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Familial dysautonomia (FD) patients have reduced levels of the modified wobble nucleoside mcm^{5}s^{2}U in tRNA

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ABSTRACT

Familial dysautonomia (FD) is a recessive neurodegenerative genetic disease. FD is caused by a mutation in the *IKBKAP* gene resulting in a splicing defect and reduced levels of full length IKAP protein. IKAP homologues can be found in all eukaryotes and are part of a conserved six subunit protein complex, Elongator complex. Inactivation of any Elongator subunit gene in multicellular organisms cause a wide range of phenotypes, suggesting that Elongator has a pivotal role in several cellular processes. In yeast, there is convincing evidence that the main role of Elongator complex is in formation of modified wobble uridine nucleosides in tRNA and that their absence will influence translational efficiency. To date, no study has explored the possibility that FD patients display defects in formation of modified wobble uridine nucleosides as a consequence of reduced IKAP levels. In this study, we show that brain tissue and fibroblast cell lines from FD patients have reduced levels of the wobble uridine nucleoside 5-methoxycarbonylmethyl-2-thiouridine (mcm^{5}s^{2}U). Our findings indicate that FD could be caused by inefficient translation due to lower levels of wobble uridine nucleosides.

1. Introduction

Familial dysautonomia (FD), initially called Riley-Day syndrome is a neurodegenerative autosomal recessive genetic disorder primarily found amongst the Ashkenazi Jewish population [1,2]. FD patients display a variety of clinical features such as cardiovascular dysfunction, decreased pain and temperature sensation, blood pressure variability, vomiting crises, lack of overflow tears and increased sweating [3,4]. Nearly all FD patients have a mutation in the donor splice site of intron 20 of the *IKBKAP* gene which leads to aberrant splicing. The missplicing results in skipping of exon 20 in a tissue specific manner and consequently reduced levels of the full length IKAP protein [5–8].

The IKAP protein homologue Elp1p has been extensively studied in the yeast *Saccharomyces cerevisiae*. The Elp1p is part of a six subunit protein complex Elp1p–Elp6p and this complex was initially described as a histone acetyltransferase (HAT) that associates with the hyperphosphorylated elongating form of RNA polymerase II (Pol II) [9]. Therefore the complex was named Elongator complex [9–12]. The HAT activity of Elongator complex reside in the Elp3p subunit since *in vitro* this subunit transfer acetyl groups from acetyl-CoA to histones [13,14]. Consistent with a role in transcription, null mutants of genes encoding Elongator subunits results in defects in Pol II transcription [9,10,13–15]. In addition to defects in Pol II transcription, deletions of genes encoding Elongator subunits in yeast show multiple phenotypes including defects in DNA repair, telomeric gene silencing, exocytosis and formation of the 5-carbamoylmethyluridine (ncm^{5}U), 5-methoxycarbonylmethyluridine (mcm^{5}U) and 5-methoxycarbonylmethyl-2-thiouridine (mcm^{5}s^{2}U) nucleosides at wobble position in tRNAs [16–18]. It was controversial whether Elongator complex is involved in multiple cellular processes or if it participate in one key process influencing multiple downstream cellular processes. In yeast, there is convincing evidence that the primary role of Elongator complex is in formation of the ncm^5 and mcm^5 side chains on wobble uridines in tRNA [18,19]. In Elongator mutants, overexpression of various combinations of tRNA$^{Lys}_{s^2UUU}$, tRNA$^{Gln}_{s^2UUG}$ and tRNA$^{Glu}_{s^2UUC}$ which in wild type have the mcm^{5}s^{2}U nucleoside restored all defects mentioned above but not the defect in formation of the ncm^{5}U, mcm^{5}U and mcm^{5}s^{2}U nucleosides [19,20]. Presence of ncm^{5}U, mcm^{5}U and mcm^{5}s^{2}U nucleosides are important for proper decoding of A- and G-ending codons in

Abbreviations: cm^{5}U, 5-carboxymethyluridine; ncm^{5}U, 5-carbamoylmethyluridine; mcm^{5}U, 5-methoxycarbonylmethyluridine; mcm^{5}s^{2}U, 5-methoxycarbonylmethyl-2-thiouridine.

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mRNAs [21–24]. Thus, elevated levels of hypomodified $tRNA^{Lys}_{s^2UUU}$, $tRNA^{Gln}_{s^2UUG}$ and $tRNA^{Glu}_{s^2UUC}$ most likely compensate for inefficient codon/anticodon interaction during translation due to the absence of modifications at wobble position. Likely translational targets are mRNAs enriched in these codons or having these codons in a certain context. This was recently verified for the *cdr2*$^+$ gene of *Schizosaccharomyces pombe* where changes of the Elongator tRNA modification dependent AAA lysine codons to Elongator independent AAG lysine codons significantly improved expression of the protein encoded by the modified *cdr2*$^+$ gene [23].

Elongator complex is conserved in multicellular eukaryotes and six subunit protein complexes have been purified from humans and plants [12,25–27]. Mutations in the *ELP1*/*IKBKAP* gene homologues of the plant *Arabidopsis thaliana*, the worm *Caenorhabditis elegans* and mouse *Mus musculus* causes defects in formation of wobble uridine modifications [28–30]. As in yeast, depletion of Elongator subunits in multicellular organisms show pleiotropic phenotypes implicating a role in very many cellular processes [29–39]. Phenotypes observed in fibroblasts derived from FD patients are reduced histone H3 acetylation and reduced Pol II transcription of several genes encoding proteins required for proper cell motility [25,31]. However, in FD fibroblasts it is unknown if reduced histone H3 acetylation could be a consequence of reduced amounts of the mcm^5s^2U nucleoside at wobble position in tRNA.

In yeast, the Elongator complex is required for formation of the first intermediate, likely to be 5-carboxymethyluridine (cm^5U), in formation of the ncm^5U, mcm^5U and mcm^5s^2U nucleosides at wobble position in tRNA [40–44]. In essentially all archaea, homologues to the Elp3 protein are found but not the other subunits of the Elongator complex [45,46]. Recently, the archaea *Methanocaldococcus infernus* Elp3 protein produced in *Escherichia* coli was shown to catalyse the formation of cm^5U by transfer of an acetyl radical originating from acetyl-CoA in the presence of S-adenosylmethionine (SAM) [46]. Thus, this reaction utilizes both the HAT and Radical SAM domains found in Elp3p. In mammals, a fraction of the Elongator complex dependent mcm^5U wobble nucleoside is further converted into the diastereomeric modifications (*R*)- and (*S*)-5-methoxycarbonylhydroxymethyluridine ($mchm^5U$) [47,48] whereas in plants and worms the (*S*)-$mchm^5U$ nucleoside is found [48–50].

In this study, we analyzed the levels of the mcm^5s^2U nucleoside in brain tissue and fibroblast cell lines derived from healthy individuals and FD patients. Our results show that FD patients have reduced levels of the mcm^5s^2U nucleoside in tRNA.

2. Materials and methods

2.1. Tissue specimens, cell lines and media

Tissue samples were from the parietal- or frontal-cortex of cerebrum from patients with UMB#: M3697M (FD), M3783M (FD), 1670 (non-FD), 880 (non-FD), 1744 (non-FD) and 5120 (non-FD) were from NICHD Brain and tissue bank for developmental disorders. Fibroblasts GM03348 (non-FD), GM04959 (FD), AG08498 (non-FD) and GM04899 (FD) were from Coriell Cell Repositories. Fibroblasts were grown in DMEM Glutamax-I media (Gibco) with 15% FBS (Sigma-Aldrich), sodium pyruvate (Gibco), MEM non-essential amino acids (Gibco) and Penicillin-Streptomycin (Gibco) or Antibiotic-Antimycotic (Gibco) at 37 °C in a humidified atmosphere with 5% CO_2.

2.2. tRNA isolation and analysis by HPLC

Approximately 1 g of tissue from the parietal- or frontal-cortex of cerebrum was cut into small pieces. The tissue was homogenized by grinding in the presence of liquid nitrogen. The homogenized tissue was dissolved in 7 mL TRIzol (Life Technologies) and samples were vortexed for 20 min at room temperature (RT). Samples were centrifuged for 20 min at 12,000*g* and the supernatant was mixed with an equal volume of chloroform and vortexed 5 min at RT. Samples were subjected to centrifugation at 12,000*g* for 20 min and the aqueous phase was mixed with 5 mL of water saturated phenol and 0.5 mL of chloroform. Samples were vortexed for 5 min at RT and centrifuged for 20 min at 12,000*g*. Total RNA was precipitated with 0.7 volumes of isopropanol, centrifuged at 12,000*g* for 20 min and separated from high molecular weight RNA as earlier described [20]. Fibroblasts were collected at 90–95% confluency from at least 14 culture flasks with the bottom area of 75 cm^2. Fibroblasts were detached using Trypsin and collected by centrifugation at 390*g* for 5 min. Total tRNA was prepared as earlier described [20] using PBS instead of 0.9% NaCl to resuspend harvested cells. tRNA was digested to nucleosides using nuclease P1 (Sigma–Aldrich) and bacterial alkaline phosphatase (Sigma–Aldrich) and analyzed as earlier described [51].

2.3. Western blot

Fibroblasts were grown to 80–95% confluency. Cells were scraped off in the presence of RIPA buffer (150 mM sodium chloride, 1.0% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris pH 8.0) supplemented with cOmplete Protease Inhibitor Cocktail Tablet (Roche). Cells were agitated for 30 min at 4 °C and centrifuged for 20 min at 12000 rpm at 4 °C. Proteins in the supernatant were separated on 7.5% SDS–PAGE and transferred to an Amersham™ Hybond ECL membrane (GE Healthcare). Membranes were incubated with antibodies in 5% non-fat dried milk in PBS. IKAP was detected using a monoclonal Anti-IKBKAP antibody (Sigma–Aldrich, 0.5 mg/ml, mouse, 1:200) and actin by anti-beta Actin antibody (Abcam, 1 mg/ml, mouse, 1:4000). Secondary antibody was a horseradish peroxidase-conjugated anti-mouse IgG antibody (GE Healthcare, 1:4000). Signals were detected using Amersham ECL Western blotting detection reagents (GE Healthcare). Signals were quantified using the ImageJ software.

3. Results and discussion

To investigate if FD patients show lower levels of the mcm^5s^2U nucleoside in tRNA we analyzed levels of this nucleoside in tissue derived from the parietal- and frontal-cortex of cerebrum of two FD patients and six non-FD individuals. Total tRNA was analysed by high-performance liquid chromatography (HPLC). Quantification of the mcm^5s^2U nucleoside levels revealed that tRNA extracted from FD brain tissue has 65–71% of the mcm^5s^2U nucleoside levels observed in tRNA from the non-FD brain tissue. (Fig. 1 and Table 1). Since we only had access to brain tissue from two deceased FD patients we decided to continue our work with fibroblast cells obtained from FD patients and non-FD individuals. To confirm that our FD fibroblasts had a reduction of the IKAP-protein we performed a Western blot using an IKAP-specific antibody (Fig. 2). The Western blot show a distinct reduction in levels of IKAP in the FD derived fibroblasts compared to fibroblasts derived from non-FD individuals. HPLC analysis of the mcm^5s^2U nucleoside in total tRNA extracted from non-FD and FD fibroblasts revealed that FD fibroblasts have 64% of the mcm^5s^2U nucleoside levels observed in the non-FD fibroblasts (Fig. 3 and Table 1). We also tried to analyze levels of the modified nucleoside ncm^5U which is dependent on Elongator complex for its formation [18,28–30]. However, the amount of ncm^5U could not be quantified by HPLC analysis due to co-migration with an unrelated peak (data not

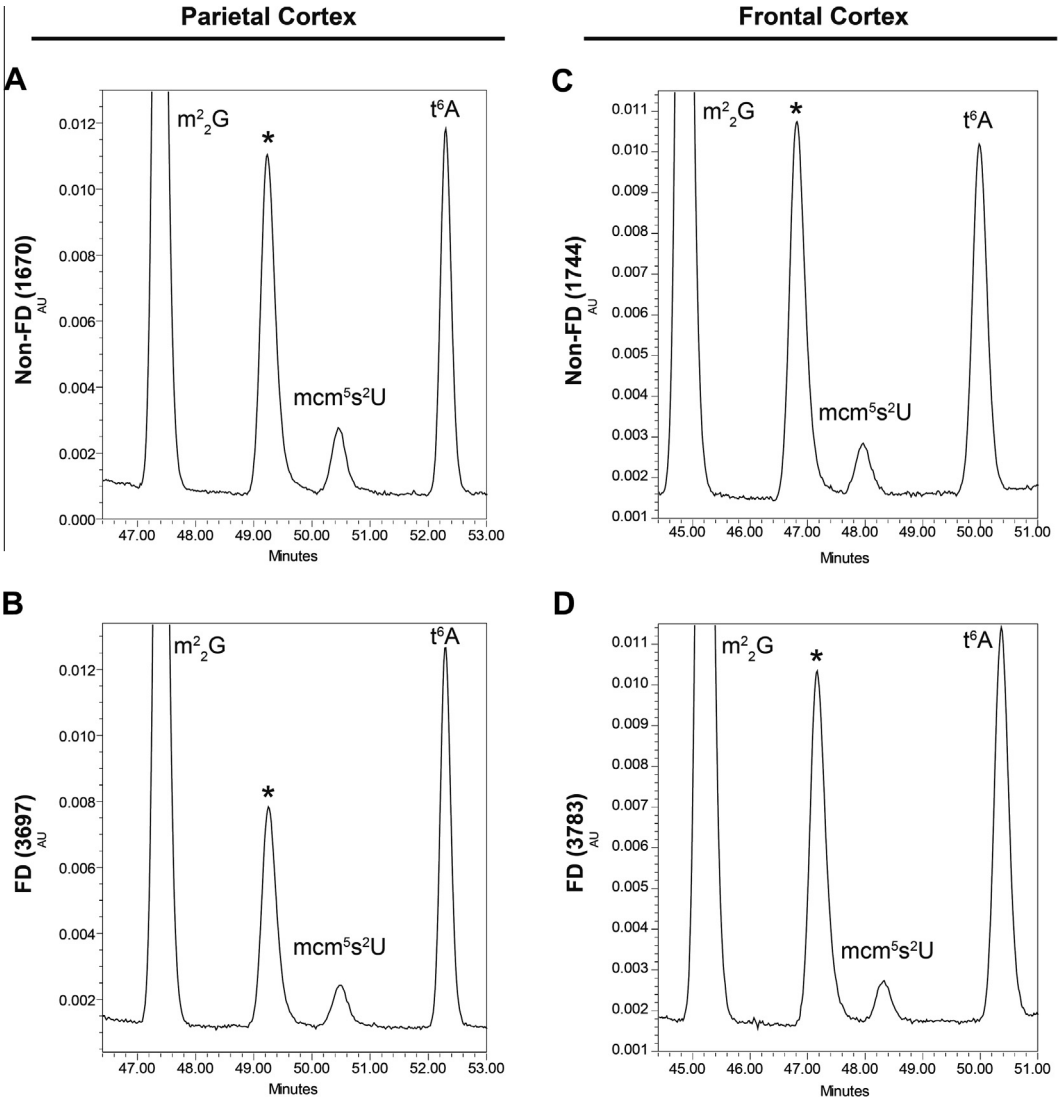

Fig. 1. Brain tissue from familial dysautonomia (FD) patients show reduced levels of the mcm^5s^2U modified wobble nucleoside. HPLC analysis of modified tRNA nucleosides from parietal cortex. (A) Non-FD individual (1670). (B) FD patient (3697). HPLC analysis of modified tRNA nucleosides from the frontal cortex. (C) Non-FD individual (1744). (D) FD patient (3783). The part of the chromatogram between the retention times 46.5 and 53 min (A and B) and 44.5 and 51 min (C and D) are shown. Chromatograms are monitored at 254 nm. *Abbreviations:* ($m^2$2G) N^2,N^2-dimethylguanosine; (mcm^5s^2U) 5-methoxycarbonylmethyl-2-thiouridine, (t^6A) N^6-threonylcarbamoyladenosine. Asterisk (∗) indicates an unknown peak.

Table 1
Content of modified nucleosides in total tRNA isolated from brain tissue and fibroblast cell lines from familial dysautonomia (FD) patients and non-FD individuals.

Samples			Ψ	Cm	m^1G	m^2G	m^2_2G	mcm^5s^2U	% of Non-FD
Parietal Cortex	Non-FD	1670	1.00	1.00	1.00	1.00	1.00	**1.00**	**65%**
	FD	3697	1.00	0.95	1.11	1.11	1.09	**0.65**	
Frontal Cortex	Non-FD	880	1.00	1.00	1.00	1.00	1.00	**1.00**	**71%**
		1744	1.00	1.12	0.96	0.98	0.99	**1.00**	
		5120	1.00	1.09	0.97	1.00	0.99	**0.88**	
	FD	3783	1.00	1.05	0.99	0.99	1.02	**0.68**	
Fibroblast	Non-FD	GM03348	1.00	1.00	1.00	1.00	1.00	**1.00**	**64%**
		AG08498	1.00	1.07	0.97	0.90	1.06	**0.90**	
	FD	GM04899	1.00	0.97	1.08	1.01	1.04	**0.67**	
		GM04959	1.00	1.06	1.03	0.87	1.06	**0.54**	

Pseudouridine (ψ) was used as an internal standard. The numbers are ratios of various modified nucleosides (modified nucleoside/ψ) in total tRNA isolated from fibroblasts or brain tissue (parietal cortex and frontal cortex). In each set, levels of modified nucleosides from GM03348, 1670 and 880 were set to 1. Values for mcm^5s^2U are shown in bold. Percentage of mcm^5s^2U in FD relative to non-FD samples (% of non-FD) is calculated by the average value for FD derived samples divided by the average value from the non-FD samples in each set (Fibroblasts, Parietal cortex and Frontal cortex).
Abbreviations: (ψ) pseudouridine; (Cm) 2′-*O*-methylcytidine; (m^1G) 1-methylguanosine; (m^2G) N^2-methylguanosine; ($m^2$2G) N^2,N^2-dimethylguanosine; (mcm^5s^2U) 5-methoxycarbonylmethyl-2-thiouridine.

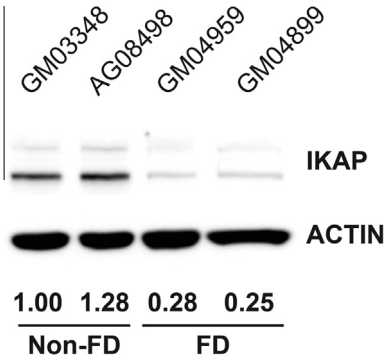

Fig. 2. Fibroblasts from familial dysautonomia (FD) patients display reduced levels of the IKAP protein. Protein was extracted from fibroblasts derived from non-FD individuals (GM03348, AG08498) and FD patients (GM04899, GM04959). Levels of IKBKAP were determined by Western blot analysis with an IKBKAP specific antibody. Actin levels were used as loading control. Ratio IKAP/actin was set to 1.00 in GM03348 (non-FD) and other samples were normalized to GM03348.

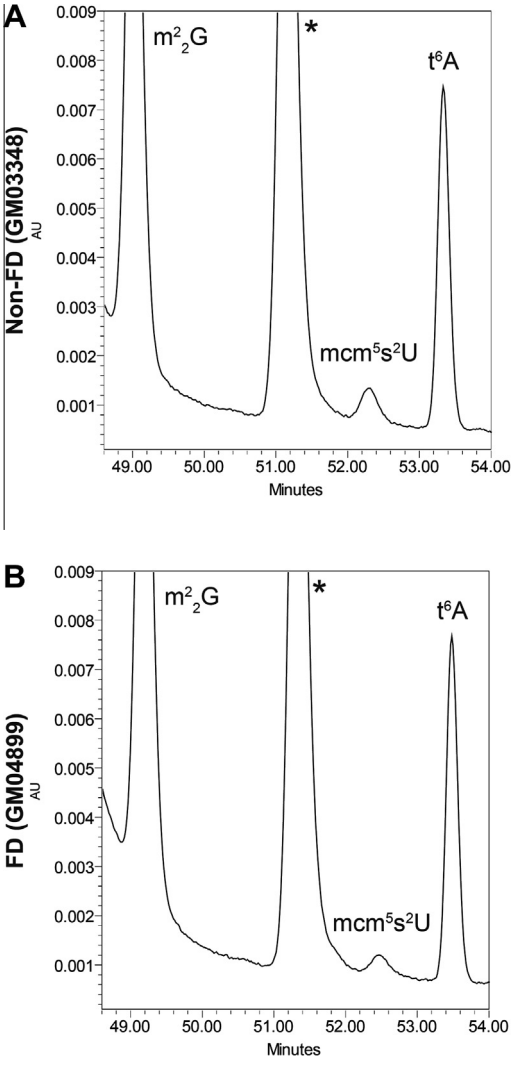

Fig. 3. Fibroblasts derived from familial dysautonomia (FD) patients show reduced levels of the mcm^{5}s^{2}U modified wobble nucleoside. HPLC analysis of modified tRNA nucleosides from fibroblast cell lines. (A) Non-FD individual (GM03348). (B) FD patient (GM04899). The part of the chromatogram between the retention times 48.8 and 54 min are shown. Chromatograms are monitored at 254 nm. *Abbreviations:* (m^{2}2G) N^2,N^2-dimethylguanosine; (mcm^{5}s^{2}U) 5-methoxycarbonylmethyl-2-thiouridine, (t^{6}A) N^6-threonylcarbamoyladenosine. Asterisk (∗) indicates an unknown peak.

shown). To verify that the reduction in mcm^{5}s^{2}U levels were FD specific we compared levels of other modified nucleosides in total tRNA isolated from FD patients and non-FD individuals. We found that of the nucleosides analysed, only mcm^{5}s^{2}U was reduced in FD brain tissue as well as FD fibroblasts (Table 1). The reduction in mcm^{5}s^{2}U levels we observed is similar to the reduction of mcm^{5}s^{2}U observed in studies of yeast *kti13* null mutants [52]. Strikingly, *kti13* null mutants display similar but weaker phenotypes than Elongator null mutants [19,53]. These results suggest that partial loss of the modified wobble nucleosides in tRNA is sufficient to reduce translational efficiency causing multiple phenotypes. Furthermore, a study in *C. elegans* where the *IKBKAP* homologue *ELPC-1* was inactivated cause defects in translation [29]. In addition, the same study observed that *ELPC-1::GFP* reporters are strongly expressed in a subset of chemosensory neurons required for salt chemotaxis learning. Inactivation of the *elpc-1* gene causes a defect in salt chemotaxis learning, associated with posttranscriptional reduction of neuropeptide and decreased accumulation of acetylcholine in the synaptic cleft [29]. Thus in *C. elegans* a functional *ELPC-1* is relevant for proper neurological function. Similarly, inefficient translation in nervous tissues of FD patients due to partial loss of the mcm^{5}s^{2}U nucleoside in tRNA may be the cause for the neurodegenerative nature of the disease.

In conclusion, we demonstrate that brain tissue and fibroblasts from FD patients show reduced levels of the mcm^{5}s^{2}U nucleoside at wobble position in tRNA. We found that brain tissue from FD patients has 65–71% of the mcm^{5}s^{2}U nucleoside levels observed in tRNA from non-FD brain tissue. Furthermore, fibroblasts from FD patients have 64% of the mcm^{5}s^{2}U nucleoside levels observed in the non-FD fibroblasts. These results suggest that lower levels of the IKAP protein due to aberrant splicing of *IKBKAP* cause reduced amounts of the mcm^{5}s^{2}U nucleoside in tRNA. A complete loss of modified wobble uridine nucleosides in FD patients was not expected as mice homozygous *ikbkap*$^{-/-}$ knockouts are embryonic lethal [54]. Our results show that IKAP is required for formation of the mcm^{5}s^{2}U nucleoside at wobble position in tRNA implicating that FD may be the result of inefficient translation.

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