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Review

Pseudoexons of the DMD Gene

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Since *DMD* was discovered there have been numerous reported cases of pseudoexons (PEs) arising in the mature *DMD* transcripts of some individuals, either as the result of mutations or as low-frequency errors of the spliceosome. In this review, I collate from the literature 58 examples of *DMD* PEs and examine the diversity and commonalities of their features. In particular, I note the high frequency of PEs that arise from deep intronic SNVs and discuss a possible link between PEs induced by distal mutations and the regulation of recursive splicing.

Keywords: Cryptic Splice Sites, Muscular Dystrophy, Duchenne, DNA Mutational Analysis, RNA Splicing

CHARACTERISTICS OF THE DMD GENE

The *DMD* gene is the largest gene in the human genome. Situated on the *p*-arm of the X chromosome, *DMD* spans over 2.22Mb, more than 99% of which is intronic sequence, with the coding sequence of its largest isoform totalling 11,058 bases across 79 exons. Eight unique alternative promoters [1], alternatively spliced exons, and an alternative polyadenylation site [2] produce at least 17 *DMD* transcript variants [3], one or more of which are expressed and translated in all types of muscle as well as various other cell types throughout the body, including myoblasts, lymphocytes and retinal cells.

The *Dp427 m* transcript of *DMD* encodes the muscle isoform of dystrophin, the *DMD* protein. In XY individuals, who carry just a single *DMD* copy, mutations that fully disrupt the function of the *DMD* gene (resulting in functionless or absent dystrophin protein) give rise to Duchenne muscular dystrophy, while mutations that only partially diminish the gene's function and/or quantity of product give rise to Becker Muscular Dystrophy.

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THE MAJOR SPLICEOSOME

The vast majority of RNA splicing in humans is achieved via the major spliceosome, a ribonucleoprotein complex responsible for excising introns from pre-mRNA molecules [4]. In order for the spliceosome to process a transcript correctly it first must accurately recognize the transcript's exon-intron boundaries. This recognition is achieved through a network of mechanisms, including sequence-specific interaction with conserved acceptor and donor splice site motifs in the RNA, silencer and enhancer binding motifs both proximal and distal to the splice junctions, and RNA secondary structure [5, 6]. Mutations to a gene that alter the interactions of these factors with its transcripts can lead to errors in the processing of those transcripts, such as the expansion, truncation or loss of canonical exons, or the initiation of pseudoexons (PEs) within its introns [7]. These incorrectly spliced transcripts may be degraded prior to translation or may be translated to less functional or even harmful protein isoforms, with deleterious consequences for the health of the patient.

Thirty-six of the 78 introns in *DMD* are more than ten times the human median intron length of 1334bp [4], and of these 36, three are more than 100 times the median size. A transcript of this size and complexity

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presents a unique challenge to the major spliceosome, and as a result is arguably more vulnerable to splicing errors such as pseudoexons.

PSEUDOEXONS: ONE DEFINITION OF MANY

In the literature, the terms "pseudoexon" and "cryptic exon" are often applied interchangeably and inconsistently in reference to a wide range of splicing errors. For the sake of clarity, I hereby define a pseudoexon as: *Any continuous tract of a transcribed gene that: (1) does not overlap, adjoin or duplicate any sense-strand sequence of that gene's canonical exons; (2) bears an acceptor splice site motif at its 5′ end and a donor splice site motif at its 3′ end; and, (3) via both these motifs, is spliced into a measurable proportion of the mature transcripts of that gene in at least one proband.*

Though this definition of "pseudoexon" may not agree with every prior usage of the term, it includes the majority of prior use while excluding splicing events that are better described by other terms, such as cryptic splice sites and whole exon duplications.

While some PEs are observable as rare splicing events in normal individuals, the majority are created by mutations that give the PE site an exon-like profile, resulting in the spliceosome falsely recognizing it and splicing it into an increased proportion of transcripts. When PE-splicing levels are high compared to normal splicing, these inclusions are likely to bear negative consequences for the phenotype of the affected organism, as the majority of PEs will disrupt the transcript's open reading frame and/or encode premature stop codons. Consequently, the resulting transcript, if it is not degraded by nonsense-mediated decay, will be translated to a non-functional or truncated protein. Even in cases where a pseudoexon preserves the reading frame and does not encode a premature stop codon, it is likely that the amino acids it encodes will disrupt the secondary structure of the protein and thereby abrogate its function.

Numerous *DMD* PEs have been reported over the last few decades, perhaps more than have been described for any other single gene. When considered as a body of research, these reports comprise a unique opportunity for generating new insights into the splicing of *DMD* and other large genes.

In this review, I catalog the characteristics of 58 reported *DMD* PEs. Where possible, I describe the origins of these rare splicing events and draw inferences from their common features.

PSEUDOEXONS OF THE DMD GENE

Following a thorough search of the literature, I compiled a catalog of all 58 known PEs of the *DMD* gene (Table 1). In order to consistently record highly similar PEs, I adopted the criteria of *unique local sequence:* I assigned a separate catalog entry to each PE that was unique in at least one nucleotide of its sequence or splice motifs (eg. PE09 vs. PE10, PE15 vs. PE16); and listed as single entries all PEs with locally identical sequence (eg. PEs 11 and 12).

PSEUDOEXONS ARISING WITHOUT MUTATIONS

Six *DMD* pseudoexons have been reported as low-frequency splicing events in normal cells lacking any known mutation: PEs 04, 07, 08, 11, 21 and 44. Interestingly, four of these PEs are of lengths that do not shift the reading frame of the transcript – 162bp for PE04, 357 bp for PE11, 66bp for PE21 and 84bp for PE44 – and of these four, only PEs 04 and 11 contain stop codons.

In addition to their splicing profile in normal cells, PEs 04, 07, 08 and 11 are also spliced at much higher frequencies in the cells of some patients with other *DMD* mutations. For PEs 08 and 11, this behavior was observed for the cells of only a single patient each (see below, subsection 'Pseudoexons arising from duplications'). However, the behavior of PEs 04 and 07 – referred to in some prior literature as exons 1a [10–14] and 2a [16, 17] respectively – is somewhat more complex. Though the inclusion of PE04 in muscle cell *DMD* transcripts is rare [14], this PE is included in approximately 50% of *DMD* transcripts in lymphocytes [11], and is included at higher frequencies in both cell types as a result of a frame-shifting single nucleotide deletion in exon 5 [12] and, in a different proband, an exon 2 tandem duplication [13]. Given the frequency of its inclusion in mature *DMD* transcripts, especially in normal lymphocytes, it may be that PE04 would be better classified as a canonical, alternatively spliced exon rather than a pseudoexon. However, at the time of writing no functional role for PE04 has been conclusively determined [10]. Similarly, PE07 has also been observed as a predominant inclusion in the muscle *DMD* transcripts of multiple patients with deletions and duplications in the 5′

Table 1

Details of 58 known pseudoexons (PEs) arising from mutations within the human *DMD* gene. Bold, upper-case sequence denotes the PE itself, while regular lower-case sequence indicates flanking intron. Inverted sequence is italicized. Duplications and insertions are underlined. Single base transitions are shown in the form of "(X>Y)," where X is the reference nucleotide and Y is the mutant. Shapiro-Senapathy splice scores are also shown, before and after mutation where appropriate (see supplementary materials for splice score calculator). "RNA Source" lists the cell or tissue types where the PE was detected. Genomic co-ordinates pertain to human genome assembly accession GCA_000001405.27, chromosome X, except where otherwise stated. Transcript co-ordinates pertain to *DMD* reference sequence NG_012232.1(DMD_v001)

PE #	Intron	Sequence	Genomic Co-ordinates (chrX.)	Size (bp)	Acceptor Score	Donor Score	Mutation(s)	Orientation of Mutation to PE and Splice Sites	ORF	RNA Source	Reference
01	1	tctcttccttggttttgc(g>a)g**C TTCTCGAGTTCATAGGAGACTTTCA GTTTCCAGTGACCTGGAAACTCACC ATTCCTCATCACCATCCTTTACTGT AGTAACTTCCTTTTACCTGACCACC CTGCATAGTCACAGAAGATGCACTC CTGACAAGTGATCCTCAAAACAG**gt agtaatctctttgagaag	33174185-33174333	149	71.37 -> 87.94	72.72	c.31+36947G >A	Proximal	Frameshift	Skeletal muscle	[9]
02	1	ctcaattaaaatatatttag**TCAAA GCCAGATGCAGTGGCTCACACCTGT GATCCCAGAACTTTGGGAGGCGGAG GCGGATGGATCATTTGAAGTCAGGA GTTCGACACCAGCTTGATCAACATG** gtgagacccctgtctctact	33088244-33088348	105	62.92	81.59	c.exon3_6del	Distal (3′)	Premature stop codon	Heart, skeletal muscle	[10]
03	1	tttttacatcatggcataag**ATCAA CACTAAATTTTAGGAGTGCATA**gta agtattaccatagtgta	33085901-33085927	27	58.89	78.81	c.exon3_6del	Distal (3′)	Premature stop codon	Heart, skeletal muscle	[10]
04	1	tctgttttcttttgtaacag**GACAC CATTGGAGAAATTGGTCATTTTACC AAGGCTTTGACTGGAATGGCATGCT TCCTTTAAAGAATCAAAGTTGACTT ATAGAGCCATTTAAAGCCCGTTGGG GAATCTGGCCTCATACCTTGTCCAC ACAGAGTCCCTGTACAAGGTTCCTG ACCTGTG**gtaagtaaagaatgtcac tt	33078186-33078347	162	96.38	88.39	Multiple mutations, also observed in normal cells (see main text)	Distal (3′)	Premature stop codon	Lymphocytes	[10 - 14]
05	2	ttccttgtttctctacat(t>a)g**G TTGAATCTGTTCCTGCAGCAACTAG TAACCCCAACAATCTGACTG**gtgag aaattcaatctccgt	33014502-33014547	46	68.17 -> 84.74	82.07	c.93+5590T>A	Proximal	Frameshift	Lymphocytes	[15]

(*Continued*)

Table 1
(*Continued*)

PE #	Intron	Sequence	Genomic Co-ordinates (chrX.)	Size (bp)	Acceptor Score	Donor Score	Mutation(s)	Orientation of Mutation to PE and Splice Sites	ORF	RNA Source	Reference
06	2	ttccttgtttctctacat(t>a)g**GTTGAATCTGTTCCTGCAGCAACTAGTAACCCCAACAATCTGACTGGTGAGAAATTCAATCTCCGTAACATTTTGCATTCTTCTACTTCTTCCCACTTCCATTCCAAAGGGCCAGGTAGAGTCCAGAATACT**gtaagtgtcctggttttcag	33014416-33014547	132	68.17 -> 84.74	77.7	c.93+5590T>A	Proximal	ORF preserved	Lymphocytes, skeletal muscle	[15]
07	2	tgttagtgttttttgttcag**GTCTGTGCAGGCCAAGGTTATGGCCTAGCTGAGAAGAGGGCTCAGAGGAGCCTAGCTAAAGTTTGGTCAAGGGGAGCGTCTTGGTGGAGCCTCACTCCTGTTCAAGTAAAGAAGACACATTATTCATCCTTCTGAACAGT**gtaagtccatttgtcattcg	32960208-32960347	140	100	77.84	Multiple deletions and duplications [17]; also observed in normal cells [16]	Distal (3′)	Frameshift	Skeletal muscle	[16, 17]
08	2	agtcttggtctgtcgcctag**GCTAGAGTGCAGTGGTGCTGTCTCAACTCAATGCAACCTCCGCCTCCCGGGTTCAAGCAACTCTCCTGCCTCAGCCTCCCCAGTAGCTGGGATTACAG**gtacctgccactgtgcccgg	32878883-32878980	98	72.84	76.65	c.exon2dup; also observed in normal cells	Distal (3′)	Frameshift	Multiple tissue types	[13]
09	2	tgatttggaacttcctgtag**ACAGACCCTTACAGGCATGGAAGAAGAAAGAATGAATAAACCAAGGATGACTTCCCAGTAGGTGGAGGATGGGAATAATTAGGAAGAAGCTGTGTCTTGTCACCTATATTGTCCATACACAACTGCACCGTAGGGTAATTGAGAAATTATAAGAATG**gtaataaatacgttttacat	32863759-32863915	157	70.85	73.68	c.exon8_11dup	Distal (3′)	Frameshift	Lymphocytes	[18]
10	2	ttggaacttcctgtagacag**ACCCTTACAGGCATGGAAGAAGAAAGAATGAATAAACCAAGGATGACTTCCCAGTAGGTGGAGGATGGGAATAATTAGGAAGAAGCTGTGTCTTGTCACCTATATTGTCCATACACAACTGCACCGTAGGGTAATTGAGAAATTATAAGAATG**gtaataaatacgttttacat	32863759-32863911	154	79.35	73.68	c.exon8_11dup	Distal (3′)	Frameshift	Lymphocytes	[18]

11	3	aatcttactctgtcgcccag**GCTGG AGTCAGCGACATGATCTTGGCTCAC TGCAACCTCTGCCTCCTGGGTTCAA GCAATTTTCCTGCCTCGGCCTCCCA AGTAGCTGAGACTACAGGCATGTTC CATCATGCCTGGCTAAGTTTTGTAT TTTTAGTAGACATGGGGTTTCACCA TGTTGGCCAGGCTGGTCTTGAGCTC CTGATCTCAATGAGCCACCTGCCTC AGCCTCCCCAAATGTCTGGCTGTTG CTTTTTTTTTTTTTTTTTTTTTTT AAATCTAAAGTCTTATTTTTTCCT CTTTTTGGTGGAAAGTGGGAGAAAT ATCAGAATGTAAACCAACATCATTC TGACATTCTTGGAGGAAAATCTAAG AG**gtaaaaatggagattctggt	32846622-32846978	357	81.06	79.88	c.exon8_11dup; also observed in normal cells	Distal (3′)	Premature stop codon	Lymphocytes	[18]
12	3	ttcctctttttggtggaaag**TGGGA GAAATATCAGAATGTAAACCAACAT CATTCTGACATTCTTGGAGGAAAAT CTAAGAG**gtaaaaatggagattctg gt	32846622-32846683	62	72.62	79.88	c.280Adel [12], c.exon 8_11 dup [18]	Distal (3′)	Frameshift	Lymphocytes	[12, 18]
13	4	ttcatatgcttgcttttcag**ATGCT TGTGTTTAACTTTACTCCACCTTAA ACATTTGAGGAGTGTGAAGGACAGG AGACACAGAGATTTGCCTTGATTAA GGCAAATAAAACCCTGCCAGATTTT CATTTCCAAACACAGTCCTAGACAG AG**(a>g)taagagagctggcagttt g	32823851-32823982	132	90.56	74.59 -> 91.82	c.265-463A>G	Proximal	Premature stop codon	Skeletal muscle	[19]
14	7	atattctaattgaatttcag**(A>C) GTGAATTTCACACCTCTCCTTTTGA AAGATTCATTTCTATGAATTTGGGA CAGCTTCCTAGTATGATATTCCATC T**(a>g)taagtatatccatatcatc	32738792-32738868	77	79.91 -> 78.04	56.78 -> 74.00	c.650-39575A>C; c.650-39498A>G	Proximal	Frameshift	Skeletal muscle	[20]
15	9	ttcattttcttcattta(a>c)ag**A GATTGATCATATTGGGATAAAGACG TGTTTTGGAATCCAAACAACCTGGT TTTAAGTCCCAGAACCACCAGTTAC CTTTGTGACCTTTG**gcaagtcatct aatttttct	32650985-32651074	90	83.55 -> 93.28	70.04	c.961-5925A>C	Proximal	Premature stop codon	Skeletal muscle	[21]

(*Continued*)

Table 1
(*Continued*)

PE #	Intron	Sequence	Genomic Co-ordinates (chrX.)	Size (bp)	Acceptor Score	Donor Score	Mutation(s)	Orientation of Mutation to PE and Splice Sites	ORF	RNA Source	Reference
16	9	ttcattttcttcatttaaag**AGATTGATCATATTGGGATAAAGACGTGTTTTGGAATCCAAACAACCTGGTTTTAAGTCCCAGAACCACCAGTTACCTTTGTGACCTTTG**g(c>t)aagtcatctaatttttct	32650985-32651074	90	83.55	70.28 -> 87.27	c.961-5831C>T	Proximal	Premature stop codon	Skeletal muscle	[9]
17	11	cttttctctctctacctaag**ACAGGGTTTGGATAGATCCAGTCGGAAGCCATTATTTCCTGCCTAAGCC**[del: 11631bp]**AAACCTTTTCTACAAGAAATGGTAAAGGGCGTTCTTCAATCTTAAAGAAAAGGATGTTAATGAGCAATGAGTCATCATCTGAAGTAACAAAACTCACTGGTGATAGTAAG**gtttgttactattctgttac	32641751-32641799; 32630010-32630119	159	73.92	79.54	c.1331+2382_1331+14010del	Proximal	Premature stop codon	Multiple tissue types	[22 - 25]
18	11	ctctttttttttccccccaag**TGTCTATTTGACTCTGGAAATAAGATGGCATATGTGAGAGTGGATAGAGAAAAGGAGGTGCTGAACAAATGAGTGGGTTATTTTCTCCCAAGTGCAGTGAAGTTTGCGTATGTGAATATATGAATGAATAGATGAACAACTAAATTAAGTTATTCAG**gtaattccaatcttggaatt	32638732-32638888	157	79.76	87.83	c.1336_1337del	Distal (3′)	Frameshift	Lymphocytes	[26]
19	11	agttttgttctttcacccag**GCTGGAGTGAAGTGGTGTGATCTCAGCTCACTGCAACCTCTGTCCCCCCGGGTTCAAGTGATTCTCCTGCCTCAGCCTC**(c>g)tgagtagccgggattaaaa	32626363-32626441	79	90.08	56.56 -> 73.78	c.1332-11909C>G	Proximal	Frameshift	Skeletal muscle	[27]
20	18	ttttttccacctgccttag**TGGAAGAGGCTATCCTTCACCTGGTTGAGGCTCATCTCTGGGTGTGTGTTCTCAGCAGCATCACTGACTATGTATTAAGCCACCTGGTTCCATTCAGCTGTATATCCAGATTGTCAAAAATCTACATCCC****AG**gtctttatcattagctttta	32514091-32514222	132	68.21	67.88	c.2622+1G>A	Distal (3′)	Premature stop codon	Lymphocytes	[10]

21	21	tttatttttaatacatctag**GCAAAGGAATGTTTTGTTCCAGTAGACACATAATCTGTTGCATTGCTCCTTCACTCCAGAAACTAG**gtaaactgtttgtaaatgtt	32480880-32480945	66	80.03	78.22	None	N/A	ORF preserved	Skeletal muscle	[14]
22	25	tatctgtgcttttcctaca(a>g)**GTATCACTCTGGCCATGTTCTGACTTTGTAGCCAAATGAGTTAGGTTGTAAAAGGAAGGAACAATGGCGCTCAAGGAGAAGAAGAAGACGATGCG**gtaaaaacaaggaagccata	32461308-32461402	95	79.15 -> 95.72	70.75	c.3432+2036A>G	Proximal	Frameshift	Skeletal muscle	[28]
23	25	atctgtgcttttcctacaag**TATCACTCTGGCCATGTTCTGACTTTGTAGCCAAATGAGTTAGGTTGTAAAAGGAAGGAACAATGGCGCTCAAGGAGAAGAAGAAGACGATGCGGTAAAAACAAGGAAGCCATATGTGAATATTGTTACCAATTCAGCATTCCAGAGAGAATAATGGAAATGAAGTGTAAATCTATGCATTACACAGAAATATCTACAGACAAA**(a>g)taagtgtgtgatacactct	32461200-32461401	202	76.71	70.14 -> 87.36	c.3432+2240A>G	Proximal	Frameshift	Skeletal muscle	[29]
24	25	gccatgttctgactttgtag**CCAAATGAGTTAGGTTGTAAAAGGAAGGAACAATGGCGCTCAAGGAGAAGAAGAAGACGATGCGGTAAAAACAAGGAAGCCATATGTGAATATTGTTACCAATTCAGCATTCCAGAGAGAATAATGGAAATGAAGTGTAAATCTATGCATTACAGAAATATCTACAGACAAA**(a>g)taagtgtgtgatacactct	32461200-32461371	172	82.72	70.14 -> 87.36	c.3432+2240A>G	Proximal	Frameshift	Skeletal muscle	[29]
25	26	cttctctctctgcttatcag**AAAACTGCAATTCCCAGATA(G>C)GTCAAATGATTTAGCCATAGTCACAGACTTTATTTGTGGTAGAGCCCACAGGATTGAAG**gtattttattctatttcatc	32452550-32452629	80	86.2	77.21	c.3603+2053G>C	Proximal	Frameshift	Skeletal muscle	[30]

(*Continued*)

Table 1
(*Continued*)

PE #	Intron	Sequence	Genomic Co-ordinates (chrX.)	Size (bp)	Acceptor Score	Donor Score	Mutation(s)	Orientation of Mutation to PE and Splice Sites	ORF	RNA Source	Reference
26	27	catattcttatgtgttacag**ATGTTGCAGTTGTTTTCTTCTATTTTGAGTCTGTTTTTCAATTACTTGATGGTGTCCTTTGATGAATAGAAGGTCCTAATTTTAATGTAGTCGAATGTATTCATCTCTTTTTTCTTACG**gt(c>a)agttttttacgagatgt	32442160-32442278	119	87.46	80.58 -> 90.39	c.3787-843C>A	Proximal	Frameshift	Lymphocytes or skeletal muscle (unspecified)	[31]
27	29	catttttttctatcaacag**AGCTGAATGAGTGCCAGGAAGCTGCGAAATCTGTCTTACAAAAAG**gtgattgtggaagagtctag	32412019-32412063	45	90.17	83.09	c.3613delG	Distal (5′)	Premature stop codon	Lymphocytes	[10]
28	34/42	caaatgttcccgttttatag**ATGTTCCCGTTTTATAGATGAACAAATACAAATACAATA**[del:78031bp]**CAGCCAAGAAGATATGTTGGTGCACGTTTCTGGTACCTGACCTAATCAGGCTAGCGGAGTAGCCCTAAACATTCCACCCAAGACTCCAGGCTTGGAGCCATCAGAAGATGGTAGTAAAATTCTACCAG**gtaaataaaagtaaagaaat	32372085-32372098; 32293942-32294069	167	83.07	88.06	c.4846-6885_6118-6369delinsATACAATA; c.4846-6900_4846-6899ins17	Proximal	Frameshift	Unspecified	[32]
29	37	gaatttgctctccaccacag**ATGTGACAGACCCAGCCAATACAAGTTTGTGACCAAGACAAGTTTGTGAGTTTTCATTTCACATTCTGCATGAATTT**gtaagtatacttctggaaat	32360933-32361009	77	77.72	74.58	c.5325+1740_5325+1757del	Distal (5′)	Frameshift	MyoD transformed fibroblasts	[33]
30	37	tgaattgtactatcttgca(t>g)**GTATGTTCAGCTCTGGTGATGTGAAATGTTTCTCCTTATTTGCATCCTCAG**gtacttttccagttgtattt	32348692-32348742	51	71.85 -> 88.42	77.01	c.5326-215T>G	Proximal	Premature stop codon	Skeletal muscle	[34]
31	43	agtgattttgtcaccaccag**GCCTGGCTTACAAGAGCTCCTGAAGGAATCACTAAACATGGAAAGGAAAAACCGGTACCAGCCACTGAGAGAAACATACCAAATTGTAAAGACCATCGACCCTATGAAGAAACTGCCTCAACTAACAG**g(c>t)aaaataactaaccaacat	32256577-32256704	128	86.94	65.72 -> 82.94	c.6290+30954C>T	Proximal	Frameshift	Skeletal muscle	[35]

32	43	ttttatttatttttattctag**ATTTCCTGCTCTTCTAAGACACAGTGATTTAGAATTTCTGTTCAAGCAAGAGAACTAAAGACTTCTTTAAAGAAGAGAAG**gtaagtgcaagagattaaaa	chr4.182041664-182041743	80	90.26	99.52	Complex insertion/deletion (see cited work)	Encompassing	Frameshift	Skeletal muscle	[36]
33	43	tgtctcccatcacccccag**ATGGGACCATCTAGCTGCAGGAAAGGAAGCTCAAGGCTCCCACTGAATCTACATTATG**gtgagttgtataattatttc	32235147-32235204	58	76.18	86.71	c.6291-21015_6438+98743dupinsA; c.6291-21008_6291-21007insCTCCCCTGAACATGG	Distal (5′ and 3′)	Frameshift	Unspecified	[32]
34	44	*ttttttttaattgcaatcag**ATTCCAGATGGACCCTAAGAGCTCAGTTACGGAAGTGGAAGGGGAAAAACAGCAGCAGCAGCAGCAACAACAACAACGACAACAACAACAACGACAACAACAAACCGAAGAGGAAAAGAGAGCTGAGTGGAAAAAGTCTTTGAAAATTTAGTCCTAAAACTTATATTTACATTCTCATGCTCTCCTGAAGTCACAGAAAGAGGTTCGGAACTTCTGCTTTAAGAGGACATTTGAATCGACTGGAGAGGAACAAAGCATTGAAGAGAGAGGAAGTGAACTCCTCAGATAAAGCTCTAATATGAGAAACATAGTTCCAGAGAGAAGTTAATGTTACTTCATTTTTCTGTCTCATTTCCTTCAAGCTCAAAGCATGAGTAAGTGATAATG**gtaagtaaatatgtattctc*	32074802-32075188	387	75.04	90.97	Complex inversion (see cited work)	Encompassing	Premature stop codon	Skeletal muscle	[37]
35	44	ttttttttttttttttgag**AGGGAGTTTCGCTCTTTCGCCCAGGCTGGAGTGAGGTGGCGCAATCTCAGCTGACTGTAACCTCTGCCCCCACCAGGTTCGA**gt[del:121167bp]aagtatgaaaaaaataaa	32024402-32024483	82	85.52	62.43 -> 78.86	c.6439-55921_6912+26400del	Proximal	Frameshift	MyoD transformed fibroblasts	[38]

(*Continued*)

Table 1
(*Continued*)

PE #	Intron	Sequence	Genomic Co-ordinates (chrX.)	Size (bp)	Acceptor Score	Donor Score	Mutation(s)	Orientation of Mutation to PE and Splice Sites	ORF	RNA Source	Reference
36	45	tttcttctggagtattctag**GAGAAGACATACCAGTCGAGGGGTTCTGGGGAGCCAGGCCTTCAAGCAATGGATTGCTGACAACATAATGAAGAGGATTTTACTTAGAATAATGTCAGTTGATAAAAGTTTGAATGGGAGACGGAAGCAAGGCAGTG**g(g>t)aagtggaattcctaaatt	31965031-31965167	137	73.52	71.16 -> 88.39	c.6614+3310G>T	Proximal	Frameshift	Skeletal muscle	[27]
37	47	aattatggtaatccccaca(t>g)**GTCAAGGGTGGAGCCAGGTGCAGATAATTGAATCATGGAAGAGGATCCCCCATACTGTTCTCCTGATAATCA**gtaagtttcacaagatctga	31879338-31879409	72	61.61 -> 78.18	74.53	c.6913-4037T>G	Proximal	Premature stop codon	Skeletal muscle	[27]
38	48	*aaagctgctctttccgctag**GGTCCTGGGATGAGAAGACATTTAGAGCAGATCCATATTCTAGAATGAAAAGACCTTAACTTGATTCATAGTGTGGAGTAAAGCTGCAGCTGATCTGCAGACACATGA**gtgagtaataaatgtttgtt*	31850637-31850744	108	81.97	70.41	Complex inversion (see cited work)	Encompassing	Premature stop codon	Cultured myogenic cells	[39]
39	48	*gacagtgggctctattttag**GCAATGTGAGCACAGAAGACTGCTCCTCTCTTCAGGAAATGGAATGAAGTGAAGAAATGAGTCATGTCAACATACTGAAGACTATTTCAGGCAGAGGGAAAAGCAAGTTCCATTGCCAAAAGGCG**gtgaggagttttgcttgtat*	31845152-31845276	125	75.76	78.66	Complex inversion (see cited work)	Encompassing	Frameshift	Cultured myogenic cells	[39]
40	49	*atgtatttgacctttacag**GAAGTCCTCAAAGAAAATAAAGAAGTTGGTTAGTCAGAGATTTTAAAAAAGAAACCAGAAATATACCATCTTTGAAAATAAAAGAAATCTTAAAGAAGTTGACGAAGTGAAGGAAAGAAGAAAGAGGAAAGAAACTGAGCAGGGATGAACCCAGCTTCCAATCAGAACTCAGCTTGGGAG**gtgagcgtaggagctggaca*	31833539-31833718	180	93.4	87.02	Complex inversion (see cited work)	Encompassing	Premature stop codon	Cultured myogenic cells	[39]

41	49	*atgatgctgtcattttgcag**TTCAT ATCTGAGGAAATGATGCCTGGAAGA TTATAATTGCCCTCTTGACCATTTG TCTACAGCAATTATTTTGTGTTAAA GGTTGGGATGGAACGCTTTGGCCAA ATACAGAAAGTTCCTCACACCTTTT TCTGTTACATTGAAAAATAATTCAA GAGCA**gtaagtaaaacatattacta*	31832500-31832659	160	87.27	75.66	Complex inversion (see cited work)	Encompassing	Frameshift	Cultured myogenic cells	[39]
42	49	*tattgttatcttctttctag**AAGTC CTCTCCCAACTTATTCTGGTGTTAT TGGCATGGAAATTGTGCTCAATAAA TGAGTCAGTCAAAATAGACTTGTTC ATTTCAGGGGACAATTTTTGTCTGT TATCTCTCTATTGATTTCTGTTGCT ACAAGTGAAGTTACACCAG**gtaaat gagattgtacgcat*	31831990-31832138	149	88.15	88.06	Complex inversion (see cited work)	Encompassing	Frameshift	Cultured myogenic cells	[39]
43	49	*ctttctgaattcctccttag**AATCA TGCAGCCAGCTGAACTCTGTAAGAA GCTTTTTCCAATGCCCTCTTACTGA GATGCCCACACTCTGAAGTGTTTTA CGCTCTTGCTGCAATAAG**gtaaata ttcttaatttttt*	31831041-31831138	98	73.62	87.58	Complex inversion (see cited work)	Encompassing	Frameshift	Cultured myogenic cells	[39]
44	51	aaatggtcttttggttacag**GGTGA AAGAGACCCAACAATACACCTTTCC CACTTCCGGAGGCCTTTGGTTAAAC CATGTCTGCCACAAGGACACAGGAG CCTG**gtatgactggttgttttttg	31752441-31752524	84	96.74	76.2	None	N/A	ORF preserved	Skeletal muscle	[14]
45	51	tgcttttatgatttttaaag**AATTC GGAGGAGGAGCCAAGATGGCCGAAT AGGAACAGCTCCGGTCTACAGCTCC CAGCGTGAGCGACGCAGAAGACGGG TGATTTCTGCATTTCCATCTGAG**gt accgggttcatctcaata...	31765009-31765013	103	79.68	68.7	c.7542+8951_7542+8952 ins6091	Proximal	Frameshift	Skeletal muscle	[40]

(*Continued*)

Table 1
(*Continued*)

PE #	Intron	Sequence	Genomic Co-ordinates (chrX.)	Size (bp)	Acceptor Score	Donor Score	Mutation(s)	Orientation of Mutation to PE and Splice Sites	ORF	RNA Source	Reference
46	53	*tttatcctgaggaattatag**ACTAC TAAGCAGACAGATATTTGAGGAAAT TTCAGAGGAAAGAGAAAGAAGAGAA GAATGAGCTGGGCTGGAG**gtaagag aatgggggagaa*	31678558-31678630	73	64.29	91.82	Complex inversion (see cited work)	Encompassing	Frameshift	Skeletal muscle	[41]
47	55	ttttacttttctatttaca(a>g)**A AATGGAACACCACCAGAAAAACAAG AATTTGAAAGACGAGATGAGAAAA**g taagttgtaattggaaaca	31609571-31609620	50	79.61 -> 96.18	87.36	c.8217+ 18052A>G	Proximal	Frameshift	Skeletal muscle	[33]
48	55	ttttgtttttccttttta**gAGTTC TTGCTAATGATGGGCCCAAAGTTAT ATTAAGAACTGCAAAGTAAATTTCA ACCAATTACTTTATTCAG**g(g>t)g agtcattaaattgaggt	31595572-31595644	73	90.26	78.52 -> 95.74	c.8217 +32103G>T	Proximal	Frameshift	Skeletal muscle	[35]
49	56	catccaatttttctaccag**AAATA TTAAGAATTGTTGACTACAACAGTA TGGAAAAGCAATAGATTCCAGTGTG TATTTCATGCCAAAAGTCTCAGCAT TCTGCATGTGGAAATAAACATATGG CTAAACACTGCCTTTTCTCAAAATT GCCATCAAACTATCCTCTGTTTTGT GGCTCTCAAAA**gtaagtagccagat ttttat	31497079-31497244	166	94.32	87.36	c.8391-1419_8391-828del; c.8391-102_8391-76del	Distal (5′ and 3′)	Frameshift	Skeletal muscle	[37]
50	60	ttgattgattattgttgcag**GTTGA GTCCTCCAAGAAGCAGATGCCAGGG CAGATTTATTGCAGAATAACACCTG TGAAGAAATAGGGGTGGAAGCAGAA TTGAACAAG**g(g>t)aagccatcag atcacaat	31364155-31364243	89	88.65	76.64 -> 93.87	c.9085-15519G>T	Proximal	Frameshift	Skeletal muscle	[9]
51	62	ttttgttctttttgtggcag**GAGCT GATAGCCAGCAACCACACTTCAAGA AATGGAAGACAGCTGTGAATGCTTC ATTCAGGCCCA(A>G)**gtaaatata ggaagaggtgt	31261663-31261729	67	96.61	75.91 -> 88.06	c.9225-647A>G	Proximal	Frameshift	Skeletal muscle	[9, 42]

52	62	tgtcggtgtcctttctgtag**TGTTCCACATTGGATGGGTGAAGAAGTCCTGATAGTCGATTATTGATCACATAACAAG**gtca(a>g)tttatcataactgaa	31261306-31261363	58	81.83	77.76 -> 89.70	c.9225-285A>G	Proximal	Frameshift	Skeletal muscle [28]; Lymphocytes or skeletal muscle (unspecified) [31]	[28, 31]
53	63	tgtttccactacatctgcag**TTACATCCTCCACTGAAGTCTTGAACCCCTGACAGTCATCCATGAGGGTTGGAATCAACGTCTTCCAAACTCCTG**gtaatgttgatattttgacg	31247694-31247768	75	76.44	74.39	Unknown	Unknown	Premature stop codon	Lymphocytes	[10]
54	65	tggcactgttttctttgcag**ATGACATGTGAATGCATTCTGAATGTATAACTTCCTTCTACCTGACTGAAAAGTATTTGGTGACAATTTTAACTCCTTGAAGACCTGAGTTGCTGTATAAAGTGGATTTGTTAAATTTTGATCTACCTTTTCTTAAGAGGGAGAAAG**(a>g)taagaaaattttccagtga	31208284-31208430	147	96.18	77.17 -> 94.40	c.9563+1215A>G	Proximal	Premature stop codon	Skeletal muscle	[33, 43]
55	65	attaattttctttcaataag**GGGCAATCTGATGAAGATCTGAGCATTTAAGAGGGCTGAGCAGTTAGTTGCTG**gtaa(t>g)ttttttggcttccat	31207099-31207151	53	83.88	79.28 -> 91.45	c.9564-427T>G	Proximal	Frameshift	Skeletal muscle	[19, 45]
56	67	ttgctttttctctgtcgtag**TCAGTGCCCCAGAAACACCCTCTTCTTCCCAAATGCCTGAGCACTCCACTG**gtagacattcatcttgttct	31203344-31203394	51	81.16	63.87	Unknown	Unknown	ORF preserved	Lymphocytes	[10]
57	67	gtgttttgttttgttttcag**AAGGGGTCTAACTTCGTCACCCAGGCTGGAGTGCAGTGGCACGATCACAGCTCATTGCAGCCTCGACCTCTGGGCTCAAGTGATCCTCCCACCTCAGCCTCCTGAGTAGCTGGGACTACAG**g(c>t)atggaccgccacgcctgg	31201249-31201369	121	96.18	67.75 -> 84.98	c.9807+2714C>T	Proximal	Frameshift	Lymphocytes or skeletal muscle (unspecified)	[31]
58	77	gtacttatggtcaattgcag**ATACAGAACTCCAAAGAAATTCAAATCACAGTACAATCATTGATTACTGTATGATCCTGGCCCCTTGAACTGGCAGCTTGCTTACCTGCTCTGGAAAGTTGCTGGCTGCCCTGTTGGCACCCTGGGCATTTCTTCCACATCTAAACAAGAG**gttagtagagaagaagctac	31132483-31132633	151	76.72	87.1	Unknown	Unknown	Frameshift	Lymphocytes	[10]

exons of the gene – though, as with PE04, it is yet to be determined whether these inclusions indicate a functional role for PE07 [17].

PSEUDOEXONS OF UNKNOWN ORIGIN

Underlying genomic mutations were not identified for three of the pseudoexons catalogued (PEs 53, 56 and 58). However, as these pseudoexons were exclusively detected in the RNA of specific DMD patient cells, they are believed to be pathogenic and therefore were not classed as arising *sans* mutation.

PSEUDOEXONS ARISING FROM DELETIONS

Of the 54 known mutation-obligate *DMD* PEs, ten arose from genomic deletions: PEs 02, 03, 12, 17, 18, 27, 28, 29, 35, and 49. For some of these cases, PE initiation can easily be explained as a direct result of the deletion event bringing into conjunction tracts of sequence that, when transcribed, present a strong exon signal to the spliceosome. Pseudoexons appearing to fit this description are PEs 17, 28 and 35, though it should be noted that PE28 also has two small insertions (17bp and 8bp) near its junction site. Two additional but less obvious examples can be seen with PEs 29 and 49 – in these cases, the sequence of the pseudoexons and their splice sites are unaltered from normal individuals, but their inclusions in mature transcripts are initiated by deletions of immediately flanking intronic regions, which presumably contain essential splicing silencers.

For the remaining five deletion-initiated PEs, the link between mutation and pseudoexon is less clear. PEs 12 (i3), 18 (i11) and 27 (i29) all arose from frameshifting deletions of one, two and one bases in exons 5, 12 and 27 respectively, and PE02 (i1) and PE03 (i1) (both from the same patient) were purportedly initiated by a deletion of exons 3 to 6. Though a more detailed explanation of these PEs may not be possible at present, they appear to support the general theory that splicing of a given *DMD* intron is often interdependent on the correct processing of distant elements of the same transcript [46].

PSEUDOEXONS ARISING FROM DUPLICATIONS

Five *DMD* pseudoexons arose from genomic duplications: PEs 08, 09, 10, 11, and 33. PE08 (i2), which has also been observed as a low-frequency inclusion in normal skeletal muscle RNA, was converted to a pseudoexon by a tandem duplication of exon 2. PE09 (i2), PE10 (i2) and PE11(i3) were reported in the same proband as a result of an exon 8–11 duplication, and PE33 (i43) arose from an exon 44 duplication. These cases offer further support to the theory of correct *DMD* splicing occurring through coordination of distant elements. At this point, however, it is not clear whether these PEs are induced specifically by alterations to the canonical exon order, or whether they arise from disruptions to intronic sequences that would normally act as distal pseudoexon silencers.

PSEUDOEXONS ARISING FROM INVERSIONS

Eight *DMD* pseudoexons arose from inversion mutations: PEs 34, 38, 39, 40, 41, 42, 43, and 46. In all these cases, each PE was completely internal to the inverted region. PE34 arose from an inversion internal to intron 44 – i.e. no canonical exons were directly affected. PEs 38 to 43 (i48 and i49) were reported from a single patient with a complex inversion of exons 49 and 50, while PE46 (i53) arose in a patient with a deletion of exons 48–52 and an inversion of exon 53. It is perhaps unsurprising that such dramatic rearrangements of large tracts of transcribed sequence would result in splicing disturbances of some kind, but these cases nevertheless serve to illustrate that, in addition to recognition of canonical exons, the silencing of pseudoexons is an equally essential component of spliceosome function, and one that is likely to be achieved through orientation-dependent sequence motifs in the intron.

PSEUDOEXONS ARISING FROM INSERTIONS

Two *DMD* pseudoexons arose from insertion mutations, PEs 32 and 45. PE32 was created by an insertion into intron 43 of two large tracts (88.0kb and 2.6kb) of intragenic sequence from chromosome 4, the PE itself originating within the larger of these two tracts, while PE45 was created by a 6096bp LINE-1 retrotransposon with a potential donor site at its 5′ end inserting immediately 3′ of a latent acceptor site in *DMD* intron 51.

PSEUDOEXONS ARISING FROM SINGLE BASE-PAIR SUBSTITUTIONS

Single base-pair substitutions were the most commonly observed cause of *DMD* PEs, accounting for

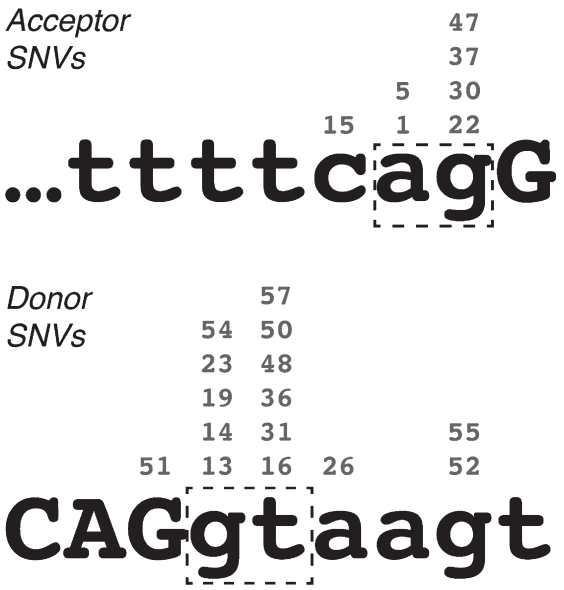

Fig. 1. Locations of pseudoexon-initiating single-nucleotide variations in the *DMD* gene, relative to acceptor and donor splice site consensus sequences. Numbers above each nucleotide indicate the exemplar pseudoexons. Lower-case letters indicate intron sequence, upper-case letters indicate exon sequence. Dash-line boxes highlight the essential "ag" and "gt" of the acceptor and donor site motifs respectively.

26 of the 58 catalogued, through 24 unique mutations. In most of these cases, the etiology of the PE appears to stem from the creation or enhancement of a latent mid-intron splice site – of the 24 unique mutations, 7 created new acceptor splice sites (PEs 01, 05/06, 15, 22, 30, 37 and 47) and 15 created new donor splice sites (PEs 13, 14, 16, 19, 23/24, 26, 31, 36, 48, 50, 51, 52, 54, 55 and 57). All 22 of these acceptor-motif and donor-motif mutations greatly enhanced the Shapiro-Senapathy splice score of the mutated site, and in every case the new nucleotide was the most common consensus base for that position in the splice site (Fig. 1). While a possible exception to this rule was noted at the acceptor site of PE14 (c.650-39575A>C), this mutation was found to be a common SNP (rs113593006, dbSNP build 151 – see ref. 44) that only marginally decreased the Shapiro-Senapathy score of the acceptor site (from 79.91 to 78.04). I therefore judged that this SNP was likely to be incidental to the pathology of this pseudoexon and did not constitute a true counterexample to the prevailing pattern of splice site enhancement.

Only two PEs arose from SNVs outside of the PE consensus splice sites, PE20 and PE25. PE20 (i18) arose from a G-to-A substitution at the first base of intron 20, suggesting that the correct splicing of these two introns may be interdependent. In this way, PE20 is qualitatively similar to PEs 12 and 18, which also arose from small mutations distal to the pseudoexon, although the mutation that initiated PE20 did not directly alter the *DMD* coding sequence. PE25 (i26) is a unique case that arose from a G-to-C substitution internal to the PE that altered the predicted binding of splicing enhancer SRp55 [30].

PSEUDOEXONS AND RECURSIVE SPLICING

Multi-step or recursive splicing was first described in *Drosophila* in 2005 [47] and has more recently been discovered to be prevalent in the genes of the human transcriptome [48, 49], including *DMD* [50, 51]. While conventionally spliced introns are removed with a single splicing event, recursively spliced introns are excised from their maturing transcripts in two or more segments, via intronic acceptor splice sites called 'ratchet points'. Sibley et al. [48] have also reported that recursive splicing in vertebrates is facilitated by recognition of evolutionarily conserved donor-like splice sites downstream of acceptor-like ratchet points.

Georgomanolis et al. [49] have postulated that some of the low-frequency pseudoexons observed in the transcripts of normal cells may be a natural byproduct of the spliceosome incorrectly recognizing exon-like intronic ratchet points. I suggest that this hypothesis can reasonably be extended to include mutation-induced PEs – i.e. mutations that enhance the exon-like characteristics of intronic ratchet points may thereby convert them into pathogenic PEs. Evidence supporting this hypothesis has already been described by Bouge et al. [14], who noted the alignment of six pseudoexon splice sites with six of the *DMD* intron ratchet points predicted by Gazzoli et al. [51]. Seeking to expand upon these observations, I cross-referenced the splice sites of all eligible PEs in Table 1 with all of the intronic ratchet points predicted by Gazzoli et al. This analysis excluded the splice sites of the *DMD* inversion PEs (34, 38, 39, 40, 41, 42, 43 and 46), the chromosome 4 insertion PE (32), and the *de novo* donor site for PE45, as these sites could not be sensibly compared to any part of the *DMD* reference sequence. Splice sites shared by multiple PEs (acceptor sites for PEs 5 and 6, 15 and 16, and donor sites for PEs 9 and 10, 11 and 12, and 15 and 16) were included but were counted only once each to avoid bias. Using these criteria, including the matches noted by Bouge et al. I confirmed 12 Gazzoli matches out of 47 unique acceptor sites and 14 Gazzoli matches out of 44 unique donor sites (Table 2).

Table 2
DMD pseudoexon splice sites coinciding with recursive splicing ratchet points predicted by Gazzoli et al. (2016). Co-ordinates listed are for genomic reference sequence NC_000023.10, as used by the cited authors. Dotted-line boxes enclose pairs of split reads that match to the same pseudoexon splice site. Asterisks (*) indicate the six coinciding splice sites previously noted by Bouge et al. [14]

Intron	Genome positions of split read	Recursive Splicing Type	PE Match	Donor/Acceptor
1	chrX:33190805-33192302	nested	1	D
1	chrX:33096464-33229399	5'RS	4*	A
1	chrX:33038317-33096303	3'RS	4*	D
2	chrX:32978464-33038256	5'RS	7	A
2	chrX:32978004-32978325	nested	7	D
	chrX:32867937-32978325	3'RS		
2	chrX:32897097-33038256	5'RS	8	A
2	chrX:32867937-32897000	3'RS	8	D
3	chrX:32862977-32864739	3'RS	11, 12	D
18	chrX:32532339-32536125	5'RS	20	A
	chrX:32532339-32533049	nested		
18	chrX:32519959-32532208	3'RS	20	D
21	chrX:32499062-32503036	5'RS	21*	A
21	chrX:32490426-32498997	3'RS	21*	D
27	chrX:32460395-32466573	5'RS	26	A
27	chrX:32459431-32460277	3'RS	26	D
29	chrX:32430180-32456358	5'RS	27	A
	chrX:32430180-32430279	nested		
29	chrX:32430030-32430136	3'RS	27	D
43	chrX:32253321-32305646	5'RS	33	A
43	chrX:32235180-32253264	3'RS	33	D
51	chrX:31770641-31792077	5'RS	44*	A
51	chrX:31747865-31770558	3'RS	44*	D
56	chrX:31515061-31515196	3'RS	49	D
63	chrX:31265885-31279072	5'RS	53	A
63	chrX:31241238-31265811	3'RS	53	D
67	chrX:31221511-31222078	5'RS	56	A
77	chrX:31150750-31152219	5'RS	58	A
77	chrX:31144790-31150600	3'RS	58	D

Several interesting features were apparent in this set of Gazzoli-matched splice sites. Firstly, most of the matched PEs matched at both their acceptor and donor splice sites. Only PEs 1, 11/12 and 49 matched at their donor sites alone, and only PE56 matched at its acceptor site alone. Secondly, a clear bias was evident in the mutation categories of the matched PEs, as the majority of the Gazzoli-matched sites were from PEs induced either without mutations or by mutations distal to the PE and its splice motifs, PEs 1 and 26 being the only exceptions to this rule. Lastly, of the 15 PEs where inducing distal mutations were identified, 11 arose exclusively from mutations that were 3′ to the PE (PEs 2, 3, 4, 7, 8, 9, 10, 11, 12, 18 and 20). Only PEs 27 and 29 were induced exclusively by 5′ mutations, while PEs 33 and 49 were each induced by flanking mutations.

PSEUDOEXONS AND RECURSIVE SPLICING REGULATION

Canonical splicing of a donor-acceptor pair is often dependent on distal regulatory elements, including

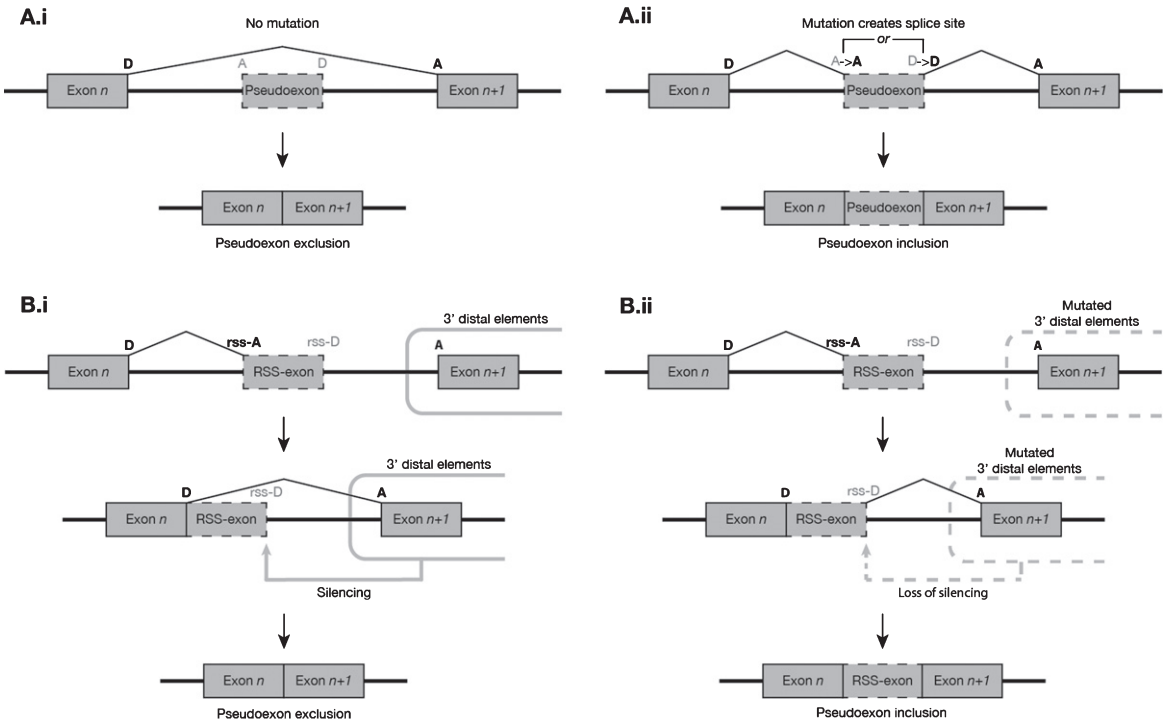

Fig. 2. Suggested model of the two most common modes of pseudoexon initiation observed in the *DMD* gene. (A) Proximal mutations at non-RS sites. (i) In the absence of mutation, a putative pseudoexon presents a weak exon-like profile to the spliceosome and is predominantly excluded from mature transcripts. (ii) The presence of a mutation, usually a splice-site-creating SNV, increases the exon-like profile of the putative pseudoexon, resulting in its inclusion in a much higher proportion of transcripts. (B) Mutations 3′ distal to RS sites. (i) In the absence of mutation, the exon *n* donor site and RS-acceptor site are used to excise the 5′ segment of an intron. Silencing elements distal (usually 3′) to the RS-exon prevent spliceosome recognition of its donor-like motif, and the RS-exon is subsequently removed from the transcript along with the rest of the 3′ intron segment. (ii) When mutations to the distal silencing elements impair their function, the intron segment 5′ to the RS-exon is spliced as normal, but the RS-exon donor-like site escapes silencing and is more readily recognised by the spliceosome, leading to a much higher frequency of inclusion of the RS-exon in the mature transcript population.

but not limited to other canonical splice sites [51, 52, 53]. Mutations that alter or destroy these distal elements can impede exon definition and decrease the frequency of inclusion of the affected exons in the mature transcript [42, 43]. It may be that spliceosome recognition of recursive splice sites (which necessarily exhibit a strong exon-like profile in their local motifs) is regulated by a similar system of mostly 3′ distal elements, but a system that acts to silence rather than promote the inclusion of its targets. Mutations that impaired these distal silencing elements might thereby permit an increase in the erroneous processing of recursive splice sites, converting them to PEs via a distinctly different pathway than PEs created by proximal mutation (Fig. 2). If valid, this model would explain the high coincidence of Gazzoli predicted recursive splice sites with the splice sites of PEs induced by distal mutations. However, while the mutations collated in this report may offer broad clues as to the locations of some such suppressive distal elements in the *DMD* gene, any further analysis of their common features awaits the assembly and analysis of a much larger dataset of PEs and recursive splice sites – one that will have to encompass multiple other genes besides *DMD*.

CONCLUSIONS

The 58 *DMD* pseudoexons collated from published reports exhibit great diversity in their sizes, locations, and pathologies. Surprisingly, PEs arising either from no mutation, or from mutations distal to the pseudoexon and its splice sites, exhibited a high coincidence with predicted recursive splice sites in the *DMD* introns, suggesting that some such pseudoexons may arise from disruptions to recursive splicing regulation. This finding may represent an important new insight into the etiology of pseudoexons in *DMD* specifically and human disease genes generally.

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CONFLICT OF INTEREST

The author has no conflict of interest to declare.

SUPPLEMENTARY MATERIAL

The supplementary material is available in the electronic version of this article: https://dx.doi.org/10.3233/JND-190431.

REFERENCES

[1] Nudel U. Alternative Promoters: Duchenne Muscular Dystrophy (DMD) Gene. In eLS, (Ed.). 2005.
[2] Feener CA, Koenig M, Kunkel LM. Alternative splicing of human dystrophin mRNA generates isoforms at the carboxy terminus. Nature. 1989;338(6215):509-11.
[3] Doorenweerd N, Mahfouz A, van Putten M, Kaliyaperumal R, t'Hoen PAC, Hendriksen JGM, et al. Timing and localization of human dystrophin isoform expression provide insights into the cognitive phenotype of Duchenn muscular dystrophy. Sci Rep. 2017;7(12575).
[4] Wahl MC, Will CL, Lührmann R. The Spliceosome: Design Principles of a Dynamic RNP Machine. Cell. 2009;136:701-18.
[5] Berget SM. Exon recognition in vertebrate splicing. J Biol Chem. 1995;270(6):2411-4.
[6] De Conti LM, Baralle M, Buratti E. Exon and intron definition in pre-mRNA splicing. Wiley Interdiscip Rev RNA. 2013;4(1):49-60.
[7] Anna A, Gos M. Splicing mutations in human genetic disorders: examples, detection, and confirmation. J Appl Genet. 2018;59(3):253-68.
[8] Hong X, Scofield DG, Lynch M. Intron size, abundance, and distribution within untranslated regions of genes. Mol Biol Evol. 2006;23(12):2392-404.
[9] Beroud C, Carrie A, Beldjord C, Deburgrave N, Llense S, Carelle N, et al. Dystrophinopathy caused by mid-intronic substitutions activating cryptic exons in the DMD gene. Neuromuscular disorders: NMD. 2004;14(1):10-8.
[10] Zhang Z, Habara Y, Nishiyama A, Oyazato Y, Yagi M, Takeshima Y, et al. Identification of seven novel cryptic exons embedded in the dystrophin gene and characterization of 14 cryptic dystrophin exons. Journal of human genetics. 2007;52(7):607-17.
[11] Roberts RG, Bentley DR, Bobrow M. Infidelity in the structure of ectopic transcripts: a novel exon in lymphocyte dystrophin transcripts. Human mutation. 1993;2(4):293-9.
[12] Suminaga R, Takeshima Y, Adachi K, Yagi M, Nakamura H, Matsuo M. A novel cryptic exon in intron 3 of the dystrophin gene was incorporated into dystrophin mRNA with a single nucleotide deletion in exon 5. Journal of human genetics. 2002;47(4):196-201.
[13] Tran VK, Zhang Z, Yagi M, Nishiyama A, Habara Y, Takeshima Y, et al. A novel cryptic exon identified in the 3′ region of intron 2 of the human dystrophin gene. Journal of human genetics. 2005;50(8):425-33.
[14] Bouge AL, Murauer E, Beyne E, Miro J, Varilh J, Taulan M, et al. Targeted RNA-Seq profiling of splicing pattern in the DMD gene: exons are mostly constitutively spliced in human skeletal muscle. Sci Rep. 2017;7:39094.
[15] Yagi M, Takeshima Y, Wada H, Nakamura H, Matsuo M. Two alternative exons can result from activation of the cryptic splice acceptor site deep within intron 2 of the dystrophin gene in a patient with as yet asymptomatic dystrophinopathy. Human genetics. 2003;112(2):164-70.
[16] Dwi Pramono ZA, Takeshima Y, Surono A, Ishida T, Matsuo M. A novel cryptic exon in intron 2 of the human dystrophin gene evolved from an intron by acquiring consensus sequences for splicing at different stages of anthropoid evolution. Biochemical and biophysical research communications. 2000;267(1):321-8.
[17] Gualandi F, Rimessi P, Trabanelli C, Spitali P, Neri M, Patarnello T, et al. Intronic breakpoint definition and transcription analysis in DMD/BMD patients with deletion/duplication at the 5′ mutation hot spot of the DMD gene. Gene. 2006;370:26-33.
[18] Ishibashi K, Takeshima Y, Yagi M, Nishiyama A, Matsuo M. Novel cryptic exons identified in introns 2 and 3 of the human dystrophin gene with duplication of exons 8-11. The Kobe journal of medical sciences. 2006;52(3-4):61-75.
[19] Sedláčková J, Vondráček P, Hermanová M, Zámečník J, Hrubá Z, Haberlová J, et al. Point mutations in Czech DMD/BMD patients and their phenotypic outcome. Neuromuscular Disorders. 2009;19(11):749-53.
[20] Zaum AK, Stuve B, Gehrig A, Kolbel H, Schara U, Kress W, et al. Deep intronic variants introduce DMD pseudoexon in patient with muscular dystrophy. Neuromuscular disorders: NMD. 2017;27(7):631-4.
[21] Tuffery-Giraud S, Saquet C, Thorel D, Disset A, Rivier F, Malcolm S, et al. Mutation spectrum leading to an attenuated phenotype in dystrophinopathies. European journal of human genetics: EJHG. 2005;13(12):1254-60.
[22] Ferlini A, Muntoni F. The 5′ region of intron 11 of the dystrophin gene contains target sequences for mobile elements and three overlapping ORFs. Biochemical and biophysical research communications. 1998;242(2):401-6.
[23] Ferlini A, Galie N, Merlini L, Sewry C, Branzi A, Muntoni F. A novel Alu-like element rearranged in the dystrophin gene causes a splicing mutation in a family with X-linked dilated cardiomyopathy. American journal of human genetics. 1998;63(2):436-46.
[24] Nasim MT, Chernova TK, Chowdhury HM, Yue BG, Eperon IC. HnRNP G and Tra2beta: opposite effects on splicing matched by antagonism in RNA binding. Human molecular genetics. 2003;12(11):1337-48.
[25] Rimessi P, Fabris M, Bovolenta M, Bassi E, Falzarano S, Gualandi F, et al. Antisense modulation of both exonic and intronic splicing motifs induces skipping of a DMD pseudo-exon responsible for x-linked dilated cardiomyopathy. Human gene therapy. 2010;21(9):1137-46.
[26] Malueka RG, Takaoka Y, Yagi M, Awano H, Lee T, Dwianingsih EK, et al. Categorization of 77 dystrophin exons into

5 groups by a decision tree using indexes of splicing regulatory factors as decision markers. BMC genetics. 2012;13:23.

[27] Gurvich OL, Tuohy TM, Howard MT, Finkel RS, Medne L, Anderson CB, et al. DMD pseudoexon mutations: splicing efficiency, phenotype, and potential therapy. Annals of neurology. 2008;63(1):81-9.

[28] Tuffery-Giraud S, Saquet C, Chambert S, Claustres M. Pseudoexon activation in the *DMD* gene as a novel mechanism for Becker muscular dystrophy. Human mutation. 2003;21(6):608-14.

[29] Ikezawa M, Minami N, Takahashi M, Goto Y, Miike T, Nonaka I. Dystrophin gene analysis on 130 patients with Duchenne muscular dystrophy with a special reference to muscle mRNA analysis. Brain & development. 1998;20(3):165-8.

[30] Trabelsi M, Beugnet C, Deburgrave N, Commere V, Orhant L, Leturcq F, et al. When a mid-intronic variation of DMD gene creates an ESE site. Neuromuscular Disord. 2014;24(12):1111-7.

[31] Takeshima Y, Yagi M, Okizuka Y, Awano H, Zhang Z, Yamauchi Y, et al. Mutation spectrum of the dystrophin gene in 442 Duchenne/Becker muscular dystrophy cases from one Japanese referral center. Journal of human genetics. 2010;55(6):379-88.

[32] Ishmukhametova A, Van Kien PK, Mechin D, Thorel D, Vincent MC, Rivier F, et al. Comprehensive oligonucleotide array-comparative genomic hybridization analysis: new insights into the molecular pathology of the DMD gene. European Journal of Human Genetics. 2012;20(10):1096-100.

[33] Bovolenta M, Neri M, Fini S, Fabris M, Trabanelli C, Venturoli A, et al. A novel custom high density-comparative genomic hybridization array detects common rearrangements as well as deep intronic mutations in dystrophinopathies. BMC genomics. 2008;9.

[34] Gonorazky H, Liang M, Cummings B, Lek M, Micallef J, Hawkins C, et al. RNAseq analysis for the diagnosis of muscular dystrophy. Ann Clin Transl Neurol. 2016;3(1):55-60.

[35] Cummings BB, Marshall JL, Tukiainen T, Lek M, Donkervoort S, Foley AR, et al. Improving genetic diagnosis in Mendelian disease with transcriptome sequencing. Sci Transl Med. 2017;9(386):eaal5209.

[36] Baskin B, Gibson WT, Ray PN. Duchenne muscular dystrophy caused by a complex rearrangement between intron 43 of the DMD gene and chromosome 4. Neuromuscular Disorders. 2011;21:178-82

[37] Khelifi MM, Ishmukhametova A, Khau Van Kien P, Thorel D, Mechin D, Perelman S, et al. Pure intronic rearrangements leading to aberrant pseudoexon inclusion in dystrophinopathy: a new class of mutations? Human mutation. 2011;32(4):467-75.

[38] Greer K, Mizzi K, Rice E, Kuster L, Barrero RA, Bellgard MI, et al. Pseudoexon activation increases phenotype severity in a Becker muscular dystrophy patient. Molecular genetics & genomic medicine. 2015;3(4):320-6.

[39] Madden HR, Fletcher S, Davis MR, Wilton SD. Characterization of a complex Duchenne muscular dystrophy-causing dystrophin gene inversion and restoration of the reading frame by induced exon skipping. Human mutation. 2009;30(1):22-8.

[40] Goncalves A, Oliveira J, Coelho T, Taipa R, Melo-Pires M, Sousa M, et al. Exonization of an Intronic LINE-1 Element Causing Becker Muscular Dystrophy as a Novel Mutational Mechanism in Dystrophin Gene. Genes. 2017;8(10).

[41] Cagliani R, Sironi M, Ciafaloni E, Bardoni A, Fortunato F, Prelle A, et al. An intragenic deletion/inversion event in the DMD gene determines a novel exon creation and results in a BMD phenotype. Human genetics. 2004;115(1):13-8.

[42] Juan-Mateu J, Gonzalez-Quereda L, Rodriguez MJ, Verdura E, Lazaro K, Jou C, et al. Interplay between DMD point mutations and splicing signals in Dystrophinopathy phenotypes. PloS one. 2013;8(3):e59916.

[43] Deburgrave N, Daoud F, Llense S, Barbot JC, Recan D, Peccate C, et al. Protein- and mRNA-based phenotype-genotype correlations in DMD/BMD with point mutations and molecular basis for BMD with nonsense and frameshift mutations in the DMD gene. Human mutation. 2007;28(2):183-95.

[44] Sherry ST, Ward MH, Kholodov M, Baker J, Phan L, Smigielski EM, et al. dbSNP: the NCBI database of genetic variation. Nucleic Acids Res. 2001;29:308-11.

[45] Magri F, Del Bo R, D'Angelo MG, Govoni A, Ghezzi S, Gandossini S, et al. Clinical and molecular characterization of a cohort of patients with novel nucleotide alterations of the Dystrophin gene detected by direct sequencing. BMC medical genetics. 2011;12:37.

[46] Suzuki H, Aoki Y, Kameyama T, Saito T, Masuda S, Tanihata J, et al. Endogenous Multiple Exon Skipping and Back-Splicing at the DMD Mutation Hotspot. International Journal of Molecular Sciences. 2016;17(10).

[47] Burnette JM, Miyamoto-Sato E, Schaub MA, Conklin J, Lopez AJ. Subdivision of large introns in Drosophila by recursive splicing at nonexonic elements. Genetics. 2005;170(2):661-74.

[48] Sibley CR, Emmett W, Blazquez L, Faro A, Haberman N, Briese M, et al. Recursive splicing in long vertebrate genes. Nature. 2015;521(7552):371-5.

[49] Georgomanolis T, Sofiadis K, Papantonis A. Cutting a Long Intron Short: Recursive Splicing and Its Implications. Front Physiol. 2016;7:598.

[50] Suzuki H, Kameyama T, Ohe K, Tsukahara T, Mayeda A. Nested introns in an intron: evidence of multi-step splicing in a large intron of the human dystrophin pre-mRNA. FEBS Lett. 2013;587(6):555-61.

[51] Gazzoli I, Pulyakhina I, Verwey NE, Ariyurek Y, Laros JF, t Hoen PA, et al. Non-sequential and multi-step splicing of the dystrophin transcript. RNA Biol. 2016;13(3):290-305.

[52] Ke S, Chasin LA. Intronic motif pairs cooperate across exons to promote pre-mRNA splicing. Genome Biol. 2010;11:R84.

[53] Kim SW, Taggart AJ, Heintzelman C, Cygan KJ, Hull GH, Wang J, et al. Widespread intra-dependencies in the removal of introns from human transcripts. Nucleic Acids Res. 2017;45(16):9503-13.

[54] Shapiro MB, Senapathy P. RNA splice junctions of different classes of eukaryotes: sequence statistics and functional implications in gene expression. Nucleic Acids Res. 1987;15(17):7155-74.

[55] Sheth N, Roca X, Hastings ML, Roeder T, Krainer AR, Sachidanandam R. Comprehensive splice-site analysis using comparative genomics. Nucleic Acids Res. 2006;34(14):3955-67.