DOI 10.1002/mnfr.201100670 *Mol. Nutr. Food Res.* 2012, *56*, 570–579

RESEARCH ARTICLE

Nutraceutical-mediated restoration of wild-type levels of *IKBKAP*-encoded IKAP protein in familial dysautonomia-derived cells

Sylvia L. Anderson[1], Bo Liu[1], Jinsong Qiu[1,2], Andrea J. Sturm[1], Jamie A. Schwartz[3], Austin J. Peters[1], Kerry A. Sullivan[1] and Berish Y. Rubin[1]

[1] Laboratory for Familial Dysautonomia Research, Department of Biological Sciences, Fordham University, Bronx, NY, USA
[2] Department of Cellular & Molecular Medicine, University of California (San Diego), La Jolla, CA, USA
[3] Department of Surgery, St. Luke's-Roosevelt Hospital Center, Columbia University College of Physicians and Surgeons, New York, NY, USA

Received: October 5, 2011
Revised: November 10, 2011
Accepted: December 8, 2011

Scope: The reported ability to modulate the production of the wild-type transcript in cells bearing the splice-altering familial dysautonomia (FD)-causing mutation in the *IKBKAP* gene prompted an evaluation of the impact of commonly consumed nutraceuticals on the splicing of this transcript.

Methods and results: Screening efforts revealed the ability of the isoflavones, genistein, and daidzein, to impact splicing and increase the production of the wild-type, exon-20-containing, transcript, and the full-length *IKBKAP*-encoded IKB kinase complex associated protein (IKAP) in FD-derived cells. Genistein was also found to impact splicing in neuronal cells, a cell type profoundly impacted by FD. The simultaneous exposure of FD-derived cells to genistein and epigallocatechin gallate (EGCG) resulted in the almost exclusive production of the exon-20-containing transcript and the production of wild-type amounts of IKAP protein.

Conclusion: This study represents the first demonstration that the isoflavones, genistein and daidzein, possess splice-altering capabilities and that simultaneous treatment with genistein and EGCG reverses the splice-altering impact of the FD-causing mutation. These findings support the clinical evaluation of the therapeutic impact of the combined administration of these two commonly consumed nutraceuticals on this patient population and suggest a broader evaluation of the impact of these nutraceuticals on the in vivo RNA splicing process.

Keywords:
Alternative splicing / Familial dysautonomia / Genistein / Isoflavone / Nutraceutical

1 Introduction

Familial dysautonomia (FD) is an autosomal recessive disorder that impacts the development and survival of sensory, sympathetic, and some parasympathetic neurons [1, 2]. Individuals with FD exhibit decreased sensitivity to pain and temperature, cardiovascular instability, recurrent pneumonias, vomiting/dysautonomic crises, gastrointestinal dysfunction, and defective lacrimation [1–4]. FD is a life-threatening disorder with a high mortality rate. Two FD-causing mutations have been identified in individuals of Ashkenazi Jewish descent [5, 6]. The more common FD-causing mutation is a C→T transition in the sixth position of the 5′-splice donor site of intron 20 (termed as IVS20$^{+6T\rightarrow C}$) of the *IKBKAP* gene. This mutation alters the splicing process, resulting in the almost exclusive production of an exon-20-lacking transcript. Translation of this transcript produces a truncated nonfunctional protein.

The *IKBKAP*-encoded IKB (Ikappa B)-kinase-complex-associated protein (IKAP), initially named on the basis of

Correspondence: Professor Berish Y. Rubin, Department of Biological Sciences, Fordham University, 441 E. Fordham Rd., Bronx, NY 10458, USA
E-mail: rubin@fordham.edu
Fax: +1-718-817-2792

Abbreviations: **EGCG**, epigallocatechin gallate; **FD**, familial dysautonomia; **IKAP**, IKB kinase complex associated protein; ***IKBKAP***, inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase complex-associated protein

its reported ability to bind and serve as a scaffold protein for the IkB kinase complex [7], was subsequently found to not be associated with this complex [8, 9]. IKAP plays multiple cellular functions. It has been reported to be a subunit of the Elongator complex, where it plays a role in transcriptional elongation [10], and it has been demonstrated to be involved in regulation of the c-Jun N-terminal kinase (JNK) signaling pathway [11], exocytosis [12], neuronal development during embryogenesis [13], tRNA modification [14], and actin cytoskeleton regulation [15].

The observed production of a small, but detectable, amount of the exon-20-containing *IKBKAP* transcript in cell lines and tissues from individuals with FD [16] prompted a search for therapeutic modalities capable of facilitating the production of the exon-20-containing *IKBKAP* transcript in those affected. In 2003, we reported that the tocotrienols, members of the vitamin E family, elevate the production of the exon-20-containing transcript in FD-derived cells by increasing the rate of transcription of the *IKBKAP* gene [16]. Clinical analysis of the impact of the tocotrienol administration in individuals with FD revealed the tocotrienol-mediated elevated production of the full-length *IKBKAP* transcript in peripheral blood cells, improvement of cardiac function, a reduced incidence of dysautonomic crises, and increased eye moisture [4, 17–19, http://emedicine.medscape.com/article/1200921]. Additional screening efforts have demonstrated that epigallocatechin gallate (EGCG), a flavonoid present in plants, and kinetin, a plant cytokinin, facilitate the inclusion of exon 20 in the transcript generated in FD-derived cell lines [20, 21]. Experiments examining the impact of the combined treatment of cells with EGCG and tocotrienols revealed the synergistic production of the exon-20-containing transcript and functional IKAP protein [20]. Recent studies have demonstrated that the treatment of FD-derived cells with phosphatidylserine elevates *IKBKAP* gene expression, resulting in the increased production of the full-length transcript from the IVS20$^{+6T\rightarrow C}$-bearing allele [22].

The demonstrated therapeutic potential of EGCG and tocotrienols, two commonly consumed nutraceuticals, in individuals with FD encouraged us to develop a screening program to examine the impact of hundreds of commonly consumed plant or animal-derived compounds on the splicing of the *IKBKAP*-derived transcript in FD-derived cells. Our focus on nutraceuticals reflects the growing understanding of the biochemical and physiological impact of these food-based compounds that have known safety profiles.

Two of the compounds, genistein and the related daidzein, which are extensively studied plant-derived isoflavones found in soy, were found to modulate the splicing of the *IKBKAP*-derived transcript in FD-derived cells, producing an increased amount of the wild-type, exon-20-containing, transcript. We further report that treatment of FD-derived cells with a combination of genistein and EGCG results in the production of cellular levels of the IKAP protein that are comparable to that present in normal cells.

2 Materials and methods

2.1 Cell lines and reagents

The GM04589 and GM04663 cell lines, homozygous for the IVS20$^{+6T\rightarrow C}$ FD-causing mutation, and the GM02912 cell line, derived from a healthy individual, were obtained from the NIGMS Human Genetic Mutant Cell Repository (Camden, NJ, USA). The normal human FS_4 fibroblast cell line was kindly provided by Dr. Jan Vilcek. The LA1-55n, BE(2)-C, and CB-JMN neuronal cell lines were kindly provided by Dr. Robert A. Ross. All cell lines were maintained at 37°C in 5% CO_2 in Minimal Essential Medium (MEM)(fibroblast lines) or Dulbecco's Modified Eagle's Medium (DMEM) (neuronal lines) containing 10% fetal bovine serum (FBS). The HEK293 cell line was purchased from the ATCC (Manassas, VA, USA). Purified genistein and EGCG were purchased from Calbiochem (La Jolla, CA, USA) and daidzein from Sigma-Aldrich (St. Louis, MO, USA).

2.2 RNA preparation and reverse transcriptase polymerase chain reaction (RT-PCR)

Total RNA was purified using RNeasy Plus Mini Kits (Qiagen) according to the manufacturer's directions. A total of 25 ng of total RNA were amplified in 20 μL RT-PCR reactions using OneStep RT-PCR Kits (Qiagen, Valencia, CA, USA). One-step RT-PCR was carried out as follows: one cycle of 50°C × 30 min and 95°C × 15 min, followed by 42 cycles of 94°C × 20 s, 58–60°C × 30 s, and 72°C × 30 s, and then a final extension of 72°C × 2 min. PCR products were analyzed on 2% agarose gels. Primers, used at a concentration of 0.5 μM, are shown in Table 1. Quantitect SYBR Green RT-PCR Kits (Qiagen) were used for real-time RT-PCR (Quantitative RT-PCR (qRT-PCR)) analysis of the relative quantities of the exon-20-containing transcript (wild-type). ABI PRISM 7000 and 7500 Sequence Detection Systems (Applied Biosystems, Foster City, CA, USA), programmed as follows, were used to perform the real-time RT-PCR and analysis: 50°C × 30 min and 95°C × 15 min for one cycle, followed by 40 cycles of 94°C × 15 s, 57–60°C × 30 s, and 72°C × 30–34 s. Primers, used at a concentration of 0.5 μM, are shown in Table 1. Results obtained are in the form of threshold cycle, or C_T, which refers to the PCR cycle at which the fluorescence of the PCR is increased to a calculated level above background. A change of 1.0 in C_T, assuming 100% PCR efficiency, reflects a two-fold change in the starting amount of the RNA template that was amplified. To present relative amounts of PCR product obtained, results are expressed as "fold induction" of the wild-type exon-20-containing transcript in treated cells over that present in untreated cells. To control for the amount of RNA present in the samples, RT-PCR amplification of actin mRNA was performed and used to standardize the *IKBKAP* results.

Table 1. Oligonucleotide primers used

Template	Application	Primer	Sequence (5′→3′)	Accession #/template	Position
IKBKAP ex 19–21	Minigene construction	Forward	cacccattacaggccggcctgag	NT_008470	40 827 187–40 827 167
		Reverse	cttagggttatgatcataaatcag	NT_008470	40 825 483–40 825 506
pcDNA-*IKBKAP*	Minigene RT-PCR	Forward	ctggctagttaagcttggtacc	vector (pcDNA3.1)	892–913
		Reverse	ctcatgcattcaaatgcctc	NM_003640	2757–2738
IKBKAP ex 19–23	RT-PCR	Forward	gcagcaatcatgtgtccca	NM_003640	2553–2571
		Reverse	tagcatcgcagacaaggtc	NM_003640	2987–2969
IKBKAP Wild-type	qRT-PCR	Forward	agttgttcatcatcgagc	NM_003640	2671–2688
		Reverse	catttccaagaaacaccttaggg	NM_003640	2819–2585
ACTB	qRT-PCR	Forward	atgggtcagaaggattcc	NM_001101	223–240
		Reverse	gtgttgaaggtctcaaac	NM_001101	470–453
CLK1 ex 2–5	RT-PCR	Forward	gtcctgattgggatgacaag	NM_004071	107–226
		Reverse	gatcgatgcactccacaac	NM_004071	722–704
IHPK2 ex 1–3	RT-PCR	Forward	ggtgcgagagaaacaatagg	NM_001005910	69–88
		Reverse	ggatggccagctaacaagg	NM_001005910	489–471

2.3 Western blot analysis

Cells treated as described were washed twice with PBS and lysed in NuPAGE LDS sample buffer (Invitrogen, Carlsbad, CA, USA). Western blot analysis was performed essentially as described [23]. Equal amounts of protein fractionated on a 7% NuPAGE tris-acetate gel (Invitrogen) were blotted onto nitrocellulose (Bio-Rad, Hercules, CA, USA) and probed overnight with a monoclonal antibody raised against amino acids 796–1008 of IKAP (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). The blots were then washed and probed with a goat antimouse antibody conjugated to alkaline phosphatase (Promega, Madison, WI, USA), followed by detection with Western Blue Substrate solution (Promega). The loading of equal amounts of protein was confirmed by Coomassie Blue staining of a 7% NuPAGE tris-acetate gel that was run in parallel. Quantification of bands on Westerns was accomplished by scanning the blots, then determining the densities of the bands using ImageJ software [24].

2.4 Construction of the FD mutation-bearing *IKBKAP* minigene (*IKBKAP*-FD) and the generation of HEK293 cells constitutively expressing the *IKBKAP* minigene

A fragment of DNA containing *IKBKAP* genomic sequence spanning exons 19 to 21 was generated from DNA derived from a cell line homozygous for the IVS20$^{+6T\rightarrow C}$ FD-causing mutation, using PfuUltra II Fusion HS DNA polymerase (Stratagene, La Jolla, CA). Primers used are listed in Table 1. This DNA fragment was inserted into the pcDNA3.1/V5-His-Topo vector (Invitrogen) according to the manufacturer's instructions. The resulting minigene was purified using a QIAprep Spin Miniprep Kit (Qiagen) and sequenced by Sanger dideoxy sequencing (Genewiz, Inc., South Plainfield, NJ, USA). HEK293 cells seeded for 24 h in a 75 cm^2 flask were transfected with 400 ng of the minigene construct in the presence of Lipofectamine 2000 (Invitrogen) in Opti-MEM-reduced serum medium (Invitrogen) according to the manufacturer's directions. Six hours after transfection, the media was changed to fresh culture media containing 1 mg/mL G418. Surviving cells were isolated following a 4-week exposure to the G418 and the selected cells were maintained in media containing 1 mg/mL G418.

2.5 *IKBKAP* minigene splicing assay

HEK293 cells constitutively expressing the *IKBKAP*-FD minigene were seeded into wells 24 h before exposure to the compounds being studied. Following a 48 h incubation period, the cells were harvested and their RNA purified as described above. RT-PCR was performed as described above using primers (Table 1) that specifically amplified the minigene-derived *IKBKAP* exon-20-containing and exon-20-lacking transcriptst.

2.6 Analysis of *IKBKAP-*, *CLK1-*, and *IHPK2*-encoded RNAs in fibroblast and neuronal cells

Cells were seeded into 25 cm^2 flasks 1 week (fibroblast cell lines) or 24 h (neuronal cell lines) before treatment. Fibroblasts were confluent when treated and neuronal cells were approximately 25% confluent when treated. Following 48 h of treatment with 50 μg/mL genistein, cells were washed with PBS and total RNA was isolated as described above. Primers that spanned regions of alternative splicing in *IKBKAP*, *CLK1*, or *IHPK2* transcripts were used to simultaneously amplify both transcript forms. RT-PCR products were analyzed on 2% agarose gels.

3 Results

3.1 Construction of *IKBKAP*-FD minigene for compound screening

To characterize the impact of large numbers of compounds on the splicing of the exon-20-encoded sequence of the

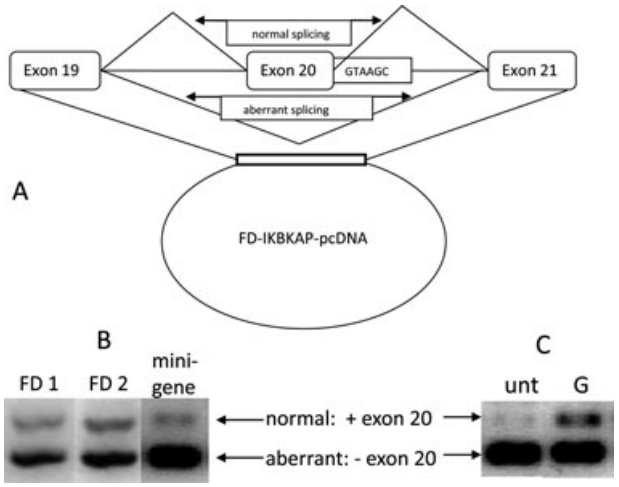

Figure 1. An FD minigene construct expressing normal and aberrant *IKBKAP* RNA splice variants expresses more normal transcript in response to genistein. (A) A pcDNA3.1/v5-His-TOPO vector construct containing a genomic DNA fragment encoding exons 19–21 of *IKBKAP* that contains the FD-causing IVS20$^{+6T\rightarrow C}$ mutation was stably transfected into HEK293 cells. The diagram depicts the two *IKBKAP*-encoded transcripts constitutively expressed from the minigene: (i) the normal (wild-type), exon-20-containing, transcript in which introns 19 and 20 are spliced out and exon 20 is retained; and (ii) the aberrant, exon-20-lacking transcript, in which introns 19 and 20 as well as exon 20 are spliced out. (B) RNA purified from two FD-derived cell lines, GM04589 (FD1) and GM04663 (FD2), and from the minigene-containing HEK293 cell line was subjected to RT-PCR with primers spanning exons 19–23 (for the FD cell-line-derived RNA) or with a vector-specific primer and a primer in exon 21 (for the minigene-transfected HEK293 cell-line-derived RNA) of the *IKBKAP* transcript under conditions as described in Section 2. Primer sequences are listed in Table 1. RT-PCR products were separated on agarose gels with a typical experiment depicted. (C) RNA, isolated from minigene-transfected HEK293 cells incubated in the absence (unt) or presence (G) of 50 μg/mL genistein for 48 h, was purified and subjected to RT-PCR as described above.

IKBKAP transcript produced by the allele bearing the FD-causing mutation, we generated an HEK293 cell line that contained and constitutively transcribed a minigene containing genomic DNA spanning exons 19–21 of the FD mutation-bearing *IKBKAP* allele (Fig. 1A). Using primers designed to amplify the vector-derived transcript, we observed that, like the *IKBKAP*-derived transcript produced in FD-derived cells, both an exon-20-containing (normal, i.e., wild-type) and an exon-20-lacking (aberrant) transcripts are produced by this minigene (Fig. 1B). As is the case in the FD-derived fibroblasts, the majority of the minigene-expressed *IKBKAP* transcripts lack exon 20.

3.2 Genistein impacts splicing of *IKBKAP* transcripts

The screening of several hundred extracts of commonly consumed compounds in extract or purified form revealed the ability of the isoflavone genistein to enhance the production of the exon-20-containing transcript from the *IKBKAP*-FD minigene (Fig. 1C). To characterize the effect of genistein on the *IKBKAP* transcript produced in FD-derived cell lines, two FD-derived fibroblast cell lines were incubated in the presence or absence of 50 μg/mL of genistein and the *IKBKAP* transcripts generated in these cells were characterized by RT-PCR, using primers recognizing exon 19 and 23 sequences that amplify both the exon-20-containing and the exon-20-lacking transcripts (Fig. 2A). Genistein not only increased the expression of the exon-20-containing transcript, but clearly changed the exon-20-containing: exon-20-lacking transcript ratio.

qRT-PCR was performed using primers that amplify only the exon-20-containing transcript (Fig. 2B). qRT-PCR analysis revealed an approximately two-fold increase in the level of exon-20-containing transcript produced in the genistein-treated cells as compared to untreated cells. Treatment of the FD-derived fibroblast cell lines with the related isoflavone daidzein similarly elevated the level of exon-20-containing transcript produced in the FD-derived cell lines (Fig. 2C).

3.3 Genistein increases IKAP protein levels in FD-derived cells

To determine whether the genistein-mediated increased production of the exon-20-containing transcript impacts the level of the full-length IKAP protein, Western blot analysis was performed on FD-derived cell lines incubated in the absence or presence of genistein (Fig. 3A and B). A clear elevation of the full-length protein was detected in the genistein-treated cells.

3.4 Genistein impacts splicing of *CLK1* and *IHPK2 RNA* in neuronal cells

As FD is a disorder that impacts the development and survival of sensory, sympathetic, and parasympathetic neurons, and as mRNA splicing has been demonstrated to be differentially regulated in different cell types, the observed impact of genistein on the splicing of the *IKBKAP* transcript in FD-derived fibroblast cell lines might not accurately reflect the impact of this compound on the splicing process occurring in neuronal cells. To study the impact of genistein on RNA splicing in neuronally derived cell lines, we first selected 30 genes from the kinase list on the Exonhit Therapeutics (Gaithersburg, MD) splicearray portal (http://portal.splicearray.com/PortalHome/) and designed primers to amplify the RNA resulting from114 alternative splicing events previously reported to occur in the processing of these gene transcripts. We assayed for the impact of genistein on these events by performing RT-PCR on RNA isolated from untreated and genistein treated FD-derived fibroblast cells and observed that genistein modulated two

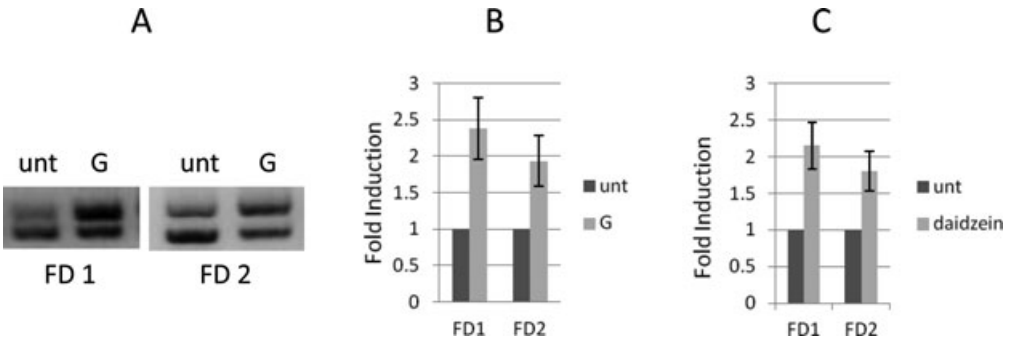

Figure 2. Genistein increases production of wild-type *IKBKAP*-encoded transcript by altering splicing. (A) RNA was isolated from two FD-derived cell lines, GM04589 (FD1) and GM04663 (FD2), incubated either in the absence (unt) or presence (G) of 50 μg/mL genistein for 48 h. The RNA was subjected to RT-PCR and analyzed on agarose gels as described in the legend to Fig. 1 and Section 2. (B) Real-time RT-PCR (qRT-PCR) was performed on the above-described RNA isolated from untreated (unt) and genistein-treated (G) FD-derived cell lines as described in Section 2. Primers used are shown in Table 1. Values obtained for the housekeeping gene, actin, were used to standardize results. "Fold induction" refers to the relative increase in exon-20 containing *IKBKAP* transcripts in genistein-treated cells as compared to untreated cells. Experiments were done three times and the results presented represent the mean ± SD. (C) RNA isolated from FD-derived cell lines that were incubated for 48 h in either the absence (unt) or the presence of 50 μg/mL daidzein was subjected to qRT-PCR as described above. Experiments were repeated three times and the results represent the mean ± SD.

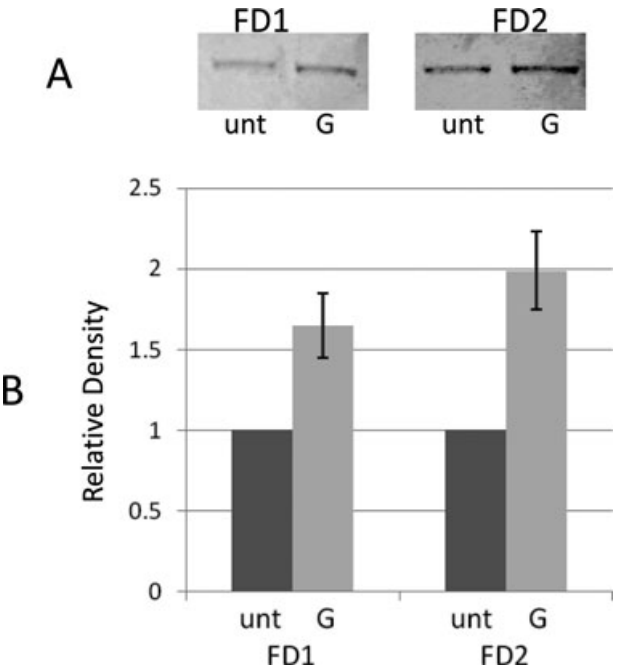

Figure 3. Genistein upregulates *IKBKAP*-encoded protein (IKAP) in FD-derived cells. (A) Two FD-derived cell lines, GM04589 (FD1) and GM04663 (FD2), were incubated in the absence (unt) or presence (G) of 50 μg/mL genistein for 48 h. Cells were harvested and protein extracts were prepared and subjected to Western blot analysis using a monoclonal antibody to IKAP as described in Section 2. (B) Blots were then scanned and the relative densities of the bands determined with ImageJ software [40]. Mean ± SD is shown.

of these splicing events. It mediated the inclusion of exon-4-encoded sequence in the CDC-like kinase 1 (*CLK1*)-encoded transcript and the inclusion of a novel 131 bp exon between exons 2 and 3 in the inositol hexaphosphate kinase 2 (*IHPK2*)-encoded transcript in the FD-derived fibroblast cells (Fig. 4A). We then examined the impact of genistein treatment on the *CLK1*- and *IHPK2*-encoded transcripts in several neuronally derived cells lines and observed that the splice-modulating activity of genistein occurs in cells of neuronal origin (Fig. 4B).

3.5 Combined treatment with EGCG and genistein synergistically upregulates wild-type *IKBKAP*-encoded RNA and protein levels

The reported ability of EGCG to modulate the splicing process and facilitate the inclusion of exon 20 in the transcript generated from the IVS20$^{+6T\rightarrow C}$-bearing *IKBKAP* allele [20] prompted an examination of the response of FD-derived cells to the combined treatment of genistein and EGCG. For this study, cells were treated in either the absence or the presence of EGCG, genistein, or a combination of these compounds and the impact on the splicing of exon 20 was examined by RT-PCR. The combined treatment of EGCG and genistein resulted in a synergistic increase in the relative amount of the exon-20-containing RNA generated in these cells (Fig. 5A). To quantify the impact of these treatments on the *IKBKAP* transcript, qRT-PCR was performed on the RNA isolated from these cells and the relative amounts of *IKBKAP* exon-20-containing (wild-type) transcripts were determined. As reported here and previously [20], treatment with genistein or EGCG elevates the level of the exon-20-containing transcript present in FD-derived fibroblasts. The combined treatment with these two compounds resulted in an approximately five-fold increase in the amount of wild-type transcript produced in these cells (Fig. 5B).

The dramatic increase in the amount of wild-type transcript present in the cells treated with the combination of

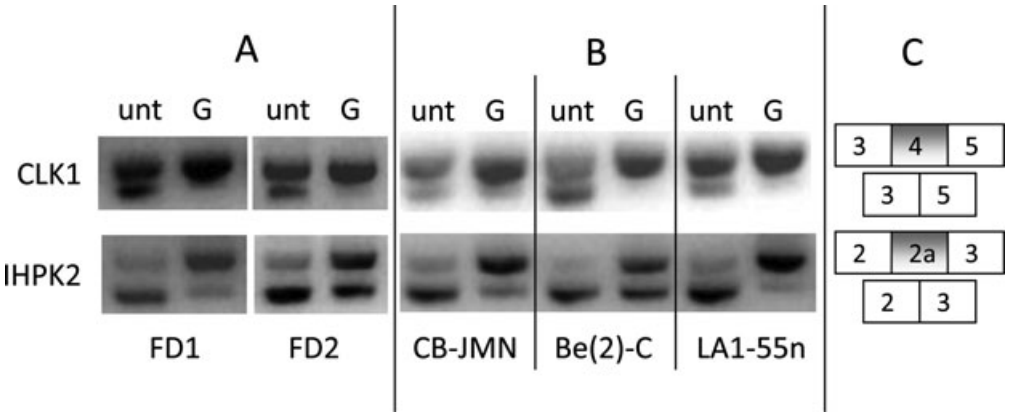

Figure 4. *CLK1* and *IHPK2* transcripts are alternatively spliced in FD-derived and neuronal cell lines treated with genistein. (A) RNA was isolated from two FD-derived cell lines, GM04589 (FD1) and GM04663 (FD2), incubated for 48 h in the absence (unt) or presence (G) of 50 μg/mL genistein and subjected to RT-PCR as described in Section 2. Primers used to amplify regions of alternative splicing in *CLK*1 and *IHPK2* transcripts are listed in Table 1. RT-PCR products were separated on agarose gels with a typical experiment depicted. (B) RNA was isolated from three neuronally derived cell lines, CB-JMN, Be(2)-C, and LA1-55n, that were incubated for 48 h in the absence (unt) or presence (G) of 50 μg/mL genistein and the *CLK1* and *IHPK*2 transcripts were analyzed as described above. (C) The schematic depicts alternatively spliced transcripts visualized on the agarose gels shown in "A" and "B" of this figure. The larger *CLK1* transcript contains exon 4, while in the smaller one, exon 4 is spliced out. The larger *IHPK*2 transcript contains an alternative exon 2a that is not present in the smaller band.

EGCG and genistein prompted an analysis of the impact of these treatments on the level of IKAP protein present in these cells. Western blot analysis revealed a synergistic increase in the level of full-length IKAP protein present in the cells treated with the combination of EGCG and genistein, and the amount of protein present in these cells is comparable to that observed in fibroblast cells derived from normal individuals (Fig. 6).

4 Discussion

The accurate removal of introns from pre-mRNA is a complex process that is regulated by *cis*-acting sequences and *trans*-acting factors. The *cis* sequences include donor and acceptor splice sites, intronic branch points, intronic polypyrimidine tracts, and auxiliary elements known as exonic and intronic splicing enhancers (ESEs and ISEs, respectively) and exonic

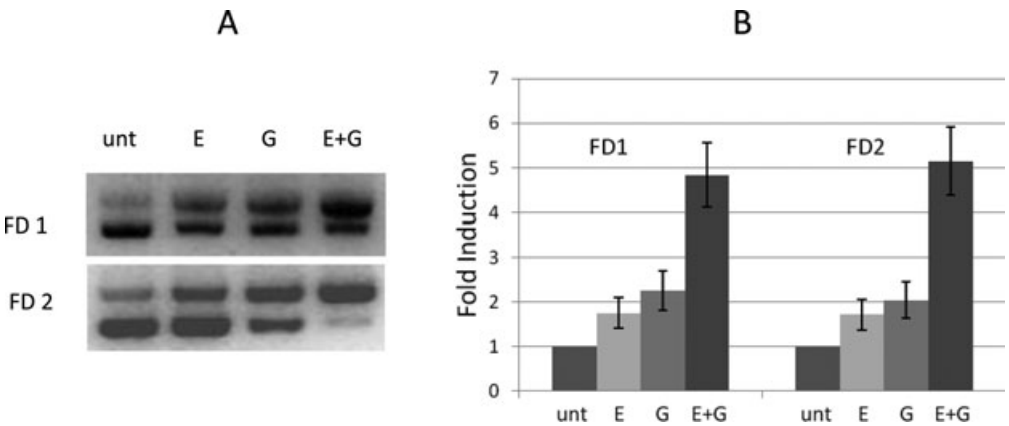

Figure 5. Exon-20-containing *IKBKAP* transcript is synergistically upregulated when FD-derived cell lines are treated with the combination of genistein and EGCG. (A) Two FD-derived cell lines, GM04589 (FD1) and GM04663 (FD2), were incubated in the absence of any reagent (unt) or with 25 μg/mL EGCG (E), 50 μg/mL genistein (G), or a combination of 25 μg/mL EGCG and 50 μg/mL genistein (E+G). After 48 h, RNA was purified and subjected to RT-PCR with *IKBKAP* primers in exons 19 and 23 as described in Section 2. Exon-20-containing (larger bands) and 20-lacking (smaller bands) RT-PCR products can be seen on a representative agarose gel showing one of three replicate experiments performed. (B) Real-time RT-PCR (qRT-PCR) analysis was performed on the above-described RNAs as described in Section 2. The mean relative quantities, ± SD, of exon-20-containing *IKBKAP* transcript in each of the two FD cell lines are shown.

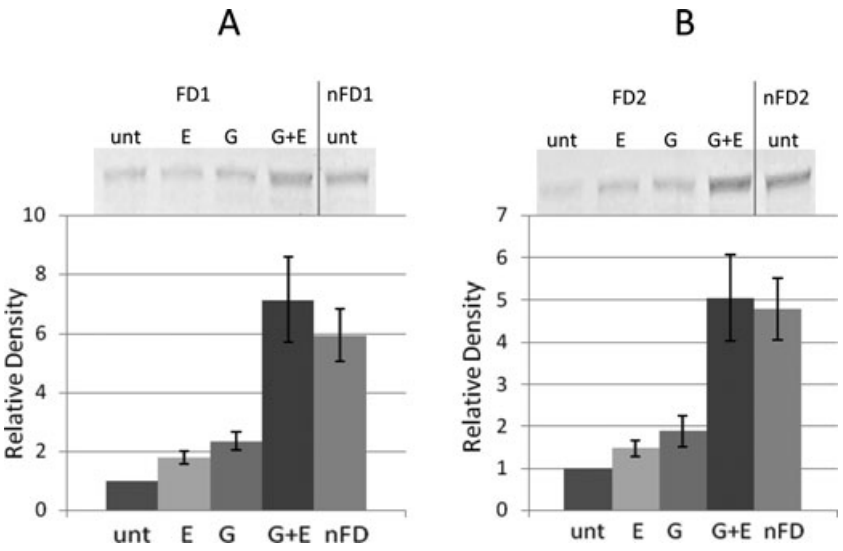

Figure 6. IKAP protein is synergistically induced in FD-derived cells treated with the combination of EGCG and genistein. (A) An FD-derived fibroblast cell line, GM04589 (FD1), was incubated in the absence (unt) or presence of 25 μg/mL EGCG (E), 50 μg/mL genistein (G), or a combination of 25 μg/mL EGCG and 50 μg/mL genistein (G+E). A cell line derived from a normal individual, GM02912 (nFD1), was untreated. After 48 h, cells were harvested, protein extracts prepared, and Western blot analysis performed as described in Section 2. A representative blot is shown. Blots were scanned and the relative densities of the bands determined with ImageJ software [40]. Mean ± SD is shown. (B) The experiment described above was performed using a second FD-derived cell line, GM04663 (FD2), and a second normal individual-derived cell line, FS_4 (nFD2). A representative blot is shown with a plot of the mean (± SD) relative densities of the IKAP bands.

and intronic splicing silencers (ESSs and ISSs, respectively) [25, 26]. C*is* elements recruit specific RNA binding proteins that either enhance or repress the use of the adjacent splice sites. The relative levels of the splicing factors during development and in different tissues regulate the splicing process and enable the generation of multiple proteins isoforms from a single pre-mRNA transcript. A survey of alternative pre-mRNA splicing events predicts that at least 74% of human genes undergo alternative splicing [27].

Disruption of exon recognition and misregulation of splicing are common causes of human disease. Estimations of the percentage of disease-causing mutations that affect the splicing process range from 15% [28] to 62% [29]. A recent study of possible splice-regulating sequence motifs suggests that 22% of known disease-causing missense mutations may mediate their deleterious effects as a result of their impact on the RNA splicing process [30]. The significant role that dysregulation of splicing plays in human diseases has encouraged the development of therapeutic approaches that target and modulate the RNA splicing machinery.

Since the identification of the genetic cause of FD and the demonstration that this disorder is almost exclusively caused by a mutation that alters the splicing process, causing the skipping of the exon-20-encoded sequence of the *IKBKAP* gene, much research has focused on the development of therapeutic approaches that facilitate the production of the correctly spliced transcript. To date, treatment of FD-derived cells with two compounds, EGCG and kinetin, has been demonstrated to alter the splicing process and to facilitate the inclusion of exon 20 into the *IKBKAP* transcript [20, 21]. As EGCG is present in commonly consumed foods, in particular, green tea, its use by the FD patient population was unimpeded and without adverse side effects. Improvement of neurological function has been recorded in individuals with FD who have ingested 700 mg of EGCG daily (Rubin et al., unpublished data). A recent study evaluating the impact of kinetin in individuals with FD has revealed that it effectively increases the production of the wild-type transcript in peripheral blood cells of these patients [31], but its use may be limited because its administration has been demonstrated to cause liver toxicity and nausea in many of the participants in this study [31].

The previous identification of commonly consumed compounds that facilitate the production of the wild-type *IKBKAP* transcript in cells derived from individuals with FD [16, 20] encouraged us to evaluate the impact of several hundred compounds, including genistein, on the production of the wild-type transcript. Genistein, a compound found in soy, has been previously reported to have protein tyrosine kinase inhibitor activity [32, 33] and neuro- and cardio-protective properties [34–42]. It may also play a therapeutic role in the treatment of cystic fibrosis (CF) through its effect on Cl^- gating and cystic fibrosis transmembrane conductance regulator (CFTR) maturation/localization [43, 44] and mucopolysaccharidoses (MPSs) by impairing glycosaminoglycan production [45]. In this study, we report for the first time the ability of genistein to alter the splicing process and facilitate the inclusion of exon 20 in the *IKBKAP*-encoded transcript produced in fibroblast cell lines derived from individuals with FD. As FD is a disorder that primarily impacts neuronal cells, and as the splicing process has been demonstrated to be differentially regulated in different cell types, we characterized the impact of genistein on the splicing of mRNAs present in neuronally derived cells. We observed that genistein treatment modulates the splicing process and facilitates increased exon inclusion in the *CLK1*- and *IHPK2*-encoded transcripts in these cells.

Characterization of the impact of genistein on more than 100 known alternative splicing events demonstrated that it modulates only a small percentage of these events.

Genistein's impact on the RNA splicing process reveals a newly identified mechanism by which it can modulate gene expression. As protein phosphorylation plays a critical role in the activity and cellular location of proteins involved in the regulation of RNA splicing [46], and as changes in splice site selection have been observed when the activity of kinases or phosphatases is manipulated [47, 48], it is possible that genistein is modulating the splicing process through its reported ability to act as a protein tyrosine kinase inhibitor [32, 33].

We report that the combined treatment of EGCG and genistein results in the almost exclusive production of the exon-20-containing transcript in FD-derived cells and the production of IKAP protein levels comparable to that found in normal human fibroblasts cells. As the FD-causing mutation impacts the structure and function of both the central and peripheral nervous system, the ability of both EGCG and genistein to cross the blood-brain barrier [49, 50] suggests that their use, individually and in combination, may benefit those with FD. Clinical studies to evaluate their combined efficacy are currently being designed.

The impact of pharmaceuticals and nutraceuticals on splicing events involved in genetic diseases has extensively been investigated in many laboratories. Much of this effort has focused on the development of therapeutic modalities to treat spinal muscular atrophy (SMA). SMA is a neurodegenerative disorder that results from homozygous deletions or mutations of part or all of the survival of motor neuron gene-1 (*SMN1*) [51]. The *SMN2* gene, which is retained in individuals with SMA, is nearly identical to the *SMN1* gene but contains a C→T base change at position +6 in exon 7 which facilitates alternative splicing and a low efficiency of exon 7 inclusion in the *SMN2* transcript [52, 53]. The exon-7-deficient *SMN2*–derived transcript generates a protein that is defective in self-oligomerization and is unstable [54]. Using cells derived from individuals with SMA, Lorson and coworkers examined the effect of polyphenolic botanical compounds on mRNA splicing and reported that treatment with EGCG resulted in an approximately 40% increase in the amount of total protein generated from the *SMN2* allele in SMA-derived cells [55]. This finding prompted us to investigate the impact of the combination of EGCG and genistein on the *SMN2*-derived transcript. We have observed that both EGCG and genistein individually increase the amount of exon-7-containing *SMN2* transcript produced in the SMA-derived fibroblast cells and that the combined treatment with these two nutraceuticals results in the exclusive production of the exon-7-containing *SMN2* transcript (Anderson et al., manuscript in preparation). This observation suggests the therapeutic potential for this combination of nutraceuticals in the treatment of individuals with SMA.

Our findings suggest that diet may influence the phenotype observed in individuals with identical splice-altering mutations. A very dramatic difference in the phenotype of individuals from two distinct populations that are homozygous for the same splice-altering IVS4$^{+4A\rightarrow T}$ mutation of the fanconi anemia gene, *FANCC,* has been reported by Futaki and coworkers [56]. Individuals of Ashkenazi Jewish descent homozygous for this mutation exhibit severe symptoms that are usually detected at birth. In contrast, individuals of Japanese descent who are homozygous for this same mutation show much milder symptoms that appear later in life. It has been suggested that environmental factors or other genes may modulate the phenotype associated with this mutation [56]. It is interesting to consider that the Japanese diet, which includes large amounts of soy, rich in genistein and daidzein, and green tea, which is high in EGCG, may provide the environmental basis for modulation of the splicing process and production of the correctly spliced transcript from the IVS4$^{+4A\rightarrow T}$-bearing *FANCC* gene.

Our findings that the ingestion of tocotrienols, a compound present in brown rice, facilitates the production of the full-length *IKBKAP* gene product [16] and mitigates some of the symptoms observed in individuals with FD [4, 18, 19, http://emedicine.medscape.com/article/1200921], clearly demonstrates the ability of food-derived compounds to impact the in vivo expression and manifestations of a splice-altering mutation. An environmental impact on the production of the functional gene product from the IVS20$^{+6T\rightarrow C}$-bearing *IKBKAP* allele is not limited to compounds found in foods. n 2005, Axelrod and coworkers reported that fludrocortisone, a synthetic corticosteroid that has been used to treat postural hypotension in patients with FD, alters the splicing process and reduces the production of the exon-20-containing *IKBKAP* transcript in cells derived from patients with FD [57]. This observation, taken together with our findings, supports the continued analysis of the effects of nutraceuticals and pharmaceuticals on the inclusion and exclusion of exon 20 sequence in the *IKBKAP* transcript produced in FD-derived cells.

We thank Dr. Robert Ross for his critical reading of the manuscript and his insightful comments. This work was funded by grants from Familial Dysautonomia Now, the Cure FD Foundation, and the Eric Alterman Foundation for F.D. Cure. We also acknowledge the generous support of NYC Council Member G. Oliver Koppell and his securing of funds from the New York City Council to support this research effort.

The authors have declared no conflict of interest.

5 References

[1] Riley, C. M., Day, R. L., Greely, D., Langford, W. S., Central autonomic dysfunction with defective lacrimation. *Pediatrics* 1949, *3*, 468–477.

[2] Axelrod, F. B., Nachtigal, R., Dancis, J., Familial dysautonomia: diagnosis, pathogenesis and management. *Adv. Pediatr.* 1974, *21*, 75–96.

[3] Axelrod, F. B., in: Robertson, D., Low, P. A., Polinsky, R. J. (Eds.), *Primer on the Autonomic Nervous System*, Academic Press, San Diego 1996, pp. 242–249.

[4] Rubin, B. Y., Anderson, S. L., The molecular basis of familial dysautonomia: overview, new discoveries and implications for directed therapies. *Neuromol. Med.* 2008, *10*, 148–156.

[5] Anderson, S. L., Coli, R., Daly, I. W., Kichula, E. A. et al., Familial Dysautonomia is caused by mutations of the IKAP gene. *Am. J. Hum. Genet.* 2001, *68*, 753–758.

[6] Slaugenhaupt, S. A., Blumenfeld, A., Gill, S. P., Leyne, M. et al., Tissue-specific expression of a splicing mutation in the *IKBKAP* gene causes familial dysautonomia. *Am. J. Hum. Genet.* 2001, *68*, 598–605.

[7] Cohen, L., Henzel, W. J., Baeuerle, P. A., IKAP is a scaffold protein of the IKB kinase complex. *Nature* 1998, *395*, 292–297.

[8] Otero, G., Fellows, J., Li, Y., de Bizemont, T. et al., Elongator, a multisubunit component of a novel RNA polymerase II holoenzyme for transcriptional elongation. *Mol. Cell* 1999, *3*, 109–118.

[9] Krappmann, D., Hatada, E. N., Tegethoff, S., Li, J. et al., The IKB kinase (IKK) complex is tripartite and contains IKKγ but not IKAP as a regular component. *J. Biol. Chem.* 2000, *275*, 29779–29787.

[10] Hawkes, N. A., Otero, G., Winkler, G. S., Marshall, N. et al., Purification and characterization of the human elongator complex. *J. Biol. Chem.* 2002, *277*, 3047–3052.

[11] Holmberg, C., Katz, S., Lerdrup, M., Herdegen, T., et al., A novel specific role for I kappa B kinase complex-associated protein in cytosolic stress signaling. *J. Biol. Chem.* 2002, *35*, 31918–31928.

[12] Rahl, P. B., Chen, C. Z., Collins, R. N., Elp1p, the yeast homolog of the FD disease syndrome protein, negatively regulates exocytosis independently of transcriptional elongation. *Mol. Cell.* 2005, *17*, 841–853.

[13] Chen, Y. T., Hims, M. M., Shetty, R. S., Mull, J. et al., Loss of mouse Ikbkap, a subunit of elongator, leads to transcriptional deficits and embryonic lethality that can be rescued by human IKBKAP. *Mol. Cell. Biol.* 2009, *29*, 736–744.

[14] Mehlgarten, C., Jablonowski, D., Wrackmeyer, U., Tschitschmann, S. et al., Elongator function in tRNA wobble uridine modification is conserved between yeast and plants. *Mol. Microbiol.* 2010, *76*, 1082–1094.

[15] Cheishvili, D., Maayan, C., Cohen- Kupiec, R., Lefler, S. et al., IKAP/Elp1 involvement in cytoskeleton regulation and implication for familial dysautonomia. *Hum. Mol. Genet.* 2011, *20*, 1585–1594.

[16] Anderson, S. L., Qiu, J., Rubin, B. Y., Tocotrienols induce IKBKAP expression: a possible therapy for familial dysautonomia. *Biochem. Biophys. Res. Commun.* 2003, *306*, 303–309.

[17] Anderson, S. L., Rubin, B. Y., Tocotrienols reverse IKAP and monoamine oxidase deficiencies in familial dysautonomia. *Biochem. Biophys. Res. Commun.* 2005, *336*, 150–156.

[18] Gold-von Simson, G., Axelrod, F. B., Familial dysautonomia: update and recent advances. *Curr. Probl. Pediatr. Adolesc. Health Care* 2006, *36*, 218–237.

[19] Rubin, B. Y., Anderson, S. L., Kapás, L., Can the therapeutic efficacy of tocotrienols in neurodegenerative familial dysautonomia patients be measured clinically? *Antioxid. Redox. Signal.* 2008, *10*, 837–841.

[20] Anderson, S. L., Qiu, J., Rubin, B. Y., EGCG corrects aberrant splicing of IKAP mRNA in cells from patients with familial dysautonomia. *Biochem. Biophys. Res. Commun.* 2003, *310*, 627–633.

[21] Slaugenhaupt, S. A., Mull, J., Leyne, M., Cuajungco, M. P. et al., Rescue of a human mRNA splicing defect by the plant cytokinin kinetin. *Hum. Mol. Genet.* 2004, *13*, 429–436.

[22] Keren, H., Donyo, M., Zeevi, D., Maayan, C. et al., Phosphatidylserine increases IKBKAP levels in familial dysautonomia cells. *PLoS One* 2010, *5*, e15884.

[23] Rubin, B. Y., Anderson, S. L., Sullivan, S. A., Williamson, B. D. et al., Purification and characterization of a human tumor necrosis factor from the LuKII cell line. *Proc. Natl. Acad. Sci. USA* 1985, *82*, 6637–6641.

[24] Rasband, W. S., ImageJ, *U.S. National Institutes of Health*, Bethesda, MD, USA, http://imagej.nih.gov/ij/, 1997–2011.

[25] Ladd, A. N., Cooper, T. A., Finding signals that regulate alternative splicing in the post-genomic era. *Genome Biol.* 2002, *3*, reviews0008.1–reviews0008.16.

[26] Zheng, Z. M., Regulation of alternative RNA splicing by exon definition and exon sequences in viral and mammalian gene expression. *J. Biomed. Sci.* 2004, *11*, 278–294.

[27] Johnson, J. M., Castle, J., Garrett-Engele, P., Kan, Z. et al., Genome-wide survey of human alternative pre-mRNA splicing with exon junction microarrays. *Science* 2003, *302*, 2141–2144.

[28] Stenson, P. D., Ball, E. V., Mort, M., Phillips, A. D. et al., Human gene mutation database (HGMD): 2003 update. *Hum. Mutat.* 2003, *21*, 577–581.

[29] López-Bigas, N., Audit, B., Ouzounis, C., Parra, G. et al., Are splicing mutations the most frequent cause of hereditary disease? *FEBS Lett.* 2005, *579*, 1900–1903.

[30] Lim, K. H., Ferraris, L., Filloux, M. E., Raphael, B. J. et al., Using positional distribution to identify splicing elements and predict pre-mRNA processing defects in human genes. *Proc. Natl. Acad. Sci. USA* 2011, *108*, 11093–11098.

[31] Axelrod, F. B., Liebes, L., Gold-von Simson, G., Mendoza, S. et al., Kinetin improves *IKBKAP* mRNA splicing in patients with familial dysautonomia. *Pediatr. Res.* 2011, *70*, 480–483.

[32] Akiyama, T., Ishida, J., Nakagawa, S., Ogawara, H. et al., Genistein, a specific inhibitor of tyrosine-specific protein kinases. *J. Biol. Chem.* 1987, *262*, 5592–5595.

[33] Akiyama, T., Ogawara, H., Use and specificity of genistein as inhibitor of protein-tyrosine kinases. *Methods Enzymol.* 1991, *201*, 362–370.

[34] Trieu, V. N., Uckun, F. M., Genistein is neuroprotective in murine models of familial amyotrophic lateral sclerosis and stroke. *Biochem. Biophys. Res. Commun.* 1999, *258*, 685–688.

[35] Zhao, L., Chen, Q., Diaz Brinton, R., Neuroprotective and neurotrophic efficacy of phytoestrogens in cultured hippocampal neurons. *Exp. Biol. Med.* 2002, *227*, 509–519.

[36] Bang, O. Y., Hong, H. S., Kim, D. H., Kim, H. et al. Neuroprotective effect of genistein against beta amyloid-induced neurotoxicity. *Neurobiol. Dis.* 2004, *16*, 21–28.

[37] Sonee, M., Sum, T., Wang, C., Mukherjee, S. K., The soy isoflavone, genistein, protects human cortical neuronal cells from oxidative stress. *Neurotoxicology* 2004, *25*, 885–891.

[38] Zeng, H., Chen, Q., Zhao, B., Genistein ameliorates beta-amyloid peptide (25–35)-induced hippocampal neuronal apoptosis. *Free Radic. Biol. Med.* 2004, *36*, 180–188.

[39] Azcoitia, I., Moreno, A., Carrero, P., Palacios, S., Garcia-Segura, L. M., Neuroprotective effects of soy phytoestrogens in the rat brain. *Gynecol. Endocrinol.* 2006, *22*, 63–69.

[40] Schreihofer, D. A., Redmond, L., Soy phytoestrogens are neuroprotective against stroke-like injury in vitro. *Neuroscience* 2009, *158*, 602–609.

[41] Altavilla, D., Crisafull, i, A., Marini, H., Esposito, M. et al., Cardiovascular effects of the phytoestrogen genistein. *Curr. Med. Chem. Cardiovasc. Hematol. Agents* 2004, *2*, 179–186.

[42] Rimbach, G., Boesch-Saadatmandi, C., Frank, J., Fuchs, D. et al., Dietary isoflavones in the prevention of cardiovascular disease—a molecular perspective. *Food Chem. Toxicol.* 2008, *46*, 1308–1319.

[43] Andersson, C., Roomans, G. M., Activation of deltaF508 CFTR in a cystic fibrosis respiratory epithelial cell line by 4-phenylbutyrate, genistein and CPX. *Eur. Respir. J.* 2000, *15*, 937–941.

[44] Schmidt, A., Hughes, L. K., Cai, Z., Mendes, F. et al., Prolonged treatment of cells with genistein modulates the expression and function of the cystic fibrosis transmembrane conductance regulator. *Br. J. Pharmacol.* 2008, *153*, 1311–1323.

[45] Piotrowska, E., Jakobkiewicz-Banecka, J., Maryniak, A., Tylki-Szymanska, A. et al., Two-year follow-up of Sanfilippo disease patients treated with a genistein-rich isoflavone extract: assessment of effects on cognitive functions and general status of patients. *Med. Sci. Monit.* 2011, *17*, CR196–202.

[46] Stamm, S., Regulation of alternative splicing by reversible protein phosphorylation. *J. Biol. Chem.* 2008, *283*, 1223–1227.

[47] Hartmann, A. M., Rujescu, D., Giannakouros, T., Nikolakaki, E. et al., Regulation of alternative splicing of human tau exon 10 by phosphorylation of splicing factors. *Mol. Cell. Neurosci.* 2001, *18*, 80–90.

[48] Allemand, E., Hastings, M. L., Murray, M. V., Myers, M. P. et al., Alternative splicing regulation by interaction of phosphatase PP2Cgamma with nucleic acid-binding protein YB-1. *Nat. Struct. Mol. Biol.* 2007, *14*, 630–638.

[49] Lin, L. C., Wang, M. N., Tseng, T. Y., Sung, J. S. et al., Pharmacokinetics of (-)-epigallocatechin-3-gallate in conscious and freely moving rats and its brain regional distribution. *J. Agric. Food Chem.* 2007, *55*, 1517–1524.

[50] Tsai, T. H., Concurrent measurement of unbound genistein in the blood, brain and bile of anesthetized rats using microdialysis and its pharmacokinetic application. *J. Chromatogr. A.* 2005, *1073*, 317–322.

[51] Lefebvre, S., Bürglen, L., Reboullet, S., Clermont, O. et al., Identification and characterization of a spinal muscular atrophy-determining gene. *Cell* 1995, *80*, 155–165.

[52] Lorson, C. L., Hahnen, E., Androphy, E. J., Wirth, B., A single nucleotide in the SMN gene regulates splicing and is responsible for spinal muscular atrophy. *Proc. Natl. Acad. Sci. USA* 1999, *96*, 6307–6311.

[53] Monani, U. R., Lorson, C. L., Parsons, D. W., Prior, T. W. et al., A single nucleotide difference that alters splicing patterns distinguishes the SMA gene SMN1 from the copy gene SMN2. *Hum. Mol. Genet.* 1999, *8*, 1177–1183.

[54] Lorson, C. L., Strasswimmer, J., Yao, J. M., Baleja, J. D. et al., SMN oligomerization defect correlates with spinal muscular atrophy severity. *Nat. Genet.* 1998, *19*, 63–66.

[55] Sakla, M. S., Lorson, C. L., Induction of full-length survival motor neuron by polyphenol botanical compounds. *Hum. Genet.* 2008, *122*, 635–643.

[56] Futaki, M., Yamashita, T., Yagasaki, H, Toda, T. et al., The IVS4 + 4 A to T mutation of the fanconi anemia gene FANCC is not associated with a severe phenotype in Japanese patients. *Blood* 2000, *95*, 1493–1498.

[57] Axelrod, F. B., Goldberg, J. D., Rolnitzky, L., Mull, J. et al., Fludrocortisone in patients with familial dysautonomia—assessing effect on clinical parameters and gene expression. *Clin. Auton. Res.* 2005, *15*, 284–291.

