the extent to which shortening of existing exons can be a natural evolutionary path for microexons is beyond the scope of this paper. Given the extensive literature on the special cis-acting sequences and trans-acting factor requirements for microexon regulation (e.g. reviewed in PMID: 32535349), we were surprised to find that an *accidentally* generated microexon was included, and even regulated by SRRM4. This may reflect a general role for SRRM4 in solving the general problem of steric constraints for the simultaneous engagement of U1 and U2 snRNPs on short exons, but once again testing this hypothesis is beyond the scope of this manuscript. We have nevertheless expanded on elements of this discussion in the text (lines 612-616).

Reviewer #2 (Remarks to the Author):

The submitted manuscript uses mutagenesis of FAS exon 6 to understand existing and introduced splicing regulatory elements (SREs). The authors characterize in detail the SREs for this specific exon and validate examples of splicing regulation impacting cryptic exons, and propose this as a broader mechanism of splicing regulation in transmembrane helices. The authors apply what they've learned using this approach to build a model to identify SREs in other exons and characterize the exons in terms of their abundance of different types of SREs as well as exon length. The authors benchmarked computational tools to predict the impact of deletions on splicing on FAS exon 6 and used the output of these models to aid the design of antisense oligonucleotides (AONs).

Generally speaking, the conclusions drawn are well supported by the experiments and analyses performed in the paper; the statistical methods seem sound and many of the findings are novel. This is a rich paper with a lot of data and creative and informative analyses. The SRE characterization exome-wide and development of an AON design software for altering splicing I especially find potentially appealing to a broad audience and novel (albeit see important caveats below).

We thank the two reviewers for their positive overall opinion and constructive suggestions to improve our manuscript.

There are however revisions that the authors should pursue to identify experimental biases in their approach and understand the scope of their conclusions. Additionally, the clarity and completeness of the supplemental figures needs to be improved generally. Specifically my major concerns are as follows:

1. Nonsense-mediated decay (NMD) is a potential source of bias in the acquired data, especially when so many of the experimental interventions are insertions and deletions. The FAS exon 6 is 63 nucleotides long, so NMD

would be predicted to act upon a subset of these transcripts where a stop codon is introduced within 50 nucleotides upstream of the last exon junction either by frameshift or inserting nucleotides that code for a stop codon. It is possible that NMD has a modest or negligible effect on the reporter, but this should be demonstrated by comparing the PSI of NMD predicted variants to nearby non-NMD predicted variants in similar locations in the exon. If these are not broadly different, then it's likely that NMD can be ignored, but if not this is a caveat that needs to be taken into consideration when drawing conclusions from these data. If the reporter isn't translated or should not be subject to NMD for other experimental design reasons, this should be explicitly addressed by the authors. Another possible way to address this would be to perform the experiments in NMD deficient cells, but this is likely a more difficult alternative.

We apologize for not having described this aspect of the design of our assays more clearly. To minimize potential effects of NMD alluded to by the reviewer, the minigenes were designed to include exons 5 to 7 of Fas/CD95 and therefore do not include the natural starting AUG codon of Fas/CD95. None of the 3 AUGs present in transcripts from the wild type minigene, nor correspond to a natural AUG start codon, nor are in a good Kozak context for translation, nor are followed by long open reading frames. Furthermore, all 3 potential AUGs are located in the flanking exons, not in alternative exon 6, and the single potential in frame termination codon within exon 6 is < 50 nucleotides from the last exon-exon junction and therefore not a classical substrate for NMD. Therefore the generation of premature stop codons (and consequently NMD) should not be relevant for the fate of the vast majority of transcripts derived from these minigenes. Furthermore, judging from the total levels of minigene-derived transcripts in a battery of 42 independently validated substitution/indel mutants, less than 15% of the mutants show decreased levels of total RNA above 20%, suggesting no major effects of RNA degradation by these mutations. We now describe this aspect of the design in the text and discuss that, while NMD is unlikely to be a major contributor to the effects of the mutations, our design cannot rule out other effects of the mutations on differential mRNA decay of the isoforms (lines 117-120).

2. In the proposal of pyrimidine rich regions being a potential mechanism driving protein localization via being soluble vs. membrane bound, there is no broad analysis of this in proteins with annotated transmembrane domains; this limits the generalizability of this proposed mechanism. Non-polar amino acids and hydrophobic domains are not exclusive to transmembrane domains, so what proportion of the exons analyzed which are transmembrane domains is unclear. One way to address this is to examine exons which overlap with annotated transmembrane domains (annotated by UniProt, Pfam, or another database) and ask whether these have either better pyrimidine tracts for splicing or are observed splice sites compared to exons encoding other protein domains (including non-transmembrane hydrophobic domains). The examples presented in Figure 4F are convincing examples of this, but how broad this may be as a mechanism of transmembrane domain regulation is still unclear given the evidence presented.

Our global analysis of exons throughout the genome indicates that 23% of all exons had a SVM-BP score greater than that of Fas exon 6, suggesting a relatively widespread mechanism of regulation of alternative exon inclusion. The shorter exons of this class encode the most hydrophobic amino acid sequences, characteristic of transmembrane domains, and displayed lower levels of inclusion correlating with the presence of a polypyrimidine tract. We have focused on single-pass transmembrane domains because of the *accidental* activation of a 3' splice site in Fas exon 6, because of a precedent in the work of García-Blanco lab on IL7R (Ref. 47, PMID: 33568552) and because one-pass transmembrane helices are often (84%, PMID: 22708632) encoded in distinct alternative exons (PMID: 14992489) and display antagonistic functions in processes spanning from programmed cell death to proteolytic ectodomain shedding (e.g. PMID: 14675776, PMID: 22708632). We then experimentally validated the phenomenon in two other transmembrane domain-encoding exons (CHODL) and CXADR. While generalizing the mechanism further to transmembrane and other hydrophobic domains would be certainly interesting, we submit that such an analysis is beyond the scope of this first report.

3. The accuracy of deletion effect predictions (Fig 5A) - if I understand correctly, this includes all deletions, short and longer. Would be prediction improve if limiting to short deletions? Add this analysis for an apples to apples comparison to substitutions and insertions would seem useful. Understanding the quality of predictions as a function of deletion length more broadly would also be informative regarding DANGO.

As requested by the reviewers, Figure 2A illustrates how deletions of progressive length help to delineate regulatory elements, which emerge more robustly when using lengths of 5 nucleotides and above, consistent with typical sequence lengths recognized by standard RNA binding domains such as RRM or KH domains (e.g. reviewed in PMID: 2466216, PMID:25625331). This is also noticeable in the complete set of deletion effects shown in Figure 5B (lower triangle plot), where the shorter deletions correspond to the layers closer to the longer diagonal, which best illustrate the alternating succession of enhancer and silencer elements. Nevertheless, the analysis of the whole set of deletions allows to better detect differences in the length / strength of the different regulatory elements.

4. The DANGO model and the AON effect analysis are interesting, but not sufficiently supported by the data. The

empirical data in Fig 7B is sparse - 9 deletions in a single exon of a single gene - and the validation of the DANGO score in Fig 7C is based on these 9 data points. In Fig 7B the correlation is quite high, but the actual PSI values don't match, which should be pointed out in the text. Additionally, it would demonstrate the robustness of both Dango and the SRE HMM if the directionality of Dango matched the splicing enhancer or silencer states from their HMM. Overall, the current data is not sufficient to support the conclusions and framing, including the highlighting of AONs in the abstract and statements like "DANGO is therefore a genome-wide resource that accurately predicts the effects of 21-nt deletions that can be used for AON selection" and "we have shown that deletion scanning accurately predicts the effects of AONs". I'm not against presenting these analyses, but it should be either toned down or supported with more (validation) data.

Following the reviewer's advice, we have benchmarked our approach against two other alternative exons for which similar AON *walks* have been reported in the literature and for which SpliceAI generated specific predictions of regulatory elements. This exercise showed that the combination of *in silico* deletion and SpliceAI predictions can be used to identify AONs that regulate alternative splicing of these genes. At the same time, the results also show that the predictive power of SpliceAI varies between alternative exons. Specifically, the approach

- provides a good overall picture of the effects of AONs on Fas exon 6 (Figure 7).
- identifies a splicing enhancer in the 5' half of SMN2 exon 7 and AONs targeting these sequences that induce potent skipping of the exon (PMID: 17355180), although it does not identify other regulatory elements in the 3' half of the exon revealed by the same AON walk (Figure S19A, shown below as Figure R5A for the reviewers' convenience).
- identifies the 5' end of Mnk2 exon e14b as the only region of this alternative exon containing regulatory elements, which have been targeted by AONs to modulate its use (PMID: 30329087), although the direction of the regulation predicted by SpliceAI is opposite to the effects of the AONs (Figure S19B, shown below as Figure R5B for the reviewers' convenience).

Clearly, further work combining machine learning with high-throughput mutagenesis of multiple other exons would be necessary to generate better predictive tools. Given these results, and following the reviewers' advice, we have toned down our statements (including in the abstract) and propose that DANGO can be used as a discovery tool to identify AONs that efficiently modulate splicing of alternative exons, but the extent to which it provides a comprehensive picture of the regulatory architecture depends on the particular exon analyzed (lines 565-568).

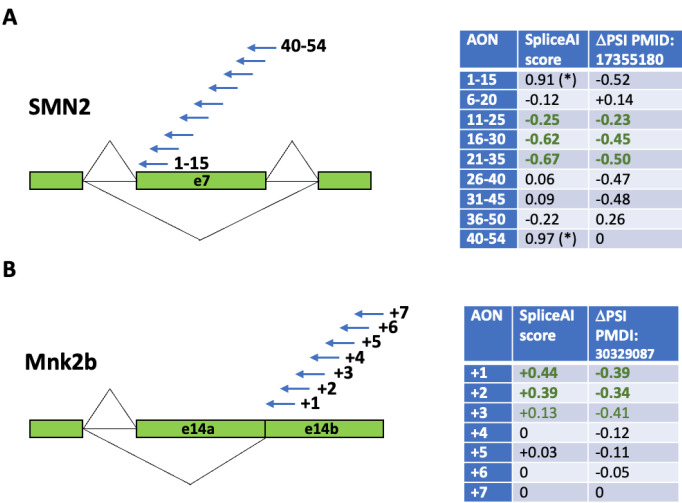


Figure R5 (also shown as figure S19). Benchmarking previously published effects on alternative splicing regulation of overlapping antisense oligonucleotides (AON walks) and SpliceAI-based predictions of the effects of deletion of exonic sequences complementary to the corresponding AONs. For each series of overlapping AONs, schematically represented above the indicated genomic regions (left schemes), SpliceAI scores for the expected effects of deletion of the complementary exonic region are tabulated along with the experimentally determined values of PSI changes upon treatment with the corresponding AON. Green indicates correlation between the two classes of values. **A. SMN2 exon 7** (Hua et al PloS Biol. 5, e73, 2007 PMID: 17355180). (*) indicates high values of SpliceAI scores likely related to effects of deletions on the sequences of the splice sites themselves -occurring upon deletions involving the first and last nucleotides of the exon-. SpliceAI detects the presence of a splicing enhancer(s) between nucleotides 11 and 35 but fails to detect other regulatory sequences located in the 3' half of the exon. **B. Mnk2b exon e14b** (Mogilevsky et al Nucleic Acids Res. 46, 11396-11404, 2018 PMID: 30329087). SpliceAI predicts the presence of regulatory sequences in only the 5' 1/3 of the exon, although it predicts opposite effects for the deletion of these sequences and the effects of AONs complementary to them.

5. There are multiple issues with the supplemental material, which make some claims difficult to interpret not matching the quality of the rest of the figures. These include:

a) Low resolution images, which are difficult to read even when zoomed in (S5-S10)

The quality of the images and font sizes in Figures S5-S10 has been improved, thank you.

b) Missing figure labels (S13)

Figure S13, now figure S14, is correctly labeled and a legend has also been included.

c) Unclear legends (i.e. what is the x-axis of figure S10? Length deciles?, if so please say explicitly)

We now clearly indicate in the legend of Figure S10 that the x-axis represents length deciles as in Figure 3B.

d) In the absence of a ladder, I'm unconvinced there aren't microexons in figure S11B. I am unsure as to whether the boxed-in parts of the gel (see attachment) are shadows of overloaded bands or microexons. A picture gel with a ladder showing the scale of separation, adjacent microexon positive controls of the expected size, or junction specific qPCR would be useful in clearing this up. To be clear, I don't believe this to be an issue with the other microexons detected in S11C, or figure 3, as these appear to be well separated.

Full gels with a ladder are now shown in Figure S11.

e) Figure S16 is referenced in the text, but not present in the supplement. It could be that S14 is missing and S15 and S16 are labeled as S14 and S15, as this seems consistent with their references in the text (line 470 doesn't match E/S state transitions in S14).

We sincerely apologize for the confusion. We have thoroughly revised the text to match the Supplementary Figures (please note that the order has been altered at the addition of new Supplementary Figures).

While most of the paper is clear, there are additional minor alterations that I believe would improve clarity and reader comprehension.

- Figure 1D; horizontal line should be labeled at reference sequence PSI somewhere (either on figure or figure legend).

We indicate in the Figure legend 1D that the horizontal line corresponds to the reference PSI value of the wild type sequence.

- A reference to Figure 2A would be useful in the paragraph beginning at line 144 to orient readers visually to the statistics mentioned in that paragraph.

A reference to Figure 2A has been included (line 137).

- Line 360-361: "Collectively, our results reveal a novel mechanism of exon skipping based upon assembly of U2 snRNP on exonic sequences that resemble (but cannot be active as) 3' splice sites." Authors should clarify how this is novel because they go on to say how U2AF2 binds pyrimidine tracts in the exon of IL7R to promote exon skipping in a separate paper (Line 366-367). Is it novel for FAS exon 6, is the proposed mechanism different from the IL7R mechanism?

The mechanism revealed by the *accidental* activation of a 3' splice site in our high throughput mutagenesis of Fas exon 6 is similar to the mechanism reported by García-Blanco and co-workers on IL7R (Ref. 47, PMID: 33568552). Our global analysis of exons throughout the genome then showed that 23% of all exons have a SVM-BP score greater than that of Fas exon 6, suggesting this could be a relatively widespread mechanism of regulation of alternative exon inclusion. As the shorter exons of this class encode the most hydrophobic amino acids, characteristic of transmembrane domains, and are encoded by pyrimidine-rich sequences, we propose that exons encoding transmembrane domains are inherently regulated by the presence of 3' splice site-like sequences, as observed in Fas and in IL7R, which we could then also document in two other transmembrane domain-encoding exons (CHODL and CXADR). These observations are interesting because such exons are frequently involved in regulating the expression of membrane-bound versus soluble isoforms, which typically display antagonistic functions in processes spanning from programmed cell death (e.g. Fas/CD95 exon 6), cell growth signaling (e.g. FGFR3 exon 9) to proteolytic ectodomain shedding (e.g. syndecan-4 exon V-a) (e.g. PMID: 14675776, PMID: 22708632) and whose deregulation has been linked to various pathologies (PMID: 22708632). We have tried to better explain the novelty of the model in the text (lines 385-399).

- Figure 5B-C being referenced in the text before Figures 3 and 4 could be very confusing for a reader depending on how the final paper is formatted.

We understand the point made by the reviewer, but the alternative would be to include the results of the computational analyses already in Figure 2, long before they are explained in the flow of the paper, which we believe would be also confusing for the readers. For that reason we would prefer to maintain the same order of Figures in the text, unless the reviewer/editor think otherwise.

- HMM identified SREs in 6C would be easier to evaluate for readers if they were aligned with FAS exon 6 experimental data (as a sub panel on top or something). This would also visually support the statement on lines 445-448: "Our model captured the regulatory architecture of FAS exon 6 as uncovered by our deep indel mutagenesis experiment: regions of the exon corresponding to inferred enhancers were predicted to be in state E, and regions corresponding to inferred silencers in state S (figure 6C)."

Thank you for this suggestion. We include now experimental 4-mer deletion data aligned to the plot in new Figure 6C. The deletion effects are centered around the position that the deletions share in common (e.g., deletions 1-4, 2-5, 3-6, 4-7 all cover position 4, therefore they are centered around position 4; deletions 10-13, 11-14, 12-15, 13-16 all include position 13, therefore those are centered around position 13 in the plot). The results illustrate the good overall concordance between the predictions of the hidden Markov model and the experimental data, to delineate regulatory elements in Fas exon 6. This is now explained in the text (lines 460-461) and in the Figure legend (lines 1304-1306).

- Figure 7C (which has Dango score on the y-axis) is referenced in the text before Dango is introduced and is a bit confusing for the reader.

Thank you. We now explain at this point that the Dango score corresponds to the SpliceAI-predicted effects of 21-nt deletions (line 560).

- Figure S12 should be labeled "UAG" instead of "TAG".

Corrected, thank you for the detailed review.

- Line 641 typo: "Quiagen"

Corrected (lines 733 and 734, also 750), thank you for the detailed review.

Separately, there are a couple of other improvements that could be made regarding code, data, and reagent availability.

- It would be useful to the community if the HMM predictions for SREs genome wide were available in a usable format for researchers (like a ucsc track or bedgraph).

This has been included in the github repository.

- The code availability on github is nice, but it would be useful for people trying to reproduce analysis if there was a README file explaining the repository contents and dependencies to reproduce the analysis.

The github repository has been completely updated for clarity.

- The authors should comment on the availability of the plasmid used in their experiments for other researchers.

Every reagent used in the paper will be, of course, available upon request. We have expanded our previous statement (lines 984-985) to reflect this.

Aside from the recommended changes mentioned, I think the experimental approach is sound, the interpretation for the data is appropriate, and exceptions are well addressed in separate experiments. I find that the statistical analysis in this manuscript is appropriate and well-described in methods and in figures. The references provided seem appropriate for the content of the paper. The abstract, introduction, and conclusion are accessible and appropriate.

Thank you.

Reviewer #3 (Remarks to the Author):

I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

[Thank you for your review.](#)

Reviewer #4 (Remarks to the Author):

This work by Baeza-Centurion et al describes a systematic exploration of the regulatory architecture of human exons. The authors built a minigene based on a specific exon of interest, FAS exon 6, and used a synthetic genetic library to introduce into it all possible deletions, all possible 1-3 nucleotide insertions plus a collection of randomly chosen 4 nucleotide insertions, together with all single nucleotide substitutions. Then they measure the exon inclusion rate using deep sequencing.

First, this high throughput approach reveals the full regulatory architecture of the exon of interest, exposing that splicing enhancers and silencers are found on the exon in an alternating pattern. They also demonstrate a strong correlation between k-mers insertion effects in the library and the effects of having these k-mers in exons in the genome, demonstrating the power of the synthetic library to predict genomic features.

Second, the authors show that some library variants lead to an alternatively spliced micro-exon, which leads them to suggest an alternative splicing regulatory mechanism through exonic cryptic splice sites. The authors also predict using bioinformatic analysis that other short exons that code for transmembrane domains (similar to the exon of interest) should have this mechanism, and demonstrate this mechanism on 3 other such exons.

Next, the authors examine the performances of 5 different computational models on predicting indels effects on splicing. After demonstrating that one of the models performs well on their indel library they use this model to generalize their observations on exonic regulatory architecture for the entire human exome.

Finally, the authors claim that the method presented in this work can be very beneficial for screening for effective antisense oligonucleotides (AONs), to treat splicing-associated genetic syndromes. And they provide a new computational model to predict AON efficiency.

We think that the parts of the paper where the authors analyze the regulatory architecture of the exon of interest and then generalize their observations over the entire human exome contribute significantly to our understanding of splicing regulation. Moreover, this work demonstrates the power of deep indel mutagenesis in revealing splicing regulatory elements, and their mechanism.

On the other hand, in the last part of the paper where they claim this method contributes to discovering new AONs with therapeutic potential, the contribution and the novelty are less clear to us. First, It's not clear to us from the text how deletion scanning is more accessible than directly performing AON scanning as was done for Figure 7B. If there are significant benefits for deletion scanning over AON scanning it should be better described in the text. Second, we think that the last section in the results which describes a new computational model named DANGO is not novel, since they merely use specific inputs (21-nt deletions) as input to an existing computational model (spliceAI). We think that naming this as "a new model" is misleading.

Since the abstract and introduction of the paper put a lot of focus on the AON discovery part we suggest this paper be revised. Either put more focus on the first parts describing the regulatory architecture, or better describe the advantage of deletion scanning as an AON discovery method over AON scanning. As for the computational model, we think it shouldn't be presented as a new model, only as a way to use an existing model and how it can help with AON discovery.

[We thank the two reviewers for their overall positive opinion on the manuscript and for the suggestions to improve it, which we have followed as detailed below. In addition, we have followed the reviewers' advice and now present the computational model as an application of SpliceAI to assist with AON discovery \(see Dango Section, lines 569-598\).](#)

Below are specific comments:

Major points:

Calculation of PSI values per variant is based on counting the frequency of mature mRNA molecules per variant, divided by the frequency of the variant in the input DNA library. There is a caveat in the experimental design since only mRNAs with fully included exons can be assigned to a library variant. When the exon is skipped, all mRNA products from the library have identical sequences. Therefore, there is no way to measure the total mRNA per variant. So it's impossible to differentiate between the mutant's effect on PSI and the mutant's effect on total mRNA expression level. The authors should address this caveat and come with means to mitigate it. The experimental validation with RT-PCR (Figure 1C) does measure both skipped and included transcripts per variant, so the authors can use this validation experiment to check for effects on total mRNA levels per variant in this validation subset. If they don't observe significant variation in total mRNA between mutants it can justify the library's experimental design.

We apologize for not having made our approach to determine PSI values more clear. We agree with the reviewers that enrichment scores determined from variant frequencies in the input and in processed mRNAs including exon 6 do not allow to parse out effects on splicing from effects on mRNA abundance. The experimental validation via RT-PCR of Figure 1C allows, as the reviewers suggested, to confidently determine PSI values as ratios between inclusion / inclusion + skipping isoforms. The excellent correlation between PSI values estimated by the two independent approaches is precisely the way in which we are confident that we can convert enrichment scores into PSI values for the whole collection of detectable mutants. Judging from the total levels of minigene-derived transcripts in a battery of 42 independently validated substitution/indel mutants, less than 15% of the mutants show decreased levels of total RNA above 20%, as would be expected from RNA degradation effects. In addition, potential effects of mutagenesis on the generation of premature stop codons and NMD have been minimized by designing minigenes that do not include the natural starting ATG codon of Fas/CD95, nor contain long ORFs, nor ATGs in a good Kozak context for translation. We now describe this aspect of the design and discuss that, while NMD is unlikely to be a major contributor to the effects of the mutations, our design cannot rule out other effects of the mutations on differential mRNA decay between isoforms (lines 117-120).

In line 530 the authors write “We reasoned that deletion mutagenesis might provide a rapid method to predict the effects of AONs binding...”. However, the authors don’t describe what are the benefits of deletion scanning over AON scanning. Is it faster? cheaper? easier to perform? The authors should elaborate on that.

Following the reviewer’s advice, we have elaborated (lines 585-595) on the benefits of deletion scanning over AON scanning, including 1) higher resolution in the definition of regulatory elements, and 2) that the good correlation between experimental and computational predictions of the effects of extensive deletion analyses opens up the possibility of predicting *in silico* AONs for any desired exon, as well as transcriptome-wide.

Minor points:

The experimental design in Figure 1B is not clear enough. From the figure, it can be seen as if mRNA levels for all library variants were measured through RT-PCR as described in line 705, which is not the case.

We apologize for this confusion. We have clarified in the text (lines 114-117 and section starting on line 737) and in the Figure legend (line 1204) that RT-PCR was carried out only for a subset of validating samples. Thank you for pointing this out.

The calculation of PSI value as described in Figure 1B and equations in lines 696-704 is not clear enough. According to the equations in Figure 1B, the ES value for WT equals 0 by definition, and then normalizing the PSI value by $\exp(ES[WT])$ is redundant. In addition, we suggest adding the ES calculation as an equation in the method section, and not only in Figure 1B.

Thank you for these suggestions. We have updated the equation in Figure 1 and updated the Methods section as well (lines 727-736).

In lines 374-387 the authors describe how due to the nature of the genetic code, exons that code for transmembrane domains are rich in pyrimidines and therefore have a high probability of including cryptic 3’ splice sites that can lead to exon skipping. Since we assume some transmembrane domains in the genome are essential, we think it would be interesting to check bioinformatically if essential domains have a way to avoid the effect of such cryptic splice sites.

Our global analysis of exons throughout the genome indicates that 23% of all exons had a SVM-BP score greater than that of Fas exon 6, suggesting a relatively widespread mechanism of regulation of alternative exon inclusion. The shorter exons of this class encode the most hydrophobic amino acid sequences, characteristic of transmembrane domains, and displayed lower levels of inclusion correlating with the presence of a polypyrimidine tract. We have focused on single-pass transmembrane domains because of the *accidental* activation of a 3’ splice site in Fas exon 6, because of a precedent in the work of García-Blanco lab on IL7R (Ref. 47, PMID: 33568552) and because one-pass transmembrane helices are often (84%, PMID: 22708632) encoded in distinct alternative exons (PMID: 14992489) and display antagonistic functions in processes spanning from programmed cell death to proteolytic ectodomain shedding (e.g. PMID: 14675776, PMID: 22708632). We then experimentally validated the phenomenon in two other transmembrane domain-encoding exons (CHODL) and CXADR. While generalizing the mechanism further to essential domains would be certainly interesting, we submit that such an analysis is beyond the scope of this first report.

In line 40 there is a typo, “that that”.

Corrected, thank you.

Reviewer #5 (Remarks to the Author):

I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Thank you for your review.

Response to reviewers' comments of NCOMMS-24-24469A

Reviewer #1 (Remarks to the Author):

The authors have addressed all points this reviewer has raised.

Reviewer #2 (Remarks to the Author):

The authors have done careful and comprehensive revisions that have improved the manuscript. All my concerns have been addressed.

Reviewer #3 (Remarks to the Author):

I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

We thank the three reviewers for their input.

Reviewer #5 (Remarks to the Author):

The authors have addressed sufficiently most of my comments.

We thank the reviewer for his/her input.

However, I am still not convinced by the benefit of deletion mutagenesis over AON walk.

First, in the rebuttal letter, the authors refer to lines 585-595 in the revised manuscript. I assume this is a mistake, as these lines describe the DANGO genome browser track. In the section above titled "Deletion scans accurately predict the effects of antisense oligonucleotides on exon inclusion" the only change I notice from the previous version relates to analyses of two previously published AONs. Could it be that a part of the revised text is missing?

Apologies for the confusion. We had included in the Results section of the submitted manuscript (lines 558-568) the following paragraph: "The correlation between the AON effects and the SpliceAI-predicted effects of 21-nt deletions (Dango score, see next section) was similarly strong ($\rho = 0.74$, **figure 7C**), suggesting that *in silico* deletion mutagenesis could be an efficient, affordable and high resolution strategy to identify regions in an exon that can be targeted by AONs to achieve a range of desired splicing outcomes for therapeutic or biotechnological applications. Additional analyses using previously published AON walks on two other exons^{64,65} confirm that SpliceAI-based predictions of the effects of exonic deletions can be used to identify AONs that efficiently modulate splicing of alternative exons, although the extent to which it provides a comprehensive picture of regulatory architectures depends on the particular exon analyzed (**figure S19**). We have now complemented this with an expanded text in the Discussion section, as described below.

Second, in the rebuttal letter, the authors provide two benefits of deletion scanning over AON scanning.

1) "higher resolution in the definition of regulatory elements"

I see how deletion scanning can provide this higher resolution, because deleted elements can be shorter than the 18-21nt AONs, hence, deletion scanning should be a better tool to explore the regulatory landscape as beautifully demonstrated in this work. However, for clinical applications, I still struggle to understand how deletion scanning is better than AON scanning for predicting AONs effect. If one performs an empirical scanning, wouldn't it be better to perform the scanning as close as possible to the treatment itself? If there are other benefits for deletion scanning (like cost or simplicity), the authors should elaborate on them.

What we meant to say is that a deletion scan like the one described in our manuscript can explore a large number of sequences and lengths (in fact, all possible deletions) in a single experiment at a relatively low cost (in fact at a negligible cost if carried out *in silico*), while high costs and experimental logistics soon become prohibitive for the synthesis and testing of a large number of AONs of different lengths at high (e.g. 1 nucleotide) resolution. Deletion scans can therefore inform the selection of a subset of AONs that, of course, will need to be tested experimentally before any clinical application. Please see the proposed text below that has been included in the Discussion section to complement the text in the Results section mentioned above.

2) "that the good correlation between experimental and computational predictions of the effects of extensive deletion analyses opens up the possibility of predicting *in silico* AONs for any desired exon, as well as transcriptome-wide."

In my opinion, the added analysis provided by the authors in Figure S19 doesn't support this claim. As noted by the authors in the figure legend, in the case of Mnk2b spliceAI predicts the opposite effect than the AON scan, and in the case of SMN2 there is a good correlation only for 3/9 AONs.

So, eventually, taking all three analyses together (Figure 7C, and Figure S19 A-B), only one of them demonstrates good correlation between *in-silico* predictions and AON effect.

To better reflect this point, as well as to address the other points mentioned by the reviewer above, we have introduced the following new text at the end of the Discussion section (lines 635 - 645):

"The good correlation between *in silico* and experimental deletion scans, as well as their high resolution and relatively low cost compared to affordable experimental AON walks, make them interesting tools to prioritise AONs to be tested for eventual clinical or biotechnological applications. Our benchmarking efforts indicate, however, that the predictive power of *in silico* deletion scans varies among different alternatively spliced events: while predicted AONs generally display effects on splicing, *in silico* deletion scans failed to predict the effects of some AONs targeting certain transcript regions or splicing events (**Fig. 7C and Supplementary Fig. 19**). Nevertheless, we envision that both *in silico* and high-throughput experimental deletion scans will play an increasingly important role in accelerating the discovery of AONs to effectively modulate splicing for many different therapeutic goals."