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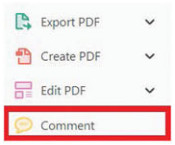

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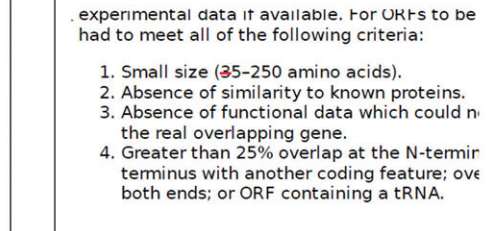

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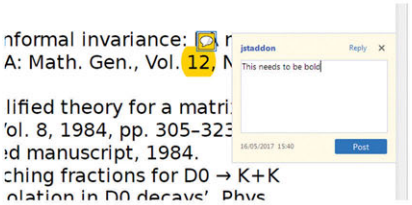

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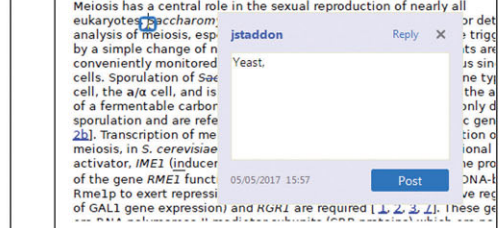

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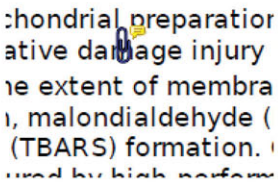

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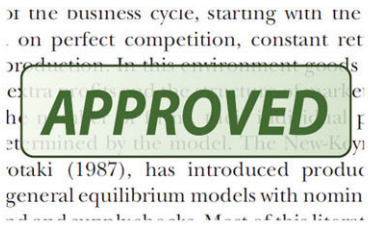

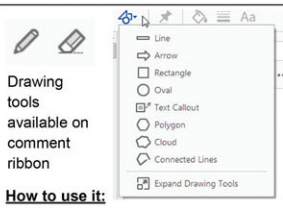

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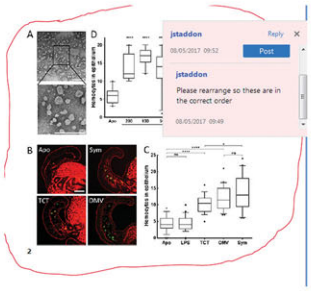

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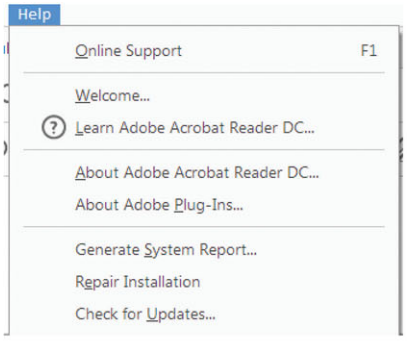

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BRIEF REPORT

WILEY HGVS HUMAN GENOME VARIATION SOCIETY

Disease-causing variants of the conserved +2T of 5′ splice sites can be rescued by engineered U1snRNAs

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Abstract

The ability of variants of the spliceosomal U1snRNA to rescue splicing has been proven in several human disease models, but not for nucleotide changes at the conserved GT nucleotide of 5′ splice sites (5′ss), frequent and associated with severe phenotypes. Here, we focused on variants at the 5′ss of F9 intron 3, leading to factor IX (FIX) deficiency (hemophilia B). Through minigene expression, we demonstrated that all changes induce complete exon 3 skipping, which explains the associated hemophilia B phenotype. Interestingly, engineered U1snRNAs remarkably increased the proportion of correct transcripts in the presence of the c.277+4A>G (~60%) and also c.277+2T>C mutation (~20%). Expression of splicing-competent cDNA constructs indicated that the splicing rescue produces an appreciable increase of secreted FIX protein levels. These data provide the first experimental evidence that even part of variants at the conserved 5′ss +2T nucleotide can be rescued, thus expanding the applicability of this U1snRNA-based approach.

KEYWORDS
ExSpeU1, hemophilia B, human disease, RNA splicing, splicing mutations

Nucleotide changes affecting the 5′ splice site (5′ss) represent approximately 9% of all mutations found to be associated with human inherited diseases (http://www.hgmd.org/; Faustino & Cooper, 2003; Ward & Cooper, 2010), and commonly causing severe clinical phenotypes. These variants are thought to elicit their detrimental effect by interfering with the interaction with the small nuclear ribonucleoprotein U1 (U1snRNP), the spliceosomal unit that in the earliest splicing step recognizes the 5′ss by complementarity with the 5′ tail of its RNA component (U1snRNA; Horowitz & Krainer, 1994).

On the basis of the frequency and relevance of these nucleotide changes and on their mechanism, we and others have devised a correction approach based on variants of the U1snRNA designed to restore complementarity with the defective 5′ss (compensatory U1snRNAs; Pinotti et al., 2008) or to target downstream intronic regions (exon-specific U1snRNAs; ExSpeU1; Alanis et al., 2012). For different human genetic disorders, in both cellular (Balestra et al., 2015; Dal Mas et al., 2015; Glaus, Schmid, Da Costa, Berger, & Neidhardt, 2011; Scalet et al., 2017; Schmid et al., 2011; Tajnik et al., 2016; van der Woerd et al., 2015) and animal (Balestra et al., 2014; Balestra et al., 2016; Dal Mas, Rogalska, Bussani, & Pagani, 2015; Donadon et al., 2018; Rogalska et al., 2016) models, the engineered U1snRNAs were shown to be effective on variants at 5′ss but also within the exon or at the 3′ss. However, these approaches failed to rescue changes at the highly conserved nucleotides +1G and +2T of the 5′ss (Alanis et al., 2012; Cavallari et al., 2012), which are the most represented (Buratti et al., 2007; Krawczak et al., 2007) and severe ones, and commonly considered to be virtually null.

Conscious of the fact that the changes at the highly conserved nucleotides +1 and +2 of 5′ss, the most detrimental ones, did not respond to engineered U1snRNAs but also of the strong dependence of alternative splicing mechanisms from the sequence context of the specific exon unit, we further extended our investigation to the panel of nucleotide changes affecting the 5′ss of *F9* (LRG_556) intron 3 (Figure 1). These naturally occurring variants are mostly associated in patients with moderate to severe hemophilia B (Factor IX Variant Database, http://www.factorix.org/; Rallapalli, Kemball-Cook, Tuddenham, Gomez, & Perkins, 2013; Supporting Information Table S1). Detailed methods for creation of *F9* minigenes and expression vectors, cell culture, RNA splicing, and secreted FIX analyses are described in the Supporting Information Methods.

The expression of *F9* minigenes (Figure 1a) in mammalian cells, a well-established approach to investigate splicing, combined with the splicing pattern analysis through denaturing capillary electrophoresis of fluorescently labeled RT-PCR products, due to the small exon 3 size (25 bp), clearly demonstrated that all variants, different from the wild-type exon 1–4 minigene construct (pFIX$^{1\text{-}4wt}$), induce complete

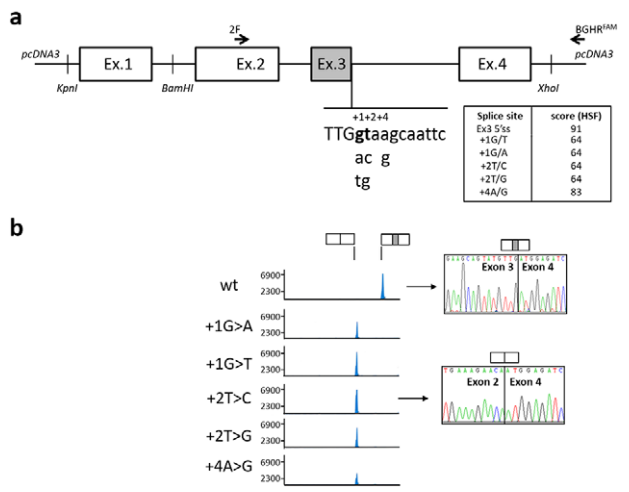

FIGURE 1 Aberrant splicing patterns triggered by nucleotide changes at the F9 exon 3 5′ss. (a) Schematic representation of the F9 genomic sequence cloned as minigene in the pcDNA3 expression vector. Exons (Ex.) and introns are represented by boxes and lines, respectively. The exon 3 is highlighted in gray. Restriction sites exploited to create the minigene are indicated together with primers (arrows) used for RT-PCR. The sequence, with exonic and intronic nucleotides in upper and lower cases, respectively, reports the authentic 5′ss with the positions of the investigated changes detailed below. The highly conserved dinucleotide GT of the authentic 5′ss is in bold. Inset: scores of the authentic and mutated 5′ss calculated by the HSF matrices at Human Splicing Finder (http://www.umd.be/HSF3/index.html). Detailed method for in silico splicing prediction is described in the Supporting Information Methods. (b) Alternative splicing patterns of F9 minigenes (indicated on the left) transiently expressed in HEK293 cells evaluated by denaturing capillary electrophoresis. The schematic representation of transcripts (with exons not in scale) is reported above. The chromatograms on the right report the sequences of transcripts.

exon skipping (Figure 1b). Taken into account the undetectable levels of correct transcripts in our experimental system and the fact that the removal of exon 3 from the mature mRNA leads to frameshift and premature translation termination, these data demonstrate the causative nature of nucleotide changes and their association with hemophilia B in patients.

Moreover, the remarkable splicing impairment is in line with the computational analysis of 5′ss scores (Figure 1a, inset), an estimate of the complementarity between the 5′ss sequence and the 5′ tail of the key spliceosomal U1snRNA. All nucleotide changes at positions +1 (c.277+1G>A, c.277+1G>T), +2 (c.277+2T>C, c.277+2T>G), and +4 (c.277+4A>G) are predicted, to variable extent, to weaken the 5′ss and therefore its efficient recognition by complementarity of the endogenous U1snRNA. Indeed, in the attempt to restore exon 3 definition in the presence of the nucleotide changes and counteract its skipping, we designed a compensatory U1snRNA on the *F9* exon 3 5′ss ($U1^{IVS3}$) and exon-specific U1-snRNAs ($U1^{+6}$) targeting the downstream intron between positions +6 and +14 (Figure 2a). The screening for efficacy was initially performed on the c.277+4A>G variant, the +4 position being less conserved and thus more prone to rescue. As shown in Figure 2b, both the $U1^{IVS3}$ and the $U1^{+6}$ remarkably promoted exon 3 definition and thus inclusion (from undetectable to ~60% of correct transcripts).

The $U1^{+6}$ that, by targeting the intron, guarantees increased gene specificity was then challenged toward the unfavorable changes at the other positions. While reducing the proportion of exon 3-skipped transcripts, the $U1^{+6}$, unexpectedly, led to appreciable usage of an exonic cryptic 5′ss located 8 nucleotides upstream of the defective 5′ss (Figure 2c, inset; Figure 2a, underlined) causing frameshift and premature translation termination. Notwithstanding, and most importantly, the $U1^{+6}$ also triggered the usage of the 5′ss affected by the c.277+2T>C variant, with appreciable rescue of correct transcripts (from undetectable to 21.5% ±1%).

To demonstrate that the above-mentioned splicing patterns were exclusively produced by the expression of the *F9* minigenes, hardly mutagenizable in the very small exon without altering the splicing regulatory elements, we exploited as additional control the naturally occurring change (c.277G>A; p.Asp93Asn) in the exon at −1 position of the 5′ss (−1G>A), also predicted to affect splicing (score 80). In minigene assays, the change induced exon skipping (Figure 2d) and was remarkably rescued by the $U1^{+6}$ (from undetectable to ~50% of correct transcripts). Importantly, direct sequencing of correct transcripts (inset) demonstrated the presence of the exonic change, thus validating our experimental setting and strengthening the overall results.

However, the minigene including only a partial coding region does not permit the evaluation of the rescue at the protein level, the key issue to extrapolate a potential therapeutic impact. Therefore, we created a splicing-competent cDNA minigene in which portions of introns have been included into the full-length FIX cDNA cassette (Figure 2a). The expression of the wild-type construct led to FIX protein in medium, which validated our experimental setting and led us to explore the rescue of secreted FIX in the presence of the c.277+2T>C and c.277+4A>G variants, and choosing the c.277+1G>T as negative control. As expected, the coexpression of the $U1^{+6}$ with the c.277+1G>T did not result in appreciable levels of secreted FIX. Noticeably, coexpression of the $U1^{+6}$ with the splicing-competent minigene resulted in a significant increase of FIX in medium for the c.277+4A>G mutant (8.7 ± 2.4% of wild-type; $P = 00002$; Figure 2e) and, most intriguingly, for the c.277+2T>C variant (4.3 ± 1.9%; $P = 00034$). It is worth noting that for hemophilia B, as well as for the other coagulation factor disorders, raising levels above 5% of normal would result in a mild bleeding phenotype, no longer associated with spontaneous bleeding (Den Uijl et al., 2011).

Taken together, these data demonstrate that at least some changes at the +2 position can be approached by ExSpeU1s, thus adding a new perspective in the rescue of changes altering the conserved 5′ss GT dinucleotide, thought to be essential for correct splicing of pre-mRNA (Sheth et al., 2006) and indeed not rescuable. However, a small proportion (0.56%) of introns has a variant of the 5′ss containing a cytosine, instead of thymine, in position +2 (Thanaraj, 2001). These introns are efficiently recognized by the U2-type spliceosome through the presence of strong consensus sequences maximized for base-pair formation with U1 and U5/U6 snRNAs. This observation, together with our data demonstrating the U1-mediated rescue, supports a mechanism

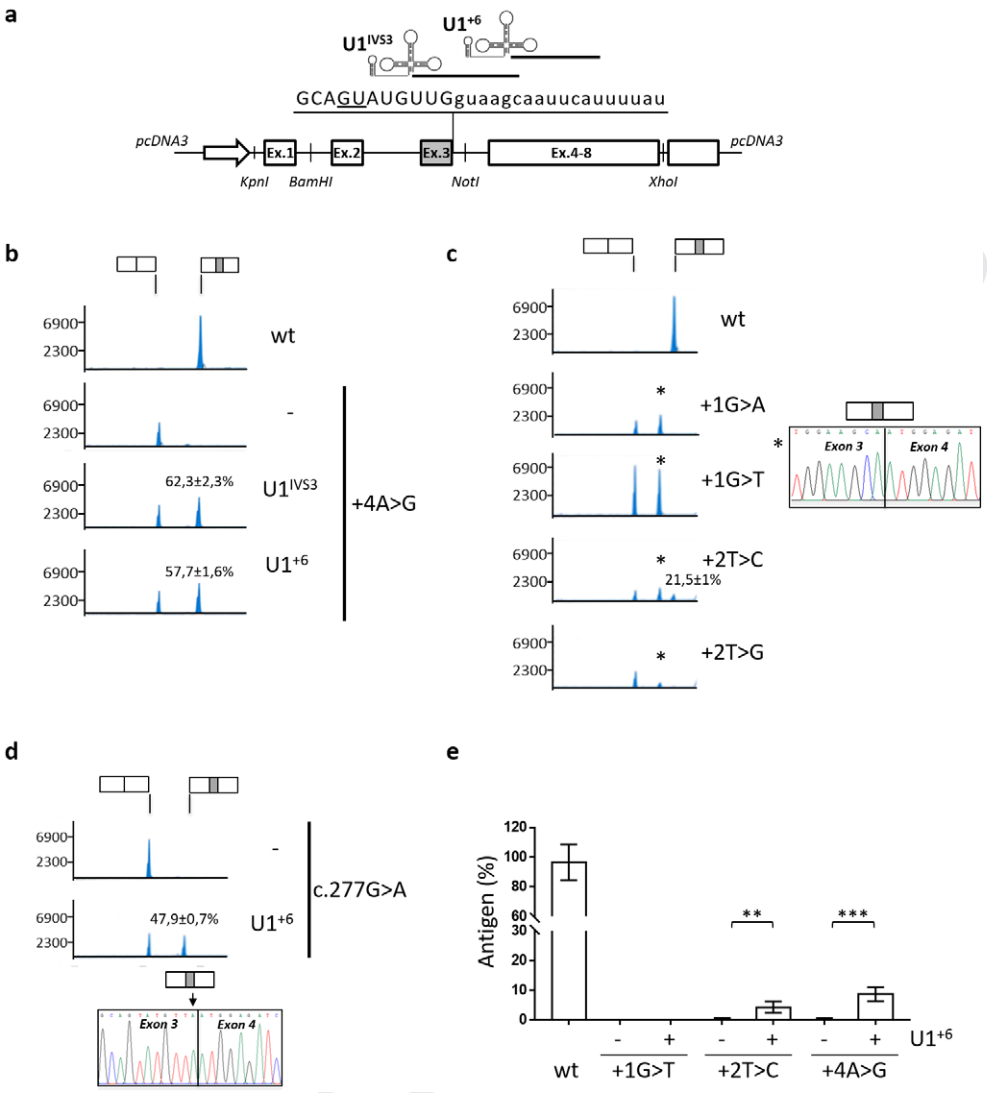

FIGURE 2 Rescue of *F9* exon 3 by modified U1snRNAs. (a) Schematic representation of the *F9* splicing-competent cDNA minigene (pSC-FIX^{wt}) and of engineered U1snRNAs with the 5′ tail located above the corresponding target sequence on *F9* pre-mRNA. Exon (Ex.) and intron sequences are represented by boxes and lines, respectively. The exon 3 is highlighted in gray. Restriction sites exploited to create the minigene are shown. The white arrow and box represent the cytomegalovirus promoter and poly-adenylation signal from human β globin gene, respectively. The sequence of the exon 3 (upper cases) and of the downstream intron 3 (lower cases) with the cryptic 5′ss (underlined) is reported. (b) Alternative splicing patterns of the *F9* minigene harboring the c.277+4A>G variant expressed in HEK293 cells alone or in combination with engineered U1snRNAs. (c) Alternative splicing patterns of the *F9* minigenes harboring the changes at the conserved GT dinucleotide expressed in HEK293 cells alone. Asterisks indicate the presence of the shorter transcript resulting from the usage of an exonic cryptic 5′ss (underlined in Figure 2a), as indicated by the sequence in the chromatogram on the right. (d) Alternative splicing patterns of the *F9* minigene harboring the c.277G>A variant expressed in HEK293 cells alone or in combination with the U1^{+6}. The chromatogram reports the sequence of the correctly spliced transcripts resulting from U1^{+6} cotransfection, and harboring the nucleotide change at −1 position (arrow). In panel B, C and D, the splicing patterns have been evaluated by denaturing capillary electrophoresis. The schematic representation of the transcripts (with exons not in scale) is reported on top. The relative proportion (%) of correctly spliced transcripts is reported as mean ± standard deviation (SD) from three independent experiments. (e) Secreted FIX protein levels measured by ELISA in medium from HEK293 cells transiently transfected with the pSC-FIX variants alone or in combination with the U1^{+6}. Results are expressed as a percentage of pSC-FIX^{wt} (96.4 ± 12.1 ng/mL, detection limit of the assay 0.78 ng/mL) and are reported (histograms) as mean and standard deviation from three independent experiments. ***, $P < 0.0005$; **, $P < 0.005$

in which nucleotide changes at +2, depending on the specific exon context, could be still recognized by the U2-type spliceosome in the presence of particular exon/intron context.

Overall, our data provide the first experimental evidence that engineered U1snRNA, and particularly their second-generation ExSpeU1, can rescue splicing, and thus proper protein expression, in the presence of variants at the highly conserved nucleotide at position +2 of the 5′ss. Our data expand the applicability of the ExSpeU1-mediated correction approach to severe forms of human genetic diseases.

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CONFLICTS OF INTEREST

D.S., I.M., A.B., and D.B. have no competing interests to declare. M.P. and F.B. are founders of the start-up company RareSplice and M.P. is inventor of a patent (PCT/IB2011/054573) on modified U1snRNAs.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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