Human Molecular Genetics, 2023, Vol. 32, 15, 2455–2463
https://doi.org/10.1093/hmg/ddad072
Advance access publication date 5 May 2023
Original Article

Inhibition of nonsense-mediated mRNA decay may improve stop codon read-through therapy for Duchenne muscular dystrophy

Adi Amar-Schwartz[1,†], Yuval Cohen[1,†], Antony Elhaj[1], Vered Ben-Hur[1], Zahava Siegfried[1], Rotem Karni[1] and Talya Dor[2,*]

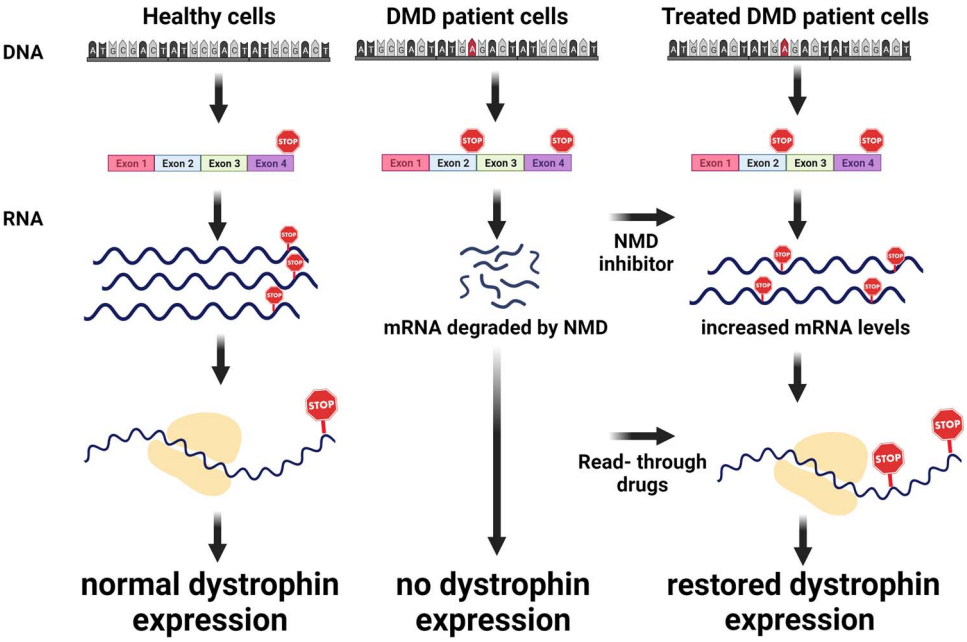
[1]Department of Biochemistry and Molecular Biology, The Institute for Medical Research Israel-Canada, Hebrew University-Hadassah Medical School, Jerusalem 9112001, Israel
[2]Pediatric Neurology Unit, Hadassah Medical Center, Faculty of Medicine, Hebrew University of Jerusalem, Jerusalem 9112001, Israel

*To whom correspondence should be addressed at: Neuropediatric Unit, Hadassah-Hebrew University Medical Center, Ya'Akov Man Street, Jerusalem 9112001, Israel. Tel: +972 25844751; Fax - 02 6778921; Email: talyad@hadassah.org.il
†These authors contributed equally to this work.

Abstract

Duchene muscular dystrophy (DMD) and Becker muscular dystrophy (BMD) are genetic neuromuscular disorders that affect skeletal and cardiac muscle resulting from mutations in the *dystrophin* gene (*DMD*), coding for dystrophin protein. Read-through therapies hold great promise for the treatment of genetic diseases harboring nonsense mutations, such as DMD/BMD, as they enable a complete translation of the affected mRNA. However, to date, most read-through drugs have not achieved a cure for patients. One possible explanation for the limitation of these therapies for DMD/BMD is that they rely on the presence of mutant dystrophin mRNAs. However, the mutant mRNAs containing premature termination codons are identified by the cellular surveillance mechanism, the nonsense-mediated mRNA decay (NMD) process, and are degraded. Here, we show that the combination of read-through drugs together with known NMD inhibitors have a synergistic effect on the levels of nonsense-containing mRNAs, among them the mutant dystrophin mRNA. This synergistic effect may enhance read-through therapies' efficacy and improve the current treatment for patients.

Graphical Abstract



Received: December 15, 2022. **Revised:** April 23, 2023. **Accepted:** April 28, 2023.

Introduction

Duchene muscular dystrophy (DMD) is a severe genetic neuromuscular disorder that affects skeletal and cardiac muscle resulting from mutations in the *dystrophin* gene (*DMD*), coding for dystrophin protein (1). Dystrophin protein plays a major structural role in muscle, linking the internal cytoskeleton to the extracellular matrix, thus protecting the sarcolemma from stress during muscle contraction (2). Dystrophin deficiency leads to myofiber necrosis as well as progressive muscle weakness and fatigability. The progression of the disease is rapid with DMD patients usually wheelchair-bound by the age of 12 and dead by their third decade due to respiratory or cardiac failure (3). Another dystrophin-associated muscular dystrophy, Becker muscular dystrophy (BMD), is a milder disease with slower progression (4). Patients with BMD have a later age of onset than DMD patients, and heart failure is the most common cause of death. The damaging effects of mutations in the *dystrophin* gene leading to BMD are less severe than those of DMD, due to the fact that dystrophin protein is produced in BMD patients, albeit in smaller amounts or with reduced function (1).

The *DMD* gene is one of the largest genes in the genome. The gene extends over 2.4 megabases on the X chromosome and contains 79 exons that are transcribed into a 14-kb mRNA and translated into a 427-kD protein (5). Mutations in the *dystrophin* gene usually create premature termination codons (PTCs), either by nonsense mutations that introduce a PTC or by duplication or deletion mutations that affect the reading frame (6). Depending on the cohorts studied, the fraction of DMD/BMD patients with deletions has been estimated to be 60–80%, 7–11% for duplications and 10–30% for more subtle DNA changes including nonsense mutations, splice-site mutations and small insertions/deletions that disrupt the reading frame (4). Approximately one-quarter of all patients with dystrophin gene mutations are classified as BMD. In conjunction with individuals diagnosed with DMD who possess nonsense mutations, comprising ∼10–11% of the patient population, a total of 35% of those affected by DMD/BMD could potentially benefit from mRNA stabilization therapy, either alone or in conjunction with read-through drugs. To date, the standard of care is aimed at inhibiting muscle inflammation by steroid treatment and, if possible, the restoration of dystrophin function. Currently, the only the US Food and Drug Administration (FDA)-approved treatments aimed at restoring dystrophin function are exon-skipping therapies (7–9) for exons 45, 51 and 53 and only 12%, 14–20% and 10–13% of the patients, respectively, can potentially benefit from this approach. This only represents a small fraction of DMD patients (10,11). A read-through therapy drug, PTC124 (Ataluren), has been tested both in primary muscle cells from humans and *mdx* mice expressing dystrophin nonsense alleles in a mouse model, with promising results. The read-through of dystrophin mRNA premature nonsense codons was evident in primary muscle cell cultures from DMD patients or *mdx* mice at all PTC124 concentrations tested. *Mdx* animals treated with PTC124 had dystrophin levels (assessed by staining) ∼20–25% that of muscles from wild-type mice (12). However, these reported results were mostly unreproducible in many labs worldwide. This drug was approved by the European Medicines Agency (EMA) and initially showed some success in slowing the progression of the disease. Subsequent Phase IIb and Phase III trials did not show a significant improvement in the 6-minute walk test after daily treatment with PTC124 for a period of 48 weeks according to the FDA (13). A possible explanation for the limited effectiveness of PTC124 is that read-through therapies rely on the presence of mutant dystrophin mRNAs. However, mutant mRNAs containing PTCs are identified by the cellular surveillance mechanism, the nonsense-mediated mRNA decay (NMD) process, and are degraded (14,15). The NMD pathway is a cytoplasmatic post-transcriptional translation-dependent surveillance mechanism that prevents the synthesis of proteins carrying PTCs. This phenomenon may diminish the success of PTC124 or other read-through drug treatments. Previous studies have provided evidence that the administration of NMD inhibitors can result in a significant increase in the expression of functional CFTR protein in cases where nonsense mutations are present (16,17). Synergy between read-through and NMD inhibition was tested using a mouse model of cystic fibrosis nonsense mutations (18). However, a comprehensive analysis of the combined effect of read-through agents and NMD inhibitors has yet to be conducted. Our working hypothesis is that inhibition of the NMD pathway can increase the amount of dystrophin mRNA, which can then be translated into a full-length protein using read-through drugs. Here, we present experiments using DMD/BMD patient-derived skin fibroblasts, and either genetic or pharmacological perturbation of NMD, to stabilize mutant dystrophin mRNA. These results support the possibility of using the inhibition of NMD to improve stop codon read-through therapy for the treatment of Duchenne muscular dystrophy.

Results

Dystrophin mRNA is unstable and degraded via NMD in DMD/BMD patient–derived skin fibroblasts

It is known that dystrophin mRNA levels are lower in DMD/BMD patient cells compared to healthy individuals (19). To investigate the levels of dystrophin mRNA in different DMD and BMD patients, we collected patient-derived skin fibroblasts from two BMD patients, one harboring an in-frame duplication of exons 2–7 (BMD dup ex 2–7) and one with an in-frame deletion of exons 45–49 (BMD del ex 45–49), and three DMD patients, with nonsense mutations in exon 11 (DMD stop ex 11), exon 53 (DMD stop 53) and exon 66 (DMD stop 66) (Table 1). We observed reduced dystrophin mRNA levels in the DMD patient-derived fibroblasts compared to three healthy fibroblast samples (Fig. 1A). We observed a range of dystrophin mRNA levels between the healthy individuals, but all are significantly higher than dystrophin mRNA levels in DMD patients. In addition, we observed higher mRNA levels in BMD patients compared to DMD patients, as expected. In order to determine if these reduced dystrophin mRNA levels are due to NMD, we exposed the cells to an indirect NMD inhibitor, cycloheximide (CHX) (20). We observed a significant increase in dystrophin mRNA levels in all patient-derived fibroblasts after treatment with CHX, regardless of the mutation. The increase in dystrophin mRNA levels in healthy samples can be explained by dystrophin transcripts that are potential NMD targets (Fig. 1B–G, Supplementary Material, Fig. S1). We conclude that mutated dystrophin mRNA undergoes NMD and that the inhibition of NMD could stabilize the dystrophin mRNA.

Dystrophin mRNA is stabilized by either genetic or pharmacological perturbation of NMD

Previous studies have identified UPF1 as a crucial component of the NMD machinery and have shown that its downregulation inhibits the NMD process (14). To examine whether the downregulation of UPF1 can stabilize dystrophin mRNA in patient-derived

Table 1. BMD/DMD patient-derived fibroblasts used in this study

Donor	Mutation type	Exon	Mutation	Age (Year)	6 min walk (m)
BMD dup ex 2–7	Duplication	Exons 2–7		7.5	520
BMD del ex 45–49	Deletion	Exons 45–49		7	540
DMD stop ex 11	Nonsense	Exon 11	c.907>T; p.303Q>STOP	7.5	460
DMD stop ex 53	Nonsense	Exon 53	c.7683G>A; p.2561W>STOP	3.5	NA
DMD stop ex 66	Nonsense	Exon 66	c.9645C>G; p.3215Y>STOP	4.5	NA

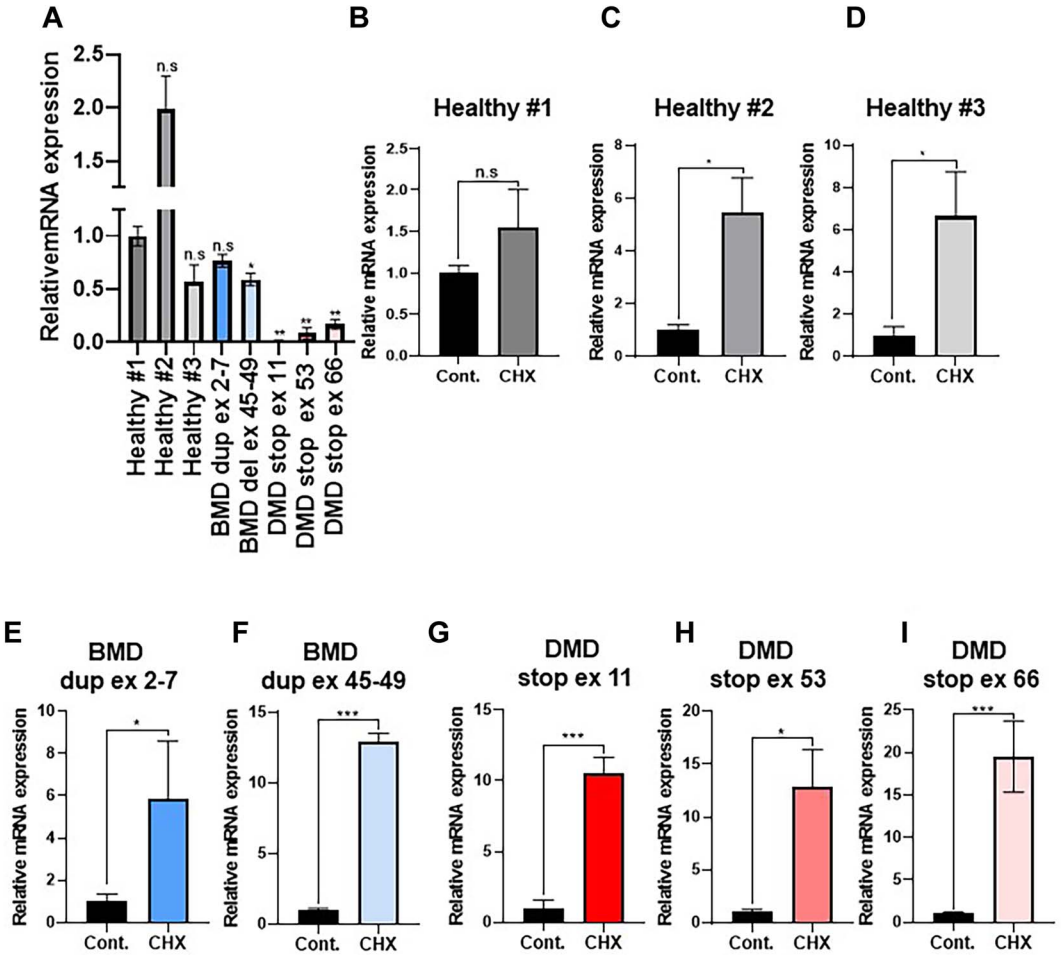


Figure 1. Dystrophin mRNA is unstable in DMD and BMD patient-derived skin fibroblasts. (**A**) q-RT-PCR of dystrophin mRNA levels from healthy, BMD and DMD patient-derived skin fibroblasts using primers specific for exons 65–66 of *DMD* gene. (**B**–**I**) q-RT-PCR of dystrophin mRNA levels in healthy, BMD and DMD patient-derived skin fibroblasts, with or without exposure to cycloheximide (CHX 9 $\mu g/\mu l$) for 24 hours, using primers specific for exons 65–66 of *DMD* gene. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. *P*-values were calculated using Student's *t*-test (two-tailed). Data represent the means ± SD of three biological repeats.

skin fibroblasts, we transfected patient-derived skin fibroblasts (DMD stop 53 and BMD dup ex 2–7) with two different siRNAs against UPF1 (Fig. 2A and E). Dystrophin mRNA was stabilized in DMD/BMD patient-derived skin fibroblasts transfected with either siUPF1 (Fig. 2B and F). To validate that siUPF1 inhibits global NMD, we measured the mRNA levels of two known NMD targets—ATF4 and PISD (21). The transfection of either siUPF1 in DMD/BMD patient-derived skin fibroblasts stabilized both known NMD-prone transcripts (Fig. 2C, D, G and H).

To date, no read-through drug is available in the USA for DMD patients. Read-through drugs (such as PTC124 (12)) have a limited effect for some patients (5). We hypothesized that PTC-containing mRNAs are unstable and undergo degradation via the NMD pathway. We therefore wanted to test if the combination of read-through drugs together with known NMD inhibitors will have a synergistic effect on the levels of nonsense-containing mRNAs. To this end, we examined the effect of two NMD inhibitors, 5′-Aza cytidine (5-aza) (22) and Amlexanox (Amx) (23); both are FDA-approved drugs for different indications. 5-aza and Amx were tested alone and in combination with read-through drug PTC124 for their effect on dystrophin mRNA stability in DMD patient-derived skin fibroblasts (DMD stop ex 53 and BMD dup ex 2–7). As can be seen in Figure 3, we observed an increase in dystrophin mRNA (Fig. 3A and D), as well as other NMD-prone mRNAs (ATF4 and PISD) (Fig. 3B, C, E, and F), with all treatments. We did not detect a significant difference in the effect of the various NMD

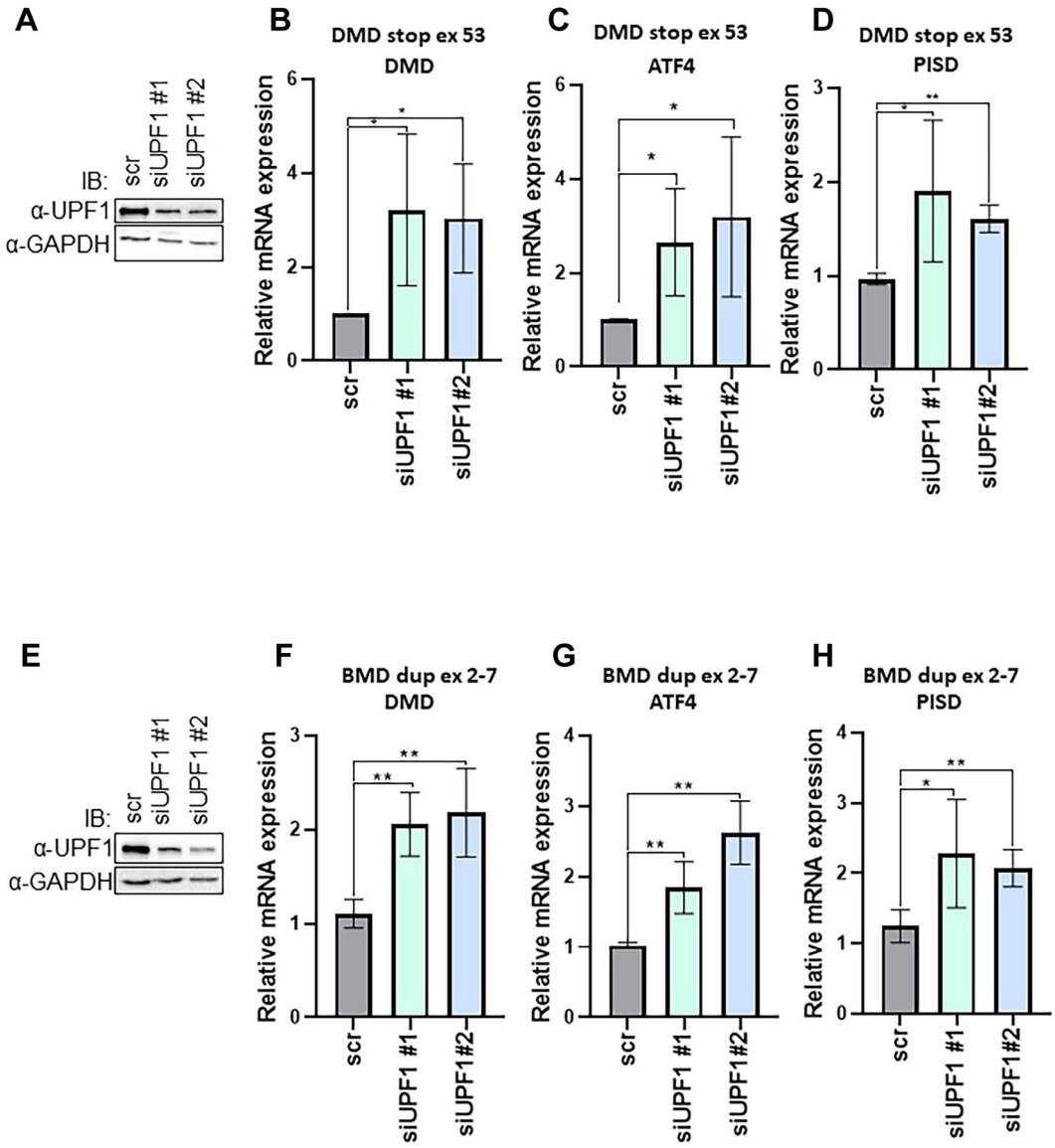


Figure 2. Knockdown of UPF1 elevates dystrophin mRNA levels in DMD and BMD patient-derived skin fibroblasts. (**A**) DMD stop ex 53 patient-derived skin fibroblasts were transfected with two different siRNAs against UPF1 (siUPF1 #1,2). 48 hours after transfection cells were harvested for western blotting. (**B**–**D**). q-RT-PCR of dystrophin mRNA (B) and known NMD-prone targets (ATF4 and PISD) (C, D) in cells described in (A). (**E**) BMD dup ex 2–7 patient-derived skin fibroblasts were transfected with two different siRNAs against UPF1 (siUPF1 #1,2). 48 hours after transfection cells were harvested for western blotting. (**F**–**H**) q-RT-PCR of dystrophin mRNA (**f**) and known NMD-prone targets (ATF4 and PISD) (G, H) in cells described in (E). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. *P*-values were calculated using Student's *t*-test (two-tailed). Data represent the means ± SD of three biological repeats.

inhibitors (5-aza and Amx). We did not expect to detect a greater effect on mRNA levels by the combination of NMD inhibitors and read-through drugs since read-through drugs mainly affect protein levels. Although these experiments showed an increase in NMD-prone transcripts (ATF4 and PISD), we were not able to determine the effect of the drugs on protein levels of these genes due to low levels of protein expression.

Combination of 5-aza and PTC124 increase SRSF1 and SRSF6 protein levels

Dystrophin is a skeletal muscle protein and is expressed mainly in muscle cells (6). In order to examine the potential therapeutic effect of a combination of the NMD inhibitor and read-through therapy on protein levels, we needed to identify an NMD-prone mRNA of a ubiquitously expressed protein that can be detected in fibroblasts. SR proteins are members of a family of splicing factors that autoregulate their expression by alternative splicing—coupled to NMD (24,25). We focused on two SR proteins: SRSF6, which contains a poison cassette exon that introduces an in-frame PTC, and SRSF1, which undergoes alternative splicing of its 3' UTR that leads to the introduction of a new exon–exon junction that marks the original stop codon as a PTC. The splicing in the 3' UTR of SRSF1 does not affect the protein-coding sequence and therefore does not alter the protein size, while the included poison cassette exon in SRSF6 is in the protein coding sequence resulting in a truncated protein (24) (Fig. 4A). We exposed DMD patient-derived fibroblasts to either 5-aza alone or in combination with PTC124 for 72 hours and determined SRSF1 and SRSF6 mRNA and protein levels. Both SRSF1 and SRSF6 mRNA levels were increased after exposure to 5-aza, PTC124 or in combination (Fig. 4B and C). A combination of 5-aza with PTC124 significantly increased the full-length protein

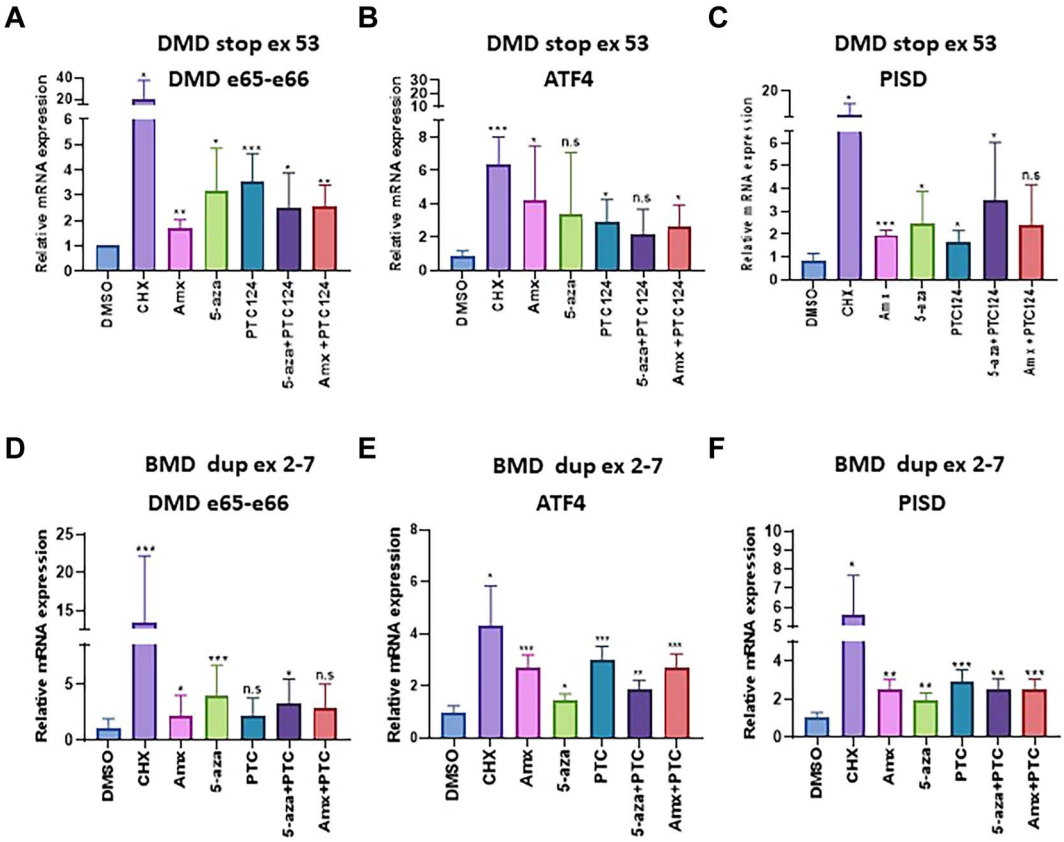


Figure 3. Treatment of DMD and BMD patient-derived skin fibroblasts with read-through and NMD-inhibiting compounds. (**A–F**) DMD stop ex 53 patient-derived skin fibroblasts (A–C) or BMD dup ex 2–7 patient-derived skin fibroblasts (D–F) were exposed to either DMSO, cycloheximide (CHX 9 μg/μl), Amlexanox (Amx, 25 μM), 5-azacytidine (5-aza, 4 μM) or PTC124 (5 μ M) alone, or in combination (5-aza + PTC124 or Amx + PTC124). Cells were harvested 72 hours later and RNA analyzed by q-RT-PCR to determine mRNA levels of dystrophin (A, D) and two known NMD-prone targets (ATF4 and PISD) (B, C, E, F). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. *P*-values were calculated using Student's *t*-test (two-tailed). Data represent the means ± SD of three biological repeats.

levels of SRSF6, while treatment with either drug alone did not (Fig. 4D and F, Supplementary Material, Fig. S2). In contrast, SRSF1 protein levels increased after treatment with either 5-aza alone or in combination with PTC124 (Fig. 4E and F and Supplementary Material, Fig. S2). One possible explanation for this could be that the location of the PTC mutation in SRSF1 is in the 3'UTR of the transcript (Fig. 4A). These results suggest that a combination of the NMD inhibitor with read-through therapy may increase the efficacy of the treatment, increasing protein levels of NMD-prone transcripts.

Discussion

Approximately 10–11% of all genetic disease-causing mutations, including DMD, are nonsense mutations that cause PTCs. Read-through therapies hold great promise for the treatment of genetic diseases harboring nonsense mutations as they enable a complete translation of the affected gene. However, to date, most read-through drugs have failed in the clinic (26–28). Since PTCs lead to the degradation of the mutant mRNA by the NMD pathway (15), we hypothesized that read-through therapies fail in the clinic due to degradation of the substrate mRNA by NMD. Thus, NMD inhibition is required for effective read-through therapy. To test this hypothesis, we examined the stability of dystrophin mRNA in skin fibroblasts from several DMD/BMD patients, with and without NMD inhibitors. Moreover, we combined NMD inhibitors with the read-through drug PTC124 to examine if the combination of these drugs enhances the protein production of NMD-prone transcripts of SR proteins.

One important point to consider is that read-through therapy introduces an amino acid at the PTC position that might be different from the original encoded amino acid. The identity of the inserted amino acid plays a critical role in determining the characteristics of the resulting complete protein, such as protein stability and/or function. This is a crucial factor that determines the outcome of any strategy aimed at suppressing nonsense mutations. Specifically, for PTC124, it was reported that it does indeed promote the insertion of near-cognate RNAs at nonsense codons and that this yields functional proteins (29–31). In theory, enhanced read-through might cause some escape from NMD if the process is rapid and especially if it interferes with the first round of translation. In some cases, read-through drugs may enhance the stability of NMD-prone transcripts (Figs 3 and 4). We believe that this mechanism is not very significant biologically and most transcripts are still degraded by NMD even in the presence of read-through drugs. This is, in our opinion, the reason these drugs show very minor clinical benefit.

It has been previously reported that both wild-type and mutant dystrophin mRNAs are unstable (32). Surprisingly, even though there are no PTCs or out-of-frame indels in some BMD patients, dystrophin mRNA in these patients is unstable and can be stabilized by pharmacological NMD inhibition, cycloheximide

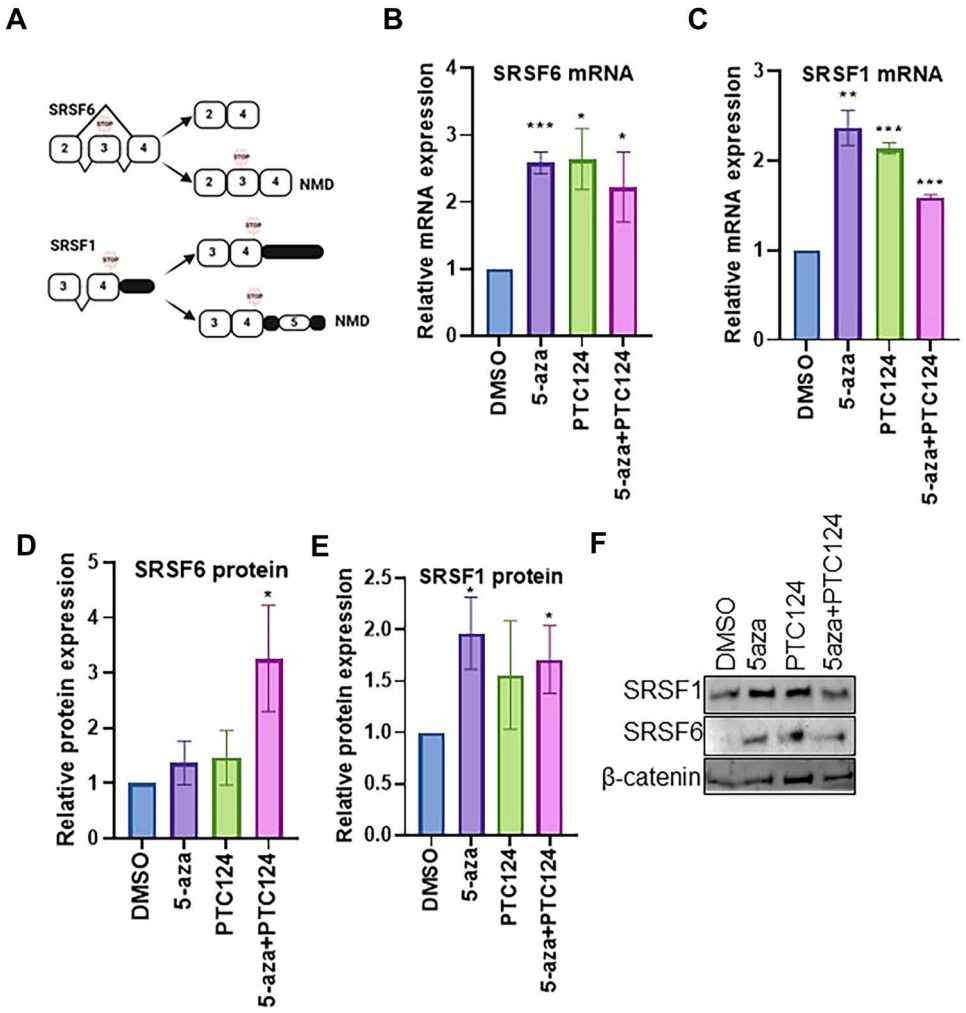


Figure 4. Treatment of DMD patient-derived skin fibroblasts with read-through and NMD-inhibiting compounds elevates protein levels of NMD-prone transcripts. (**A**) Schema representing the alternative splicing of SRSF1 and SRSF6 transcripts. The inclusion of a poison exon in SRSF6 leads to an unstable transcript that undergoes degradation via NMD. The splicing of SRSF1 results in an exon junction downstream to the natural stop codon leading to an unstable transcript. Intron retention in the 3'UTR will produce a stable transcript. (**B**, **C**) DMD stop ex 53 patient-derived skin fibroblasts were exposed to either DMSO, 5-azacytidine (5-aza, 4 μM), PTC124 (5 μM) alone or in combination (5-aza + PTC124) for 72 hours. RNA was analyzed by q-RT-PCR to determine mRNA levels of SRSF6 (B) and SRSF1 (C). (**D–F**) SRSF1 and SRSF6 protein levels in cells described in (B) were measured by western blot analysis (representative blot f, Supplementary Material, Fig. S2) and quantified (D, E). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. *P*-values were calculated using Student's *t*-test (two-tailed). Data represent the means ± SD of three biological repeats.

treatment (Fig. 1) or genetic manipulation, the knockdown of the essential NMD factor UPF1 (Fig. 2). Moreover, NMD inhibitors and read-through drugs enhanced dystrophin mRNA stability, although these drugs were less effective than cycloheximide treatment (Fig. 3). These results suggest that improved drugs can be developed to be more potent dystrophin mRNA stabilizers. To examine the effect of combining NMD inhibitors with read-through drugs, we examined the production of two SR proteins, SRSF1 and SRSF6. These proteins were chosen due to the fact that, unlike dystrophin, which is only produced in muscle cells, they are ubiquitously expressed (33,34) and prone to NMD by an autoregulatory negative feedback loop (24). Both of these proteins contain an alternatively spliced poison exon/cassette, which introduces a PTC when included. SRSF1 has a poison cassette in the 3′ untranslated region (UTR) resulting in a premature stop codon in the 3' UTR, thus not altering protein size. In contrast, SRSF6 has an alternative poison exon in the coding region. The inclusion of this poison exon results in a truncated protein (Fig. 4A). Our results suggest that NMD inhibition enhances the production of both SRSF1 and SRSF6 mRNA levels (Fig. 4B and C). The inhibition of NMD by 5-aza increased protein levels of only SRSF1, due to the location of the poison exon in the 3' UTR, while the combination of NMD inhibition with read-through PTC124 enhanced protein levels of both SRSF1 and SRSF6 (Fig. 4D–F and Supplementary Material, Fig. S2). These results demonstrate that the principle of combining NMD inhibitors and read-through drugs may benefit read-through therapies and enhance efficacy. The next step will be to trans-differentiate DMD patient-derived fibroblasts into myocyte cells and examine both dystrophin mRNA and protein levels after the combined treatment.

Another important finding that emerges from these experiments is that NMD inhibition and read-through drugs enhance the production of splicing factors (SRSF1 and SRSF6) that affect the splicing of hundreds of pre-mRNAs (34–38). Further investigation is required to determine the clinical impact of such splicing perturbations. The development of improved and more specific

NMD inhibitors, in combination with read-through drugs, will enable the treatment of patients with genetic diseases harboring nonsense mutations.

Materials and Methods

Patient-derived skin fibroblast samples

Studies were approved by the Hadassah Medical Center Helsinki/IRB Committee (study HMO-0650-20 and study b14-14/02/06) and conducted according to the Declaration of Helsinki protocol. Written informed consent was obtained from all participants. All procedures were conducted according to the protocol approved by the World Medical Association Declaration of Helsinki.

Cells were cultured in Eagle's Minimum Essential Medium (EMEM) supplemented with 1% L-glutamine and 10% fetal bovine serum (FBS) (Biological Industries).

Cell lines

Healthy #2 (1092sk (ATCC: CRL-2114)) and Healthy #3 (1077sk (ATCC: CRL-2094)) were purchased from ATCC. The cells were cultured in EMEM supplemented with 1% L-glutamine and 10% FBS (Biological Industries).

Quantitative RT-PCR

Total RNA was isolated using the TRI Reagent (Sigma #T9424). For cDNA synthesis, 1 μg of total RNA was reverse-transcribed to cDNA with the iScript™ cDNA Synthesis kit (Bio-Rad #1708891). The resulting cDNA was diluted 5–25-fold before use. Quantitative PCR was conducted on 1 μL of cDNA using iTaq universal SYBR Green Supermix (Applied BIO-RAD #1725124) using the CFX96 (Bio-Rad) real-time PCR machine. Samples were normalized to GAPDH expression (the value was arbitrarily set to 1). Samples were compared to a standard curve, which was established by serial dilutions of a known concentration of cDNA. Primers are listed below.

Primers		
	Forward	**Reverse**
e65-e66 (DMD)	CTGGCTGCTGAATGTT-TATGATA	CTTGC-CACTTGCTTGAAAAG
DMD-233	TGGGCCTCCTTCTGCAT-GAT	TTTAGACTCCTGTA-CAAATT
GAPDH	TGAGCTTGACAAAGTG-GTCG	GGCTCTCCAGAACAT-CATCC
ATF4	ATGTCCCCCTTCGACCA	CCATTTTCTCCAACATC-CAATC
PISD	TCCCTGATGTCAGT-GAACCCT	TGGTGTGCGTCAC-GAAGC
SRSF1 NMD	GGAGGCAATGGTTTG-GATT	CGCTCCATGAATCCTG-GTAA
SRSF6 NMD	GACGGCTACAGCTACG-GAAG	ATAGGGCAAGGGTCACA-CAA

Western blotting

Cells were lysed in Laemmli buffer, and lysates were separated in 8% SDS-PAGE gels and then transferred to PVDF membranes (Invitrogen). The membranes were probed with primary antibodies: UPF1 (1:1000, Abcam #133504), GAPDH (1: 5000, Sigma #G9545), SRSF1 (1:200, mAb AK96 culture supernatant), SRSF6 (1:500, mAb 8–1-28 culture supernatant), tubulin (1:5000, Abcam #ab6160), β-catenin (1:10 000, Abcam #ab6302) and β-actin (1:200, Santa Cruz #sc-1616). The secondary antibodies were HRP-conjugated goat anti-mouse (#AB_10015289), goat anti-rabbit (#AB_2307391), donkey anti-rat (#AB_2340639) or donkey anti-goat (#AB_2340390) IgG (H + L) (1:10 000, Jackson Laboratories). Quantification was performed using Image Lab 5.0 (Bio-Rad).

siRNA treatment

Double-stranded siRNAs (Sigma) were used at specified concentrations to deplete UPF1 from cells. siRNA Universal Negative Control (Sigma) was used as a control at specified concentrations. Lipofectamine 2000 reagent (Invitrogen 11 668 019) was used for transfection as per the manufacturer's instructions.

siRNA	Sequence
siUPF1 #1	GAGAUAUGCCUGCGGUACA[dT][dT] UGUACCGCAGGCAUAUCUC[dT][dT]
siUPF1 #2	AAUGGAGCGGAACUGCAUC[dT][dT] GAUGCAGUUCCGCUCCAUU[dT][dT]

Treatment with NMD inhibitor compounds

Primary skin fibroblasts were seeded at 70% confluence; 24 hours later, cells were treated with either cycloheximide 9 μg/μl (Sigma, cat #C7698) for 16 hours or Amlexanox 25 μM (Abcam, cat #152825), 5-Azacytidine 4 μM (Sigma, cat #A2385) or PTC124 5 μM (AdooQ, cat #A10758) for 72 h. Cells were harvested either for RNA using TRI Reagent (Sigma) or protein analysis using Laemmli buffer.

Acknowledgement

We thank the genetic unit of Hadassah medical center for extraction and maintenance of patient derived fibroblasts.

Supplementary Material

Supplementary Material is available at *HMG* online.

Data Availability

The authors confirm that the data supporting the findings of this study are available within the article and its Supplementary material. Raw data that support these findings are available from the corresponding author, upon reasonable request.

Conflict of Interest statement. R.K. is a consultant for RNAble and SKIP therapeutics (https://www.futurx.co.il/portfolio/). The remaining authors have no relevant financial or nonfinancial interests to disclose.

Funding

Little Steps association in Israel for children with Duchenne muscular dystrophy (DMD)/Becker muscular dystrophy (BMD), by A.D.I. (Association Duchenne Israel) and by Israel Precision Medicine Program (IPMP) ISF Research Grant No. 2039/20 (to R.K.). Figures were created with https://biorender.com/.

Author Contributions

A.A.-S., Y.C., R.K. and T.D. conceived the project. A.A.-S., Y.C. and AE performed the experiments. T.D. collected samples. A.A.-S., Y.C., Z.S., T.D. and R.K. wrote the paper with input from all the authors.

References

1. Duan, D., Goemans, N., Takeda, S., Mercuri, E. and Aartsma-Rus, A. (2021) Duchenne muscular dystrophy. *Nat. Rev. Dis. Prim.*, **71**(7), 1–19.
2. Petrof, B.J., Shrager, J.B., Stedman, H.H., Kelly, A.M. and Sweeney, H.L. (1993) Dystrophin protects the sarcolemma from stresses developed during muscle contraction. *Proc. Natl. Acad. Sci. U. S. A.*, **90**, 3710–3714.
3. Nowak, K.J. and Davies, K.E. (2004) Duchenne muscular dystrophy and dystrophin: pathogenesis and opportunities for treatment. *EMBO Rep.*, **5**, 872–876.
4. Duan, D., Goemans, N., Takeda, S., Mercuri, E. and Aartsma-Rus, A. (2021) Duchenne muscular dystrophy. *Nat. Rev. Dis. Prim.*, **71**(7), 1–19.
5. Pichavant, C., Aartsma-Rus, A., Clemens, P.R., Davies, K.E., Dickson, G., Takeda, S., Wilton, S.D., Wolff, J.A., Wooddell, C.I., Xiao, X. *et al.* (2011) Current status of pharmaceutical and genetic therapeutic approaches to treat DMD. *Mol. Ther.*, **19**, 830–840.
6. Muntoni, F., Torelli, S. and Ferlini, A. (2003) Dystrophin and mutations: one gene, several proteins, multiple phenotypes. *Lancet Neurol.*, **2**, 731–740.
7. Syed, Y.Y. (2016) Eteplirsen: first global approval. *Drugs*, **7617**(76), 1699–1704.
8. Heo, Y.A. (2020) Golodirsen: first approval. *Drugs*, **80**, 329–333.
9. Santorelli, M., Politano, L., Eser, G. and Topalo, H. (2022) Current outline of exon skipping trials in Duchenne muscular dystrophy. *Genes*2022, **13**, 1241.
10. Matsuo, M. (2021) Antisense oligonucleotide-mediated exon-skipping therapies: precision medicine spreading from Duchenne muscular dystrophy. *Japan Med. Assoc. J.*, **4**, 232–240.
11. Roshmi, R.R. and Yokota, T. (2019) Viltolarsen for the treatment of Duchenne muscular dystrophy. *Drugs Today*, **55**, 627–639.
12. Welch, E.M., Barton, E.R., Zhuo, J., Tomizawa, Y., Friesen, W.J., Trifillis, P., Paushkin, S., Patel, M., Trotta, C.R., Hwang, S. *et al.* (2007) PTC124 targets genetic disorders caused by nonsense mutations. *Nature*, **447**, 87–91.
13. Lim, K.R.Q. and Yokota, T. (2023) Current strategies of muscular dystrophy therapeutics: an overview. *Methods Mol. Biol.*, **2587**, 3–30.
14. Maquat, L.E. (2004) (2004) nonsense-mediated mRNA decay: splicing, translation and mRNP dynamics. *Nat. Rev. Mol. Cell Biol.*, **52**(5), 89–99.
15. Isken, O. and Maquat, L.E. (2008) The multiple lives of NMD factors: balancing roles in gene and genome regulation. *Nat. Rev. Genet.*, **9**, 699–712.
16. Martins-Dias, P. and Romão, L. (2021) Nonsense suppression therapies in human genetic diseases. *Cell. Mol. Life Sci.*, **78**, 4677–4701.
17. Linde, L., Boelz, S., Nissim-Rafinia, M., Oren, Y.S., Wilschanski, M., Yaacov, Y., Virgilis, D., Neu-Yilik, G., Kulozik, A.E., Kerem, E. *et al.* (2007) Nonsense-mediated mRNA decay affects nonsense transcript levels and governs response of cystic fibrosis patients to gentamicin. *J. Clin. Invest.*, **117**, 683–692.
18. McHugh, D.R., Cotton, C.U. and Hodges, C.A. (2020, 2021) Synergy between readthrough and nonsense mediated decay inhibition in a murine model of cystic fibrosis nonsense mutations. *Int. J. Mol. Sci.*, **22**, 344.
19. García-Rodríguez, R., Hiller, M., Jiménez-Gracia, L., van der Pal, Z., Balog, J., Adamzek, K., Aartsma-Rus, A. and Spitali, P. (2020) Premature termination codons in the DMD gene cause reduced local mRNA synthesis. *Proc. Natl. Acad. Sci. U. S. A.*, **117**, 15664–16464.
20. Schneider-Poetsch, T., Ju, J., Eyler, D.E., Dang, Y., Bhat, S., Merrick, W.C., Green, R., Shen, B. and Liu, J.O. (2010) Inhibition of eukaryotic translation elongation by cycloheximide and lactimidomycin. *Nat. Chem. Biol.*, **63**(6), 209–217.
21. Nickless, A., Jackson, E., Marasa, J., Nugent, P., Mercer, R.W., Piwnica-Worms, D. and You, Z. (2014) Intracellular calcium regulates nonsense-mediated mRNA decay. *Nat. Med.*, **20**, 961–966.
22. Bhuvanagiri, M., Lewis, J., Putzker, K., Becker, J.P., Leicht, S., Krijgsveld, J., Batra, R., Turnwald, B., Jovanovic, B., Hauer, C. *et al.* (2014) 5-azacytidine inhibits nonsense-mediated decay in a MYC-dependent fashion. *EMBO Mol Med*, **6**, 1593–1610.
23. Gonzalez-Hilarion, S., Beghyn, T., Jia, J., Debreuck, N., Berte, G., Mamchaoui, K., Mouly, V., Gruenert, D.C., Déprez, B. and Lejeune, F. (2012) Rescue of nonsense mutations by amlexanox in human cells. *Orphanet J. Rare Dis.*, **7**, 58.
24. Lareau, L.F., Inada, M., Green, R.E., Wengrod, J.C. and Brenner, S.E. (2007) Unproductive splicing of SR genes associated with highly conserved and ultraconserved DNA elements. *Nature*, **446**, 926–929.
25. Sun, S., Zhang, Z., Sinha, R., Karni, R. and Krainer, A.R. (2010) SF2/ASF autoregulation involves multiple layers of post-transcriptional and translational control. *Nat. Struct. Mol. Biol.*, **173**(17), 306–312.
26. Konstan, M.W., VanDevanter, D.R., Rowe, S.M., Wilschanski, M., Kerem, E., Sermet-Gaudelus, I., DiMango, E., Melotti, P., McIntosh, J. and De Boeck, K. (2020) Efficacy and safety of ataluren in patients with nonsense-mutation cystic fibrosis not receiving chronic inhaled aminoglycosides: the international, randomized, double-blind, placebo-controlled Ataluren confirmatory trial in cystic fibrosis (ACT CF). *J. Cyst. Fibros.*, **19**, 595–601.
27. Mercuri, E., Muntoni, F., Osorio, A.N., Tulinius, M., Buccella, F., Morgenroth, L.P., Gordish-Dressman, H., Jiang, J., Trifillis, P., Zhu, J. *et al.* (2020) Safety and effectiveness of ataluren: comparison of results from the STRIDE registry and CINRG DMD natural history study. *J. Comp. Eff. Res.*, **9**, 341–360.
28. McDonald, C.M., Campbell, C., Torricelli, R.E., Finkel, R.S., Flanigan, K.M., Goemans, N., Heydemann, P., Kaminska, A., Kirschner, J., Muntoni, F. *et al.* (2017) Ataluren in patients with nonsense mutation Duchenne muscular dystrophy (ACT DMD): a multicentre, randomised, double-blind, placebo-controlled, phase 3 trial. *Lancet (London, England)*, **390**, 1489–1498.
29. Roy, B., Friesen, W.J., Tomizawa, Y., Leszyk, J.D., Zhuo, J., Johnson, B., Dakka, J., Trotta, C.R., Xue, X., Mutyam, V. *et al.* (2016) Ataluren stimulates ribosomal selection of near-cognate tRNAs to promote nonsense suppression. *Proc. Natl. Acad. Sci. U. S. A.*, **113**, 12508–12513.
30. Politano, L. (2021) Read-through approach for stop mutations in Duchenne muscular dystrophy. An update. *Acta Myol.*, **40**, 43–50.
31. Xue, X., Mutyam, V., Thakerar, A., Mobley, J., Bridges, R.J., Rowe, S.M., Keeling, K.M. and Bedwell, D.M. (2017) Identification of the amino acids inserted during suppression of CFTR nonsense

mutations and determination of their functional consequences. *Hum. Mol. Genet.*, **26**, 3116–3129.
32. Spitali, P., Van Den Bergen, J.C., Verhaart, I.E.C., Wokke, B., Janson, A.A.M., Van Den Eijnde, R., Den Dunnen, J.T., Laros, J.F.J., Verschuuren, J.J.G.M., Peter, P.A. *et al.* (2013) DMD transcript imbalance determines dystrophin levels. *FASEB J.*, **27**, 4909–4916.
33. Hanamura, A., Cáceres, J.F., Mayeda, A., Franza, B.R. and Krainer, A.R. (1998) Regulated tissue-specific expression of antagonistic pre-mRNA splicing factors. *RNA*, **4**, 430–444.
34. Twyffels, L., Gueydan, C. and Kruys, V. (2011) Shuttling SR proteins: more than splicing factors. *FEBS J.*, **278**, 3246–3255.
35. Ben-Hur, V., Denichenko, P., Siegfried, Z., Maimon, A., Krainer, A., Davidson, B. and Karni, R. (2013) S6K1 alternative splicing modulates its oncogenic activity and regulates mTORC1. *Cell Reports*, **3**, 103–115.
36. Cohen-Eliav, M., Golan-Gerstl, R., Siegfried, Z., Andersen, C.L., Thorsen, K., Ørntoft, T.F., Mu, D. and Karni, R. (2013) The splicing factor SRSF6 is amplified and is an oncoprotein in lung and colon cancers. *J. Pathol.*, **229**, 630–639.
37. Karni, R., De Stanchina, E., Lowe, S.W., Sinha, R., Mu, D. and Krainer, A.R. (2007) The gene encoding the splicing factor SF2/ASF is a proto-oncogene. *Nat. Struct. Mol. Biol.*, **14**, 185–193.
38. Das, S. and Krainer, A.R. (2014) Emerging functions of SRSF1, splicing factor and oncoprotein, in RNA metabolism and cancer. *Mol. Cancer Res.*, **12**, 1195–1204.