Phosphatidylserine Increases IKBKAP Levels in Familial Dysautonomia Cells

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Abstract
Familial Dysautonomia (FD) is an autosomal recessive congenital neuropathy that results from abnormal development and progressive degeneration of the sensory and autonomic nervous system. The mutation observed in almost all FD patients is a point mutation at position 6 of intron 20 of the IKBKAP gene; this gene encodes the IκB kinase complex-associated protein (IKAP). The mutation results in a tissue-specific splicing defect: Exon 20 is skipped, leading to reduced IKAP protein expression. Here we show that phosphatidylserine (PS), an FDA-approved food supplement, increased IKAP mRNA levels in cells derived from FD patients. Long-term treatment with PS led to a significant increase in IKAP protein levels in these cells. A conjugate of PS and an omega-3 fatty acid also increased IKAP mRNA levels. Furthermore, PS treatment released FD cells from cell cycle arrest and up-regulated a significant number of genes involved in cell cycle regulation. Our results suggest that PS has potential for use as a therapeutic agent for FD. Understanding its mechanism of action may reveal the mechanism underlying the FD disease.

Introduction
Familial dysautonomia (FD) is an autosomal recessive congenital neuropathy that occurs almost exclusively in the Ashkenazi Jewish population with a carrier frequency between 1 in 27 to 1 in 32 [1,2]. Ashkenazi Jews of Polish descent have a higher carrier frequency of 1 in 18 [3]. FD results from abnormal development and progressive degeneration of the sensory and autonomic nervous system. Patients are severely affected with a variety of symptoms in most body systems. Among these symptoms are gastrointestinal and cardiovascular dysfunction, vomiting crises, abnormal sensitivity to pain and temperature, and recurrent pneumonia. Despite recent advances in patient management, about 50% of patients die before the age of 40 [4,5,6].

The gene associated with the disease was linked to chromosome 9q31 and identified as the IKBKAP gene. This gene encodes the IκB kinase complex-associated protein (IKAP; for simplicity, IKAP is used rather than IKBKAP to refer to the mRNA encoded by this gene). The point mutation observed in almost all FD patients (>99.5%) is a change from T to C at position 6 of the 5′ splice site of intron 20 [7,8]. The mutation results in a shift from constitutive inclusion to alternative splicing of exon 20 (Figure 1A). The splicing defect in FD is tissue specific. Tissues from the brain and nervous system express primarily mutant IKAP mRNA (skipping of exon 20), while other tissues express both wild-type and mutant mRNA in different ratios [4,9,10]. The skipped isoform has a frameshift relative to the wild-type mRNA that results in a premature stop codon, leading to considerably reduced IKAP expression [9,10]. The mutant transcript is a potential target for degradation by the nonsense mediated decay (NMD) pathway [11]. In our system, treatment with cycloheximide, an inhibitor of NMD, did not alter the level of the mutant transcript (data not shown); however, this is not consistent with observations from another study [12].

The IKAP protein is a 1332 amino acid, 150-kDa protein that is highly conserved in eukaryotes [13,14]. The function of IKAP has been a subject of much research but is still obscure. Based on homology to a yeast protein, ELP1, and co-purification with human elongator [13], IKAP is thought to be a subunit of the elongator complex, which assists RNA polymerase II in elongation of transcription in the nucleus [13,15,16]. There is evidence that in the cytosol IKAP is involved in regulation of the c-Jun N-terminal kinase (JNK) signaling pathway [14], tRNA modification [17], exocytosis [18], cell adhesion, migration of cells and reorganization of actin in the cytoskeleton [19,20]. IKAP may also play a role in oligodendrocyte differentiation and/or myelin formation [21] and in p53 activation [22]. IKAP is also crucial for vascular and neural development during embryogenesis [23].

Based on our current knowledge of FD and what is known so far about IKAP, we presume that the key for effective therapy of FD is increasing the amount of the normal, functional IKAP protein. We found that an FDA-approved food supplement, phosphatidylserine (PS), increased the amount of wild-type IKAP mRNA in FD cell lines. Further, long-term treatment of FD cells led to a significant increase in the amount of IKAP protein. Untreated FD cells accumulated at the G1 state with lower levels of cells in S and G2
states. PS treatment released this blockage, and this was associated with elevation in expression of genes involved in cell cycle regulation. Overall, our data indicate that PS has promise for treatment of FD patients.

Results

To examine the effect of potential drugs on the splicing of the IKAP mRNA we used three FD cell lines derived from three FD patients (termed FDA, FDB and FDD). In addition, a cell line derived from a parent of an FD patient, and therefore heterozygous for the FD mutation, and four matched cell lines derived from healthy individuals were used as controls. Analysis of the splicing pattern of exon 20 was performed by RT-PCR using primers to the endogenous IKAP mRNA. Figure 1B reveals that exon 20 was constitutively spliced in the control cell lines (lanes 1–4); 96% inclusion was observed in the heterozygous cells (lane 5), and from 38% to 52% inclusion was observed in the three FD cell lines (lanes 6–8). Figure 1B also demonstrates that IKAP mRNA was either expressed at higher levels or was considerably more stable in the control compared to the FD-derived cell lines. The levels of the wild-type and mutant isoforms were further quantified by real-time quantitative PCR (QPCR) analysis (Figure 1C). FD cells expressed considerably lower amounts of wild-type IKAP mRNA compared to control cells (Figure 1C, left side). The heterozygous cell line expressed 2.5-fold less wild-type IKAP mRNA than did control cells. FD cells, FDA, FDB and FDD, expressed 5.7-, 7.2- and 3.8-fold less, respectively, wild-type IKAP mRNA than did control cells. As shown in Figure 1C, right side, the skipped IKAP isoform was expressed at different levels in the FD cell lines compared to the heterozygous cell line. No skipped isoform was detected in control cells (data not shown).

In order to characterize the IKAP protein levels, a western blot analysis was conducted (Figure 1D). The control cell lines and the heterozygous cell line expressed the wild-type IKAP protein at the expected size of 150 kDa at similar levels (1.2 less IKAP protein in heterozygous cells). However, despite the presence of both wild-type and mutant mRNA isoforms as shown by RT-PCR, in FD cells only a very faint 150-kDa band was observed. Between 4 and 5 fold less IKAP protein was observed in FD cells than in control cells. No product of the size expected for the truncated protein, 79 kDa, was detected, in agreement with previous reports [10,24].

FD is characterized by dysfunction of the autonomic and sensory nervous system resulting from incomplete neuronal development and progressive neuronal degeneration [1]. We therefore sought substances that would affect neuronal function and would be safe for immediate testing in FD patients. We first examined two food supplements, one based on choline and one based on serine. The choline-based substance had little effect on the level of IKAP mRNA. However, the serine-based substance, phosphatidylserine (produced by Enzymotec under the SharpPS® brand), significantly increased IKAP mRNA and IKAP protein levels in cells derived from FD patients. PS, a nutritional supplement, is a major component of every living cell, especially neuronal cells [25], and slows cognitive degeneration in human subjects [26,27,28]. We found that in FD cell lines PS significantly raises IKAP mRNA and protein levels. PS, shown in Figure 2A, is used worldwide and is considered by the FDA as a safe and lawful dietary supplement. PS was added to FD cells at concentrations ranging from 0–300 μg/ml. The cells were harvested and RNA was extracted 24 and 48 hr following the addition of PS for each cell line; IKAP mRNA was analyzed by RT-PCR analysis and QPCR. The highest effect of PS on IKAP mRNA levels was obtained at different time points for each cell line. After 24 hr of treatment the best results were obtained for cell line FDA; we observed about a 5-fold increase in the amount of wild-type IKAP mRNA after treatment with 5, 10 or 100 μg/ml PS compared to levels in FDA cells treated with the solvent only (Figure 2B). At 200 μg/ml PS, the increase was 1.5 fold, and at 300 μg/ml a 1.2-fold increase in wild-type IKAP mRNA levels was observed. A possible explanation for lower efficacy at higher PS concentration is the toxicity of the solvent at high volumes, although the solvent itself did not affect IKAP mRNA levels (data not shown). After 48 hr of treatment, best results were obtained for cell line FDB; IKAP mRNA levels peaked at about 3-fold higher than levels in untreated cells at 100 and 200 μg/ml PS (Figure 2C).

In order to test whether expression of genes in addition to IKAP was altered as a result of PS treatment, we performed a human gene expression microarray analysis (Human Gene 1.0, Affymetrix) of cDNA samples from FDB cells treated with 100 μg/ml PS. Using SAM (Significance Analysis of Microarrays) analysis, we identified 877 genes with significantly different levels of expression following PS treatment: 441 genes were up-regulated and 436

Figure 1. Expression of IKAP mRNA and protein in FD cells. (A) Schematic diagram illustrating the area in IKAP responsible for FD. The FD mutation at position six of exon 20 splice donor site is shown by an arrow. In FD patients, two mRNA isoforms, one containing exon 20 and one without, can be present. (B) RT-PCR analysis of IKAP mRNA. RNA was extracted from control, heterozygous and FD cells and the endogenous splicing products were separated on a 2% agarose gel after RT-PCR reaction using primers to exon 19 and 21. Isoforms were quantified using ImageJ. GAPDH was used as control for cDNA amounts. (C) QPCR analysis of the IKAP mRNA. Left side: Level of exon 20 inclusion isoform (wt). Relative quantity represents normalization to control cells. Right side: Level of exon 20 skipped isoform (mut). Relative quantity represents normalization to heterozygous (Htz) cells. All values were normalized to HPRT mRNA. QPCR experiments were amplified in triplicate; results shown are mean values ± SD. (D) Analysis of IKAP protein levels. Western blotting of extracts from the indicated cell lines using an anti-IKAP antibody (Santa Cruz Biotechnology, D-17). Band intensities were quantified using ImageJ.

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genes were down-regulated. These genes are listed in Figure S2 of the Supplementary Material. We confirmed the effect of PS on six significantly up-regulated genes (YWHAH, TM4SF1, MYC, DCTPP1, BLM and BRIP1) and four significantly down-regulated genes (RCAN2, ROBO2, CYP7B1, and ITGB8) using QPCR (Figure 4). All values were normalized to levels of LZIC, which were unchanged by PS treatment. A gene ontology (GO) enrichment analysis of these genes was performed using the Database for Annotation, Visualization, and Integrated Discovery (DAVID) [29]. A significant GO enrichment was observed for the up-regulated genes coding for proteins involved in regulation of the cell cycle and DNA metabolic processes (Table 1). Complete tables are presented in Figure S3. As a group, the down-regulated genes did not present highly significant GO enrichment; most of them function in developmental processes (data not shown). Using the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways database in DAVID we also observed a significant enrichment for genes involved in the signaling pathways for pyrimidine and purine metabolism, as well as for genes known to be involved in base excision repair (Table 2).

Due to the fact that a significant number of genes up-regulated by PS treatment are involved in cell cycle regulation, we tested the effect of PS on the cell cycle distribution of FD cells using propidium iodide (PI) staining and flow cytometry. Cell cycle analysis of untreated FD cells revealed that a significantly higher fraction of the cells were in the G1 stage compared to control or heterozygous cells (Figure 5A). A lower fraction of the FD cells were in the S+G2 stages, 1.5-fold less than in the control cells, indicating that a low number of FD cells are in the dividing state (Figure 5B). Treatment of FD cells with PS significantly raised the fraction of cells in S+G2 stages by 1.5 fold compared to untreated FDA cells (Figure 5C) and 1.7 fold compared to untreated FDB cells (Figure 5D). These results indicate that PS releases FD cells from cell cycle arrest.

Figure 2. PS raises IKAP mRNA levels in FD cell lines. (A) Chemical structure of PS. (B) FDA cells or (C) FDB cells were treated with 0, 5, 10, 100, 200 and 300 μg/ml PS. RNA was extracted after 24 hr for FDA cell line and after 48 hr for FDB cell line. Left side: QPCR analysis of the level of exon 20 inclusion isoform (wt). Data were normalized to levels in untreated control cells. Right side: RT-PCR analysis of the splicing of the endogenous IKAP mRNA in FD cells. All splicing products were separated on a 2% agarose gel after RT-PCR reaction using primers to exons 19 and 21. The PCR products were eluted and sequenced. All experiments were repeated independently three times, and the results shown are representative of an average experiment. QPCR experiments were amplified in triplicate; results shown are mean values ± SD.

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Since PS treatment increased the amount of the wild-type Ikap mRNA and protein present in FD cells, we obtained three additional food supplements and tested their effect on splicing. The first is produced under the name Sharp-PS GOLD4508P and is a proprietary conjugate of PS and docosahexaenoic acid (DHA), an omega-3 fatty acid. Sharp-PS GOLD resembles the functional form of natural (brain) PS, increases DHA availability in the brain, and acts to increase cognitive abilities [30]. The second substance tested was L-alpha-glycerophosphorylcholine (GPC), a dietary supplement reported to improve mental performance that is marketed as SharpGPC 85F. GPC serves as a precursor for reconstituting a nerve cell membrane component. Clinical trials over the past two decades have demonstrated that treatment of subjects with adult-onset dementia disorders with GPC at 1,000–1,200 mg per day protects against...
Third, we tested Krill oil + 4225F, a proprietary complex of marine-derived DHA and eicosapentanoic acid (EPA), delivered as triglycerides or attached to phospholipids. Krill oil + 4224F also contains a significant amount of astaxanthin. A similar combination improves blood-lipid markers, including LDL, HDL and triglycerides, and in clinical studies, Krill oil has greater potency than omega-3, due to its unique structure and composition [32,33].

FD cell lines were treated with these three formulations, and the effects on the splicing of IKAP mRNA were analyzed by RT-PCR and QPCR (Figure 6). At 5 μg/ml, the Sharp-PS GOLD supplement increased the level of IKAP mRNA level 4.2 fold compared to levels in untreated cells (Figure 6A). Sharp-PS GOLD increased the level of the wild-type IKAP mRNA at one-twentieth the effective concentration of PS. Treatment with GPC did not significantly increase the amount of wild-type IKAP mRNA.

| Table 1. GO analysis for up-regulated genes. |
|---------------------------------------------|-------------------------------------|-----------------|----------------|
| Cluster 1 | Enrichment score=33.994 | GO term | p-value | # genes |
| Cell cycle | 7.15E-41 | 96 |
| Cell cycle phase | 6.16E-35 | 69 |
| Cell cycle process | 2.08E-34 | 77 |
| M phase | 6.40E-34 | 61 |
| Mitotic cell cycle | 6.44E-30 | 60 |
| Mitosis | 4.02E-29 | 48 |
| Nuclear division | 4.02E-29 | 48 |
| M phase of mitotic cell cycle | 9.58E-29 | 48 |
| Organelle fission | 2.76E-28 | 48 |
| Cell division | 3.79E-24 | 49 |
| Cluster 2 | Enrichment score=25.16 | GO term | p-value | # genes |
| DNA metabolic process | 5.93E-35 | 74 |
| Response to DNA damage stimulus | 2.65E-21 | 51 |
| DNA repair | 5.23E-21 | 45 |
| Cellular response to stress | 1.89E-13 | 51 |
| Cluster 3 | Enrichment score=22.12 | GO term | p-value | # genes |
| Nuclear lumen | 3.65E-27 | 104 |
| Intracellular organelle lumen | 2.43E-22 | 108 |
| Membrane-enclosed lumen | 4.98E-22 | 110 |
| Organelle lumen | 1.60E-21 | 108 |
| Nucleoplasm | 1.44E-13 | 62 |
| Nucleolus | 1.05E-11 | 52 |
| Cluster 4 | Enrichment score=21.08 | GO term | p-value | # genes |
| Chromosome | 2.63E-31 | 65 |
| Chromosomal part | 7.37E-29 | 58 |
| Chromosome, centromeric region | 5.19E-18 | 29 |
| Condensed chromosome | 2.93E-15 | 27 |
| Condensed chromosome, centromeric region | 4.83E-15 | 21 |
| Condensed chromosome kinetochore | 6.45E-15 | 20 |
| Condensed chromosome kinetochore | 1.44E-13 | 21 |
| Chromosome segregation | 2.21E-12 | 21 |

Enriched categories were identified using DAVID to cluster differentially up- and down-regulated genes into functional categories using GO identification terms. Significant GO enrichment (p-value < 0.05 after FDR multiple testing correction) was observed only for the up-regulated genes.

1FDR multiple testing correction.
2Number of identified genes in Gene Ontology (GO) category.

Enriched categories were identified using DAVID; See supplementary file 3 for complete tables.

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animals at a higher dose than recommended for use in humans at 520 mg per day. The substance has been tested in humans and orally to adults at a dosage of 200–300 mg per day and to children effective for treatment of age-related cognitive decline in an open (ADHD) in children [40]. Plant-derived PS was shown to be effective in treating attention deficit hyperactivity disorder in some cases improve cognitive function, especially in adults risk of dementia in the elderly [27]. Use of PS derived from bovine may improve the risk of cognitive dysfunction and may reduce the FDA has allowed two qualified health claims for the use of PS: PS increased the levels of normal IKAP [12,24,34] and in patients heterozygous for the FD mutation [35]. A significant increase in wild-type IKAP mRNA levels and a shift towards a higher level of exon 20 inclusion was observed with an optimal concentration of 100 μM of kinetin (Figure 7A), with no toxicity observed. In contrast, tocotrienol, a member of the vitamin E family that was reported to induce IKAP expression in FD cells [36,37,38], did not affect IKAP mRNA expression in our system (Figure 7B); this observation is similar to that of another publication [39]. These results indicate that the FD cells used in this study are sensitive to kinetin treatment but not to tocotrienol.

**Discussion**

FD is caused by a mutation in the IKAP gene that leads to aberrant splicing. The skipped isoform produced from the mutant gene is unable to produce normal IKAP protein, and the levels of normal IKAP mRNA in FD patients fail to provide sufficient amounts of IKAP protein. Subjects that are heterozygous for the FD mutation express a lower level of wild-type IKAP mRNA than do normal subjects (Figure 1B, 1C) but do not display any of the clinical symptoms of FD [35]. We sought substances already proven safe for clinical use that would increase the levels of wild-type IKAP mRNA in cell lines created from cells taken from FD patients in order to identify potential treatments.

We demonstrated that phosphatidylserine significantly increased the levels of normal IKAP mRNA and protein. The FDA has allowed two qualified health claims for the use of PS: PS may improve the risk of cognitive dysfunction and may reduce the risk of dementia in the elderly [27]. Use of PS derived from bovine brain cortex has been shown to slow cognitive degeneration and in some cases improve cognitive function, especially in adults suffering from mild dementia [26,27,28]. The substance is also effective in treating attention deficit hyperactivity disorder (ADHD) in children [40]. Plant-derived PS was shown to be effective for treatment of age-related cognitive decline in an open trial study [41]. PS derived from soy lecithin is currently given orally to adults at a dosage of 200–300 mg per day and to children at 520 mg per day. The substance has been tested in humans and animals at a higher dose than recommended for use in humans with no toxicity observed [42,43,44,45]. The PS/DHA conjugate (Sharp-PS GOLD) increased the level of IKAP mRNA at much lower concentrations than the effective concentrations of PS in the FD cells used in our study. This is probably due to the combined, and possibly synergistic, effects of PS and DHA. This implies that analogs of PS should be further investigated.

The effect of PS on IKAP levels was not related to changes in the ratio between exon 20 inclusion and skipping. Rather PS acted by increasing levels of transcription of this gene; increased levels of mRNA led to elevation of the level of the normal IKAP protein. In an attempt to elucidate the mechanism of action of PS, we compared gene expression in FD cells in the presence and absence of PS. A significant number of the genes up-regulated following PS treatment are involved in cell cycle regulation. That FD cells have improper regulation of the cell cycle was confirmed by examining the cell cycle distribution of FD cells. Compared to control or heterozygous cells, a lower percentage of FD cells were in growth phases. A significant fraction of FD cells were in G1 compared to control cells; this suggests that exit from G1 into the S phase is impaired in FD cells. This may mean that the IKAP protein is directly or indirectly involved in cell cycle checkpoint regulation, and the reduced levels of IKAP in FD cells results in abnormal growth of cells. PS treatment of FD cells elevated wild-type IKAP mRNA and protein levels and resulted in a cell cycle distribution similar to that of the control cells. A significant number of genes up-regulated following PS treatment are also involved in DNA metabolic processes and in DNA repair mechanisms. Upon increases in cellular metabolism and synthesis of nucleotides, transcription by RNA polymerase II increases. This enhancement of RNA polymerase II activity results in a meaningful increase in levels of mRNAs from genes that are usually under expressed, such as IKAP in FD-derived cells. Increasing the expression level of IKAP mRNA over a certain threshold leads to an increase in full-length IKAP protein levels. Although the increase in transcription level was not specific to the IKAP gene, its positive effect on the level of IKAP is unquestionable. As PS has been proven safe, it should be tested in FD patients.

The gene expression array analysis revealed that levels of expression of a large number of genes were altered due to PS treatment. It is not surprising that PS affects genes other than IKAP, and these effects may be direct or indirect. Kinetin, for example, was shown to affect splicing of other genes [24], and many genes change their expression level in IKAP-deficient cells [20,21,39]. Of particular interest in the list of genes demonstrated

![Image](https://example.com/image.png)

**Table 2.** Signaling pathways enrichment of genes up-regulated by PS treatment.

| Cluster 1 | Enrichment score | Term | p-value | # genes |
|----------|------------------|------|---------|---------|
|          | 4.42             | Pyrimidine metabolism | 0.001268 | 13 |
|          |                  | Purine metabolism | 0.03833 | 14 |

| Cluster 2 | Enrichment score | Term | p-value | # genes |
|----------|------------------|------|---------|---------|
|          | 4.05             | Base excision repair | 0.011218 | 8 |

Enriched signaling pathways were identified using the KEGG pathways database in DAVID to cluster up- and down-regulated genes. Significant KEGG enrichment (p-value <0.05 after FDR multiple testing correction) was observed only for the up-regulated genes.

1FDR multiple testing correction.

2Number of identified genes in the KEGG pathway category.

Enriched categories were identified using DAVID.

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to be affected is the TM4SF1 gene, a transmembrane 4L six family member, also known as L6-Ag, that influences cell motility [46,47]. It is possible that the PS-induced increase in TM4SF1 mRNA levels increases IKAP levels through regulation involving cell migration, which is impaired in Elongator-depleted cells [20]. Also of interest is the increase in MYC expression; this v-myc myelocytomatosis viral oncogene homolog functions as a transcription factor [48]. Since FD is characterized by defects in the Elongator complex, involved in transcription elongation [13,15], PS may affect IKAP levels through regulation of transcriptional elongation.

Several gene expression profiling involving IKAP were reported. Analysis of cerebrum samples of FD patients revealed down regulation of genes involved in oligodendrocyte differentiation and myelination compared to controls [21]. These genes do not correlate with genes up-regulated after PS treatment. This is probably because PS affects transcription rather than IKAP function directly. It is also possible that these differences are related to the type of cell tested (fibroblasts compared to cerebrum). Also, gene expression analysis of HeLa cells treated with RNAi directed against IKAP revealed that a significant fraction of down-regulated genes encode proteins regulating cell motility, cell proliferation, cellular processes such as autophagy, metabolism and DNA repair; the up-regulated genes were involved in metabolism, transcription and apoptosis [20]. PS presumably does not influence IKAP directly; therefore, it is not

Figure 5. PS alters the cell cycle distribution of FD cells. One day prior to treatment 750,000 cells were seeded. The cells were treated with 100 μg/ml PS for 24 hr and then fixed and stained with PI according to the flow cytometry protocol. The percentage of cells (A) at the G1 stage and (B) at the growth stages, S+G2, of untreated control, heterozygous, FDA and FDB cells. The percentages of cells at the growth stages, S+G2, of (C) FDA or (D) FDB cells following PS treatment compared to untreated FD cells. All experiments were repeated independently four times, and the results shown are mean values ± SD. P-values of significant results are indicated.

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unexpected that genes with altered expression as a result of PS treatment are different from genes affected by silencing of IKAP. Microarray analysis performed on induced pluripotent stem cells from FD patients compared to normal controls revealed that down-regulated genes are involved in peripheral neurogenesis and neuronal differentiation [39]. However, the fibroblast cells used in this study were already differentiated, as are most of the cells in the human body. The divergence of our altered genes due to PS treatment from those reported in the literature in cells without IKAP indicate the unique mechanism of action that results in the PS-induced increase in IKAP levels. This body of data also emphasizes the fact that IKAP is a multi-functional protein that contributes to many processes in the cell.

Compounds that increase levels of correctly spliced transcripts have been reported in studies of several disorders that result from exon skipping. Most examples are found in experiments involving models of the neuromuscular disorder spinal muscular atrophy (SMA). Valproic acid [49], sodium butyrate [50], hydroxyurea [51], aclarubicin [52], benzamide M344 [53] and a tetracycline-like compound [54] have all been shown to promote inclusion of exon 7 in the SMN2 transcript. For FD, several drugs, including kinetin and tocotrienol, increase the levels of wild-type IKAP mRNA and protein. In our system, kinetin significantly elevated the level of the normal IKAP isoform. It also altered the ratio between the included and the skipped isoforms, shifting the ratio toward inclusion. Kinetin is a plant hormone from the cytokinin family that promotes cell division [55]. It is used in the cosmetic industry as an anti-oxidant with anti-ageing effects [56]. However, kinetin has cytotoxic activity [57], especially at high concentrations [58]. Additionally, in an in vivo study treating carriers of FD with kinetin, some adverse effects resulted from treatment [35]. Therefore, kinetin has potential, but its toxicity must be further assessed. Tocotrienols are members of the vitamin E family considered to have neuroprotective and antioxidant properties [59]. The toxicity levels for humans are presently unknown, although estimates have been made based on studies in rats [60]. Tocotrienol, however, did not have an effect in our FD systems (Figure 7B) or in another study [39]. Our data suggest that PS, a
non-toxic food supplement already widely used in humans, and its analogs are suitable candidates for testing in FD patients.

**Materials and Methods**

**Cell culture and treatment**

Control fibroblast cell lines were kindly provided by Prof. Aharon Razin from the Hebrew University Medical School in Jerusalem. FDA and FDB cell lines, as well as the heterozygous cell line, were obtained from the NIGMS Human Genetic Mutant Cell Repository: FDA cell line is the GM00850, FDB is GM02342 and the heterozygous cell line is GM04664 (purchased by Prof. Aharon Razin). The FDD cell line was received from Channa Maayan. All cell lines were immortalized by Ida Vig and Yaniv Lerenthal from Tel Aviv University by transducing the cells with a retroviral vector expressing the catalytic subunit of human telomerase (hTERT), as described previously [61]. HEK 293T cells were obtained from the American Type Culture Collection (CRL-11268). 293T and control fibroblast cell lines were cultured in Dulbecco’s modified Eagle’s medium (DMEM) with 10% fetal calf serum (FCS), 0.29 mg ml⁻¹ L-glutamine, 100 U ml⁻¹ penicillin and 0.1 mg ml⁻¹ streptomycin at 37°C in a humidified atmosphere with 5% CO₂. Heterozygous and FD cell lines were cultured in medium containing 20% FCS. Cells were seeded in 6-well or 10-cm plates one day prior to treatment and were 80% confluent at the time of treatment.

For the long-term treatment, the cells were seeded and on the next day 100 µg/ml PS was added. Every two days the medium was replaced and fresh PS was added. One week after the initial PS treatment, the cells were split to allow proper growth. The PS treatment was continued for an additional week, mRNA samples were taken 3, 7 and 14 days from the initial treatment.

PS (soy lecithin derived, produced under the name Sharp PS) and Sharp PS GOLD were obtained from Enzymotec (http://www.sharp-ps.com/products/ps/ps.html) as powders. According to the company’s recommendations, the powders were dissolved in 95:5 chloroform/methanol and a stock solution was made at 20 mg/ml. Sharp GPC was obtained from Enzymotec as a viscous liquid and diluted in water. Krill oil was obtained from Enzymotec as a viscous liquid and diluted in corn oil. Kinetin solution was purchased from Sigma. Tocotreinol, full spectrum E with tocotrienols, was produced by Swanson Ultra.

**Semi-quantitative RT-PCR and QPCR analysis of **IKAP** transcripts**

After the indicated time, total RNA was extracted from treated and untreated samples using TRI reagent (Sigma). RNA concentrations were determined using a NanoDrop ND-1000 spectrophotometer. For RT-PCR, 2 µg of total RNA were amplified using avian myeloblastosis virus reverse transcriptase (RT-AMV, Roche) with an oligo(dT) reverse primer. Products were amplified with Red Load Taq master mix (Larova) using primers for exon 19 (forward, 5’-CATTACAG-GCCGGCCTGAGCAGCA-3’) and exon 21 (reverse, 5’-CTTAGGGTTATGATCATAAATCAGATT-3’) of **IKAP**. The splicing products were separated on 2% agarose gels and were sequenced. Alternative splicing isoforms were quantified using ImageJ.
QPCR was performed using the Stratagene Mx3005P System using the Absolute Blue QPCR SYBR Green ROX mix (Thermo Scientific). Primers used to detect the IKAP wild-type isoform (inclusion of exon 20) were exon19F (5'-CTGTTGTGCC-3') and exon20R (5'-GGTCCTCTTTCACCACCAAGCT-3') and HPRT-F (5'-TGAACATGGCAAAACATGCA-3') and HPRT-R (5'-GGTCCTCTTTCACCACCAAGCT-3') as used in [24]. Detection of the IKAP mutant isoform (skipping of exon 20) was performed using primers bridge 19-21F (5'-CAACGATTCTCGATTGCC-3') and exon21R (5'-GTTGGTTATGATGTAATATCAG-3'). Analysis was performed using the MxPro 4.01 software. All primer pairs yielded a linear standard curve with an R² > 0.985 and efficiency of reaction between 90–105%. Data was normalized to untreated cells. All experiments were repeated at least three times, and QPCR experiments were performed in triplicate.

Western blot analysis
Total proteins were extracted from the cells using a hypotonic lysis buffer (50 mM Tris-HCl, pH 7.5, 1% NP40, 150 mM NaCl, 0.1% SDS, 0.5% deoxycholic acid, 1 mM EDTA) containing protease inhibitor and phosphatase inhibitor cocktails I and II (Sigma). After 20 min centrifugation at 14,000 g at 4°C, the supernatant was collected and protein concentrations were measured using BioRad Protein Assay (BioRad). Proteins were separated in an 8% SDS-PAGE and then electroblotted onto a Protran nitrocellulose transfer membrane (Schleicher & Schuell). The membranes were probed with either a goat anti-IKAP antibody (D-17, Santa Cruz) or a mouse anti-IKAP antibody (BD Biosciences) for 12 hr at 4°C, followed by incubation with secondary antibody, donkey anti-goat IgG HRP (Santa Cruz) or goat anti-mouse IgG (Jackson), as appropriate. Immunoblots were visualized by enhanced chemiluminescence (SuperSignal West Pico chemiluminescent substrate; Thermo scientific) and exposure to X-ray film.

Microarray analysis
Six Human Gene 1.0 microarray chips (Affymetrix) were used: Three chips were used for the analysis of cDNA prepared from FDB cells treated with PS, and three chips were used for the analysis of cDNA prepared from FDB cells treated with the solvent only (used as controls). Cells were treated with 100 μg/ml PS. RNA was extracted 24 hr later using a Qiagen RNeasy Plus mini kit, according to the manufacturer’s protocol. cDNA and microarray chips were prepared and hybridized by the Bioinformatics Unit of Tel Aviv University. One chip from each assay was omitted from the analysis because of unsatisfying clustering results.

Analysis of microarray results was done using SAM. The results were validated using QPCR. Primers for microarray QPCR validation are presented in Figure S4. Enrichment analysis of these genes was done using DAVID to cluster differentially up and down-regulated genes into functional categories using GO identification terms, as well as enrichment in signaling pathways using the KEGG pathways database.

Flow cytometry
Cells were trypsinized, collected in PBS and centrifuged for 10 min at 282 g at 4°C. The cells were then fixed by dropwise addition of the cell suspension into an ice-cold 70% ethanol in PBS with gentle vortexing, and kept overnight at −20°C. Next, the cells were washed with PBS, left for 30 min at 4°C and then suspended in PBS containing 5 μg/ml DNase-free RNase and stained with PI. Sorting was carried out using FACSsort flow cytometry (Becton Dickinson) at 10,000 events per sample. Cell cycle analysis was performed with the ModFit software.

Supporting Information
Figure S1 PS effect on IKAP mRNA levels in FDD cell line. FDD cells were treated with 0, 5, 10, 100, 200 and 300 μg/ml PS. RNA was extracted after 24 hr (A) and 48 hr (B). Left side: QPCR analysis of the level of exon 20 inclusion isoform (wt). Data were normalized to levels in untreated control cells. Right side: RT-PCR analysis of the splicing of the endogenous IKAP mRNA in FD cells. All splicing products were separated on a 2% agarose gel after RT-PCR reaction using primers to exons 19 and 21. The PCR products were clutered and sequenced. All experiments were repeated independently three times, and the results shown are representative of an average experiment. QPCR enrichment experiments were amplified in triplicate; results shown are mean values ± SD.

Figure S2 Genes up- and down-regulated after PS treatment in FD cells. Gene expression microarray analysis was performed on cDNA samples from FDB cells treated with PS; data were compared to that from FDB cells treated with the solvent only. All 441 up-regulated genes and 436 down-regulated genes identified by significant analysis of microarray (SAM) are listed.

Figure S3 Complete Gene Ontology enrichment for up-regulated genes. Continued from Table 1. GO analysis for up-regulated genes revealed by microarray analysis following PS treatment of FDB cells. Enriched categories were identified using DAVID to cluster differentially genes into functional categories using GO identification terms. Significant GO enrichment (p-value < 0.05 after FDR multiple testing correction) was observed only for the up-regulated genes.

Figure S4 Primers used for validation of microarray results. Primers forward and reverse used for validation by QPCR of 11 genes from the microarray analysis.

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