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ORIGINAL INVESTIGATION

Skipping of an exon with a nonsense mutation in the *DMD* gene is induced by the conversion of a splicing enhancer to a splicing silencer

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Abstract

Modulation of dystrophin pre-mRNA splicing is an attractive strategy to ameliorate the severe phenotype of Duchenne muscular dystrophy (DMD), although this requires a better understanding of the mechanism of splicing regulation. Aberrant splicing caused by gene mutations provides a good model to study splicing regulatory cis-elements and binding proteins. In this study, we identified skipping of in-frame exon 25 induced by a nonsense mutation (NM_004006.2:c.3340A > T;p.Lys1114*) in the *DMD* gene. Site-directed mutagenesis study in minigenes suggested that c.3340A > T converts an exonic splicing enhancer sequence (ESE) to a silencer element (ESS). Indeed, RNA pull-down and functional study provided evidence that c.3340A > T abolishes the binding of the splicing enhancer protein Tra2β and promotes interactions with the repressor proteins hnRNP A1, hnRNP A2, and hnRNP H. By carefully analyzing the sequence motif encompassing the mutation site, we concluded that the skipping of exon 25 was due to disruption of a Tra2β-dependent ESE and the creation of a new ESS associated with hnRNP A1 and hnRNP A2, which in turn increased the recruitment of hnRNP H to a nearby binding site. Finally, we demonstrated that c.3340A > T impairs the splicing of upstream intron 24 in a splicing minigene assay. In addition, we showed that the correct splicing of exon 25 is finely regulated by multiple splicing regulators that function in opposite directions by binding to closely located ESE and ESS. Our results clarify the detailed molecular mechanism of exon skipping induced by the nonsense mutation c.3340A > T and also provide information on exon 25 splicing.

Introduction

Splicing, which involves the removal of introns from pre-mRNA and the joining of exons together, is a critical step for gene expression (De Conti et al. 2013). This step is catalyzed by the spliceosome and requires accurate exon definition through the interaction of multiple RNA-binding proteins with splicing signals on pre-mRNA (Matera and Wang 2014). The 5′ splice donor site (5′ss), 3′ splice acceptor site (3′ss), and the branch site are important splicing signals present at every exon–intron boundary. Exons and their flanking introns also contain additional sequence features known as exonic/intronic splicing enhancers (ESEs/ISEs) and silencers (ESSs/ISSs), to facilitate exon selection by the spliceosome (De Conti et al. 2013). ESEs and ESSs have been well studied. Most known ESEs act as binding sites for splicing factors belonging to the serine/arginine-rich (SR) protein or SR-like protein family to stimulate exon inclusion, whereas ESSs are usually recognized by splicing factors of the heterogeneous nuclear ribonucleoprotein (hnRNP) family to repress exon inclusion (Dreyfuss et al. 2002; Long and Caceres 2009). Mutations, which alter splicing regulatory elements, may affect the splicing pathway and cause diseases in human (Kataoka 2017).

The authors wish it to be known that, in their opinion, Masafumi Matsuo and Zhujun Zhang should be regarded as joint last authors.

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The *DMD* gene is one of the largest human genes, spanning 2.4 Mb on the X chromosome. In contrast to this gene's huge size, the full-length transcript is only 14 kb in length, which contains 79 exons and encodes the important cytoskeletal structure protein dystrophin (Hoffman et al. 1987; Koenig et al. 1987, 1988). More than 99% of the sequence of this gene consists of noncoding introns that have to be removed during splicing. Because splice site-like sequences are abundant in the human genome (Black 1995), it is a challenge for the splicing machinery to properly define the exons, which are tiny relative to the huge *DMD* pre-mRNA. ESEs have been proposed to play particularly important roles in the recognition of exons in such large genes (Takeshima et al. 1995).

The extremely large size of the *DMD* gene also makes it a prominent target for mutations. According to the TREAT-NMD DMD Global database, large deletions and duplications account for 68% and 11% of the total mutations analyzed, and small mutations account for 20% of the mutations (Bladen et al. 2015). Mutations that disrupt the reading frame of the *DMD* transcript can cause severe and lethal Duchenne muscular dystrophy (DMD, OMIM 310200), while mutations that preserve the reading frame are related to the milder Becker muscular dystrophy (BMD, OMIM 300376) (Monaco et al. 1988). Artificial manipulation of exon skipping to restore the reading frame is a plausible strategy to reduce the severity of DMD. Currently, the use of antisense oligonucleotides (AONs) against splicing signals, in particular ESE, is a major approach to induce exon skipping (Aartsma-Rus and van Ommen 2009; Matsuo 1996; Wood et al. 2010). In addition, small chemical compounds targeting the phosphorylation of splicing factors have also attracted attention (Nishida et al. 2011, 2016; Sako et al. 2017). However, to successfully apply all of these approaches, there is a need to obtain a better understanding of the splicing regulatory mechanism, which is still largely unknown in the *DMD* gene.

Mutations that affect splicing represent 12.8–26.6% of small mutations and corresponding to 3.1–6.3% of total mutations in the *DMD* gene (Tuffery-Giraud et al. 2017). Nonsense mutation, a single-nucleotide substitution that creates a premature stop codon, account for 50% of the small mutations (Bladen et al. 2015), and it is usually associated with the DMD phenotype. Occasionally, nonsense mutations can alter exonic elements and affect the splicing pathway. Such mutations in in-frame exons can be identified by the loss of correlation between phenotype and genotype, and therefore, provide a good model to study exonic elements and related binding factors necessary for exon definition and splicing regulation (Flanigan et al. 2011; Juan-Mateu et al. 2013). In this study, we report that a nonsense mutation, c.3340A > T in exon 25 of the *DMD* gene, induces the in-frame skipping of exon 25, resulting in a mild BMD phenotype. We extensively investigated the molecular mechanism underlying this aberrant splicing event.

Materials and methods

Case report

The patient is a 15-years-old Chinese boy. No neuromuscular diseases were identified in his family history. He started to walk at 1 year and had apparently normal motor development in the first year. His mother mentioned that he had run slowly and been unable to jump since childhood. After entering elementary school at the age of 7 years, he presented muscle weakness, especially with difficulty in climbing stairs. He has low academic scores at school, IQ was not tested. At the time of writing, he can still walk independently, but with a mild waddling gait. Physical examination revealed positivity for Gowers' sign and calf pseudohypertrophy. His creatine kinase (CK) level was in the range of 991–4700 IU/L (normal: < 200 IU/L). Muscle biopsy was not available. This patient was suspected of having BMD based on the clinical presentation and elevated CK level. Informed consent for molecular analysis of the *DMD* gene was obtained from the patient's parents. This study was approved by the Ethics Committee of Nankai University.

Mutation detection

Genomic DNA was isolated from peripheral blood of the patient using the Wizard genomic DNA purification kit (Promega, Madison, WI, USA). First, multiple ligation-dependent probe amplification (MLPA) analysis was conducted to identify exon deletion or duplication in the *DMD* gene using Salsa MLPA Kit P034/P035 (MRC-Holland, Amsterdam, The Netherlands). Then, screening for small mutations was performed by PCR amplification of each dystrophin exon, including the exon–intron boundaries, followed by direct sequencing using the DNA sequencer ABI PRISM 310 (Applied Biosystems, Foster City, CA, USA). To analyze the *DMD* transcript, lymphocytes were isolated from peripheral blood of the patient using Ficoll-Paque density gradient centrifugation (GE Healthcare Life Sciences, Piscataway, NJ, USA). Total RNA of lymphocytes was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). cDNA was synthesized as described previously (Matsuo et al. 1991). A single round PCR (35 cycles) was carried out to amplify a fragment encompassing exons 24–26 using a forward primer located in exon 24 (24F: 5′-AATCACATACAAACCCTG-3′) and a reverse primer in exon 26 (26R: 5′-CTTCATCTCTTC AACTGC-3′). PCR products were analyzed in Bioptic Inc. using the Qsep100 automatic nucleic acid analysis

system for quantification (Bioptic, New Taipei City, Taiwan), in accordance with the manufacturer's instructions. The percentage of exon skip was calculated as the proportion of skipped transcripts relative to total transcripts, in accordance with the measured peak areas of PCR products. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control. In addition, the amplified products were subcloned into vector pMD19-T (Takara Bio, Inc., Dalian, China) and sequenced using ABI PRISM 310 genetic analyzer (Applied Biosystems).

Plasmid construction

To construct hybrid minigenes carrying wild-type and c.3340A > T mutant exon 25 (dysEx25WT and dysEx25 M), an NheI–BamHI fragment containing dystrophin exon 25 and flanking intronic sequences was PCR-amplified from the genomic DNA of a control individual and the patient using the following primers: In24F-NheI, 5′-CTAGCTAGCAAAAGACAAAAATCCATATGCAAT-3′, and In25R-BamHI, 5′-CGCGGATCCAACGGTGAAGGGAGACATTAG-3′. The amplified products were cloned into the NheI–BamHI restriction site of H492, a splicing reporter minigene vector, as described previously (Tran et al. 2006). Point mutations were introduced into exon 25 of H492-dysEx25WT and H492-dysEx25 M by PCR-based site-directed mutagenesis (Qi and Scholthof 2008).

To construct the *DMD* exon 24–25 minigene, the genomic sequence containing exon 24, full-length intron 24 (991 bp), and exon 25 was PCR-amplified from the normal individual and the patient, and then inserted between Hind III and Xho I sites of the pcDNA3.1(+) vector (Invitrogen). To construct the exon 25–26 minigene, a genomic fragment containing either wild-type or mutant exon 25 with flanking intron 25 was PCR-amplified and ligated with another amplified fragment covering exon 26 and upstream flanking intron 25 by overlap-extension PCR (Liu et al. 2011); then, the ligated product was inserted between Kpn I and Xho I sites of the pcDNA3.1(+) vector. As intron 25 has 8606 nucleotides, we only included sequences of 460 and 486 nucleotides at each end of this intron. Minigenes containing wild-type exon 19 (dysEx19WT) and dystrophin Kobe (exon 19 lacking a well-characterized ESE, dysEx19DK) were generated previously (Habara et al. 2008). A minigene dysEx19E25(50–79), in which the well-characterized ESE in exon19 was replaced by exon 25 nucleotides 50-79, was generated by overlap-extension PCR based on the minigene plasmid dysEx19DK. Tra2β expression plasmid (pFlagCMV-4-Tra2β) was constructed by replacing the hnRNP A2 sequence of pFlagCMV-4-hnRNP A2 vector (kindly provided by Marco Baralle, ICGEB, Trieste, Italy) with human Tra2β cDNA. The identity of all constructs was confirmed by direct sequencing before use.

Cell cultures, transfection, and splicing analysis

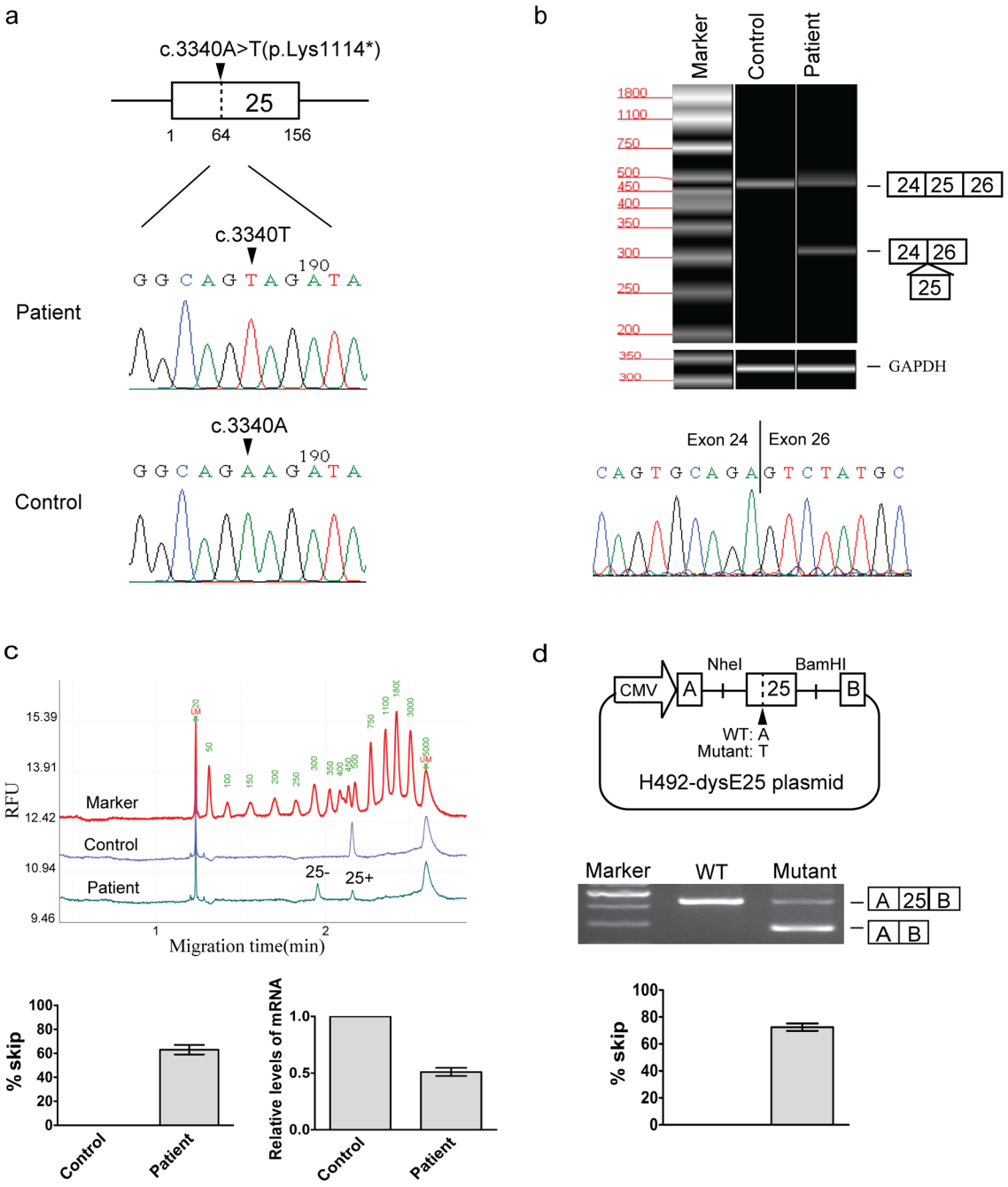
HeLa and C2C12 cells were maintained as described previously (Disset et al. 2006; Habara et al. 2008). A total of 0.5 μg of hybrid minigene was transfected or cotransfected with 0.5 μg of the indicated expression plasmid into cells in six-well plates using Lipofectamine 2000 Reagent (Invitrogen), in accordance with the manufacturer's instructions. To induce the myogenic differentiation of C2C12 myoblasts, the culture medium was replaced by DMEM containing 2% horse serum (Gibco, Gaithersburg, MD, USA). Cells were harvested at 48 h post-transfection and total RNA was extracted using TRIzol. cDNA was synthesized using 1 μg of total RNA as a template. A fragment encompassing upstream exon A, exon 25, and downstream exon B from the H492-derived transcript was PCR-amplified using the primers YH307 and YH308, as described previously (Habara et al. 2008). For cells transfected with two-exon minigenes, RT-PCR was performed using 1 μg of total RNA as a template. In addition, a RT reaction without reverse transcriptase, and a reaction without RNA template were used as negative controls to rule out the possibility of plasmid contamination. The thermocycle is 25 cycles for quantification of the splicing products, and 30 cycles for regular PCR.

RNA pull-down and mass spectrometry

RNA probes with wild-type and mutant exon 25 sequences, as well as a nonspecific probe GST-80 (Cavaloc et al. 1999), were synthesized by RiboBio Co. Ltd. (Guangzhou, China) and labeled using Pierce RNA 3′ End Desthiobiotinylation kit (Thermo Scientific, Waltham, MA, USA), in accordance with the manufacturer's instructions. HeLa cell nuclear extract (NE) was prepared using NE-PER Nuclear and Cytoplasmic Extraction Reagents (Thermo Scientific). RNA pull-down experiments were performed using Pierce Magnetic RNA–Protein Pull-down kit, following the manufacturer's instructions (Thermo Scientific). The eluted RNA-binding proteins were subjected to SDS-PAGE and visualized by silver staining (Beyotime Fast Silver Stain Kit, Haimen, China). Selected bands were excised and analyzed by mass spectrometry at Tsinghua University Center of Biomedical Analysis (Beijing, China).

siRNA knockdown of splicing factors

siRNAs for the downregulation of splicing factors were synthesized at RiboBio Co. Ltd. (Guangzhou, China). The siRNA sequences were as follows: si-hnRNP A1: 5′-CAGCUGAGGAAGCUCUUCA-3′, si-hnRNP A2: 5′-GGAACAGUUCCGUAAGCUC-3′, si-hnRNP H: 5′-AGCGGUGGUGCUUACGAACUU-3′, si-Tra2β: 5′-CAGCAGUCUAGGCGUUCAA-3′, si-Tra2α: 5′-GAAUCCCAUUCUCGA



UCAA-3′, 5′-GAGGGAUCUUCGUGAAGUA-3′, 5′-CGAGAUUCUUACUAUGAUA-3′). HeLa cells were seeded in six-well plates and cotransfected with the indicated siRNA (100nM) and 0.5 µg of minigene using Lipofectamine 2000 in each well the following day. Cells were harvested 72 h after transfection for RNA or protein extraction.

MS2-mediated tethering of splicing factors

For the tethering of splicing factors, an MS2 coat protein-binding site inserted into a splicing reporter minigene was used to tether a particular MS2 coat protein-tagged splicing factor to the targeted site, to evaluate its function in regulating minigene splicing (Rahman et al. 2015). Briefly, the MS2 coat protein-binding sequence (5′-ATGCACGATCACGGCATAA-3′) was introduced into the minigene dysEx25WT by replacing the exon 25 sequence of 5′-ATGAAGGTGGGCAGAAGATAAAGAATGAAG-3′ using PCR-based site-directed mutagenesis to generate dysEx25MS2. The bacteriophage MS2 coat protein-coding sequence was synthesized by RiboBio Co. Ltd. (Guangzhou, China), and inserted immediately after Tra2β into the expression vector

◂**Fig. 1** A nonsense mutation, c.3340A > T in the *DMD* gene, induces the skipping of exon 25. **a** gDNA analysis revealed a nonsense mutation (c.3340A > T) in *DMD* exon 25 from a patient suspected of having BMD. Upper panel: Schematic representation of exon 25 (open box) and flanking introns (thick lines). The size of exon 25 is reported (1 and 156 indicate the first and last nucleotides of exon 25, respectively). The position of c.3340A > T in exon 25 is indicated by an arrowhead and marked by nucleotide number 64. At the bottom, sequencing results of the patient and a normal control individual are shown. **b** Analysis of the *DMD* transcript. The RT-PCR products from lymphocytes of the patient and control were analyzed using a Qsep100 automatic nucleic acid analysis system. Two products were identified in the patient. The identity of these products is schematically indicated on the right. Sequencing of the shorter product revealed the skipping of exon 25 in the patient. **c** Quantification of the RT-PCR products. Upper panel: capillary gel electrophoresis of RT-PCR products from the patient and control. Lower left panel: Quantification of exon 25 skipping. The percentage of exon skipping was calculated as the rate of skipped transcripts relative to total transcripts, in accordance with the measured peak areas of PCR products. Lower right panel: quantitative analysis of total *DMD* transcripts (skipped and non-skipped). *y* axis indicates the relative level of *DMD* transcripts normalized to GAPDH. The relative level of *DMD* transcripts in a control individual was set as 1. **d** c.3340A > T induced exon 25 skipping in a splicing minigene assay. Upper panel shows a schematic of the hybrid minigene plasmids carrying either wild-type (WT) or c.3340A > T mutant (Mutant) exon 25 of the *DMD* gene. Middle panel shows the splicing pattern of minigenes in HeLa cells as revealed by RT-PCR; the identity of each product is schematically indicated on the right. Skipping of exon 25 was observed only in the mutant minigene. Lower panel shows the quantification of exon 25 skipping

pFlagCMV-4-Tra2β to construct pFlagCMV-4-Tra2β-MS2 containing fusion cDNA, or replaced Tra2β to generate pFlagCMV-4-MS2. Then, the minigene H492-dysEx25MS2 was cotransfected with either pFlagCMV-4-Tra2β-MS2 or pFlagCMV-4-MS2 into HeLa cells, and the splicing of the minigene was analyzed by RT-PCR.

Western blotting

The following commercial antibodies were used for western blot analysis: anti-hnRNP A1 (4B10), anti-hnRNP A2/B1 (DP3B3), anti-hnRNP H (N-16), anti-TDP43 (H-8) (Santa Cruz Biotechnologies, TX, USA), anti-SF2/ASF (Invitrogen, Carlsbad, CA, USA), anti-SFRS7 (9G8), anti-Tra2β (Abcam, Cambridge, UK), and anti-Tra2α (Absin Bioscience, Shanghai, China). Anti-actin (C4; Santa Cruz Biotechnologies, TX, USA) was used as a loading control.

Results

c.3340A > T induced skipping of exon 25 in the lymphocytes of a patient with BMD

To identify the pathogenic mutation of the index case, we first examined the *DMD* gene for any large deletion or duplication using multiplex ligation-dependent probe amplification (MLPA) analysis. No such rearrangements were detected. Next, we screened all 79 dystrophin exons for point mutations by direct sequencing. A single-base substitution of A to T was identified at nucleotide position 64 of exon 25, which corresponds to c.3340A > T in Dp427 m (NM_004006.2) (Fig. 1a). This point mutation altered an AAG codon for lysine to a premature TAG stop codon (p.Lys1114*), which is predicted to produce a truncated, nonfunctional protein, suggesting a DMD phenotype. However, our patient manifested a late onset and slow disease progression, which are incompatible with the DMD phenotype. Moreover, another patient carrying c.3340A > T was previously reported in the Leiden DMD Pages (http://www.dmd.nl) to have BMD. To explain the apparent discrepancy between the genotype and the exhibited phenotype, we analyzed the *DMD* transcripts extending from exon 24–26 in peripheral lymphocytes of the index case because no muscle biopsy was available. Comparison of the RT-PCR products from the patient with those from a normal control subject revealed the presence of an additional transcript that is shorter. Sequencing has been performed after subcloning of the PCR products. Sequencing results of the short product revealed its lack of exon 25 (Fig. 1b), while the sequencing data of the longer transcript from the patient showed normal exon content from exon 24–26 with c.3340A > T in exon 25 (data not shown). Because exon 25 has 156 nucleotides, we thus suppose that the milder phenotype of the index case was due to elimination of the nonsense mutation by the skipping of this in-frame exon, and that c.3340A > T was responsible for this skipping of exon 25.

Quantitative analysis of the RT-PCR products revealed that the rate of transcript lacking exon 25 in the patient was two times higher than that of the full length transcript (67% vs 33%) (Fig. 1c). As the full length transcript with premature stop codon might be targeted for degradation by nonsense-mediated mRNA decay (NMD) (Maquat 2004), we evaluated the relative amount of total *DMD* transcript (skipped and non-skipped) normalized to GAPDH, and found a 49% decrease in the patient compared with that of normal individual (Fig. 1c). It's likely that NMD affected the amount of full length transcript with nonsense mutation, so the proportion of exon skipping in the patient lymphocytes may not be as much as it appears.

c.3340A > T induced skipping of exon 25 in a splicing minigene assay

To demonstrate that exon 25 skipping is caused by c.3340A > T mutation, we inserted either the wild-type or c.3340A > T-mutated exon 25, along with the nearby intron sequences, into the H492 splicing reporter minigene (Tran et al. 2006), and analyzed the splicing pattern

in HeLa cells. The wild-type minigene resulted in a single transcript corresponding to mature mRNA, in which exon 25 was correctly spliced between exons A and B. The mutated minigene, however, produced two mature transcripts: a larger one with the inclusion of mutated exon 25 between exons A and B, and a shorter one with the skipping of exon 25 (Fig. 1d). We also observed exon 25 skipping in mouse myoblast C2C12 cells transfected with mutant minigene (Supplementary Material, Fig. S1). Taking these findings together, the splicing pattern of the mutant minigene in HeLa and C2C12 cells recapitulated the aberrant splicing of our patient, which indicated that the c.3340A > T mutation induces exon 25 skipping.

c.3340A > T affected splicing regulatory elements of exon 25

Since c.3340A > T at nucleotide position 64 of exon 25 did not affect the splicing donor nor the acceptor site, exon 25 skipping was more likely due to the mutation altering an exonic cis-element that acts in regulating splicing. To test this possibility, we introduced a series of single point mutations into the region including the mutation site (nucleotide positions 61–67 of exon 25) in the minigene construct, and analyzed their splicing pattern in HeLa cells. Replacement of each of the nucleotide at positions 63–67 (GAAGA) with other nucleotides induced skipping of exon 25 to various extents, indicating that this region possesses an ESE (Fig. 2a). Interestingly, the patient's mutation (T at nucleotide position 64) induced the highest level of exon skipping, which appeared to be more compatible with the generation of an ESS rather than the disruption of an ESE. We, therefore, characterized the 64T-based putative ESS motif by introducing a series of point mutations at upstream or downstream nucleotides. Any substitution in 65A and 66G remarkably restored the inclusion of exon 25, indicating that TAG at position 64 to 66 is essential for ESS function (Fig. 2b).

We also used a computational program, Human Splicing Finder (HSF, http://umd.be/HSF3), to predict the effect of c.3340A > T on the splicing signal in exon 25 (Desmet et al. 2009). HSF predicted overlapping ESEs in the wild-type sequence recognized by two SR proteins, SF2/ASF (CAGAAGA) and 9G8 (GCAGAA and GAAGAT), as well as an SR-like protein, Tra2β (AAGAT). The c.3340A > T mutation was predicted to disrupt all of these ESEs and create a potential ESS for the binding of a negative regulator protein, hnRNP A1 (GTAGAT). Taken together, our results from both mutational study and computational prediction indicated that c.3340A > T not only disrupts an ESE, but also creates an ESS.

Splicing factors involved in the skipping of exon 25

Next, we attempted to identify the splicing regulatory proteins responsible for the skipping of exon 25. We performed an RNA pull-down experiment using a biotin-labeled RNA probe carrying either the wild-type or the c.3340A > T mutant sequence (Fig. 3a), and compared their pull-down products. Silver staining of the pulled-down products revealed that three protein bands corresponding to ~ 50, 36, and 33 kDa were more intense in samples pulled down with mutated probe than that with the wild-type probe (Fig. 3b, c). None of them was detected in the sample with a non-specific GST-80 probe. Mass spectrometry analysis of the three bands revealed that they represented hnRNP H, hnRNP A2, and hnRNP A1, three well-known splicing repressors (Supplementary Material, Fig. S2). Western blot analysis of the same samples as well as independent pulled-down samples again confirmed the stronger signal with mutant probe than with wild-type probe for antibodies against hnRNP A1, hnRNP A2, and hnRNP H (Fig. 3d). We also examined the pulled-down products using antibodies against SF2/ASF, 9G8, and Tra2β, the three splicing enhancer proteins predicted by HSF, whose potential binding site was disrupted by the point mutation c.3340A > T. A signal of Tra2β was found only in the sample pulled down with the wild-type probe, but not with the mutant. No signal of SF2/ASF and 9G8 was detected in either wild-type or mutant sample (Fig. 3d).

To normalize the amount of RNA probes, we also conducted pull-down experiments using an alternative probe set containing a TDP43-binding sequence (Goina et al. 2008) (Supplementary Material, Fig. S3). Western blot analysis of RNA-binding proteins confirmed the increased binding of hnRNP A1, hnRNP A2, and hnRNP H to the mutant probe. In addition, a very weak signal of Tra2β was observed with the wild-type probe, but not with the mutant. Taken together, our pull-down results show that wild-type exon 25 is bound by multiple splicing regulatory proteins, including the SR-like protein Tra2β, and three hnRNP family members hnRNP A1, hnRNP A2, and hnRNP H. c.3340A > T in exon 25 disrupted the binding of Tra2β and simultaneously increased the binding of all three hnRNPs.

To investigate the functional role of four identified RNA-binding proteins in c.3340A > T-induced exon 25 skipping, we first performed siRNA-mediated downregulation of hnRNP A1, hnRNP A2, and hnRNP H in HeLa cells transfected with the mutant minigene. Decreased expression of the three hnRNP proteins in HeLa cells was confirmed by western blotting (Fig. 4a). In siRNA-control-transfected cells, the mutant minigene produced transcripts both with (27%) and without exon 25 (73%). Depletion of hnRNP A1 or hnRNP H alone increased exon 25 inclusion from 27% to 53% and 42%, respectively. In contrast, depletion of hnRNP A2 alone had no effect on minigene splicing. Combined

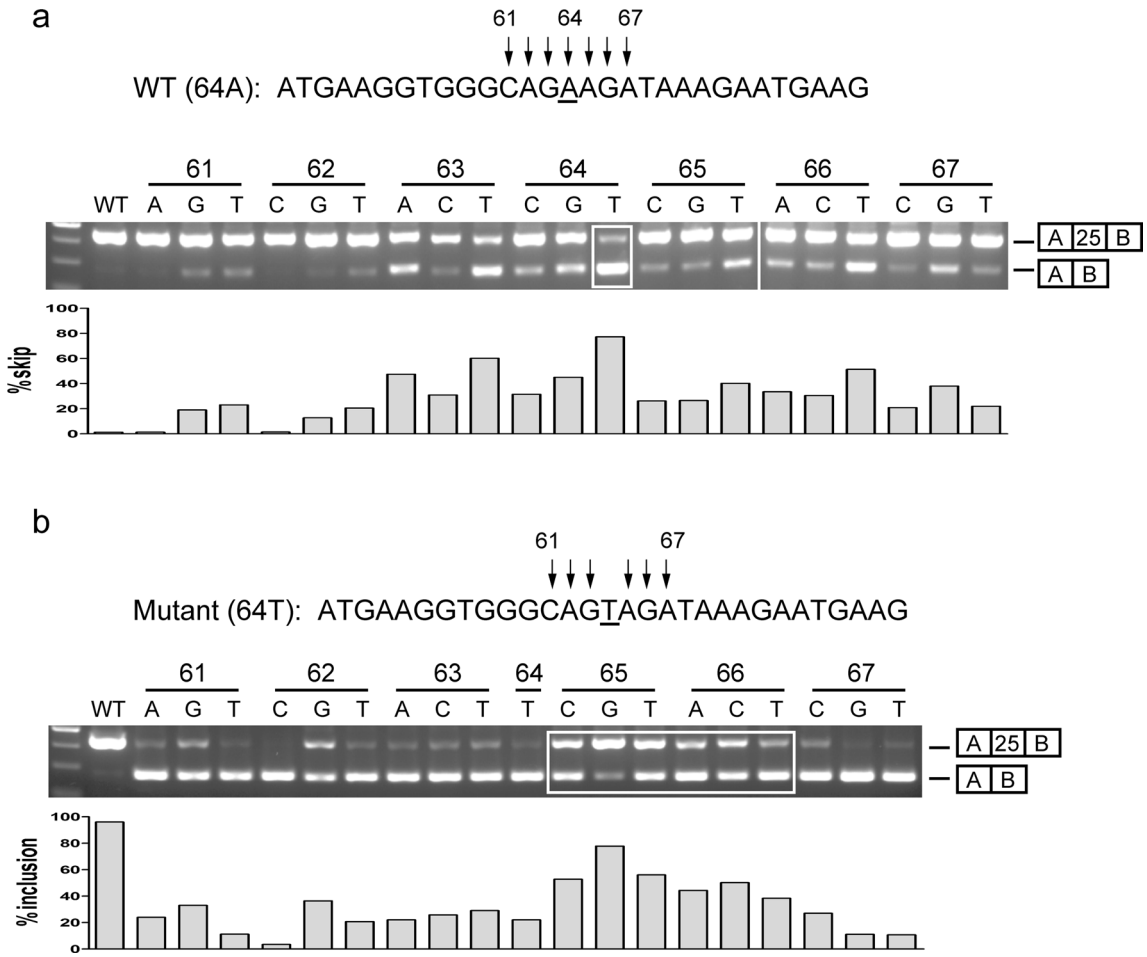


Fig. 2 c.3340A > T mutation at nucleotide position 64 of exon 25 disrupts an ESE and simultaneously creates an ESS. **a** Analysis of putative splicing regulatory element in *DMD* exon 25 by a splicing minigene assay. Upper panel shows that a single point mutation was introduced into exon 25 at nucleotide positions 61–67 (indicated by arrows) in the wild-type minigene context. Middle panel shows the splicing pattern of minigenes in HeLa cells. All variants from positions 63-67 induced the skipping of exon 25 to various extents, indicating that this region possesses an ESE. In particular, the patient's mutation (T at nucleotide position 64 as marked by an open box) induced the highest level of exon skipping, which was more compatible with the generation of an ESS rather than the disruption of an ESE. Lower panel shows quantification of exon skipping. **b** Characterization of the key nucleotides essential for putative ESS created by c.3340A > T by a splicing minigene assay. Single point mutations were introduced upstream and downstream of position 64T in the constructed mutant minigene (indicated by arrows); their effects on minigene splicing were assayed in HeLa cells. Any substitution at 65A and 66G remarkably restored the inclusion of exon 25 (marked by an open box), indicating that TAG at positions 64–66 is essential for ESS function

depletion of hnRNP A1 and A2 significantly enhanced exon inclusion compared with single depletion of hnRNP A1, while further depletion of hnRNP H only slightly increased inclusion compared with that upon double depletion of hnRNP A1 and A2 (88% vs. 84%) (Fig. 4b). These results demonstrate the synergistic role of hnRNP A1 and hnRNP A2 in c.3340A > T-induced exon skipping, and that hnRNP H also promotes mutant skipping of exon 25.

Next, we examined the effect of siRNA-mediated down-regulation of Tra2β in HeLa cells transfected with the wild-type minigene. siRNA-control had no effect on the splicing of wild-type minigene, whereas decreased expression of Tra2β slightly induced exon 25 skipping (2%) (Fig. 4a, c). As endogenous Tra2α compensated for the loss of Tra2β (Best et al. 2014), we tested the effect of double depletions of Tra2α and Tra2β on wild-type minigene splicing. The combined decrease of Tra2 proteins significantly increased exon 25 skipping (7.2%) compared with the single depletion of Tra2α (0.8%) or Tra2β (2%), which demonstrated their role in the regulation of exon 25 splicing (Fig. 4c). We also tested whether artificial tethering of Tra2β to the mutation site can rescue the splicing of exon 25. As expected, binding of MS2 coat protein-tagged Tra2β to the mutation site replaced by the MS2-binding sequence in exon 25 significantly increased exon inclusion, whereas Tra2β without MS2 coat protein-tag showed less effect on minigene

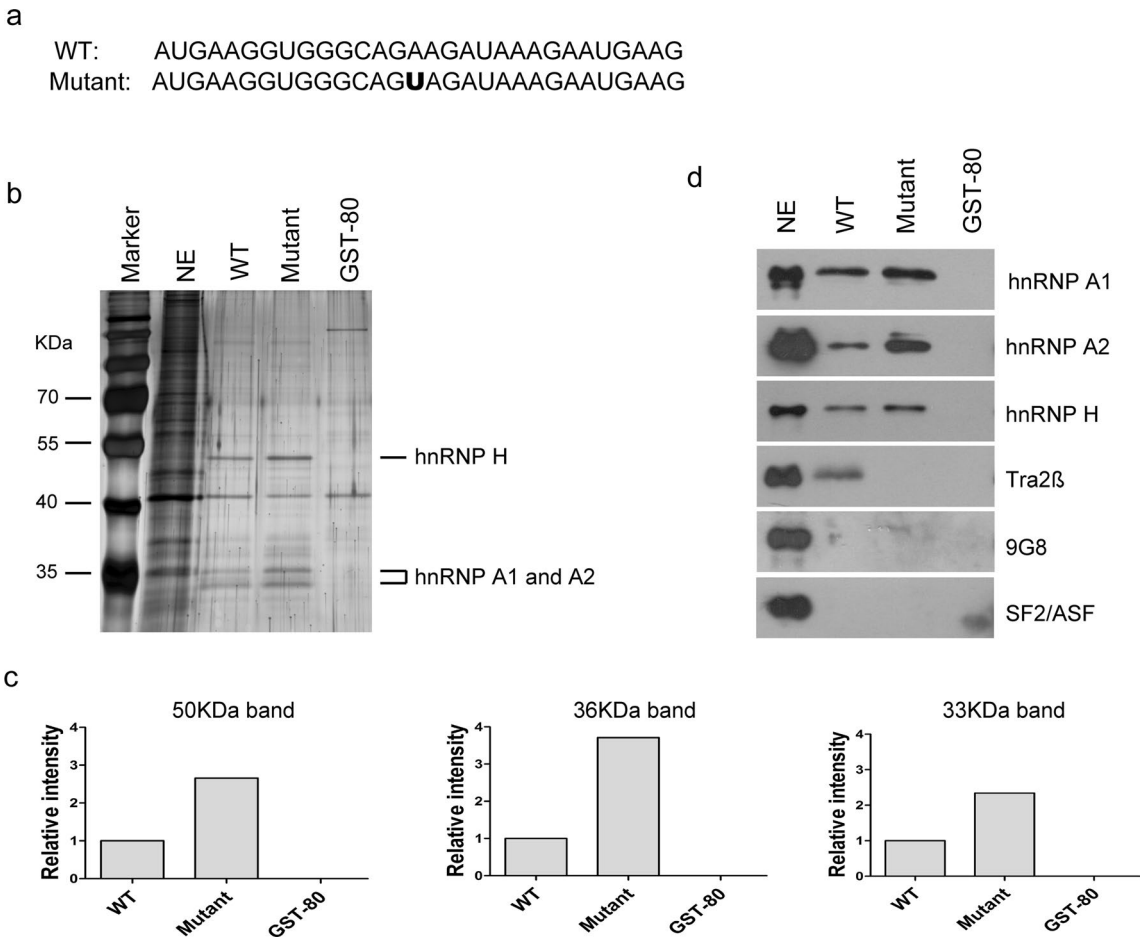


Fig. 3 c.3340A > T disrupts the binding of Tra2β and enhances the binding of hnRNP A1, hnRNP A2, and hnRNP H to exon 25. **a** Sequence of RNA probes carrying the wild type (WT) and c.3340 A > T mutation (mutant). **b** Silver staining of the pulled-down RNA-binding proteins. Three protein bands of 50, 36, and 33 kDa were more intense with the mutated probe than with the wild-type probe. None of the three bands was detected with a nonspecific GST-80 probe. Analysis of the three bands by mass spectrometry revealed that they are hnRNP H, hnRNP A2, and hnRNP A1. NE, HeLa nuclear extract. **c** Quantification analysis of the pull-down assay. The hnRNP protein bands of 50, 36, and 33 kDa in Fig. 3b were analyzed using Image J software. The intensity of each band was calculated against a loading control (a ~ 41 kDa band that nonspecifically binds to all three probes). The intensity value of each hnRNP band in wild-type probe lane was set as 1. **d** Western blot analysis of the same RNA pulled-down product (as in **b**) using specific antibodies. Increased signals of hnRNP A1, hnRNP A2, and hnRNP H were detected with mutant probe, compared with the levels with wild-type probe. In addition, the binding of splicing factor Tra2β was only detected with the wild-type probe, but not with the mutant. No signals of SF2/ASF and 9G8 were detected in either the wild-type or the mutant sample. NE, HeLa nuclear extract

splicing (Fig. 4d). Our results indicate that Tra2β indeed acts as a splicing enhancer at the site of exon 25 where the mutation occurs.

c.3340 A > T in exon 25 impaired upstream intron splicing

After characterization of the splicing regulatory elements and their binding proteins, we evaluated the effects of c.3340A > T mutation on the removal of upstream and downstream introns. In minigenes carrying exon 24–intron 24–exon 25, c.3340A > T mutation significantly suppressed the removal of intron 24 compared with the wild-type minigene (Fig. 5a). In minigenes carrying exon 25-intron 25-exon 26, there was no significant difference between the wild-type and mutant minigenes in their splicing pattern (Fig. 5b). These results could indicate that the mutation mainly affects the splicing of upstream intron 24.

Splicing enhancing element in dystrophin exon 25

The finding that both splicing enhancer and silencer proteins bound to the sequence of the wild-type probe (corresponding to nucleotides 50–79 in exon 25, Fig. 6a) led us to further investigate the overall activity of this region. We constructed a minigene carrying exon 25 with a deletion

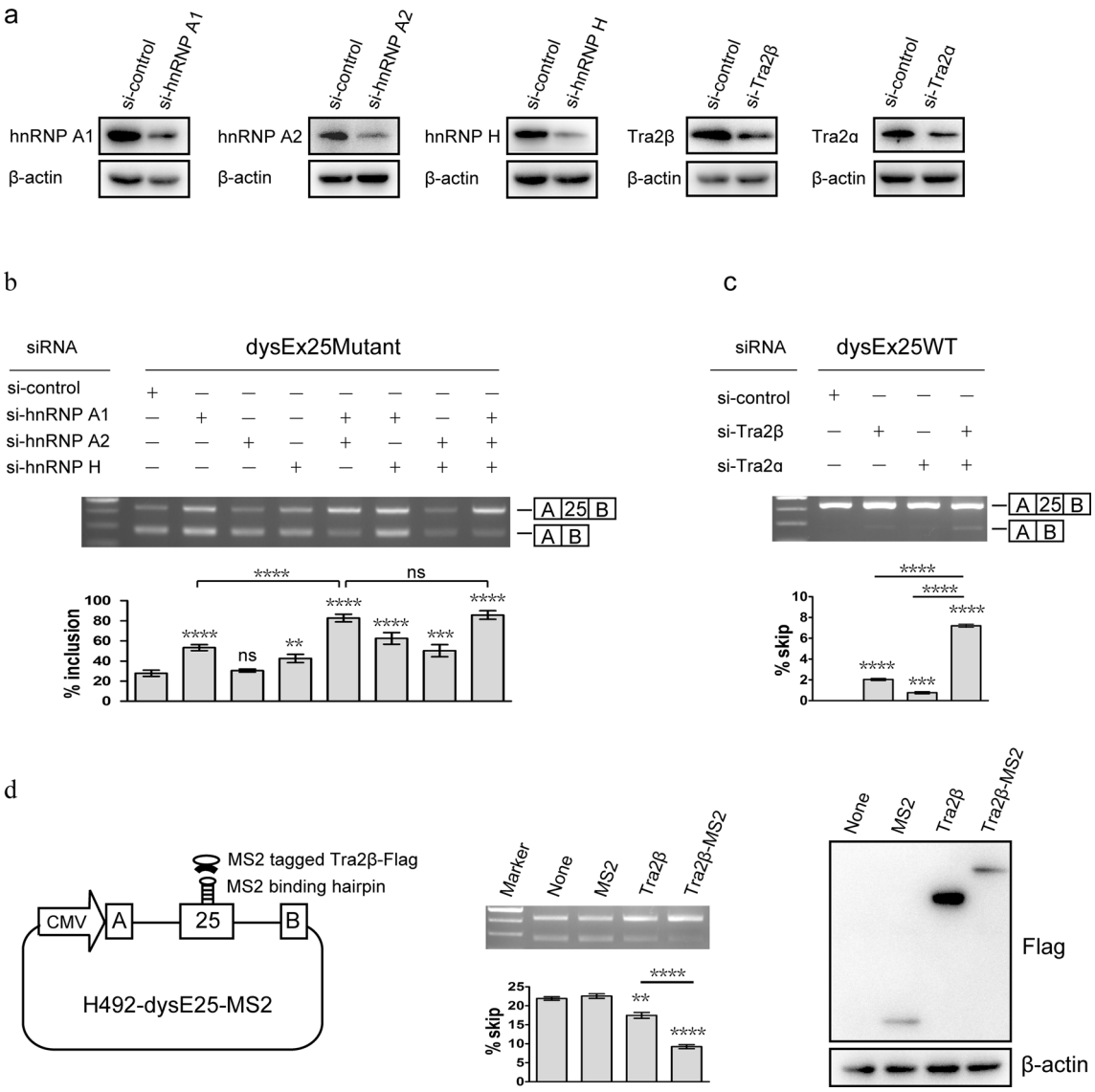


Fig. 4 hnRNP A1, hnRNP A2, and hnRNP H repress exon 25 splicing, while Tra2β promotes exon 25 splicing. **a** Confirmation by western blotting of the downregulation of hnRNP A1, hnRNP A2, hnRNP H, Tra2β and Tra2α in HeLa cells transfected with the indicated siRNAs. **b** Effects of the depletion of hnRNP A1, hnRNP A2, and hnRNP H on the splicing of mutated exon 25. HeLa cells were cotransfected with the indicated siRNA and c.3340A > T mutant minigene. Upper panel shows RT-PCR analysis of the splicing pattern of the mutant minigene. Lower panel shows the quantification of exon inclusion from three independent transfection experiments (mean ± standard deviation). **c** Effects of the depletion of Tra2β on the splicing of wild-type exon 25. Upper panel shows RT-PCR analysis of the splicing pattern of the wild-type minigene. Lower panel shows the quantification of exon skipping. **d** Effects of MS2-mediated tethering of Tra2β on the splicing of exon 25. Left panel shows a schematic of MS2-mediated tethering of Tra2β to exon 25. The sequence encompassing c.3340A > T mutation in exon 25 of the minigene was replaced by MS2 coat protein-binding sequence, which can directly tether MS2 coat protein-tagged Tra2β-Flag to the mutation site. Middle panel shows the results of RT-PCR analysis of the minigene splice pattern, the rates of exon 25 skipping are quantified. Right panel shows the expression of the Flag-tagged Tra2β using antibody against Flag. "None" indicates no transfection; "MS2" indicates MS2 coat protein expression vector; "Tra2β" indicates Tra2β expression vector; "Tra2β-MS2" indicates Tra2β-MS2 fusion protein expression vector. Statistical significance was determined by one-way ANOVA with Tukey multiple comparison test ($P < 0.05$ was considered statistically significant)

of nucleotides 50–79 (dysE25Δ50–79), and examined its splicing pattern. Loss of this 30-bp sequence resulted in the skipping of exon 25 at a rate of up to 62%, suggesting that this region may constitute an ESE, given its overall activity (Fig. 6b). To verify its splicing-enhancing capacity, we tested this sequence in the context of another minigene carrying dystrophin Kobe exon 19, the correct splicing of which depends on a well-characterized ESE (Habara et al. 2008). As expected, the minigene lacking the natural ESE in exon 19 (dysE19DK) caused exon skipping in 100% of

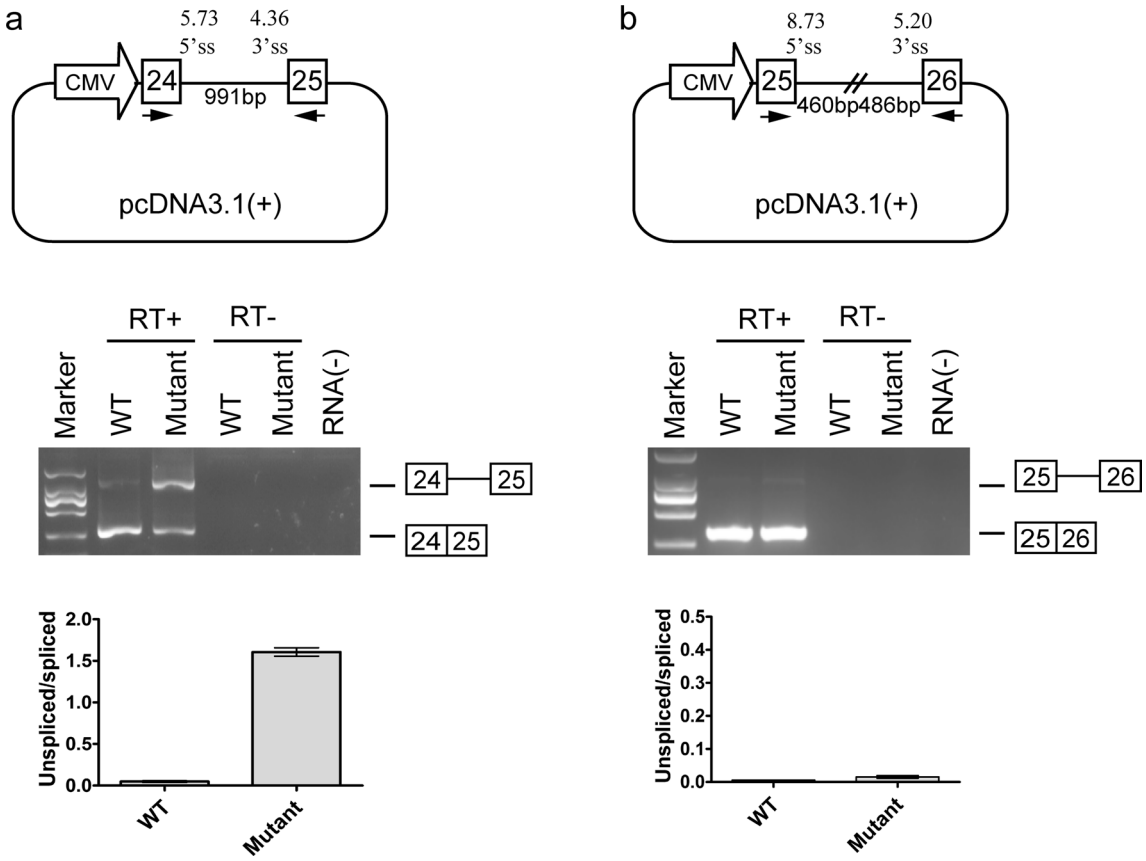


Fig. 5 c.3340A > T impairs the removal of upstream intron. **a** Splicing analysis of minigene carrying exon 24–intron 24–exon 25 in HeLa cells. **b** Splicing analysis of minigene carrying exon 25-intron 25-exon 26 in HeLa cells. Upper panel shows the structure of the minigene. The locations of primers are indicated by arrows. The 5′ss and 3′ss MaxEnt strength of intron are also shown. Middle panel shows the results of RT-PCR analysis of the minigene splicing pattern; the identity of each product is schematically indicated on the right. Lower panel shows quantification of unspliced transcripts

the transcripts (Fig. 6c). Substitution of natural ESE by our tested sequence [dysE19E25(50–79)] resulted in efficient splicing of exon 19, which further confirmed the overall activity of the sequence from nucleotide 50–79 in exon 25 and its status as a strong splicing enhancer element.

Discussion

Nonsense mutations in the *DMD* gene are highly pathogenic and usually associated with a severe DMD phenotype. However, we here identified a nonsense mutation (c.3340A > T) in exon 25 from a 15-years-old boy who was clinically suspected of having BMD (Fig. 1a). In his lymphocytes, skipping of in-frame exon 25 occurred in part of the *DMD* transcripts (Fig. 1b). Because no muscle biopsy was available in this patient, we confirmed the skipping event in experiments using a hybrid minigene (Fig. 1d). As a consequence of exon 25 skipping, in-frame transcripts that eliminate the premature stop codon created by c.3340A > T were generated, which were considered to have attenuated the severe clinical course in this patient. Exon 25 encodes part of the dystrophin rod domain that is considered to be dispensable. The functional role of in-frame transcripts lacking exon 25 on phenotype attenuation has been demonstrated in a 17-years-old Japanese boy with deletion of exon 25, who manifested only muscle pain and high CKnemia without obvious muscle weakness (Takeshima et al. 2010). In the index patient with c.3340A > T mutation, although RT-PCR revealed a high rate of transcripts with exon 25 skipping in lymphocytes, he had already presented muscle weakness. We could not rule out that NMD caused overestimation of exon skipping by degradation of nonsense-containing full-length transcripts in his lymphocytes because the amount of *DMD* transcripts was only 51% relative to that in the normal control. The *DMD* transcripts were frequently analyzed in the patient's lymphocytes because they were easy to obtain. It has been observed that, when a premature terminal codon occurs in an alternative exon, the pathogenicity of this mutation appears only within tissues that express the exon (Maillet et al.

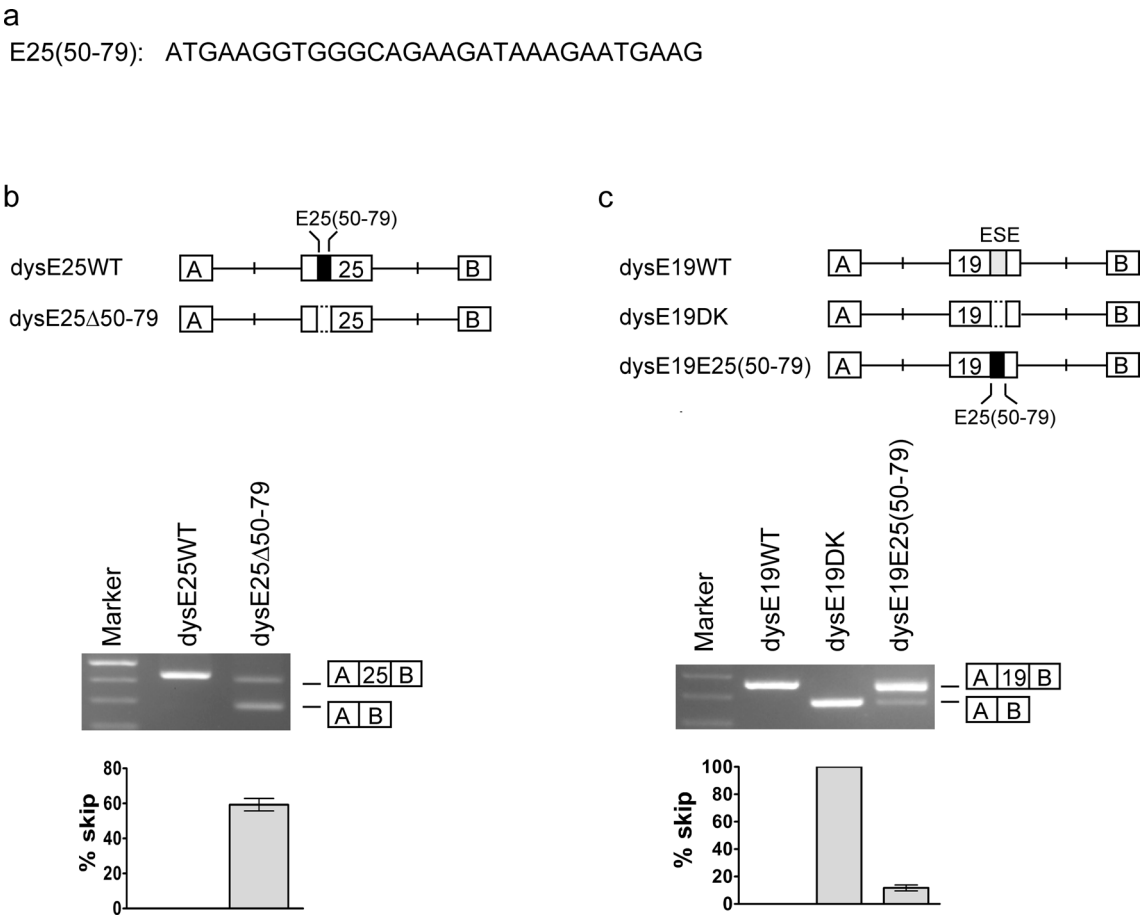


Fig. 6 The overall activity of the region corresponding to the wild-type probe (nt 50–79) in exon 25 represents an ESE. **a** The sequence corresponding to the wild-type probe and its nucleotide position in exon 25. **b** Deletion of nt 50–79 caused exon 25 skipping in a splicing minigene assay. Upper panel shows the structure of minigene carrying either wild-type exon 25 (dysEx25WT) or exon 25 with a deletion of nt 50–79 (dysEx25Δ50–79). The region covering nt 50–79 is indicated by a black box. Middle panel shows the results of RT-PCR analysis of the minigene splicing pattern in HeLa cells. Lower panel shows the quantification of exon skipping. **c** nt 50–79 of exon 25 can rescue the splicing of dystrophin Kobe (exon 19 lacking a well-characterized ESE) in a splicing minigene assay. Upper panel shows the structure of minigenes harboring wild-type exon 19 (dysEx19WT), dystrophin Kobe (dysEx19DK), and insertion of nt 50–79 of exon 25 into dystrophin Kobe [dysEx19E25(50–79)]. The characterized natural ESE in exon 19 is indicated by a gray box. Middle panel shows the results of RT-PCR analysis of the minigene splicing pattern in HeLa cells. Lower panel shows quantification of exon skiping

1999). Given that dystrophin exerts its function in muscle, further analysis of the *DMD* transcripts and proteins in the patient's muscle may help to clarify their correlation with phenotype.

Although an increasing number of reports on the large *DMD* gene have described the rescue of transcripts due to the skipping of nonsense-containing exons, only a few of them revealed the molecular basis for such splicing events (Aartsma-Rus et al. 2006; Flanigan et al. 2011; Juan-Mateu et al. 2013; Nishiyama et al. 2008; Tuffery-Giraud et al. 2009). For example, in an early study using an in vitro splicing assay, disruption of a purine-rich ESE in exon 27 (c.3839G > T) was disclosed, supporting the "ESE loss" model for skipping events. However, the nuclear factor that binds to ESE remained unclear in this case (Shiga et al. 1997). Recently, by taking advantage of the availability of mass spectrometry, the identification of RNA-binding proteins has become easier. Gain of an ESS, which interacts either with hnRNP A1 alone or with hnRNP A1, hnRNP A2, and DAZAP1, has been revealed in exon 31 (c.4250T > A) and exon 39 (c.5480T > A), respectively (Disset et al. 2006; Miro et al. 2015). In addition, another nonsense mutation in exon 31 (c.4303G > T) has been well studied by an independent group (Nishida et al. 2011). Computer prediction in combination with gel mobility shift assay revealed that this mutation not only creates an hnRNP A1-dependent ESS, but also destroys a pre-existing ESE for SRp30c binding. Similar to the latter case, our results suggest that the mutation c.3340A > T induces the skipping of exon 25 through the conversion of a Tra2β-dependent ESE to an ESS, which

is responsible for the increased recruitment of hnRNP A1, hnRNP A2, and hnRNP H.

Tra2β is an SR-like protein that preferentially binds to GA-rich ESE (Tacke et al. 1998) and is involved in the regulation of alternative splicing (Hofmann et al. 2000; Kondo et al. 2004; Tran et al. 2003). Recently, it has been observed that endogenous Tra2α functionally compensates for Tra2β. Joint depletion of Tra2α and Tra2β control splicing pattern of constitutive exon in MDA-MB-231 cells (Best et al. 2014). Tra2β mRNA is ubiquitously expressed, most highly in testis and developing brain. Heart and skeletal muscle also express high levels of it (Nayler et al. 1998; Venables et al. 2000). In the *DMD* gene, a Tra2β-responsive ESE containing a GAAGAAA motif has been identified in exon 31, the deletion of which was shown to affect splicing in both wild-type and mutant contexts (Disset et al. 2006). In exon 25, a nonsense mutation substituting T for the first A of a purine-rich motif GAAGA (nucleotides 63–67) disrupted Tra2β binding (Fig. 3d). This motif is highly similar to GAAGAA A in exon 31, and may act as a Tra2β-binding sequence. Analysis of site-directed mutants in the minigene experiment further confirmed its enhancer activity (Fig. 2a). In pull-down experiments, however, this protein was detected only by western blot, but not mass spectrometry (Fig. 3b, d). A previous study reported a similar situation upon examination of the binding of Tra2β to a purine-rich sequence in an alternative exon of HipK3 gene (Venables et al. 2005). The presence of a low concentration of Tra2β in nuclear extracts might account for the discrepancy between the results of western blotting and mass spectrometry. As some SR proteins can compensate for each other in regulating splicing (Pandit et al. 2013), we could not rule out the involvement of another SR protein or SR-like protein in exon 25 splicing in muscle.

The relationship between nonsense-associated aberrant splicing and ESS was initially established during systematic identification and analysis of ESS decamers using an in vivo splicing reporter system (Wang et al. 2004). Loss of exon identity caused by silencer gain was further linked to human inherited diseases (Sterne-Weiler et al. 2011). In the *DMD* gene, it has been observed that creation of PESS and hnRNP A1 motif tends to enrich in skipped exon (Flanigan et al. 2011). Indeed, the creation of hnRNP A1 binding ESS has been experimentally demonstrated in several nonsense mutation induced exon skipping events (Disset et al. 2006; Miro et al. 2015; Nishida et al. 2011). In *DMD* exon 25, we also found that c.3340A > T generated a new binding motif, UAGAUA, which partially matches the hnRNP A1 consensus UAGGGA and hnRNP A2 consensus UAGRGA (R represents A or G) (Burd and Dreyfuss 1994; Hutchison et al. 2002; Olsen et al. 2014). Like most known hnRNP A1 binding motifs, the core UAG triplet of UAGAUA plays a key role in the silencing effect (Fig. 2b). A synergistic effect of hnRNP A1 and hnRNP A2 on the c.3340A > T-induced skipping of exon 25 has been evidenced in a minigene by siRNA-mediated depletion (Fig. 4b). Whether hnRNP A1 and hnRNP A2 directly bind to the new ESS or act through interaction with each other remains to be elucidated. In this study, we showed for the first time that hnRNP H is involved in regulating the splicing of *DMD* exons (Figs. 3b, 4b). We noted that the UAGAUA created by c.3340A > T does not match the binding sequence for hnRNP H that recognizes the GGG motif (Schaub et al. 2007). Instead, the GGG triplet was identified 4 bp upstream of the mutation site. Therefore, the increased recruitment of hnRNP H to exon 25 observed in RNA pull-down experiments cannot be explained by the creation of a new binding site for this silencer protein. As hnRNP H has been demonstrated to interact with hnRNP A1 in live cells (Fisette et al. 2010), the close proximity of the newly created ESS to a GGG triplet may facilitate physical interaction of the newly recruited hnRNP A1 and A2 with upstream hnRNP H in mutant exon 25, and even with the pre-existing hnRNP A1 and A2 in the vicinity. The formation of a repressor complex through the interaction of three hnRNPs may contribute to the increased RNA binding affinity of hnRNP H.

Our results show that c.3340A > T is located within a region (nucleotides 50 to 79 of exon 25) containing both enhancer and silencer elements recognized by the splicing factor Tra2β, as well as hnRNP A1, hnRNP A2, and hnRNP H (Figs. 3, 4). Notably, given the overall activity of this region, it constitutes an ESE, as evidenced by deletion and rescue study using a minigene (Fig. 6). It is probable that this region also contains another enhancer sequence that interacts with unknown positive factors because artificial tethering of Tra2β to this region did not induce the inclusion of exon 25 in 100% of transcripts (Fig. 4d). It has been reported in the HIV-1 *tat* gene that exon identity was established by differential antagonism between SR proteins and hnRNP A1 (Zhu et al. 2001). Moreover, Tra2β antagonized the effects of hnRNP A1 and hnRNP H which has been reported in the splicing of a germ cell-specific exon (Venables et al. 2005). Similarly, we speculate that, in wild-type exon 25, ESE-bound Tra2β possibly together with another enhancer protein counteracts the nearby hnRNP A1-, hnRNP A2-, and hnRNP H-mediated splicing repression and eventually promotes exon 25 recognition and inclusion.

In our case, the nonsense mutation broke the fine interplay of positive and negative splicing factors by converting an ESE to an ESS. As a consequence, c.3340A > T impaired the splicing of upstream intron 24 in a two-exon minigene (Fig. 5a). Notably, intron 24 has a low splice probability score for both 3′ss and 5′ss (4.36 and 5.73 vs. median of 8.43 and 8.55 calculated by the Maximum Entropy Model) (Yeo and Burge 2004). It is likely that recognition of the weak 3′ss by the spliceosome during splicing requires an ESE in

downstream exon 25, which was disrupted by c.3340A > T mutation. A previous study showed that blocking of a Tra2β-dependent ESE in *SMN* exon 7 influences upstream 3′ splice site recognition by inhibiting U2 snRNP recruitment. hnRNP A1 also exerts an inhibitory effect on the recruitment of U2 snRNP and U2AF to *SMN2* (Martins de Araujo et al. 2009). In our case, the lack of Tra2β in combination with the gain of hnRNP A1 would further decrease the recruitment of U2 snRNP and U2AF to the 3′ splice site, thus inhibiting 3′ss recognition. In the two-exon minigene, impaired recognition of 3′ss may cause intron 24 retention. Analysis of the purified early spliceosome complex is required to confirm our hypothesis and address the precise molecular basis by which c.3340A > T affects upstream intron splicing. In the patient's lymphocytes, intron 24 retention was not detected in the *DMD* transcripts. It seems not to be simply due to NMD-mediated degradation, because full-length transcript with nonsense mutation can still be detected. As exon 25 has lower PESE density, and its 3′ss MaxEnt strength is weaker than that of the next distal exon (4.36 vs.5.20) (Flanigan et al. 2011), the lack of intron 24 retention maybe caused by competition between the 3′ss of downstream exon with the mutant exon 25 (Nogues et al. 2003), although this is highly speculative and requires experimental proof. In addition, we have to consider the possibility that from the transfection experiments, retention of intron 24 appears as an epiphenomenon. Whether this feature is fortuitous or reflects an in vivo mechanism remains to be investigated.

In conclusion, we reported that a nonsense mutation (c.3340A > T) in the *DMD* gene induced the skipping of exon 25. This mutation converts a Tra2β-dependent ESE to an ESS recognized by hnRNP A1 and hnRNP A2, which is concomitant with increased binding affinity of a pre-existing ESS for hnRNP H in the vicinity. Our results give insight into the complexity of splicing regulation of the *DMD* exon and provide detailed information about exonic elements and related binding proteins that may be useful for developing splicing modulation therapy.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Research involving human participants The study was approved by the Ethics Committee of Nankai University and was performed in accordance with the Declaration of Helsinki.

Informed consent Informed consent for molecular analysis of the *DMD* gene was obtained from the patient's parents.

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