Human Molecular Genetics, 2007, Vol. 16, No. 17 **2097–2104**
doi:10.1093/hmg/ddm157
Advance Access published on June 25, 2007

IKAP/hELP1 deficiency in the cerebrum of familial dysautonomia patients results in down regulation of genes involved in oligodendrocyte differentiation and in myelination

David Cheishvili[1], Channa Maayan[1], Yoav Smith[2], Gil Ast[3] and Aharon Razin[4,*]

[1]Department of Pediatrics, Hadassah University Hospital, Mount Scopus, Jerusalem, Israel, [2]Genomic Data Analysis Unit of the Hebrew University Medical School, [3]Department of Human Molecular Genetics, Tel Aviv University Medical School, Ramat Aviv, Israel and [4]Department of Cellular Biochemistry and Human Genetics, The Hebrew University Medical School, Jerusalem, Israel

Received May 2, 2007; Revised and Accepted June 19, 2007

The gene affected in the congenital neuropathy familial dysautonomia (FD) is IKBKAP that codes for the IKAP/hELP1 protein. Several different functions have been suggested for this protein, but none of them have been verified *in vivo* or shown to have some link with the FD phenotype. In an attempt to elucidate the involvement of IKAP/hELP1 in brain function, we searched for IKAP/hELP1 target genes associated with neuronal function. In a microarray expression analysis using RNA extracted from the cerebrum of two FD patients as well as sex and age matched controls, no genes were found to be upregulated in the FD cerebrum. However, 25 genes were downregulated more than 2-fold in the cerebrum of both the male FD child and female FD mature woman. Thirteen of them are known to be involved in oligodendrocyte development and myelin formation. The down regulation of all these genes was verified by real-time PCR. Four of these genes were also confirmed to be down-regulated at the protein level. These results are statistically significant and have high biological relevance, since seven of the downregulated genes in the cerebrum of the FD patients were shown by others to be upregulated during oligodendrocyte differentiation *in vitro*. Our results therefore suggest that IKAP/hELP1 may play a role in oligodendrocyte differentiation and/or myelin formation.

INTRODUCTION

Familial dysautonomia (FD) is an autosomal recessive congenital neuropathy that occurs in 1/3600 live births with a carrier frequency of 1 in 30 in the Ashkenazi Jewish (AJ) population (1). Pathologic studies of FD patients have revealed abnormal development and survival of the sensory and autonomic nervous system associated with demyelination at various loci in the nervous system (2). The FD condition is also described as a small fiber neuropathy that affects development and survival of unmyelinated and small fiber neurons which are responsible for impaired pain, hot and cold appreciation (3).

The IKBKAP gene that encodes the IκB kinase complex-associated protein (IKAP/hELP1) was found to be mutated in FD patients. Three mutations in the IKBKAP gene have been identified in FD patients. The major mutation is a T-to-C transition in position 6 of the 5′ splice site (donor site) of intron 20 (IVS20(+6T → C) (4). This mutation is found in >99.5% of FD patients. In a minority of FD cases (<0.5%) two other mutations were found: a G-to-C transversion in codon 696 of exon 19, predicting an arginine-to-proline substitution (R696P) (5). This mutation was shown to reduce the level of IKAP/hELP1 phosphorylation in cultured fibroblasts of an FD patient heteroallelic for the mutation (6). Another rare mutation is a P-to-L transversion in exon 26 (P914L) (7). Patients who carry the minor mutations are always found to be heterozygous for the major splicing mutation.

In cell lines derived from individuals homozygous for the major FD haplotype, the IKBKAP gene with the IVS20(+6T → C) mutation, an mRNA is produced in which exon 20 is spliced out, along with intron 20. This abnormal

*To whom correspondence should be addressed. Tel: +972 26758172; Fax: +972 26415848; Email: razina@huji.ac.il

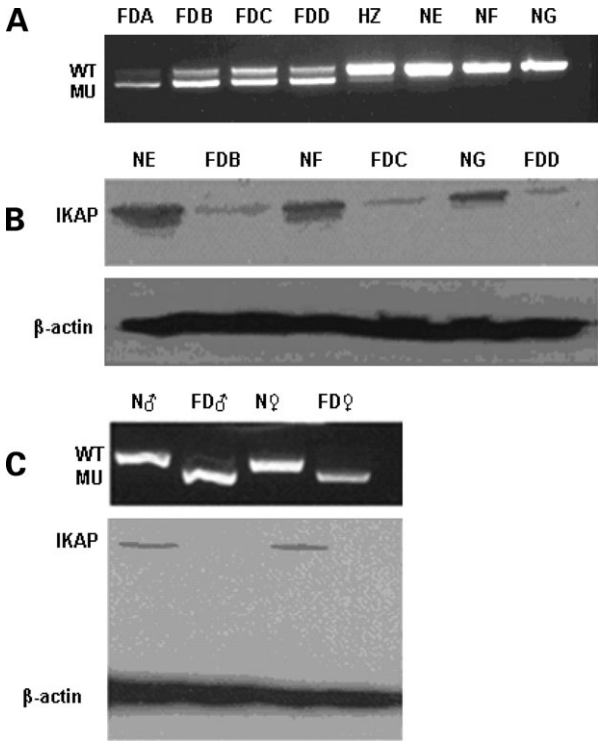

Figure 1. Expression of IKBKAP and production of IKAP/hELP1 in fibroblasts and in cerebrum of FD patients. (**A**) PCR products of normal mRNA (WT) and mutated mRNA (MU) in four fibroblast lines of FD patients (FDA-FDD), one FD heterozygote (HZ) and three fibroblast lines of normal individuals (NE-NG). PCR was performed with cDNA obtained by reverse transcription of total fibroblast RNA (see Materials and Methods). (**B**) Production of the IKAP/hELP1 protein in three of the fibroblast lines was analyzed by western blotting using cell extracts (200 μg protein/lane) of normal (NE-NG) and FD (FDB-FDD) fibroblasts. β-Actin served as a control for loading on the gels. (**C**) Expression of IKBKAP and production of IKAP/hELP1 in the cerebrum of normal individuals and FD patients of a 11-year-old male and in 47-year-old female (see text). PCR analysis was as described in (A) and western analysis with the cerebrum extract (200 μg protein/lane) was as described in (B), N-normal. β-Actin served as a control for loading on the gel.

splicing results in a frameshift that generates a truncated protein that seems to be unstable as it is undetectable (8). The mutation reduces stable base pairing between the splicing factor U1 and the 5′ splice site of exon 20, shifting splicing of exon 20 from constitutive to alternative (9). Most FD cell types examined to date express both wild-type and mutant mRNA (skipping exon 20), with a cell-specific ratio of WT to mutant mRNA (8). In contrast, the mutant IKBKAP mRNA is the exclusive mRNA species produced in brain tissue (cerebrum) and no IKAP/hELP1 protein is detected in the cerebrum (Fig. 1C).

While the genetic defect in FD has been known for more than 5 years, the biological function of the IKAP/hELP1 protein and the effect of its deficiency on brain function remains obscure. Several possible functions of IKAP/hELP1 have been suggested but none of them has been verified in human brain. Since the IKAP/hELP1 protein was originally found to be associated with the IκB kinase (IκK) complex, it was suggested that its function is in the assembly of this complex and the gene was named IKBKAP (10). However,

Table 1. Top 25 genes downregulated more than 2-fold in the FD cerebrum

	Gene symbol	Accession number	♂	♀	♂♀
1	MAG	NM_002361	−1.89	−4.70	−2.44
2	PLP1	NM_000533	−1.21	−3.24	−2.34
3	MAL	NM_022439	−2.22	−3.10	−1.40
4	EDG2	NM_057159	−1.16	−3.53	−0.75
5	PPP1R14A	NM_033256	−1.90	−2.92	−1.53
6	TMEM10	NM_033207	−1.48	−3.83	−1.07
7	APOD	NM_015964	−1.93	−2.14	−1.68
8	VGF	NM_003378	−2.01	−3.78	−1.93
9	SST	NM_001048	−1.58	−3.20	−1.01
10	GTX	NM_177400	−1.89	−3.77	−1.41
11	ERMIN	NM_020711	−1.07	−2.79	−1.71
12	TF	NM_001063	−3.38	−3.36	−2.55
13	TTYH2	NM_032646	−1.62	−2.48	−2.08
14	SREBF1	NM_004176	−1.10	−1.25	−1.26
15	EFHD1	NM_025202	−1.64	−3.04	−1.18
16	LIPE	NM_005357	−1.27	−1.74	−1.00
17	GJA12	NM_020435	−1.58	−1.19	−1.03
18	MLP	NM_023009	−1.10	−1.42	−1.69
19	SLC12A2	NM_001046	−1.05	−1.06	−1.28
20	RNASE1	NM_002933	−1.84	−2.49	−1.28
21	HRASLS3	NM_007069	−1.23	−2.27	−1.25
22	LGALS3BP	NM_005567	−2.00	−1.71	−1.20
23	HLA-DRB1	NM_021983	−1.12	−1.69	−1.31
24	AMOTL2	NM_016201	−1.52	−1.69	−1.01
25	GPRC5B	NM_016235	−1.34	−1.48	−1.16

The values in the table are log2 values obtained for the 25 genes in each microarray chip: male (♂) FD patient eliminate versus normal and 11-year-old male, female FD (♀) versus normal 42-year-old female and male (♂) FD versus normal female (♀).

subsequent reports (11,12) showed that cellular IκK complexes do not contain IKAP/hELP1. Another line of evidence showed that IKAP/hELP1 is one of six proteins that constitute the RNA polymerase II transcription elongation complex in yeast called ELP1 (elongator protein 1) (12) and in human hELP1 (13). Although solid evidence exists for IKAP/hELP1 being a member of this multiprotein complex, its role in the FD phenotype is not clear (the protein will be called hereafter IKAP/hELP1). The fact that this protein was found primarily in the cytoplasm (13,14) raised questions concerning its involvement in transcription elongation. IKAP/hELP1 was also reported to potentially activate JNK (cJun N-terminal kinase) suggesting that IKAP/hELP1 may be a physiologically important regulator of the stress associated JNK signaling pathway (15). Although this function appears attractive in light of the fact that FD patients lack the ability to cope with stress, further studies failed to disclose an effect of IKAP/hELP1 on JNK (16). Cellular loss of function experiments by siRNA or fibroblasts from FD patients did not support any involvement of IKAP in cytoplasmic kinase signaling. This, however, does not rule out the possibility that other stimuli may require IKAP/hELP1 for JNK activation (17).

Studies in which the yeast IKAP/hELP1 homologue (ELP1) was deleted suggested that this protein functions in polarized exocytosis and could therefore be involved in dysregulation of neuronal exocytosis in FD patients (14). Evidence has accumulated recently in yeast suggesting that IKAP/ELP1 might also play a role in the formation of modified wobble uridines in tRNA. These modified wobble uridines are crucial in

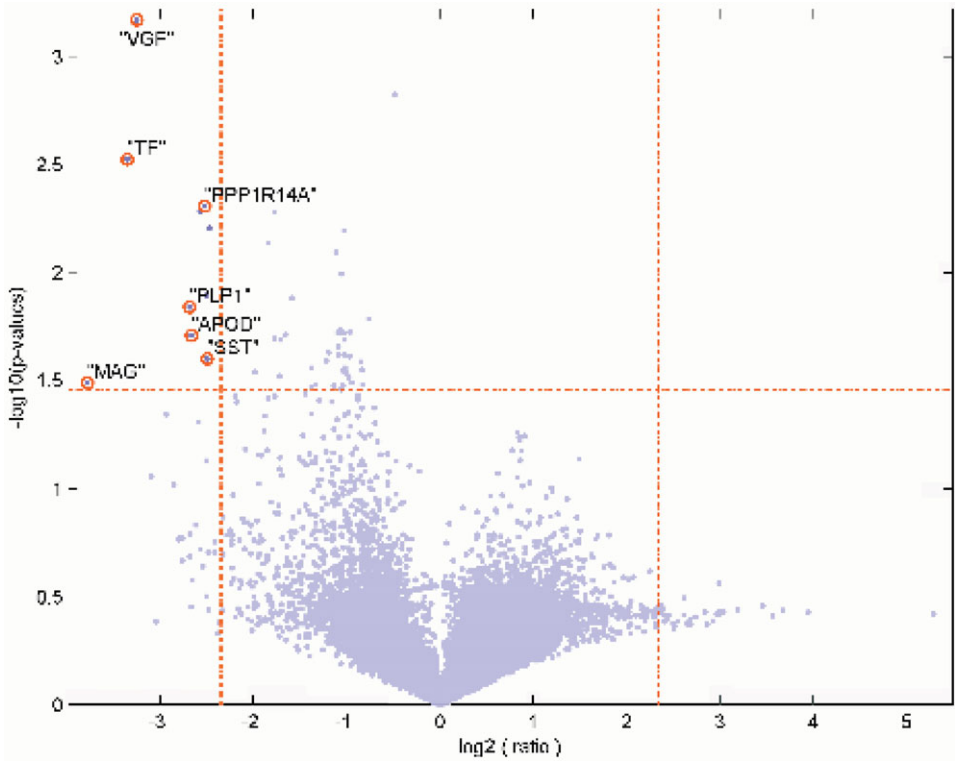

Figure 2. To challenge the statistical significance of the microarray results, we used the data obtained with the three chips (described earlier) to perform the Volcano analysis. Using thresholds of ≥2-fold decrease in expression (vertical line) and *P*-value of ≤0.035 (horizontal line), we have detected seven genes (MAG, PLP1, SST, Tf, PPP1R14A, APOD and VGF) of the 13 genes listed in Table 2. All dots in the scatter plot above the horizontal line have *P*-values less than 0.035 (calculated using the *t*-test). All points to the left of the vertical line are downregulated by a log2 ratio of more than 2.3. The dots to the right of the vertical line are upregulated by a log2 ratio of more than 2.3.

post-transcriptional expression of gene products which are important in transcription and exocytosis. A defect in translation fidelity might also contribute to the FD phenotype (18). Another interesting study performed with IKAP/hELP1 deficient Hela cells and FD fibroblasts revealed a subgroup of affected genes that are associated with cell migration (17). All the above suggested functions, however, fall short of explaining how IKAP/hELP1 functions in the nervous system and how its deficiency results in the FD phenotype.

RESULTS

The first attempts to identify IKAP/hELP1 target genes were carried out in fibroblasts (17 and unpublished data). Our results were disappointing, first because they varied with respect to the individual FD patients from which the fibroblast lines originated. Secondly, the genes that we found to be affected do not appear in the list of genes reported to be affected in Hela cells in which IKBKAP was knocked down by siRNA or in other FD fibroblast lines (17). We attribute these inconsistent results to the fact that FD fibroblasts make both the normal IKBKAP mRNA (WT) as well as the alternatively spliced mutated mRNA (MU) (Fig. 1A) and that the FD fibroblasts are not completely devoid of the IKAP/hELP1 protein (Fig. 1B). In contrast to the capacity of FD fibroblasts to produce the normal IKBKAP mRNA and the IKAP/hELP1 protein, neither normal (WT) IKBKAP mRNA nor IKAP/hELP1 protein are observed in the cerebrum of FD patients (Fig. 1C). It was therefore anticipated that a more pronounced effect of IKAP/hELP1 deficiency would be observed on target genes in the cerebrum, and these may also be more relevant to the neuronal defects observed in the FD phenotype.

To identify target genes that may be affected in the FD IKAP/hELP1 deficient cerebrum, we performed microarray expression analyses using RNA extracted from the cerebrum of an 11-year-old FD male patient and a 47-year-old female FD patient and the cerebrum of sex and age matched unaffected individuals. The individually labeled cDNAs were hybridized to the Human Operon Oligo Set version 3.0 slides representing 24 650 human genes. Two microarray chips were hybridized with cDNA from one of the FD patients and a matched normal control and the third chip was hybridized with cDNA from the 11-year-old FD patient and a normal female control (for procedure see Materials and Methods). The genes most significantly downregulated in the FD cerebrum with low *P*-values agrees with the list of 25 genes obtained by examining the three column ratios of the array after lowess normalization using the Spotfire analysis package. The results (Table 1) were obtained using a program written in Matlab (rev 2.3.0) (FD.ekmd.huji.ac.il). To challenge the statistical significance of the microarray results, we performed Volcano analysis, using a threshold of ≥2-fold decrease in expression and a *P*-value of ≤0.035. Seven genes (MAG, PLP1, SST, Tf, PPP1R14A, APOD and

Table 2. Thirteen downregulated genes that are associated with oligodendrocyte differentiation and/or myelination

	Gene symbol	Description and function	Relative expression N/FD		Microarray	Volcano	Western
			♂	♀			
1	**MAG**	Myelin-associated glycoprotein (25)	4	40	+	+	+
2	**PLP1**	Proteolipid protein-primary protein of myelin (26)	8	16.5	+	+	+
3	**EDG2 (LPA1)**	OL/myelin maintenance and formation (27)	2.5	43	+	−	ND
4	**Tf**	OL development (28), myelin formation (29)	6	11	+	+	ND
5	**PPP1R14A**	OL differentiation (19)	8	7.5	+	+	ND
6	**TMEM 10**	Transmembrane protein 10 expressed in OL (19)	10	17.5	+	−	ND
7	**APOD**	Transport of cholesterol for myelin synthesis (30). OL differentiation (19)	6	2	+	+	ND
8	MAL	Myelin and lymphocyte protein (31)	7	17	+	−	+
9	SST	Affect rate of neurotransmission in CNS, myelination process (32)	10	275	+	+	+
10	TTYH2	Expressed in OL (19)	3	12	+	−	ND
11	KIAA1189 (ERMIN)	Myelinogenesis (33)	17	9	+	−	ND
12	GTX (Nkx-6.2)	Regulator of myelin gene expression, transcription factor (34)	5	7	+	−	ND
13	VGF	Nerve growth factor inducible (35), repressed upon OL differentiation (19)	9	27	+	+	ND

The real-time PCR values, given in terms of normal (N) versus FD gene expression, are of thirteen genes as described in Figure 3. The thirteen genes are from Table 1 (Microarray +). Seven genes are from Figure 2 (Volcano +). Four genes for which protein levels were determined (Western +). ND, not determined. The functions of the individual genes were as described in the references given in the table. Genes designated in bold letters appear in the list of the top 30 genes which were upregulated during oligodendrocyte differentiation (19).

VGF) were detected to obey these criteria (Fig. 2). All these genes appear in the list of 13 genes involved in oligodendrocyte development and myelin formation (Table 2).

To further characterize the expression data, we focussed on genes with an expression change of $\geq$2-fold in the FD cerebrum. Out of 25 genes that were downregulated in all three chips (Table 1), we identified 13 genes that are involved in oligodendrocyte development and/or myelin formation (Table 2 and references within). The downregulation of these thirteen genes was verified by real-time PCR analyses (Fig. 3 and Table 2). Similar results were obtained at the protein level by using specific polyclonal antibodies against MAG, PLP1, MAL and SST in a western blot assay (Fig. 4).

DISCUSSION

In an attempt to decipher the link between the mutation in the IKBKAP gene and the FD phenotype, we searched for genes that are affected in the brain as a result of deficiency of the IKBKAP gene product, IKAP/hELP1. We anticipated that neuronal genes with relevance to the FD phenotype will be affected in IKAP/hELP1 deficient brain. To test this hypothesis, we embarked on a microarray expression study of genes expressed in the cerebrum of FD patients. Although FD brain tissue is scarce, we decided to carry out this study with available cerebrum tissue of an 11-year-old FD male patient and a 47-year-old female FD patient. These results turned out to be extremely informative. We regarded the cerebrum of both patients to be IKAP/hELP1 deficient, since only the mutant mRNA variant was detected in the cerebrum of the FD patients, and the IKAP/hELP1 protein itself was not observed (Fig. 1C).

Microarray expression analysis revealed 25 genes for which a $\geq$2-fold (log2 values $\geq$1) downregulation was found in the cerebrum of FD patients (Table 1). Since demyelination at various loci of the nervous system of FD patients had been reported in the past (2), we focussed on 13 out of the 25 genes that are known to be involved in oligodendrocyte differentiation and/or myelin formation (Table 2 and references within). For these genes, real-time PCR analysis verified their downregulation in FD cerebrum. The results of the real-time PCR, and the protein analysis correspond very well with the microarray results (Table 2).

The observation that IKAP/hELP1 deficiency affects primarily the expression of genes that are involved in the process of myelination lead us to suggest that IKAP/hELP1 either controls the process of myelination itself, or, alternatively, controls oligodendrocyte differentiation. This last possible function of IKAP/hELP1 is strongly supported by examination of the list of the top 50 genes found to be upregulated during oligodendrocyte differentiation *in vitro* (19). A recent functional genomic analysis of oligodendrocyte differentiation *in vitro* revealed that seven out of the 13 genes are activated during the induction of primordial oligodendrocyte differentiation in culture (gene symbol in bold in Table 2). None of the genes that we found affected by IKAP/hELP1 deficiency (except VGF) is expressed in undifferentiated oligodendrocyte primordial cells (19).

Using the statistical significance test (SAM), we selected the top 30 most downregulated genes based on *P*-values.We then calculated the enrichment in genes induced upon oligodendrocyte differentiation as described in (19). The striking enrichment of the IKAP/hELP1 deficiency-affected genes in the oligodendrocyte and myelination process testifies to the biological relevance of our data. The fact that seven of the top 50 genes upregulated during oligodendrocyte differentiation (14%) are genes that we have identified to be downregulated in the two FD patients may indicate that IKAP/hELP1 plays a role in oligodendrocyte differentiation. It is

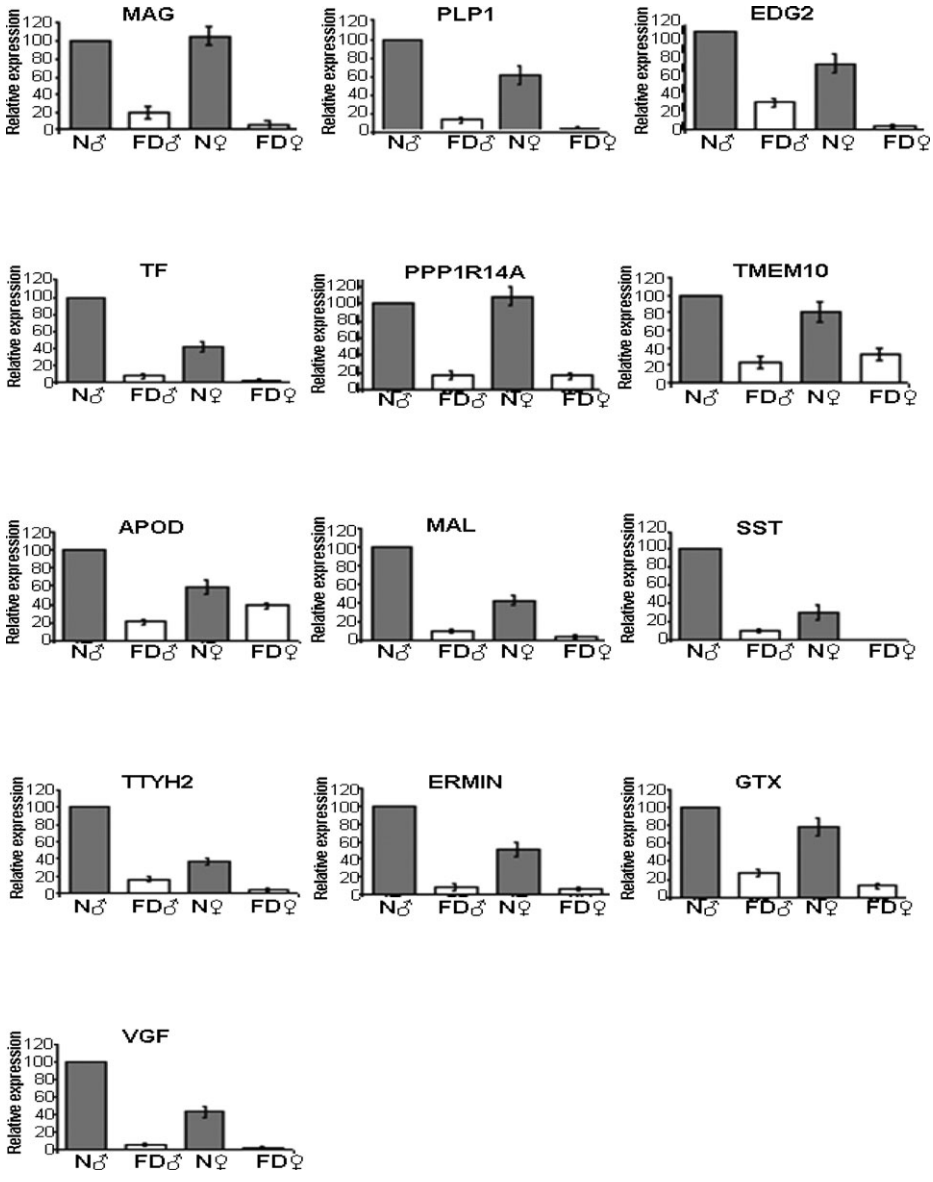

Figure 3. Expression of IKAP/hELP1 target genes (see list in Table 1) in the cerebrum of 11-year-old FD male and 47-year-old FD female and sex and age matched controls (N) were estimated by real-time PCR (see Materials and Methods). Analyses were repeated two to three times. Data presented is relative to the value obtained with the normal 11-year-old male that showed the highest value for all analyzed genes (designated 100). After correction to 18S rRNA values, the calculated ratios of normal to FD in both patients were highly reproducible (see error bars).

well known that oligodendrocyte differentiation and its resulting myelin biosynthesis takes place in the central nervous system (CNS) post-natally. However, IKAP/hELP1 deficiency in FD patients probably operates also prenatally and appears to have its primary influence in the peripheral nervous system (PNS). It is possible that the effects of IKAP/hELP1 deficiency that we observe in the cerebrum apply also to Schwann cells that produce myelin in the PNS. Alternatively, IKAP/hELP1 deficiency may affect the migration of Schwann cells and oligodendrocytes on their way to populate the PNS and CNS, respectively. Interestingly, it was previously suggested that IKAP/hELP1 may have a role in cell migration (17). The answers to all the above cardinal questions await further studies in oligodendrocytes and Schwann cell cultures *in vitro* and experiments designed to follow the migration and differentiation of these cells in a model system *in vivo*.

A recent study of myelin membrane sheath formation in a neuron glia co-culture system revealed that neurons induce lipid condensation of the oligodendroglial membrane (20). However, while oligodendrocytes cultured without neurons express the major myelin components, their sub-cellular localization and cellular morphology depend on the presence of neurons (21). Our results raise the possibility that IKAP/hELP1 controls a very complex process that is responsible for proper axon development and myelination in the CNS and PNS. The results obtained in our studies together with the data published in

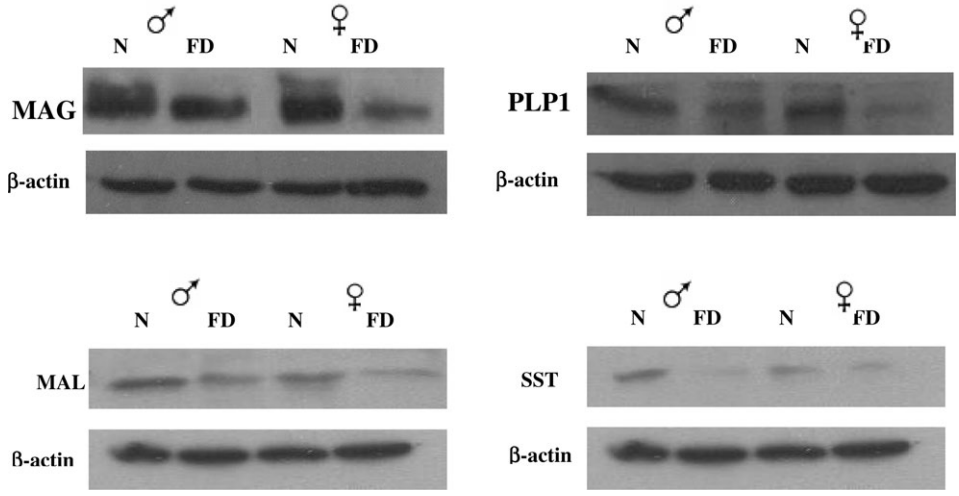

Figure 4. Western blot analysis of production of MAG, PLP1, MAL and SST proteins in extracts from normal (N) and FD cerebrum of male (♂) and female (♀) individuals. Analysis was performed as described in the Materials and Methods. The gels were loaded with 200 μg protein per lane for MAG for PLP1 and 400 μg protein/lane for SST and MAL. Antibodies were as described in the Materials and Methods. β-Actin served as a control for loading on the gels.

the literature support the notion that the expression changes that we observe in FD brain are valid and significant and suggest a possible functional link between IKAP/hELP1 and the nervous system. Studying this link in FD patients promotes understanding of how the genetic trait in FD brings about the devastating phenotype of FD patients. In addition, the results of such a study should have important implications for brain research in general with special emphasis on oligodendroglial myelin formation and neuron function.

MATERIALS AND METHODS

Tissues and cells

Brain tissue was obtained from the NICHD Brain and Tissue Bank for Developmental Disorders at the University of Maryland, Baltimore, MA. Tissues used for this study were the cerebrum of an FD 11-year-old patient (UMB #M3697M) and a 47-year-old FD female (UMB #M3783M) and age and sex matched normal brain (UMB# 616 and UMB# 1910, respectively). The cerebrum tissue includes both neurons and glial cells. Fibroblast cell lines were either cloned by us or obtained from Coriell Cell Repositories. Cells were cultured in DMEM supplemented with 15% fetal calf serum, 5% glutamine and 1% penicillin-streptomycin at 37°C with 3% CO_2.

Real-time PCR analysis

Total RNA was prepared from cells in culture or brain tissue using the Tri-reagent (MRC) and cleaned with Qiagen RNeasy mini-kit according to the manufacturer's protocol. Approximately 1 μg total RNA of each sample was reverse transcribed using the M-MLV Reverse Transcriptase (Promega) using random primers in a 20 μl reaction mixture. The reverse transcription reactions were carried out at 42°C for 30 min.

For each gene, the real-time PCR analysis was performed in triplicates with 2 μl of the cDNA primers (primer sequences available upon request) at a concentration of 0.3 μM. The SYBR Green (Applied Biosystems) was added to the 20 μl reaction mixture. An ABI PRISM 5700 Sequence Detection System (Applied Biosystems) was programmed as follows: one cycle of 10 min at 95°C, followed by 40 cycles of 15 s at 95°C and 1 min at 60°C. Data were analyzed using the 7500 SDS system software. Each experiment included cDNA of the normal and FD patient male and female. To control for the amount of RNA, amplification of 18S rRNA was performed and corrected for each cDNA sample. 18S rRNA was shown to be an adequate control for quantization of RNA in tissue (22). GAPDH in our hands and others appeared to show variable expression values and therefore could not be used here.

Processing the microarray data

Three microarray chips prepared from cDNA of male FD versus male control, female FD versus female control and male FD versus female control were color coded using cy3 and cy5 dyes. In one array, we also swapped the dye colors to avoid coloring bias. The data from the scanner were in the GPR format and we used the F635−B635 and F32−B532 column data. In each of the three microarrays, we performed flooring on the two columns setting to 20 values below that value (max values of those columns are around 65 000). We then performed a lowess normalization on the data using a 0.1 scale sliding window (plots(1/2)log2 of the product of the DataX and DataY intensities versus log2 of the intensity ratios). The corrected values from the three arrays created from the new intensity and ratio values (new_cy5 = 2. ∧ (intensity + ratio/2); new_cy3 = 2. ∧ (-intensity-ratio/2) resulted in six columns of data, three of each of the controls and three for the FD samples. We obtained *P*-values for all the genes using the *t*-test which we

then used for creating a volcano scatter plot showing significance versus gene expression ratio (fold change) of our data (Fig. 2).

To verify the statistical significance of our microarray results, we used the Stanford SAM package (23) that uses False discovery rates (FDRs) and a *P*-value, as described in (24). This method uses data permutations to provide an estimate of FDRs for multiple testing. We used an adjustable threshold $\delta = 1.265$ which determined 30 genes that were most significant (q-value and FDR = 0), out of a list of 667 genes that were downregulated $\geq$ 2-fold. Seven of these genes appear in the list presented in Table 1. The entire microarray data can be found on the website FD.ekmd.huji.ac.il.

PCR analysis of IKBKAP expression

PCR was performed with primers F cggattgtcactgttgtgc and R gactgctctcatagcatcgc that were previously published by (4). PCR amplification was carried out in a final volume of 25 μl containing: 1 μl of cDNA obtained from the RT reaction (discussed earlier), 12.5 μl ReddyMix PCR master mix for 35 cycles. Following one cycle at 95°C for 2 min, 35 cycles were performed each at 95°C for 30emsp14;s, 60°C for 30 s and 72°C for 30 s. Final extension was at 72°C for 7 min. PCR products were analyzed by electrophoresis on 2% agarose gel.

cDNA synthesis, labeling and hybridization

For the gene chip hybridization, total RNA was isolated from the cerebrum as described earlier. cDNA was prepared with oligo-dt primers (2 μg) (Amersham Biosciences) from total RNA (20 μg) using the reverse transcription enzyme superscript II (Nitrogen). The reverse transcription reaction was carried out at 40°C for 2 h. cDNA synthesis was performed with aminoallyl-dUTP. Removal of unincorporated aa-dUTP and free amines was carried out using Microcon YM-30 (Millipore) filters according to the manufacturer's recommendations. Coupling of aminoallyl labeled cDNA to Cy dye esters was performed in 0.1 M sodium carbonate buffer pH 8.6 for 1 h at room temperature. Removal of free dyes was accomplished with Qiagen PCR purification columns (Qiagen Ltd). The labeling was then quantified using an ND-1000 spectrophotometer (Nanodrop Ltd). The samples were then mixed at equal amounts of labeling (cy3 + cy5), resuspended in hybridization buffer (25% formamide, 5× SSC, 0.1% SDS, 0.4 μg/μl yeast tRNA) and applied to operon, human oligo set version 3.0 obtained from the Whitehead Institute Center for Microarray Technology. These slides were spotted with 70 new probes representing 24 650 human genes. The slides were prehybridized in 5× SSC, 0.1% BSA for 45 min. Hybridization was carried out overnight at 42°C in a hybridization chamber (Corning) submerged in a water bath. The slides were then washed in two consecutive 5 min washing solutions of low, medium and two consecutive high stringency washes. The slides were then dipped in DDW and dried by a brief centrifugation. Arrays were immediately scanned using Genepix 4000B scanner (Axon Ltd). The resulting images were analyzed using Genepix pro 4.0 (Axon Ltd).

Western blot analysis

Tissues and cells were lysed with RIPA lysis buffer (1% NP-40, 150 mM NaCl, 50 mM Tris pH 7.5, 0.1% SDS, 0.1% DOC, 1 mM PMSF). The protein concentration of the soluble fraction was measured by the Bradford assay. Proteins were separated by electrophoresis on 8–12% SDS–PAGE gels and transferred to a nitrocellulose membrane. Non-specific protein binding was prevented by treating the membranes with 10% Skim Milk in PBS (0.1% Tween-20) overnight. The membranes were subsequently incubated in PBS with 0.1% Tween20 at room temperature for 1 h with the relevant primary polyclonal antibodies. After three washes in PBS 0.1% Tween-20, the blots were incubated with appropriate secondary antibodies (final antibody concentration was according to the manufacturer's recommendation). Polyclonal antibodies to PLP1, MAL, MAG and SST were purchased from Santa Cruz Co. Polyclonal antibody to IKAP/hELP1 was purchased from BD Biosciences and monoclonal antibody to β-actin was purchased from Sigma Aldrich Co. All secondary antibodies were purchased from Jackson Laboratories.

ACKNOWLEDGEMENTS

We thank Dr. Miriam Kott-Outkowski of the Microarray Service Laboratory at the Hebrew University Medical School for performing the microarray analyses. Special thanks to Dr. Howard Cedar for helpful discussions and Helaina Skop for her technical help. CD is supported by the FD Hope Foundation and Dysantonomia Foundation Inc., USA. This work was also partially supported by a donation of the late Dina Farkash.

Conflict of Interest statement. None declared.

REFERENCES

1. Maayan, C., Kaplan, E., Shachar, S., Peleg, O. and Godfrey, S. (1987) Incidence of familial dysautonomia in Israel 1977–1981. *Clin. Genet.*, **32**, 106–108.
2. Fogelson, M.H., Rorke, L.B. and Kaye, R. (1967) Spinal cord changes in familial dysautonomia. *Arch. Neurol.*, **17**, 103–108.
3. Axelrod, F.B. (2004) Familial dysautonomia. *Muscle Nerve*, **29**, 352–363.
4. 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.
5. 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.
6. Magen, A. and Ast, G. (2005) The importance of being divisible by three in alternative splicing. *Nucleic Acids Res.*, **33**, 5574–5582.
7. Leyne, M., Mull, J., Gill, S.P., Cuajungco, M.P., Oddoux, C., Blumenfeld, A., Maayan, C., Gusella, J.F., Axelrod, F.B. and Slaugenhaupt, S.A. (2003) Identification of the first non-Jewish mutation in familial Dysautonomia. *Am. J. Med. Genet.*, **118**, 305–308.
8. Cuajungco, M.P., Leyne, M., Mull, J., Gill, S.P., Lu, W., Zagzag, D., Axelrod, 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.
9. Carmel, I., Tal, S., Vig, I. and Ast, G. (2004) Comparative analysis detects dependencies among the 5' splice-site positions. *RNA*, **10**, 828–840.
10. Cohen, L., Henzel, W.J. and Baeuerle, P.A. (1998) IKAP is a scaffold protein of the IkappaB kinase complex. *Nature*, **395**, 292–296.

11. Krappmann, D., Hatada, E.N., Tegethoff, S., Li, J., Klippel, A., Giese, K., Baeuerle, P.A. and Scheidereit, C. (2000) The I kappa B kinase (IKK) complex is tripartite and contains IKK gamma but not IKAP as a regular component. *J. Biol. Chem.*, **275**, 29779–29787.
12. Otero, G., Fellows, J., Li, Y., de Bizemont, T., Dirac, A.M., Gustafsson, C.M., Erdjument-Bromage, H., Tempst, P. and Svejstrup, J.Q. (1999) Elongator, a multisubunit component of a novel RNA polymerase II holoenzyme for transcriptional elongation. *Mol. Cell.*, **3**, 109–118.
13. Hawkes, N.A., Otero, G., Winkler, G.S., Marshall, N., Dahmus, M.E., Krappmann, D., Scheidereit, C., Thomas, C.L., Schiavo, G., Erdjument-Bromage, H. *et al.* (2002) Purification and characterization of the human elongator complex. *J. Biol. Chem.*, **277**, 3047–3052.
14. Rahl, P.B., Chen, C.Z. and Collins, R.N. (2005) Elp1p, the yeast homolog of the FD disease syndrome protein, negatively regulates exocytosis independently of transcriptional elongation. *Mol. Cell.*, **17**, 841–853.
15. Holmberg, C., Katz, S., Lerdrup, M., Herdegen, T., Jaattela, M., Aronheim, A. and Kallunki, T. (2002) A novel specific role for I kappa B kinase complex-associated protein in cytosolic stress signaling. *J. Biol. Chem.*, **277**, 31918–31928.
16. Svejstrup, J.Q. (2007) Elongator complex: how many roles does it play? *Curr. Opin. Cell. Biol*, **19**, 331–336.
17. Close, P., Hawkes, N., Cornez, I., Creppe, C., Lambert, C.A., Rogister, B., Siebenlist, U., Merville, M.P., Slaugenhaupt, S.A., Bours, V. *et al.* (2006) Transcription impairment and cell migration defects in elongator-depleted cells: implication for familial dysautonomia. *Mol. Cell.*, **22**, 521–531.
18. Esberg, A., Huang, B., Johansson, M.J. and Bystrom, A.S. (2006) Elevated levels of two tRNA species bypass the requirement for elongator complex in transcription and exocytosis. *Mol. Cell.*, **24**, 139–148.
19. Dugas, J.C., Tai, Y.C., Speed, T.P., Ngai, J. and Barres, B.A. (2006) Functional genomic analysis of oligodendrocyte differentiation. *J. Neurosci.*, **26**, 10967–10983.
20. Fitzner, D., Schneider, A., Kippert, A., Mobius, W., Willig, K.I., Hell, S.W., Bunt, G., Gaus, K. and Simons, M. (2006) Myelin basic protein-dependent plasma membrane reorganization in the formation of myelin. *EMBO J.*, **25**, 5037–5048.
21. Trajkovic, K., Dhaunchak, A.S., Goncalves, J.T., Wenzel, D., Schneider, A., Bunt, G., Nave, K.A. and Simons, M. (2006) Neuron to glia signaling triggers myelin membrane exocytosis from endosomal storage sites. *J. Cell. Biol.*, **172**, 937–948.
22. Goidin, D., Mamessier, A., Staquet, M.J., Schmitt, D. and Berthier-Vergnes, O. (2001) Ribosomal 18S RNA prevails over glyceraldehyde-3-phosphate dehydrogenase and beta-actin genes as internal standard for quantitative comparison of mRNA levels in invasive and noninvasive human melanoma cell subpopulations. *Anal. Biochem.*, **295**, 17–21.
23. Tusher, V.G., Tibshirani, R. and Chu, G. (2001) Significance analysis of microarrays applied to the ionizing radiation response. *Proc. Natl Acad. Sci. USA*, **98**, 5116–5121.
24. Storey, J.D. (2002) A direct approach to false discovery rates. *J. Roy. Stat. Soc. Ser. B.*, **64**, 479–498.
25. Barton, D.E., Arquint, M., Roder, J., Dunn, R. and Francke, U. (1987) The myelin-associated glycoprotein gene: mapping to human chromosome 19 and mouse chromosome 7 and expression in quivering mice. *Genomics*, **1**, 107–112.
26. Garbern, J.Y., Cambi, F., Tang, X.M., Sima, A.A., Vallat, J.M., Bosch, E.P., Lewis, R., Shy, M., Sohi, J., Kraft, G. *et al.* (1997) Proteolipid protein is necessary in peripheral as well as central myelin. *Neuron*, **19**, 205–218.
27. Cervera, P., Tirard, M., Barron, S., Allard, J., Trottier, S., Lacombe, J., Daumas-Duport, C. and Sokoloff, P. (2002) Immunohistological localization of the myelinating cell-specific receptor LP(A1). *Glia*, **38**, 126–136.
28. Cassia, R., Besnard, L., Fiette, L., Espinosa de los Monteros, A., Ave, P., Py, M.C., Huerre, M., de Vellis, J., Zakin, M.M. and Guillou, F. (1997) Transferrin is an early marker of hepatic differentiation, and its expression correlates with the postnatal development of oligodendrocytes in mice. *J. Neurosci. Res.*, **50**, 421–432.
29. Cabrera, E., Rojas, B., Labra, S., Matutes, E. and Puga, B. (1997) Acute myeloid leukemia MO: clinical and laboratory characteristics. *Revista medica de Chile*, **125**, 433–437.
30. Ong, W.Y., Lau, C.P., Leong, S.K., Kumar, U., Suresh, S. and Patel, S.C. (1999) Apolipoprotein D gene expression in the rat brain and light and electron microscopic immunocytochemistry of apolipoprotein D expression in the cerebellum of neonatal, immature and adult rats. *Neuroscience*, **90**, 913–922.
31. Erne, B., Sansano, S., Frank, M. and Schaeren-Wiemers, N. (2002) Rafts in adult peripheral nerve myelin contain major structural myelin proteins and myelin and lymphocyte protein (MAL) and CD59 as specific markers. *J. Neurochem.*, **82**, 550–562.
32. Carpentier, V., Vaudry, H., Laquerriere, A. and Leroux, P. (1999) Somatostatin binding sites in the white matter of the developing human brainstem: inverse relationship with the myelination process. *Neurosci. Lett.*, **264**, 49–52.
33. Brockschnieder, D., Sabanay, H., Riethmacher, D. and Peles, E. (2006) Ermin, a myelinating oligodendrocyte-specific protein that regulates cell morphology. *J. Neurosci.*, **26**, 757–762.
34. Awatramani, R., Beesley, J., Yang, H., Jiang, H., Cambi, F., Grinspan, J., Garbern, J. and Kamholz, J. (2000) Gtx, an oligodendrocyte-specific homeodomain protein, has repressor activity. *J. Neurosci. Res.*, **61**, 376–387.
35. Canu, N., Possenti, R., Rinaldi, A.M., Trani, E. and Levi, A. (1997) Molecular cloning and characterization of the human VGF promoter region. *J. Neurochem.*, **68**, 1390–1309.