Transcription Impairment and Cell Migration Defects in Elongator-Depleted Cells: Implication for Familial Dysautonomia

Pierre Close,1,4 Nicola Hawkes,4 Isabelle Cornez,1 Catherine Creppe,1 Charles A. Lambert,2 Bernard Rogister,3 Ulrich Siebenlist,5 Marie-Paule Merville,1 Susan A. Slaugenhaupt,6 Vincent Bours,1 Jesper Q. Svejstrup,1,* and Alain Chariot1,*

1 Laboratory of Medical Chemistry and Human Genetics
Center for Biomedical Integrative Genoproteomics
2 Laboratory of Connective Tissues Biology
Center for Biomedical Integrative Genoproteomics
3 Center for Cellular and Molecular Neurobiology
University of Liège
4000 Liège
Belgium
4 Clare Hall Laboratories
Cancer Research UK London Research Institute
South Mims EN6 3LD
United Kingdom
5 Laboratory of Immuno-Regulation
National Institute of Allergy and Infectious Diseases
National Institutes of Health
Bethesda, Maryland 20892
6 Center for Human Genetic Research
Massachusetts General Hospital and
Harvard Medical School
Boston, Massachusetts 02114

Summary

Mutations in IKBKAP, encoding a subunit of Elongator, cause familial dysautonomia (FD), a severe neurodevelopmental disease with complex clinical characteristics. Elongator was previously linked not only with transcriptional elongation and histone acetylation but also with other cellular processes. Here, we used RNA interference (RNAi) and fibroblasts from FD patients to identify Elongator target genes and study the role of Elongator in transcription. Strikingly, whereas Elongator is recruited to both target and non-target genes, only target genes display histone H3 hypo-acetylation and progressively lower RNAPII density through the coding region in FD cells. Interestingly, several target genes encode proteins implicated in cell motility. Indeed, characterization of IKAP/hELP1 RNAi cells, FD fibroblasts, and neuronal cell-derived cells uncovered defects in this cellular function upon Elongator depletion. These results indicate that defects in Elongator function affect transcriptional elongation of several genes and that the ensuing cell motility deficiencies may underlie the neuropathology of FD patients.

Introduction

FD is an autosomal recessive disease, ranging among the most frequent hereditary sensory and autonomic neuropathies (Slaugenhaupt and Gusella, 2002; Axelrod, 2004). Affected individuals are born with the disease and abnormally low numbers of neurons in the autonomic and sensory nervous systems, which initially triggered a search for the disease gene among candidates involved in neuronal differentiation and cell migration. FD turned out to be caused by mutations in a splice site of the IKBKAP gene, which causes tissue-specific exon skipping, and expression of a truncated mRNA transcript (Anderson et al., 2001; Slaugenhaupt et al., 2001). The predicted, shorter form of the encoded IKAP protein cannot be detected in patients (Slaugenhaupt et al., 2001), because the truncated transcript is degraded by the nonsense-mediated decay pathway (Slaugenhaupt et al., 2004). FD mutations are incompletely penetrant, so that normal IKAP protein is still synthesized in patients, albeit at lower levels, depending on cell type. Indeed, IKAP levels are very low in brain tissues from FD patients (Slaugenhaupt et al., 2001; Cuajungco et al., 2003).

IKAP was initially described as a scaffold protein of the IKK complex involved in NF-κB activation (Cohen et al., 1998). However, a role for IKAP protein in this pathway was later disproved (Krappmann et al., 2000). The IKAP protein and its yeast homolog, Elp1, are components of the highly conserved transcription elongation factor complex Elongator (Hawkes et al., 2002; Kim et al., 2002). Elongator was originally identified as a component of a hyperphosphorylated RNA polymerase II (RNAPII) holoenzyme isolated from budding yeast chromatin (Otero et al., 1999). Significantly, another subunit of Elongator, Elp3, harbors motifs found in the GNAT family of histone acetyltransferases (HATs) (Wittschieben et al., 1999). Both yeast and human Elongator have HAT activity in vitro, primarily directed toward histone H3 (Winkler et al., 2002; Hawkes et al., 2002; Kim et al., 2002), and yeast elp3 mutation results in decreased histone H3 acetylation levels in chromatin in vivo (Winkler et al., 2002; Kristjuhan et al., 2002). In agreement with a role in transcript elongation, Elongator is associated with the nascent RNA emanating from elongating RNAPII along the coding region of several yeast genes (Gilbert et al., 2004), and chromatin immunoprecipitation (ChIP) experiments have also demonstrated an association of Elongator with genes in human cells (Metivier et al., 2003; Kouskouti and Talianidis, 2005).

Surprisingly, a substantial fraction of Elongator is cytoplasmic (Hawkes et al., 2002; Holmberg et al., 2002; Kim et al., 2002), suggesting that the complex performs additional distinct functions in the cell (Gilbert et al., 2004). For example, a role as a scaffold protein involved in cytoplasmic JNK activation in response to extracellular stress has been proposed for the IKAP/hELP1 protein in mammalian cells (Holmberg et al., 2002). In yeast, genetic data have implicated the Elongator complex in processes as diverse as exocytosis and tRNA modification (Rahl et al., 2005; Huang et al., 2005). The relationship between Elongator’s role in transcription and these other processes remains poorly understood.

To gain further insight into the role played by Elongator in transcription in human cells, and concomitantly
learn about the molecular defects underlying FD, we used an RNAi strategy to deplete the IKAP/hELP1 subunit of Elongator. Using DNA microarray analysis, we then identified Elongator-dependent genes. Here, we show that Elongator depletion affects the expression of a number of genes, with correlating effects on histone H3 acetylation and transcriptional elongation. Several of the affected genes are implicated in cell motility, and cells with decreased IKAP/hElp1 levels indeed display defects in this cellular function. These data open the intriguing possibility that impaired cell motility/migration in the nervous system underlies the neuropathology of FD patients.

**Results**

**IKAP/Elongator Regulates the Expression of Genes Involved in Cell Migration**

In order to investigate the biological role of IKAP/Elongator in human cells, HeLa cells were infected with a lentivirus delivering small interfering RNAs targeting either the IKAP/hELP1 transcript or GFP as a negative control. Because IKAP/hELP1 had previously been implicated in assembling stress-induced and cytoplasmic kinase complexes (Cohen et al., 1998; Holmberg et al., 2002), we first addressed the potential role of IKAP/hELP1 in these pathways (Figure S1 available in the Supplemental Data with this article online). A number of different assays investigating the involvement of IKAP/hELP1 in Erk, p38, and JNK activation, as well as in the IKK-mediated NF-κB activation pathway, failed to uncover significant effects (Figure S1). Similarly, no effect on cytoplasmic kinase signaling was uncovered in fibroblasts derived from FD patients (Figure S1). Taken together, our data indicate that, in these cells, stress-induced MAPK and NF-κB signaling pathways can proceed through IKAP/hELP1-independent mechanisms.

Because there is substantial evidence linking IKAP/hELP1 to transcriptional elongation in the context of the Elongator complex, we next sought to identify genes whose normal expression requires IKAP/hELP1. HeLa cells were transfected with RNai oligos that target either the IKAP/hELP1 transcript or the GFP transcript as a negative control. Decreased IKAP/hELP1 mRNA and protein expression in response to IKAP/hELP1 RNAi treatment of HeLa cells was confirmed (Figures 1A and 1B, respectively). Total mRNA was then extracted from the RNai-treated cells and subjected to microarray analysis. Examples of affected genes are shown. The full list of affected genes is shown in Figure S2.

Figure 1. Identification of Genes Regulated by IKAP/Elongator (A and B) Decreased IKAP mRNA (A) and protein (B) expression in HeLa cells transfected with IKAP/hELP1 RNAi (lane 2), as compared to untransfected cells (lane 1) or cells transfected with a GFP control RNAi (lane 3), as judged by real-time PCR (A) or by anti-IKAP/hELP1 Western blot analysis (B). Error bars in (A) denote standard deviation.

We examined the validity of our microarray results by performing quantitative real-time PCR with total RNA extracted from untransfected cells or from cells transfected with RNai. Hereby, the decreased expression of several target genes in IKAP/hELP1-depleted cells was confirmed (Figure 2A). Moreover, reduced expression of paxillin and calreticulin was also confirmed at the protein level in these cells (Figure 2B).

To investigate the physiological importance and possible disease relevance of the observed effect of IKAP/hELP1 RNAi on transcription, total RNA was extracted from FD patient-derived fibroblasts (where IKAP/hELP1 levels are reduced through the IKBKAP splice site mutation) and subjected to quantitative real-time PCR analysis. In support of the results obtained with RNai-transfected cells, decreased expression of gelsolin, paxillin, lammin J3, and beclin-1, but not α-tubulin, was also observed in FD fibroblasts (Figure 2C). Interestingly, the cell cycle-dependent kinase inhibitor p57, kip2, as well as various genes coding for proteins playing critical roles in cellular processes such as autophagy (beclin-1), metabolism (transglutaminase 2), and DNA repair (hMSH2) were downregulated as well (Figure 1C). GFP RNAi, as well as from untransfected control cells. The RNA extraction was then subjected to microarray analysis. Examples of affected genes are shown. The full list of affected genes is shown in Figure S2.
some cell type specificity in the expression patterns might occur because the expression of genes such as tenascin-C and MMP2 did not appear to be significantly altered in FD fibroblasts (data not shown).

Decreased gelsolin expression in fibroblasts is known to be associated with enhanced expression of the GTPase Rac in compensation for the reduced cell motility of these cells (Azuma et al., 1998). We indeed observed increased Rac expression in FD fibroblasts compared to control cells, whereas IKAP/hELP1 expression as expected was decreased (Figure 2D, compare top two panels).

Taken together, these results demonstrate that low IKAP/hELP1 levels, resulting from either RNAi or from the splicing mutation in FD cells, similarly affect the expression of several genes.

**Impaired IKAP/hELP1 Expression Alters Cell Migration**

The gene expression data suggest that IKAP/hELP1 depletion leads to lower levels of expression of numerous genes, including several implicated in cell motility. Because normal cell motility is of crucial importance for the developing nervous system (reviewed by da Silva and Dotti [2002] and Dent and Gertler [2003]) and therefore of obvious relevance to FD, we characterized the potential role of IKAP/hELP1 in cell motility at the cellular level. IKAP/hELP1 shRNA and control shRNA cells were first compared in a wound-healing assay. This assay measures the ability of cells to migrate (cell proliferation being inhibited by mitomycin C) and fill the gap left by physical disruption of cell monolayers (West et al., 2001). Significantly, a delay in complete "wound closure" was observed in IKAP/hELP1 shRNA HeLa cells compared to control cells. Whereas complete closure was observed after 22 hr with the control cells, gaps in the cell monolayers remained open in the IKAP/hELP1 shRNA cells (Figure 3A). Next, the ability of IKAP/hELP1 shRNA cells to migrate in response to a serum gradient was investigated by using Boyden chambers (Riedy et al., 1999). Again, a clear defect in cell migration was observed in IKAP/hELP1 shRNA HeLa cells (Figure 3B).

To further correlate the observed migration defects with decreased IKAP/hELP1 expression and FD, wound-healing assays were also performed with two distinct cell lines from FD patients and control fibroblasts. Although significant closure of the gaps in cell monolayers had occurred after 14 hr in control fibroblasts, a clear defect in gap closure by cell migration was observed in FD fibroblasts (Figure 3C).

Cell motility of fibroblasts has been extensively studied by collagen gel contraction assays, in which contraction occurs as a consequence of motile activity of cells migrating through the matrix (Grinnell, 1994). We took advantage of such experiments to further characterize the cell motility defects in FD fibroblasts. Experiments over a 10 day period showed that control fibroblasts were significantly more potent than FD cells in contracting collagen gels (Figure 3D), indicating that the ability of
the mutant cells to spread and elongate is significantly perturbed. Similar results were obtained with cells in which IKAP/hELP1 levels had been depleted by RNAi 

(P.C. and A.C., unpublished data). Therefore, decreased IKAP/hELP1 expression correlates with a cell migration defect in fibroblasts from FD patients as well.

Figure 3. Cell Migration Defect in IKAP/hELP1 RNAi Cells, as Well as in FD Fibroblasts

(A) Wound-healing assays performed with shRNA GFP or shRNA IKAP/hELP1 in HeLa cells (left and right, respectively). Pictures were taken at the indicated times after wounding.

(B) Chemotaxis assays using a Boyden chamber carried out with shRNA GFP (large, open squares; Ctr) or shRNA IKAP HeLa (small, filled squares; IKAP). Cells migrating to the lower membrane were counted. The figure shows the total number of migrating cells after the indicated times. Three independent experiments were performed in triplicate, with similar results. The average, with standard deviation, of one such experiment is shown.

(C) Wound-healing assays performed with wt or FD fibroblasts (left and right, respectively). Pictures were taken at the indicated time points after wounding.

(D) Contraction of free-floating collagen lattices seeded with either wt or FD fibroblasts. Photographs taken during the course of a representative experiment are shown on the left. A graphic representation of the experiment is shown on the right, with standard deviations indicated.

(E) Top, an anti-IKAP/hELP1 Western blot performed on cell lysates derived from shRNA GFP or shRNA IKAP/hELP1 SK-N-BE cells. Bottom, as in (A) but using SK-N-BE cells.

(F) As in (B) but using SK-N-BE cells. Two independent experiments were performed in triplicate, with similar results. The average, with standard deviation, of one such experiment is shown.
Because FD mainly affects the development of neurons in the autonomic and sensory nervous systems, it was of great interest to also assess cell migration in an IKAP/hELP1-depleted neuronal cell-derived cell line. To do this, we infected neuroblastoma-derived SK-N-BE cells with the IKAP/hELP1 or GFP shRNA constructs. The infected cells were then plated on fibronectin-coated plates and subjected to wound-healing assays. Significantly, a clear delay in wound closure was observed in SK-N-BE cells expressing lower amounts of IKAP/hELP1 (Figure 3E). A similar cell migration delay was also obtained with IKAP/hELP1-depleted glioblastoma-derived U373 cells (data not shown). Moreover, cell motility defects as judged by chemotaxis assay using Boyden chambers were observed in SK-N-BE and in U373 Elongator-depleted cells as well (Figure 3F and data not shown). Taken together, these functional assays strongly suggest that the transcription defects in IKAP/hELP1 cells have cell functional consequences so that a number of different cell types with lower levels of IKAP/hELP1 have significantly reduced cell motility. In particular, the reduced motility of neuronal cell-derived cell lines may be highly relevant to the neurodevelopmental disorder of FD patients.

**Elongator and Its Association with the Coding Region of Human Genes Are Altered by the FD Mutation**

The data above show that cells with reduced levels of IKAP/hELP1 have decreased transcription of several genes and that this has consequences for cell function. However, precisely how these defects relate to the Elongator complex and its cellular role was not clear. Our previous experiments performed in the analogous yeast system showed that deletion of ELP1 leads to loss of Elp3 and Elongator integrity (Petrakis et al., 2004). Extending from yeast to human cells, this result predicts that the amount of functional Elongator should also be altered in FD cells because of their decreased IKAP/hELP1 production. Indeed, we found that the protein level of the catalytic hELP3 subunit was significantly decreased in cells where lowered IKAP/hELP1 levels were caused by either RNAi or the FD mutation (Figure 4A, left and right, respectively). This indicates that, as expected from the results in the yeast system, hElp3 levels are indeed affected by the removal of IKAP/hELP1. This suggests that the lower levels of transcription observed in cells depleted for IKAP/hELP1 are due to lower levels of Elongator. To more directly investigate this possibility, we generated a hELP3 cellular loss of function model by transfecting ELP3, or GFP, RNAi into fibroblasts. Unfortunately, although the ELP3 mRNA level was clearly reduced (data not shown), ELP3 RNAi did not lead to a very efficient depletion of this protein from cells (Figure 4B). Nevertheless, our experiments showed that gelsolin and beclin-1, but not α-tubulin, expression was consistently decreased in ELP3 RNAi fibroblasts, although to a smaller extent than in IKAP/hELP1 RNAi cells (Figure 4C). Therefore, these results suggest that cell expression deficiencies of two Elongator subunits, namely IKAP/hELP1 and ELP3, have similar consequences for gene expression.

Elongator is detected in both the cytoplasm and nucleus of human cells (see, for example, Hawkes et al. [2002], Kim et al. [2002], and Kouskouti and Talianidis [2005]). We found that, surprisingly, the protein is depleted to a similar extent in both compartments in FD cells (Figure S3). In theory, the effect of IKAP depletion on gene expression could be either indirect or direct. If the effect was direct, it would be expected that Elongator is present at the target genes and that its absence has a specific effect on transcription at these genes. If, in contrast, the effect was indirect, for example through signaling from the cytoplasm, such effects would not be expected. To address the possibility that the effect of reduced IKAP/hELP1 levels on transcription was direct, standard ChIP technique in conjunction with quantitative real-time PCR was used to detect the Elongator complex at genes in normal and FD fibroblasts. The use of FD fibroblasts served as an excellent control for the specificity of the antibodies used, as lower levels of IKAP would be expected to result in significantly decreased ChIP signals in these cells. Interestingly, we detected IKAP/hElp1 not only in the coding region (and to
a smaller extent the promoter) of the gelsolin and beclin-1 target genes, but also at the tubulin gene, whose expression is not affected by IKAP/hELP1 depletion (Figure 5B). Experiments with fibroblasts derived from FD patients showed that, as expected, the amount of IKAP on the genes was indeed significantly decreased in these cells (Figure 5B, compare wt and FD). Importantly, in agreement with the idea that IKAP is crucial for Elongator function through targeting of the catalytic hELP3 subunit, the recruitment of the hELP3 protein to the coding region of the genes was indeed dramatically affected by IKAP/hELP1 depletion. Likewise, recruitment of other Elongator subunits, such as hELP4 and hELP5, was decreased as well (Figure 5C, and Figure S4). These data further indicate that recruitment of the whole Elongator complex, not just the IKAP/hELP1 protein itself, is affected by the mutation in FD cells.

To further characterize the association of Elongator with active genes, we also investigated the relative density of IKAP/hELP1 and hELP3 across the beclin-1 gene (see Figure 5A for the location of primer pairs across the gene). The overall density profile of the proteins was remarkably similar (Figure 5D), further supporting the idea that the cellular level and function of the entire Elongator complex is affected by decreasing the level of IKAP/hELP1.

Decreased Elongator Levels Affect Histone H3 Acetylation in the Coding Region of Target Genes

To more precisely define the effects of IKAP/hELP1 mutation on transcription, we now analyzed the density of various histone modifications by ChIP analysis. In these experiments, the FD cells were used as a tool to investigate the effects of lower IKAP/Elongator levels. Interestingly, histone H3-K9 acetylation, but not histone H4 acetylation, in the coding region of the gelsolin and beclin-1 genes was indeed reduced in cells with reduced Elongator levels (Figures 6A and 6B, left and middle). A similar reduction in acetylation was observed at histone H3 K18 in the beclin-1 and gelsolin coding regions (Figure 6C). It is important to note that reduced acetylation was not due to a loss of histone H3-DNA contacts in the target genes, as histone acetylation levels were normalized for histone content (using antibodies specific to ChIP
Elongator Depletion Affects Transcript Elongation

Progressively Decreased RNAPII Density through Elongator Target Genes

We finally compared the density of RNAPII across the tested genes. We surmised that if Elongator is indeed involved in transcriptional elongation, then the density of RNAPII might be expected to be relatively lower in the 3’ end of the gene than at the promoter upon IKAP depletion. Remarkably, RNAPII density was indeed progressively decreased in FD cells across both the gelsolin and beclin-1 genes (Figure 7), with an RNAPII density similar to wild-type observed at the promoter, but only ~30% density observed at the end of the gene. In contrast, the RNAPII density at the α-tubulin control gene was largely unaffected by the decrease in IKAP/Elongator levels, as expected (Figure 7). Together, these data indicate that Elongator affects transcript elongation, but not recruitment of RNAPII to the promoter, of genes whose expression is affected by decreased IKAP/hELP1 levels. The very specific effects of IKAP depletion on histone acetylation and RNAPII density across target genes are consistent with a direct effect of Elongator on transcriptional elongation rather than with an indirect effect caused by its role in the cytoplasm.

Discussion

The data presented here provide several key insights into Elongator function. First, they demonstrate a role for Elongator in histone H3 acetylation and transcriptional elongation of human genes. Second, they indicate that the mutation carried by individuals suffering from FD causes abrogation of Elongator function, not just of IKAP/hELP1 expression. Third, normal levels of Elongator are important for normal expression of several human genes, including some implicated in cell motility. Indeed, HeLa and neuronal-derived IKAP/hELP1 RNAi cells, as well as FD fibroblasts, exhibit defects in cell motility in vitro. Cell motility is crucial for the normal development and maintenance of the nervous system, so our data also point to molecular defects that may underlie FD.
Elongator Functions in Transcript Elongation in Human Cells

The data presented here provide evidence in support of the previously proposed model for Elongator function (Otero et al., 1999; Wittschieben et al., 1999). According to this model, Elongator acetylates histones during transcription as a component of an elongating RNAPII holoenzyme. Our ChIP experiments thus clearly show that Elongator is present in the coding region of genes and that histone H3 acetylation is significantly reduced in the coding region, but not at the promoter, of affected genes in its absence. Moreover, RNAPII density is progressively lowered through the coding region of target genes. Elongator depletion leaves RNAPII recruitment to the promoter largely unaffected, and RNAPII density in the first few hundred to several thousand nucleotides of the open reading frame is also normal. This supports the idea that Elongator assists RNAPII during transcript elongation through chromatin as the polymerase moves further and further away from a promoter and the activity sphere of HATs such as PCAF and p300/CBP (Kouskouti and Talianidis, 2005), whose activity are likely overlapping with that of Elongator in the 5’ end of genes. To our knowledge, these results represent the first demonstration of a role specifically in the transcript elongation phase for an elongation factor in human cells.

Because data from ChIP experiments can only show a correlation between the presence of a factor and effects at sites of action, our ChIP data from human cells do not in themselves prove that Elongator acetylates histones during transcriptional elongation. However, it is of importance not to view these new results out of context. First, recent results have shown that both histone H3 and H4 acetylation is measurably increased in the coding region of several human genes as a consequence of active transcription (Kouskouti and Talianidis, 2005). We have shown that Elongator is primarily a histone H3 acetyltransferase in vitro (Winkler et al., 2002;...
In this context, it is therefore striking that histone H3, but not H4 acetylation, was decreased by Elongator depletion in FD cells. Second, previous data from yeast showed that mutations in Elongator are synthetic lethal with mutations in the N-terminal tail of histone H4, suggesting that Elongator function is required for normal histone H3 function in vivo (in the absence of the H4 tail, correct function of the H3 tail is essential) (Wittschieben et al. [2000] and references therein). Third, yeast cells lacking both ELPL3 and the gene encoding another histone H3 acetyltransferase, GCN5, have severe growth defects, and these growth defects can be suppressed specifically by concurrently deleting the histone deacetylases HDA1 and HOS2 (Wittschieben et al., 2000). Although the data reported here support the idea that human Elongator targets both lysine H3 K9 and H3 K18 in vivo, this does not rule out the possibility that lysine H3 K14, which is a primary target site of purified yeast Elongator in vitro, is targeted in human cells as well (see Kristjúhan et al. [2002] for a discussion of the site specificity of HATs). Taken together with the data from the yeast model, our results using human cells strongly point to a function for Elongator in histone acetylation during transcription elongation.

IKAP/Elongator Depletion Results in Downregulation of Genes Required for Normal Cell Motility
Several models for the function of IKAP/hELP1 and Elongator have been proposed. Cohen et al. (1998) and Holmberg et al. (2002) suggested that IKAP/hELP1 might be involved in cytoplasmic signaling in the NF-κB and JNK pathways, respectively. Our data, and those of others (Krappmann et al., 2000), failed to support an involvement of IKAP/hELP1 in these cytoplasmic signal transduction pathways. The name IKAP is therefore in all likelihood a misnomer, and we suggest that the protein should be designated human ELPL1 (hELP1) instead.

In yeast, Elongator has been implicated in cellular reactions as diverse as tRNA modification (Huang et al., 2005) and exocytosis (Rahl et al., 2005). Indeed, Rahl et al. proposed that FD is caused by an exocytosis defect. These authors showed that a defect in yeast exocytosis resulting from sec2-52 mutation (which creates a premature stop codon after the first 374 residues of the essential Sec2 protein) could be overcome by Elongator gene deletion. Unfortunately, the data in support of this unusual suppression effect being direct were unpersuasive. For example, an interaction between full-length Elp1 and Sec2 could only be demonstrated by the use of a potent protein-protein crosslinker. In our experience, the use of such crosslinkers in crude extracts requires several specificity controls, which were not provided. Interestingly, the reported involvement of Elongator in tRNA modification (translation fidelity) raises the possibility that the sec2-52 suppression is caused by increased stop codon readthrough in elp strains, as elp mutation may affect the recognition of ochre codons (Huang et al., 2005). elp mutation might obviously also affect expression of exocytosis genes via Elongator’s role in transcription. It thus remains unclear if the effect of Elongator on exocytosis is direct and if the yeast exocytosis data are relevant for FD. In any case, it remains a possibility that translational imprecision might contribute to FD (and to the defects resulting from Elongator disruption in yeast).

In general, the most plausible explanation for the previously reported cellular localization data seems to be that Elongator plays roles in distinct cellular processes, in distinct cellular compartments, as previously proposed (Gilbert et al., 2004). The complex nature of Elongator function and its relationship with basic cellular functions are further underscored by the data presented here. We thus demonstrated that Elongator plays a key role in transcription of several genes that regulate the actin cytoskeleton and cell motility/migration and that decreases in Elongator levels indeed result in cell migration defects in four different tested cell types, including neuronal-derived cells. It is worth noting that although the data presented here strongly argue that the cell migration defect is a consequence of lowered expression of genes required for this process, we cannot rule out the possibility that Elongator also plays a more direct role in cell motility, for example via its cytoplasmic localization.

Impaired Cell Motility May Underlie FD
FD is a neurodevelopmental and neurodegenerative genetic disorder with severe pathological consequences (reviewed by Slaugenhaupt and Gusella [2002] and Axelrod [2004]). IKAP/hELP1 mutation affects the development and maintenance of neurons, resulting in neuro-pathological and clinical progression. To appreciate the potential importance of the connection between impaired cell motility observed in cells with decreased levels of IKAP/hELP1 protein and the neuropathology of FD patients, it is important to realize that the actin cytoskeleton and cell motility play crucial roles in nerve cell growth cone motility, axon outgrowth, and guidance. Moreover, cell motility and the actin cytoskeleton also play central roles at the level of neurogenesis (the sprouting of neurites, which will later become axons and dendrites) and in the migration of neurons to their final destination in the brain (reviewed by Dent and Gertler [2003] and da Silva and Dotti [2002]). Our data thus suggest an intriguing model to explain FD at the molecular level: the mutation in the gene encoding IKAP/hELP1 results in a tissue (brain)-specific decrease in the ability of cells to migrate, which in turn leads to neuro-developmental abnormalities and the neuropathology of FD patients. Obviously, other genes that are downregulated in cells upon Elongator depletion may contribute to the disease as well.

FD is a devastating disease, and in spite of significant advances in prognosis due to better supportive treatment, only about 40% of patients are more than 20 years old (Axelrod, 2004). The data presented here will hopefully provide important clues to future treatment of the disorder.

Experimental Procedures
Cell Culture and Reagents
HeLa and 293T cells were maintained in DMEM supplemented with 10% fetal bovine serum (Life Technologies) and antibiotics, whereas fibroblasts (Slaugenhaupt et al., 2001) were cultured in DMEM supplemented with 20% FBS, 1% antibiotics, and 1% L-glutamine. HT29 cells were cultured in EMEM supplemented with 10% NEAA,
antibiotics, and L-glutamine. SK-N-BE and U373 cells were cultured in RPMI supplemented with 10% FCS and antibiotics.

Monoclonal anti-IKAP for Western blot analysis was purchased from BD Biosciences Pharmingen. Anti-IKAP antisera for ChIP analysis were raised in rabbits against an IKAP peptide. Anti-paxillin and -Rac antibodies were from Upstate Biotechnology; anti-p38, -phospho p38, -Akt, and -phospho Akt antibodies from Cell Signaling; anti-Erk1, -phospho Erk1, -JNK, -IκBα, -α-tubulin, and -calreticulin antibodies from Santa Cruz Biotechnology; rabbit polyclonal anti-actin H3 K9, anti-actin H3 K18, and anti-actin H4 antibody, as well as 4H8 antibody used to precipitate RNAPII, were from Up-state Biotechnology; and rabbit polyclonal anti-H3 (C-terminus antibody) was from Abcam. Antibodies directed against Elp3, Elp4, and Elp5 have been described previously (Petrisaki et al. [2004] and references therein). GST-c-Jun was from BIOMOL.

RNAi Transfection and Lentiviral Cell Infection
RNAi oligos were synthesized by Dharmacon Research (sequences available upon request) and were transfected into HeLa or HT29 cells by using the oligofectamine reagent (Invitrogen) or by calcium phosphate in fibroblasts. Cells were lysed 48 hr posttransfection, and anti-IKAP/hELP1 and -ELP3 Western blots performed. The pLL3.7 lentivirus and instructions on its use was kindly provided by Dr. L. van Parijs (MIT, Boston, MA) (Rubinson et al., 2003). Details are available upon request.

Total RNA Extraction, Microarray Analysis, and Real-Time PCRs
Total RNA extraction from RNAi cells was carried out by using the RNeasy Mini kit (Qiagen). Double-stranded cDNAs were generated by using the superscript II RT kit (Invitrogen). Subsequently, bioin-tained cRNA was generated with the Bioarray High Yield RNA Transcript Labeling Kit (Enzo Life Science). cRNAs were hybridized with the Human Genome U133A array, which harbors >22,000 probe sets (Affymetrix). Data were processed with GeneChip Operating Software (Affymetrix). Quantitative real-time PCR was performed with TaqMan 7000 SDS (Applied Biosystems), using SybrGreen detection. Primers sequences are available upon request.

Wound-Healing, Chemotaxis Assays, and Collagen Matrix Contraction
For wound-healing assays, HeLa, SK-N-BE, or U373 cells stably infected with lentivirus-delivering RNAi, or wild-type and FD fibroblasts, were grown until confluence. SK-N-BE and U373 cells were plated on fibronectin-coated plates. Mitomycin C (1 μg/ml) was added to the culture media 2 hr before “wounding” to inhibit cell pro-liferation. Wound areas were generated by scraping with a pipette tip, and disrupted monolayers were randomly photographed in multiple fields (T = 0). Cell migration/wound healing was similarly assessed over the following hours.

For chemotaxis assays using a Boyden chamber (Riedy et al., 1999), HeLa or SK-N-BE and U373 cells were added to serum-free medium in 24-well multiwell plates (Corning Incorporated). The lower compartment was filled with medium supplemented with 10% FBS. Cells were incubated at 37°C. Cells on the upper surface were removed, and membranes were fixed in ethanol at −20°C and stained with Giemsa 4%. Migrated cells were randomly photographed (10 fields/insert) and counted.

Collagen gel contraction experiments were performed as described (Lambert et al., 1992), using 1 mg collagen lattice.

Kinase Assays
Anti-JNK immunoprecipitates were used in kinase assays performed at 30°C for 30 min with 1 μg of purified GST-c-Jun fusion pro-tein and 10 μCi of [32P] ATP in 20 μl kinase buffer (20 mM HEPES [pH 7.4], 10 mM MgCl2, 25 mM β-glycerophosphate, 50 μM Na2VO4, and 50 μM DTT). Phosphorylated c-Jun proteins were detected by autoradiography after 10% SDS-PAGE.

ChIP Assays
ChIP assays were performed by standard techniques (see, for example, Kouskouti and Talianidis [2005]). Extracts were precleared by 1h incubation with protein A or G/Herring sperm DNA. Protein-DNA complexes were washed as per standard ChIP techniques. After elution, proteinase K treatment, and reversal of cross-links, DNA fragments were analyzed by real-time PCR with SyBr Green detection. Input DNA was analyzed simultaneously and used as normalization. For normalization of the RNAPII ChIPs, the signal obtained from a noncoding region (downstream from the albumin gene [see Kouskouti and Talianidis (2005)]) was used to compensate for possible fluctuations arising during handling. For the histone-related ChIPs, acetyl-histone-specific ChIP values were normalized according to the total H3 signal (as detected with the C terminus-specific anti-histone H3 antibody).

Supplemental Data
Supplemental Data include Supplemental References and seven figures and can be found with this article online at http://www.molecule.org/cgi/content/full/22/4/521/DC1/.

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References


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The data from the microarray experiments have been deposited in the arrayexpress database (EMBL) under accession number E-MEXP-641.