Blocking of an intronic splicing silencer completely rescues IKBKAP exon 20 splicing in familial dysautonomia patient cells

Gitte H. Bruun, Jeanne M.V. Bang, Lise L. Christensen, Sabrina Brøner, Ulrika S.S. Petersen, Barbara Guerra, Alexander G.B. Grønning, Thomas K. Doktor and Brage S. Andresen

Department of Biochemistry and Molecular Biology and The Villum Center for Bioanalytical Sciences, University of Southern Denmark, Campusvej 55, DK-5230 Odense M, Denmark

Received July 01, 2017; Revised April 24, 2018; Editorial Decision April 26, 2018; Accepted April 30, 2018

ABSTRACT

Familial dysautonomia (FD) is a severe genetic disorder causing sensory and autonomic dysfunction. It is predominantly caused by a c.2204+6T>C mutation in the IKBKAP gene. This mutation decreases the 5′ splice site strength of IKBKAP exon 20 leading to exon 20 skipping and increased amounts of full-length IKAP protein. We identified a binding site for the splicing regulatory protein hnRNP A1 downstream of the IKBKAP exon 20 5′-splice site. We show that hnRNP A1 binds to this splicing regulatory element (SRE) and that two previously described inhibitory SREs inside IKBKAP exon 20 are also bound by hnRNP A1. Knockdown of hnRNP A1 in FD patient fibroblasts increases IKBKAP exon 20 inclusion demonstrating that hnRNP A1 is a negative regulator of IKBKAP exon 20 splicing. Furthermore, by mutating the SREs in an IKBKAP minigene we show that all three SREs cause hnRNP A1-mediated exon repression. We designed splice switching oligonucleotides (SSO) that blocks the intronic hnRNP A1 binding site, and demonstrate that this completely rescues splicing of IKBKAP exon 20 in FD patient fibroblasts and increases the amounts of IKAP protein. We propose that this may be developed into a potential new specific treatment of FD.

INTRODUCTION

Familial dysautonomia (FD) (OMIM no. 223900) is a rare fatal recessive genetic disorder nearly completely restricted to Ashkenazi Jews. FD causes sensory and autonomic dysfunction due to abnormal development and progressive degeneration of the sensory and autonomic nervous system. The disease is most frequently caused by an intronic T>C mutation at position 6 in intron 20 (c.2204+6T>C) of the IKBKAP gene, which has a carrier frequency of ∼1/32 in Ashkenazi Jews. The IKBKAP c.2204+6T>C mutation causes exon 20 skipping in a tissue-specific manner with high levels of exon skipping in neuronal tissue, while e.g. lymphoblasts show less exon 20 skipping. Skipping of IKBKAP exon 20 leads to a frame-shift in the reading frame and introduction of a premature stop codon (2,4), and hence the IKBKAP transcript is a candidate for degradation by the nonsense-mediated decay (NMD) pathway.

mRNA splicing is a crucial step in gene expression and errors in mRNA splicing may lead to altered gene expression and disease. mRNA splicing depends on recognition of short well-conserved splice site sequences at the exon–intron boundaries. However, because of the degeneracy of the splice site sequences, accurate and efficient mRNA splicing is also dependent on additional splicing regulatory elements (SREs). Splicing enhancer (SE) elements attract proteins which enhance exon inclusion, while splicing silencer (SS) elements attract proteins which inhibit exon inclusion. The exact regulation of IKBKAP exon 20 splicing is not known, though some SREs with SE or SS function have been reported.

A large number of small-molecule drugs have been shown to increase the inclusion of IKBKAP exon 20. Among the most investigated drugs is the plant cytokinin kinetin, which has been shown to be efficient in a FD patient lymphoblast cell line (11), in FD-iPSC-derived neural crest cells (12), transgenic mice (13) and FD patients (14). Unfortunately, the small-molecule drugs also cause more broad effects on mRNA splicing and transcription rather than specific correction of only IKBKAP exon 20 splicing. For instance kinetin and digoxin improves exon inclusion of sev-
eral other exons (15–17). Recently, another drug, RECTAS, was shown to improve splicing of IKBKAP (18) and also EGCG has a positive effect on IKBKAP exon 20 splicing (9). Phosphatidylserine treatment affects the MAPK/ERK signaling pathway resulting in increased transcription of IKBKAP (19). Overall, the effect of these treatments is not specific to IKBKAP, and a treatment of FD, which specifically targets IKBKAP exon 20 splicing is thus highly desirable to avoid potential off-target effects.

Splice switching oligonucleotides (SSOs) can be designed to enhance exon inclusion by blocking SSs (20,21). Likewise, SSOs can be designed to decrease exon inclusion by blocking splice site sequences or by blocking SEs (22,23). SSOs may thus serve as a highly specific way of reversing aberrant splicing by targeting unique SREs. Several examples of promising SSO-based therapies have been reported (24). The most prominent example so far is the FDA-approved treatment of spinal muscular atrophy (SMA). Patients suffering from SMA lack a functional SMN1 gene; instead they depend on the highly similar SMN2 gene, which, however, predominantly skips exon 7 and therefore mainly produces a truncated protein. SSO-based blocking of an hnRNP A1-binding SS, ISS-N1, located immediately downstream of 5′RNP A1-binding sites close to the 5′RNP A1-mediated exon repression, and that intronic hnRNPs produce a protein that strongly targets IKBKAP exon 20 splicing and when administered in patients it slows down the development of disease (25,26).

hnRNP A1 is one of the major splice regulatory proteins, which traditionally has been considered a splicing repressor (27), though it may also work as a splicing activator (20,28). hnRNP A1 may repress splicing by several different mechanisms including antagonizing positive splice regulatory proteins and sterically blocking splice site recognition (27). Recently, we mapped in vivo hnRNP A1 binding sites in HeLa cells by performing individual nucleotide resolution crosslinking and immunoprecipitation (iCLIP) and identified thousands of binding sites in the human genome (20). We observed that binding of hnRNP A1 downstream of 5′ splice sites is especially important for hnRNP A1-mediated exon repression, and that intronic hnRNP A1 binding sites close to the 5′ splice sites may generally be suitable for SSO-based improvement of exon inclusion (20).

Here, we identify a fundamental hnRNP A1-binding SS located downstream of IKBKAP exon 20. Furthermore, we demonstrate that hnRNP A1 also binds to two previously reported exonic SSs, ESS1 and ESS2, which repress IKBKAP exon 20 inclusion. Knockdown of hnRNP A1 in FD patient fibroblasts improves splicing of IKBKAP exon 20 supporting that hnRNP A1 represses its splicing. Site-specific mutagenesis of an IKBKAP minigene confirms that both the exonic and the intronic hnRNP A1 binding sites cause hnRNP A1-mediated exon repression. This suggests that hnRNP A1 binds in a cooperative way to all three silencer elements to repress IKBKAP exon 20 inclusion in the FD IKBKAP gene. Finally, we show that SSO-mediated blocking of the intronic hnRNP A1 binding SS completely restores normal splicing of IKBKAP exon 20 even at low doses, and that this is accompanied by an increased IKAP protein level. We propose that this may be developed into a potent and specific future therapeutic strategy to restore IKBKAP exon 20 splicing in patients suffering from FD.

MATERIALS AND METHODS
RNA affinity purification

High-performance liquid chromatography-purified RNA oligonucleotides with 3′-biotin were purchased from LGC Biosearch Technologies. The association of hnRNP A1 and hnRNP A2/B1 with IKBKAP were analyzed using the following RNA oligonucleotides: intronic splicing silencer (ISS)-type (WT) corresponding to positions IKBKAP intron 20 +11–36: UUGUACGUUUUG CGACUAGGUAGCU-Biotin, and ISS MUT where the putative hnRNP A1 binding sites were mutated: UUGU ACGUUUUGCGACUCGUUCGU-Biotin containing the first putative hnRNP A1 binding site in IKBKAP exon 20 (ESS1), and IKBKAP exon 20 ESS1 MUT: GGAACUUCAAGGUAGUUCA-Biotin containing the second putative hnRNP A1 binding site in IKBKAP exon 20, which is mutated in the IKBKAP exon 20 ESS2 MUT: UGGUUUAGCUAGAUUCGU-Biotin.

For each purification, 100 pmol of RNA oligonucleotide was coupled to 50 µl of streptavidin-coupled magnetic beads (PureProteome™ Streptavidin Magnetic Bead System, Millipore) for 15 min in 1 × binding buffer (20 mM Heps/KOH [pH 7.9], 72 mM KCl, 1.5 mM MgCl, 1.56 mM MgAc, 0.5 mM Dithiothreitol (DTT), 4 mM glycerol, 0.75 mM adenosine triphosphate and 0.2 µg/µl bulk tRNA). The suspension was then placed in a magnet, and the supernatant was removed. The oligonucleotide–bead complexes were then resuspended in 500 µl of binding buffer containing either 100 µl of HeLa cell nuclear extract (CilibioTech) or 5 or 10 pmol pf recombinant hnRNP A1 (Origene) and incubated for 25 min at room temperature. Then, the supernatant was removed, and the beads were washed three-times in binding buffer containing 600 mM KCl or 1200 mM KCl. At last, the proteins bound to the RNA were eluted by the addition of 50 µl protein XT sample buffer (BioRad) and heated for 4 min at 90°C. The biotinylated RNA oligonucleotides were immobilized in a magnet, and the supernatant proteins were separated on a 4–12% sodium dodecyl sulphate (SDS)-polyacrylamide gel and were electroblotted onto a nitrocellulose membrane. The membranes were then probed with an anti-hnRNP A1 antibody (R9778, Sigma-Aldrich) or anti-hnRNP A2/B1 (sc-53531, Santa Cruz Biotechnology) or anti-RBM24 (ab94567, Abcam) and the control anti-hnRNP H (N16) (sc-10042, Santa Cruz Biotechnology). All RNA affinity purifications were performed at least four times employing two different batches of HeLa cell nuclear extract (CilibioTech).

Surface plasmon resonance imaging (SPRi)

Biotinylated oligonucleotides were immobilized on a Sens-eye G strep (SENS) sensorchip in a 2 × 4 × 12 array by continuous flow in a CFM 2.0 printer (Wasatch microfluidics). The oligonucleotides were diluted in 1 × Tris Buffered Saline (TBS) to a concentration of 1 µM and spotted for 20 min followed by 5 minutes washing with TBS + 0.05% Tween-20. The sensorchip was transferred to the
Hepes/Surface Plasmon Resonance imaging (SPRi) buffer (10 mM HEPES/KOH pH 7.9, 150 mM KCl, 10 mM MgCl₂ and 0.075% Tween-80). SPRi by IBIS MX-96 was used to measure the kinetics of recombinant hnRNP A1 (ab224866, Abcam) binding to the immobilized RNA oligonucleotides. Binding was measured in real time by following changes of the SPR angles at all printed positions of the array during 10 min injections of hnRNP A1 protein over the entire surface. Six injections of a 2-fold titration series from 6.25 to 200 nM hnRNP A1 were injected in sequence from the lowest concentration to the highest. Before adding protein to the chip, residual background binding was blocked by injecting 1 mg/ml bovine serum albumin in SPR buffer onto the chip for 10 min. A continuous flow of SPR buffer flowed over the surface before, between and after the hnRNP A1 injections, to measure baseline and dissociation kinetics. Dissociation was measured for 5 min, by injecting SPR buffer over the chip at a rate of 8 µl/s. Responses for a calibration curve were created after the concentration series by measuring SPR responses from defined dilutions of glycerol in running buffer (ranging from 5 to 0% glycerol) and of pure water as defined by the automated calibration routine of IBIS MX-96.

For data analysis the SPRi data were imported into SPRINTX software (v. 2.1.1.0, IBIS technologies), calibrated, reference subtracted and the baseline of the responses before all hnRNP A1 injections were zeroed. The time starting point was aligned at the beginning of each new injection. Then the data were exported to Scrubber 2 (Biology Inc.). Binding curves for all positions where binding was observed were fitted globally to the integrated rate equation that describes simple first order 1:1 binding kinetics in order to obtain kinetic association rate \((k_a)\), dissociation rate \((k_d)\) and equilibrium dissociation \((K_D = k_d/k_a)\) constants. To obtain a bimodal binding for 1:2 kinetics the simulations were calculated using the pbm package for R, and using ggplot2 for plotting the resulting binding curves.

** Knockdown of hnRNP A1 and hnRNP A2/B1 in patient fibroblasts **

FD patient fibroblasts (GMO4589, GMO4899, GMO2343 and GMO4663) (Coriell Cell Repositories) and control fibroblasts (GMO2912 (Coriell Cell Repositories)) were grown to approximately 70% confluence and transfected with siRNA smartpools targeting hnRNP A1 (L-008221-00, Dharmacon), hnRNP A2/B1 (L-011690-01, Dharmacon), the hnRNP A1 and hnRNP A2/B1 siRNAs combined or control non-targeting siRNA (D-001810-10-20, Dharmacon) using Lipofectamine RNAiMAX Transfection Reagent (Thermo Fisher Scientific) according to the manufacturer’s instructions either performing reverse transfection using 6, 12 or 24 nM of SSO or forward transfection of an SSO concentration gradient (0.046, 0.188, 0.75, 3 or 12 nM); SSOs were phosphorothioate oligonucleotides with 2′-O-methyl modification on each sugar moiety (LGC Biosearch Technologies). The IKBKAP SSOs targets IKBKAP intron 20; SSO1 (pos.+11−15): 5′-AGCUAAC UAAGUCGCAAACAGUACAA-3′; SSO2 (pos.+19−43): 5′-AAUCAACAGCUACUAGUCGCAA-3′; SSO3 (pos.+27−51): 5′-UCACAAUAAUCAAGCAACUAACUA-3′; SSO4 (pos.+44−68): 5′-UAUUACAU UAUUGCUUUCACACAGUA-3′; SSO5 (pos.+53−72): 5′-AAAUUGUAUAAUACUAAUUUGUC-3′. The control SSO does not target any human genes: 5′-GCUCUAACAUUGCUACUGCGCAUGCUUG-3′. After transfection, cells were incubated for 48 h before RNA extraction. Western blot analysis of SSO treated fibroblasts: 72 h post-transfection proteins for western blot analysis of fibroblasts was harvested using M-PER Mammalian Protein Extraction Reagent (ThermoFisher Scientific) supplemented with Complete Protease Inhibitor Cocktail (11836145001, Roche) and PMSF (93482, Sigma-Aldrich). Proteins were separated on a 4–12% SDS-polyacrylamide gel and electrophobted onto a nitrocellulose membrane. The membranes were then probed with an anti-hnRNP A1 antibody (R9778, Sigma-Aldrich) or anti-hnRNP A2/B1 (sc-53531, Santa Cruz Biotechnology) and the control anti-hnRNP H (N16) (sc-10042, Santa Cruz Biotechnology) or an anti-HPRT antibody (HPA006360, Sigma-Aldrich). The membranes were incubated with the appropriate secondary antibodies and were visualized using ECL Western Blotting detection kit (GE Healthcare).

** Transfection of splice switching oligonucleotides **

FD patient fibroblasts (GMO4663, GMO4589, GMO4899, GMO2343 (Coriell Cell Repositories)) or control fibroblasts (GMO2912 (Coriell Cell Repositories)) or an internal fibroblast control cell line FB-01) were grown to approximately 70% confluence and transfected with SSOs using Lipofectamine RNAiMAX Transfection Reagent (Thermo Fisher Scientific) according to the manufacturer’s instructions either performing reverse transfection using 6, 12 or 24 nM of SSO or forward transfection of an SSO concentration gradient (0.046, 0.188, 0.75, 3 or 12 nM); SSOs were phosphorothioate oligonucleotides with 2′-O-methyl modification on each sugar moiety (LGC Biosearch Technologies). The IKBKAP SSOs targets IKBKAP intron 20; SSO1 (pos.+11−15): 5′-AGCUAAC UAAGUCGCAAACAGUACAA-3′; SSO2 (pos.+19−43): 5′-AAUCAACAGCUACUAGUCGCAA-3′; SSO3 (pos.+27−51): 5′-UCACAAUAAUCAAGCAACUAACUA-3′; SSO4 (pos.+44−68): 5′-UAUUACAU UAUUGCUUUCACACAGUA-3′; SSO5 (pos.+53−72): 5′-AAAUUGUAUAAUACUAAUUUGUC-3′. The control SSO does not target any human genes: 5′-GCUCUAACAUUGCUACUGCGCAUGCUUG-3′. After transfection, cells were incubated for 48 h before RNA extraction. Western blot analysis of SSO treated fibroblasts: 72 h post-transfection proteins for western blot analysis of fibroblasts was harvested using M-PER Mammalian Protein Extraction Reagent (ThermoFisher Scientific) supplemented with Complete Protease Inhibitor Cocktail (11836145001, Roche) and PMSF (93482, Sigma-Aldrich). Proteins were separated on a 4–12% SDS-polyacrylamide gel and electrophobted onto a nitrocellulose membrane. The membranes were then probed with an anti-IKAP antibody (sc-13642, Santa Cruz Biotechnology) or anti-hnRNP A2/B1 (sc-53531, Santa Cruz Biotechnology) and the control anti-hnRNP H (N16) (sc-10042, Santa Cruz Biotechnology).

** Transfection of IKBKAP and SMN1/2 minigenes **

IKBKAP minigene covered the unaltered sequence of exon 19–21 of IKBKAP (genomic location 10b 900 438–108 898 593). The minigene was cloned into pJET1.2 and subcloned into pcDNA3.1 using heterozygous (IKBKAP c.2204+6T>C) genomic DNA as template (NA05044, NA05045 (Coriell). Mutagenesis of the IKBKAP minigene was performed by Genscript.
The *IKBKAP* ISS (ctagtaggct) was introduced into the pCI *SMN1* and pCI *SMN2* minigenes by in vitro mutagenesis (GeneScript) (Figure 5), replacing nucleotides +10 to +19 of intron 20 from the ISS-N1 sequence in *SMN1* and *SMN2* (ccagcattatg) (pCI *SMN1* and pCI *SMN2* were kindly provided by Prof. Adrian Krainer, Cold Spring Harbor Labory, NY, USA). The pCI SMN mutant minigenes with the *IKBKAP* ISS were named pCI SMN1 *IKBKAP* ISS and pCI SMN2 *IKBKAP* ISS. Minigenes were transfected into HeLa cells using XtremeGene9 (Roche). Forty-eight hours after transfection cells were harvested for RNA analysis.

**RNA extraction and analysis**

RNA was harvested using Isol (5Prime) or Qiazol (Qiagen) and phenol/chloroform extraction. RNA was used as template for cDNA synthesis using High Capacity cDNA Reverse Transcription Kit (Thermo Scientific). Splicing of *IKBKAP* exon 20 was examined by PCR using Tempase Hot Start DNA Polymerase (Ampliqon) and primers for analysis of endogenous *IKBKAP* splicing: *IKBKAP* F: GGGGTTCACGGATTGTCACT and *IKBKAP* R260 (exon 2): GCAAACCAGAAACCCTTG.

Splicing of exon 7 from pCI *SMN1* and pCI *SMN2* mini genes were analyzed using vector specific pCIFwdB 5′-GACTCACTATAGGCTAGCC-3′ and pCI SMN test exon 8 as 5′-GTGGTGTCATTTAGT.

Analysis of the splicing of exon 20 was performed by in vitro mutagenesis studies indicating that ESS1 has lower silencing capability than ESS2, possibly due to the existence of an overlapping SE element (17,32). We hypothesized that ESS1 and ESS2 may function by binding hnRNP A1, and that the intrinsic hnRNP A1 binding site could represent an ISS. Thus, binding of hnRNP A1 to these three silencers would have a repressive effect on *IKBKAP* exon 20 inclusion, which could result in exon 20 skipping when the *IKBKAP* exon 20 5′ splice site is compromised by the c.2204+6T>C mutation. To confirm binding of hnRNP A1 to these sites, we first performed RNA affinity purification with biotinylated RNA oligonucleotides either harboring the sequence around the ESS1, the ESS2 or the intronich background. The expression of hnRNP A1 was evaluated by including mutant oligonucleotides (Mut), where the important UAG of the ESS1 and ESS2 motifs was mutated to UCG. We have demonstrated that this type of A>C mutation dramatically reduces binding of hnRNP A1 (20,30,33,34). We initially used recombinant hnRNP A1 and analyzed the bound protein by SDS-PAGE and western blotting (Supplementary Figure S2). This showed that recombinant hnRNP A1 binds these sequences, thus hnRNP A1 is able to recognize and bind these sequences independently of other RNA binding proteins. Furthermore, the binding of hnRNP A1 was markedly reduced when using mutant oligonucleotides suggesting that hnRNP A1 interacts with these elements in a sequence-dependent way. Next, we performed the same experiment using HeLa cell nuclear extract for the binding reaction, thus better mimicking the environment of splicing in the cell nucleus, where protein binding is competitive and cooperative among several splicing regulatory proteins (35) (Figure 2 and Supplementary Figure S2). hnRNP A1 also bound to the *IKBKAP* oligonucleotides in a sequence-specific way in this context, suggesting that hnRNP A1 both on its own and in competition with other RNA binding proteins specifically binds these three regulatory elements.

**RESULTS**

HnRNP A1 binds sequences in *IKBKAP* exon 20 and sequences flanking the 5′ splice site

*IKBKAP* exon 20 is partially skipped in the c.2204+6T>C FD patient fibroblasts (Figure 1) (2). Analysis of the *IK-
Since hnRNP A1 and hnRNP A2/B1 have been reported to have overlapping binding motifs (28), we also characterized the binding of hnRNP A2/B1 in a manner similar to hnRNP A1 (Figure 2), suggesting that hnRNP A1 and hnRNP A2/B1 both regulate IKBKAP exon 20 splicing. Recently, it has been proposed that RBM24 can stimulate IKBKAP exon 20 inclusion from the c.2204+6T>C mutant splice site through binding to an intronic SE located in the region +13 to +29 (36). Consequently, we also checked for binding of RBM24 to all six different biotinylated RNA oligonucleotides, but we were unable to observe RBM24 binding above background (results not shown), possibly because RBM24 expression is low in HeLa cells (https://www.proteinatlas.org/ENSG00000112183-RBM24/cell).

In order to further investigate the binding of hnRNP A1 to the ISS and ESS’s in IKBKAP exon 20 and intron 20, the biotinylated oligonucleotides used for RNA affinity analysis were analyzed by SPRi, using recombinant hnRNP A1 protein to analyze the kinetics that occur during RNA–protein interaction. For all three set of oligonucleotides, we observe specific and concentration dependent increase in hnRNP A1 binding (Figure 3 and Supplementary Figure S3). For the ISS containing oligonucleotides binding of hnRNP A1 was completely disrupted in the mutant oligonucleotide, and when fitting the association curves to a bimodal binding model, we observe two different associations (Figure 3 and Supplementary Figure S3) which is consistent with the two hnRNP A1 binding motifs located closely to each other in the IKBKAP ISS (37). When we abolish both sites in the mutant ISS oligonucleotide all hnRNP binding is lost, and only a very poor fit can be made (Figure 3 and Supplementary Figure S3).

For the two ESSs in IKBKAP exon 20, we observe very different binding reactions. The ESS1 containing oligonucleotide shows some binding of hnRNP A1, which is completely lost in the mutant oligonucleotide. The ESS2 containing oligonucleotide shows a very strong bimodal binding of hnRNP A1 that is not completely disrupted in the mutant oligonucleotide. While binding is severely decreased the mutant ESS2 oligonucleotide can still bind more hnRNP A1 than the WT ISS oligonucleotide, consistent with previous reports of ESS2 being a strong SS (32) (Figure 3 and Supplementary Figure S3). We were a bit puzzled by the fact that the mutant ESS2 oligonucleotide still had significant affinity toward hnRNP A1, but a closer examination shows that a potential second low score hnRNP A1 binding site (UCAGAUU) is present immediately downstream. It is thus likely that the strong binding to the ESS2 oligonucleotide is caused by the simultaneous presence of two hnRNP A1 binding sites, perhaps working synergistically.

hnRNP A1 regulates the splicing of IKBKAP exon 20

To evaluate whether the binding of hnRNP A1 and hnRNP A2/B1 regulate IKBKAP exon 20 splicing, we performed siRNA-mediated knockdown of hnRNP A1, hnRNP A2/B1 or both proteins combined in FD patient fibroblast (Figure 4; Supplementary Figures S4 and 5). Knockdown of hnRNP A1 increases the inclusion of IKBKAP exon 20 in all four tested patient cell lines, supporting that hnRNP A1 has a negative effect on IKBKAP exon 20 splicing. This reflects the established role of hnRNP A1 as a repressive splicing regulatory protein. The fact that knockdown of hnRNP A1 does not fully restore splicing suggests that also other splicing repressors can inhibit exon 20 inclu-
**Figure 2.** hnRNP A1 and hnRNP A2/B1 interact with IKBKAP inside exon 20 and downstream of the 5′ splice site. (A) The position of the ESS1 and ESS2 in IKBKAP exon 20 and the position of the ISS downstream of the exon 20 5′ splice site. hnRNP A1 motives (TAG) are indicated. The sequences analyzed by RNA affinity purification are shown. For evaluation of the sequence-specific binding, the important hnRNP A1-binding UAG triplet is mutated to UCG. (B) RNA affinity purification of HeLa cell nuclear extract using the IKBKAP oligonucleotides. Purified proteins were analyzed by western blotting using anti-hnRNP A1 or anti-hnRNP A2/B1 antibodies. hnRNP H was used as loading control. Results are representative for four experiments. (C) Western blot (anti-hnRNP A1 antibody) a gel with a lane for input (binding solution with NE before addition of beads with oligonucleotides) and lanes with output from each of the pull downs, demonstrating equal content of hnRNP A1 in all binding assays. (D) Quantification of bands using IMAGE J from three representative blots. WT was set to 100% and the corresponding mutant expressed in % of WT. Error bars indicate SEM, n = 3, **P-value < 0.01, *P-value > 0.05 (one-sample t-test).

Disruption of hnRNP A1 binding sites restores splicing of IKBKAP exon 20 in a FD-IKBKAP minigene

To further characterize the regulation of IKBKAP exon 20 splicing, we constructed an IKBKAP minigene harboring the region from exon 19 to exon 21 cloned into pcDNA3.1. Splicing of the FD-IKBKAP minigene containing the c.2204+6T>C mutation mimicked the endogenous FD-IKBKAP gene when transfected into HeLa cells (Figure 5). Therefore, we constructed FD-IKBKAP minigenes with mutations in the hnRNP A1 binding sites disrupting the hnRNP A1 binding silencer motifs (Figure 5). Disruption of the exonic splicing silencers ESS1 and ESS2 completely restored splicing, and deletion of the ISS hnRNP A1 binding...
motif leads to a nearly complete restoration, whereas mutation of the ISS (ISS MUT) causes a high, but less efficient correction (Figure 5). It has been proposed that hnRNP A1 binds high-affinity binding sites and through protein-protein interactions spread across an exon to inhibit exon recognition (38). We therefore hypothesized that hnRNP A1 binds cooperatively to all three silencers and spreads across the exon to inhibit exon recognition. Thus disruption of any of these silencers may reduce the association of hnRNP A1 with IKBKAP exon 20 allowing the U1 snRNP to bind to the weakened c.2204+6T>C 5' splice site of FD-IKBKAP. Disrupting the ISS silencer by UAG>UCG mutations is less efficient in restoring splicing of IKBKAP exon 20 than deleting the hnRNP A1 ISS motif, suggesting that additional overlapping inhibitory sequences were disrupted in the deleted sequence or that introduction of the UAG>UCG mutations in the ISS does not abolish binding of positive factors, like for instance RBM24 to the intronic SE (36).

In order to further characterize the IKBKAP ISS, we tested its effect by using it to substitute one of the best-known ISSs, ISS-N1, in SMN1/2 genes. ISS-N1 also func-
Repressed splicing of +10 to +20 of the ISS-N1 in the intron of a weak 5′ acceptor (Figure 5) confirming that the stronger than the known hnRNPA1 binding ISS-N1 si-silencer (ESEs), moreover blocking of the hnRNPA1 binding ISS-N1 silencer (32) in SMN2 using an SSO corrects splicing of exon 7 and is currently in clinical use.

Therefore we designed SSOs located downstream of IKBKAP exon 20 (Figure 6). The overlapping SSO1, SSO2 and SSO3 all cover the ISS hnRNPA1 binding site, whereas SSO4 and SSO5 are located further downstream (Figure 6). We transfected FD patient fibroblast cell lines with the IKBKAP SSOs or a control SSO (Figure 6). All three SSOs that cover the hnRNPA1 binding ISS resulted in a dramatic correction of the impaired splicing of IKBKAP exon 20 in FD patient cells, and also increased total IKBKAP mRNA amounts (Figure 6) as the transcript without exon 20 is subject to degradation by the NMD pathway (40).

In contrast, the two SSOs that did not block access, had no (SSO5) or even a slightly negative effect (SSO4). SSO1 performed slightly better than SSO2 and SSO3. This could indicate that the effect of the SSOs is also influenced by blocking other regulatory sites and indicate that the effect of the SSOs is also influenced by blocking other regulatory sites and therefore we designed SSOs located downstream of IKBKAP exon 20. Knockdowns of hnRNPA1 or hnRNPA2 decreases IKBKAP exon 20 inclusion. Knockdown of the ISS completely corrects deregulated splicing (20,21,39). SSOs targeting exonic splicing silencers may unintentionally interfere with recognition of other important regulatory elements such as exonic splicing enhancers (ESEs), moreover blocking of the hnRNPA1 binding ISS-N1 silencer (32) in SMN2 using an SSO corrects splicing of exon 7 and is currently in clinical use.

In contrast, the two SSOs that did not block access, had no (SSO5) or even a slightly negative effect (SSO4). SSO1 performed slightly better than SSO2 and SSO3. This could indicate that the effect of the SSOs is also influenced by blocking other regulatory sites and/or that they bind with different efficiencies. Examination of the SSO designs by the IDT software (https://eu.idtdna.com/calc/analyzer) did not reveal any obvious differences, except that SSO1 has the highest melting temperature (56.2°C), perhaps indicating a more efficient binding.

Figure 4. hnRNPA1 and hnRNPA2/B1 regulate inclusion of IKBKAP exon 20. Knockdowns of hnRNPA1 or hnRNPA2/B1 in four different FD patient fibroblasts GMO4663, GMO4589, GMO4899 and GMO2343. (A) hnRNPA1 knockdown increases IKBKAP exon 20 inclusion, while hnRNPA2/B1 decreases IKBKAP exon 20 inclusion. (B) IKBKAP exon 20 inclusion was quantified using the fragment analyzer. Error bars indicates SEM, n ≥ 2 ***P-value < 0.001, **P-value < 0.01, *P-value < 0.05 knockdown was validated by qPCR where expression of hnRNPA1 (C) and hnRNPA2/B1 (D) was normalized to the expression of TBP. Error bars indicates SEM, n ≥ 3 ***P-value < 0.001, **P-value < 0.01, *P-value < 0.05. (E) Knockdown of hnRNPA1 and hnRNPA2/B1 was confirmed by western blotting (GMO4663) and HPRT was used as a loading control.
To further characterize the potency of the IKBKAP SSO1, we performed a gradient transfection using decreasing concentrations of SSOs (Figure 7). This generated a dose-response curve, which reached 100% inclusion already at ~3 nM SSO1. Compared with other SSOs used in our laboratory this is a very potent SSO (20). SSO1 treatment at 12 nM caused complete correction of splicing in all four patient cell lines and does not disturb splicing in non-FD fibroblasts. Importantly, we also observe that the positive effect of SSO1 treatment is reflected in a dramatic increase in IKAP protein levels in FD patient cells, indicating that this SSO could potentially be used to increase the level of functional IKAP protein in patients.

At last, we wanted to evaluate downstream effects of SSO based IKBKAP splicing correction in patient cells. One of the hallmarks of FD, which is also observed in a mouse model (41), is a reduction of pain and temperature-sensing TrkA+ neurons in the dorsal root ganglia (DRG), which is a primary site of IKBKAP expression. Interestingly, Neuregulin 1 (NRG1) knock-out mice also show a reduction of pain and temperature-sensing TrkA+ neurons in the DRG (42), and NRG1 has been reported to be downregulated in FD patient nasal mucosa cells (43). Both SSO1 and SSO2, but not SSO5 or a control SSO increased mRNA levels were increased between 1.9- and 4.7-fold. The effect of SSO1 treatment on the IKAP protein is shown in Figure 8. As previously observed, the IKBKAP SSO1 completely restores IKBKAP exon 20 splicing in patient cells and does not disturb splicing in non-FD fibroblasts. Importantly, we also observe that the positive effect of SSO1 treatment is reflected in a dramatic increase in IKAP protein levels in FD patient cells, indicating that this SSO could potentially be used to increase the level of functional IKAP protein in patients.

Figure 5. Mutation of ESS1 or ESS2 or ISS corrects splicing of a FD-IKBKAP minigene and insertion of the IKBKAP ISS in SMN1/2 minigenes confirms that it is a strong SS. (A) Schematic of the IKBKAP minigenes. (B) Splicing of IKBKAP exon 20 after transfection of IKBKAP minigenes into HeLa cells. Disruption of the hnRNP A1 motifs restores IKBKAP exon 20 splicing. The transfections were done twice in triplicates. (C) Schematic of the SMN1/2 minigenes where the ISS-N1 SS was disrupted by insertion of the IKBKAP ISS sequence. The IKBKAP ISS sequence inhibits inclusion of SMN1/2 exon 7 stronger than the endogenous hnRNP A1 binding ISS-N1. (D) Inclusion of SMN1/2 exon 7 was quantified using the fragment analyzer. Error bars indicates SEM, n ≥ 3 ***P-value < 0.001, **P-value < 0.01, *P-value < 0.05.
**DISCUSSION**

An estimated 25% of all disease-causing mutations may interfere with mRNA splicing (44,45). Therefore, the ability to control mRNA splicing has great potential in disease therapy. In recent years, a growing number of cases proves that manipulation of mRNA splicing using SSOs is a feasible approach for correcting disease-associated deregulated splicing (24). However, for specific and efficient design of SSOs insight into the mechanism of splicing regulation is necessary.

Here, we show that hnRNP A1 binds two silencer elements ESS1 and ESS2 inside IKBKAP exon 20 and to an ISS immediately downstream of the 5′ splice site. Knockdown of hnRNP A1 increases the inclusion of IKBKAP exon 20 in FD patient fibroblasts, and disruption of any of the hnRNP A1 binding sites in an FD-IKBKAP minigene restores exon 20 splicing, showing that hnRNP A1 plays an important role in repressing exon 20 in FD fibroblasts. The two exonic splicing silencers have previously been described, and the functionality of these were analyzed by minigene mutagenesis studies and in vitro splicing (32). Both silencers were shown to inhibit exon 20 inclusion, though ESS2 appeared to be a stronger inhibitor than ESS1, which is consistent with the fact that our SPRi analysis demonstrated more hnRNP A1 binding to the ESS2 than to ESS1. Furthermore, in another study investigating splicing of deletion mutants of an FD-IKBKAP minigene, only a deletion that disrupted ESS2, but not a deletion that disrupted the ESS1 silencer, restored splicing of exon 20 (17). We show that specifically mutating the hnRNP A1 binding sequence in either of the two exonic silencers, ESS1 and ESS2, completely restores splicing. The divergence regarding ESS1 may be caused by different experimental conditions. Our UUAG>UUCG ESS-disrupting mutations introduced into the FD-IKBKAP minigene may be a much more efficient disruption of the silencer element than the previously investigated UUAG>AUAG mutation in ESS1 (32). Moreover, deletion of the ESS1 silencer may not increase exon inclusion, because this at the same time disrupts the ESE1 SE (32), which overlaps the ESS1 silencer. Our study suggests that the ESS1, ESS2, as well as the ISS downstream of the 5′ splice site all inhibit inclusion of FD-IKBKAP exon 20. We propose that hnRNP A1 cooperatively binds to the three

---

**Figure 6.** SSOs that block the IKBKAP ISS restores splicing of IKBKAP exon 20 in FD patient cell lines. (A) Schematic of IKBKAP intron 20 and the binding sites for the SSOs. (B) Transfection of 12 nM IKBKAP SSOs or a control SSO into FD patient fibroblasts (GMO4663) was analyzed by gel electrophoresis of PCR products and inclusion of exon 20 was measured on the fragment analyzer. (C) Transfection of 12 nM IKBKAP SSO1, SSO2 or SSO5 or a control SSO into three different FD patient fibroblasts (GMO2343, GMO4663 and GMO4899) was analyzed by gel electrophoresis of PCR products and inclusion of exon 20 was measured on the fragment analyzer. The total IKBKAP expression was quantified by qPCR. Error bars indicates SEM, n ≥ 2 ***P-value < 0.001, **P-value < 0.01, *P-value < 0.05. Compared to control SSO.
Figure 7. SSO1 treatment of FD patient cell lines restores splicing of \textit{IKBKAP} exon 20. (A) FD-patient cells GMO4589 were transfected with increasing amounts of \textit{IKBKAP} SSO1 or control SSO. The splicing of \textit{IKBKAP} exon 20 was then analyzed by PCR. The inclusion of exon 20 was quantified using the fragment analyzer. The range of inclusion for two biological replicates in duplicates is shown. (B) Transfection of 12 nM \textit{IKBKAP} SSO1 or a control SSO into four different FD patient fibroblasts (GMO2343, GMO4663, GMO4589 and GMO4899) or two control fibroblasts (FB01 and GMO2912) was analyzed by gel electrophoresis of PCR products. (C) The total \textit{IKBKAP} expression was quantified by qPCR. Error bars indicate SEM, \( n \geq 3 \), *** \( P \)-value < 0.001, ** \( P \)-value < 0.01, * \( P \)-value < 0.05. (D) Inclusion of exon 20 was measured on the fragment analyzer. Error bars indicate SEM, \( n \geq 2 \), *** \( P \)-value < 0.001, ** \( P \)-value < 0.01, * \( P \)-value < 0.05.

Figure 8. \textit{IKBKAP} SSO treatment increases IKAP protein expression. Transfections of \textit{IKBKAP} SSO (12 nM) into control (GMO2912) or FD fibroblasts (GMO4589, GMO4663) restore splicing of \textit{IKBKAP} exon 20 (top). Western blotting shows an increase in IKAP protein expression after transfection with \textit{IKBKAP} SSO into FD fibroblasts but not control fibroblasts. hnRNP H was used as a loading control. Transfections were performed in triplicates.
NRGI/TBP

Figure 9. SSO treatment of FD patient cell lines restores NRGI expression. Transfection of 12 nM IKBKAP SSOs or a control SSO into three different FD patient fibroblasts (GMO2343, GMO4663 and GMO4899) or a control fibroblast (FB01) was analyzed for NRGI expression by qPCR and normalized to TBP. Left and right panel represent different experiments. Left: error bars indicates SEM, \( n \geq 3 \) ***P-value < 0.001, **P-value < 0.01, *P-value < 0.05 compared to control SSO. Right: error bars indicates SEM, \( n \geq 2 \) ***P-value < 0.001, **P-value < 0.01, *P-value < 0.05 compared to control SSO.

elements and spreads across the exon to prevent recognition of the weakened FD c.2204+6T>C 5′ splice site of IKBKAP exon 20 and replace positive splicing factors (Figure 10) (38). Disruption of any of the hnRNP A1-binding silencers may shift a finely tuned balance between positive and negative splicing elements and prevent the cooperative spreading of hnRNP A1. The loss of hnRNP A1 binding may then enable binding of positive splicing factors, like SR proteins or RBM24 (36), to SEs, which would help attract U1 snRNP to the weak 5′ splice site of c.2204+6T>C IKBKAP exon 20 and thereby restore splicing. On the other hand, the WT 5′ splice site is sufficiently strong to overcome hnRNP A1 binding allowing U1 snRNP to bind and it may therefore also prevent the cooperative spreading of hnRNP A1 across the exon. We have previously shown that splicing is a delicate process and single nucleotide variants outside the canonical splice sites can have strong effects on splicing. In the ACADM gene, a pathogenic mutation disrupting an ESE element causes exon skipping, but this can be counteracted by a nearby Single Nucleotide Polymorphism (SNP) that disrupts an hnRNPA1 binding ESS and thus restores the balance between positive and negative splicing elements (30).

To further analyze the WT and the FD 5′ splice site, we extracted 284213 donor splice sites from the Ensembl v79 annotation of the hg38 genome and found that of these, 1319 matched the CAAgtaagt sequence of the WT IKBKAP exon 20 and only 254 matched the CAAgtaagc site of the FD 5′ splice site indicating that the WT splice site is indeed a more frequently used splice site. We counted the number of UAG motifs in the immediate downstream intronic region (+10 to +40) where the IKBKAP ISS is located and found that 413 of the 1319 (31.3%) WT splice sites harbored at least one UAG motif, whereas only 58 (22.8%) of the 254 exons with the weaker FD 5′ splice site harbored one UAG motif, suggesting that the stronger WT splice site may generally tolerate a higher number of silencer elements downstream of the splice site. This further illustrates that exon inclusion depends on a finely tuned balance of SREs in addition to splice site strength, and as we show here, that splicing can be affected by changing this balance of positive and negative SREs.
In our previous hnRNP A1 iCLIP study, we showed that hnRNP A1 binding immediately downstream of the 5′ splice site is generally important for exon repression (20), and that SSO-mediated blocking of hnRNP A1 binding sites in this region is effective in improving exon inclusion. In addition, SSOs targeting intronic regions have lower risk of interfering with important regulatory elements than SSOs targeting exons. An SSO targeting downstream of exon 16 of the CFTR gene corrects splicing in a c.2657+5G>A mutant minigene (46), and close inspection revealed a UAG motif in the sequence blocked by the SSO. The FDA-approved SSO correcting the c.2657+5G>A mutation in the SMN2 gene when it substitutes the ISS-N1 in the SMN1/2 genes. This further strengthens the notion that blocking of hnRNP A1 binding sites downstream of weak 5′ splice sites is an efficient way of increasing exon inclusion, and it demonstrates how our previously generated iCLIP hnRNP A1 binding map can be used to pinpoint efficient target sites for SSO-based modulation of splicing. The fact that we were unable to completely restore IKBKAP exon 20 splicing by hnRNP A1 knockdown and by mutating the IKBKAP ISS in a minigene, may suggest that also other splicing inhibitory proteins can bind to the ISS or the flanking sequences in the region blocked by the SSOs.

Compared to current treatment strategies against FD, the IKBKAP SSO approach is very specific. Many of the small-molecule drugs that have been shown to improve IKBKAP exon 20 splicing, have broad non-specific effects on mRNA splicing or transcription (15–17,19). These drugs therefore pose the risk of off-target effects. Moreover, the successful therapy against SMA indicates that SSO-based therapy is well-tolerated in vivo (25), and that delivery of SSOs to CNS, which is also the primary target site for the IKBKAP SSO, is possible in vivo.

EGCG has been shown to rescue IKBKAP exon 20 splicing. Since EGCG treatment decreases hnRNP A2/B1 expression, it was suggested that hnRNP A2/B1 is a negative

---

Figure 10. Model of the hnRNP A1-dependent regulation of IKBKAP exon 20. WT: hnRNP A1 binds ESS1, ESS2 and ISS, however, the WT 5′ splice site is strong enough to attract the U1 snRNP, which prevents hnRNP A1 multimerization across the exon and ensures complete inclusion of IKBKAP exon 20. FD: the reduced splice site strength of the FD 5′ splice site makes U1 snRNP less capable of competing with hnRNP A1 for binding, resulting in multimerization of hnRNP A1 across the exon, inhibiting U1 snRNP and SR protein binding, which results in only partial exon inclusion. In addition, SSOs targeting intronic regions have lower risk of interfering with important regulatory elements than SSOs targeting exons. An SSO targeting downstream of exon 16 of the CFTR gene corrects splicing in a c.2657+5G>A mutant minigene (46), and close inspection revealed a UAG motif in the sequence blocked by the SSO. The FDA-approved SSO correcting the c.2657+5G>A mutation in the SMN2 gene when it substitutes the ISS-N1 in the SMN1/2 genes. This further strengthens the notion that blocking of hnRNP A1 binding sites downstream of weak 5′ splice sites is an efficient way of increasing exon inclusion, and it demonstrates how our previously generated iCLIP hnRNP A1 binding map can be used to pinpoint efficient target sites for SSO-based modulation of splicing. The fact that we were unable to completely restore IKBKAP exon 20 splicing by hnRNP A1 knockdown and by mutating the IKBKAP ISS in a minigene, may suggest that also other splicing inhibitory proteins can bind to the ISS or the flanking sequences in the region blocked by the SSOs.
regulator of IKBKAP exon 20 splicing (9). Interestingly, we observed that knockdown of hnRNP A2/B1 has a negative effect on IKBKAP exon 20 inclusion, showing that hnRNP A2/B1 is a positive regulator of IKBKAP exon 20 splicing. This suggests that the positive effect of EGCG is most likely mediated through a different mechanism than hnRNP A2/B1 downregulation. HnRNP A1 and hnRNP A2/B1 have previously been shown to have opposite effects on the same splicing events (28,48). When hnRNP A2/B1 and hnRNP A1 bind the same splicing elements, hnRNP A2/B1 may have a positive effect on splicing by competing with and inhibiting the binding of hnRNP A1 to the SSs. This correlates with the improvement of IKBKAP exon 20 splicing in the FD- IKBKAP minigene when disrupting the ESS1, ESS2 or the ISS, suggesting that predominantly negative splicing factors bind these splicing elements. In particular the positive effect of blocking the ISS with SSOs indicates that the ISS mainly binds negative splicing factors.

At last, we demonstrate that SSO-based blocking of the ISS increases IKAP protein levels in FD patient cell lines, and interestingly, that SSO treatment increases the suppressed neutreulin 1 (NRG1) expression in patient cell lines investigated. Decreased expression of NRG1 in FD patient nasal mucosa cells cells has previously been reported (43). Neureulin 1 is a key regulator of the peripheral nervous system and has been shown to be essential for survival of TrkA+ sensory neurons. Because increased death of TrkA+ sensory neurons is a hallmark of FD, it could be speculated that some of the clinical symptoms in FD may be due to IKBKAP deficiency-mediated NRG1 downregulation.

Altogether, we demonstrate that hnRNP A1 is an important inhibitor of inclusion of FD- IKBKAP exon 20 carrying the weak c.2204+6T>C 5’ splice site, which is the most frequent cause of FD. We take advantage of this by SSO-mediated blocking of the hnRNP A1-binding ISS, which completely restores splicing of IKBKAP exon 20 in FD patient cell lines.

However, before this approach can be developed into an efficient therapy for FD patients it is clear that further preclinical testing for instance in the available FD mouse model (49) is required. Although a BLAST search did not reveal any other potential targets in the human genome, testing for potential side effects resulting from unspecific binding of SSO1 to other targets should also be evaluated for instance by RNA-seq analysis of human cells.

SUPPLEMENTARY DATA
Supplementary Data are available at NAR Online.

ACKNOWLEDGEMENTS
We thank Tanja Bruun, Kristian Traantoft Rasmussen, Margrethe Thusholdt, Krystyna Giemza and Aleksandra Kulus for technical assistance.

FUNDING
Natur og Univers, Det Frie Forskningsråd [4181-00515 to B.S.A.]; Novo Nordisk Fonden (DK) [61310-0128, NNF17OC0029240 to B.S.A.]. Funding for open access charge: University of Southern Denmark.

Conflict of interest statement. None declared.

REFERENCES
1. Dong,J., Edelmann,L., Bajuwa,A.M., Kornreich,R. and Desnick,R.J. (2002) Familial dysautonomia: detection of the IKBKAP IVS20(+6T → C) and R696P mutations and frequencies among Ashkenazi Jews. Am. J. Med. Genet., 110, 253–257.
2. Slaugenhaupt,S.A., Blumenfeld,A., Gill,S.P., Leyne,M., Mull,J., Cuajungco,M.P., Liebert,C.B., Chadwick,B., Idelson,M., Reznik,L. et al. (2001) Tissue-specific expression of a splicing mutation in the IKBKAP gene causes familial dysautonomia. Am. J. Hum. Genet., 68, 598–605.
3. Cuajungco,M.P., Leyne,M., Mull,J., Gill,S.P., Lu,W., Zaggag,D., Axellod,F.B., Maayan,C., Gusella,J.F. and Slaugenhaupt,S.A. (2003) Tissue-specific reduction in splicing efficiency of IKBKAP due to the major mutation associated with familial dysautonomia. Am. J. Hum. Genet., 72, 749–758.
4. Anderson,S.L., Coli,R., Daly,I.W., Kichula,E.A., Rork,M.J., Volpi,S.A., Ekstein,J. and Rubin,B.Y. (2001) Familial dysautonomia is caused by mutations of the IKAP gene. Am. J. Hum. Genet., 68, 753–758.
5. Shapiro,M.B. and Senapathy,P. (1987) RNA splice junctions of different classes of eukaryotes: sequence statistics and functional implications in gene expression. Nucelic Acids Res., 15, 7155–7174.
6. Roca,X., Olson,A.J., Rao,A.R., Enery,L., Kristensen,V.N., Borresen-Dale,A.L., Andresen,B.S., Kraier,A.R. and Sachidanandam,R. (2008) Features of 5′-splice-site efficiency derived from disease-causing mutations and comparative genomics. Genome Res., 18, 77–87.
7. Wang,Z. and Burge,C.B. (2008) Splicing regulation: from a parts list of regulatory elements to an integrated splicing code. RNA, 14, 802–813.
8. Lee,G., Ramirez,C.N., Kim,H., Zeltner,N., Liu,B., Radu,C., Bhdner,Y., Kim,Y.J., Choy,I.Y., Mukherjee-Clavin,B. et al. (2012) Large-scale screening using familial dysautonomia induced pluripotent stem cells identifies compounds that rescue IKBKAP expression. Nature Biotechnol., 30, 1244–1248.
9. Anderson,S.L., Qiu,J. and Rubin,B.Y. (2003) EGCG corrects aberrant splicing of IKAP mRNA in cells from patients with familial dysautonomia. Biochem. Biophys. Res. Commun., 301, 627–633.
10. Anderson,S.L., Liu,B., Qiu,J., Sturm,A.J., Schwartz,J.A., Peters,A.J., Sullivan,K.A. and Rubin,B.Y. (2012) Nutraeutical-mediated restoration of wild-type levels of IKBKAP-encoded IKAP protein in familial dysautonomia-derived cells. Mol. Nutr. Food Res., 56, 570–579.
11. Slaugenhaupt,S.A., Mull,J., Leyne,M., Cuajungco,M.P., Gill,S.P., Hims,M.M., Quintero,F., Axcellod,F.B. and Gusella,J.F. (2004) Rescue of a human mRNA splicing defect by the plant cytokinin kinetin. Hum. Mol. Genet., 13, 429–436.
12. Lee,G., Papapetrous,E.P., Kim,H., Chambers,S.M., Tomishima,M.J., Pasano,C.A., Ganat,Y.M., Menon,J., Shimizu,F., Viale,A. et al. (2009) Modelling pathogenesis and treatment of familial dysautonomia using patient-specific iPSCs. Nature, 461, 402–406.
13. Shetty,R.S., Gallager,C.S., Chen,Y.T., Hims,M.M., Mull,J., Leyne,M., Pickel,J., Kwok,D. and Slaugenhaupt,S.A. (2011) Specific correction of a splice defect in brain by nutritional supplementation. Hum. Mol. Genet., 20, 4093–4101.
14. Axellod,F.B., Liesb,L., Gold-Von Simson,G., Mendoza,S., Mull,J., Leyne,M., Nordcliff-Kaufmann,L., Kaufmann,H. and Slaugenhaupt,S.A. (2011) Kinetin improves IKBKAP mRNA splicing in patients with familial dysautonomia. Pediatr. Res., 70, 480–483.
15. Hims,M.M., Ibrahim,E.C., Leyne,M., Mull,J., Liu,L., Lazaro,C., Shetty,R.S., Gill,S., Gusella,J.F., Reed,R. et al. (2007) Therapeutic potential and mechanism of kinetin as a treatment for the human splicing disease familial dysautonomia. J. Mol. Med. (Berl), 85, 149–161.
16. Pors,E., Fernandez-Rodriguez,J., Benito,L., Ravella,A., Capella,G., Blanco,I., Serra,E. and Lazaro,C. (2010) Modulation of aberrant NF1 pre-mRNA splicing by kinetin treatment. Eur. J. Hum. Genet., 18, 614–617.
17. Liu,B., Anderson,S.L., Qiu,J. and Rubin,B.Y. (2013) Cardiac glycosides correct aberrant splicing of IKBKAP-encoded mRNA in...
