

IKAP/Elp1 involvement in cytoskeleton regulation and implication for familial dysautonomia

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Deficiency in the IKAP/Elp1 protein leads to the recessive sensory autosomal congenital neuropathy which is called familial dysautonomia (FD). This protein was originally identified as a role player in transcriptional elongation being a subunit of the RNAPII transcriptional Elongator multi-protein complex. Subsequently, IKAP/Elp1 was shown to play various functions in the cytoplasm. Here, we describe experiments performed with IKAP/Elp1 downregulated cell lines and FD-derived cells and tissues. Immunostaining of the cytoskeleton component α -tubulin in IKAP/Elp1 downregulated cells revealed disorganization of the microtubules (MTs) that was reflected in aberrant cell shape and process formation. In contrast to a recent report on the decrease in α -tubulin acetylation in IKAP/Elp1 downregulated cells, we were unable to observe any effect of IKAP/Elp1 deficiency on α -tubulin acetylation in the FD cerebrum and in a variety of IKAP/Elp1 downregulated cell lines. To explore possible candidates involved in the observed aberrations in MTs, we focused on superior cervical ganglion-10 protein (SCG10), also called STMN2, which is known to be an MT destabilizing protein. We have found that *SCG10* is upregulated in the IKAP/Elp1-deficient FD cerebrum, FD fibroblasts and in IKAP/Elp1 downregulated neuroblastoma cell line. To better understand the effect of IKAP/Elp1 deficiency on *SCG10* expression, we investigated the possible involvement of RE-1-silencing transcription factor (REST), a known repressor of the *SCG10* gene. Indeed, *REST* was downregulated in the IKAP/Elp1-deficient FD cerebrum and IKAP/Elp1 downregulated neuroblastoma cell line. These results could shed light on a possible link between IKAP/Elp1 deficiency and cytoskeleton destabilization.

INTRODUCTION

IKBKAP is the gene mutated in the neurodevelopmental, autosomal-recessive genetic disorder—familial dysautonomia (FD). The major mutation in the *IKBKAP* gene (99.5% occurrence) is a single nucleotide (T→C) transition in the donor splice site of intron 20. This mutation leads to the skipping of exon 20 by alternative splicing, producing both wild-type and short (exon 20 skipped) transcripts in a tissue-specific manner with full exon 20 skipping and consequently elimination of the protein product IKAP/Elp1 in the nervous tissues (1).

While the biological consequences of the *IKBKAP* mutations have been described previously (1–4), the link between IKAP/Elp1 deficiency, caused by these mutations and the FD phenotype, is still not fully understood. IKAP/Elp1 is predominantly a cytoplasmic protein (5). Several possible roles played by IKAP/Elp1 in the cytoplasm are currently under investigation. It was suggested that IKAP/Elp1 plays a role in cytosolic stress signaling (5), in the regulation of exocytosis (6) and in tRNA modification (7,8). A suspected major target of the IKAP/Elp1 in the cytoplasm is the cytoskeleton. The indirect effect of IKAP/Elp1 on the cytoskeleton was first proposed by experiments in IKAP/Elp1-deficient HeLa and

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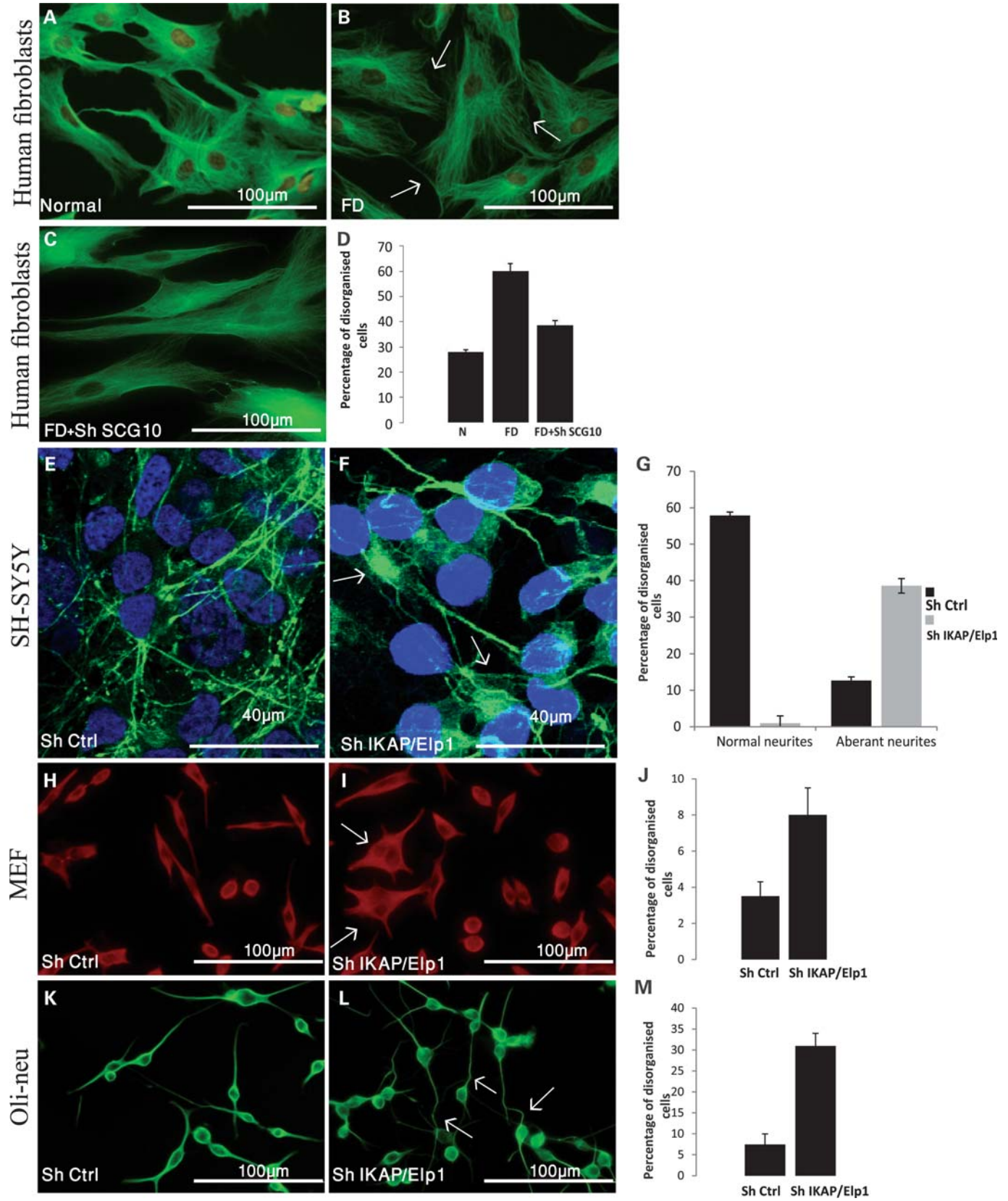


Figure 1. Morphological changes in IKAP/Elp1-deficient cells. (A) Normal human fibroblasts and (B) IKAP/Elp1-deficient fibroblasts obtained from FD patients were stained with anti-tyrosinated α -tubulin (green) and visualized by fluorescent microscopy at $\times 40$ magnification (see Materials and Methods). (C) FD fibroblasts rescued by Sh RNA-mediated *SCG10* downregulation. (D) Effect of IKAP/Elp1 deficiency on cytoskeleton organization. Cytoskeleton organization in

FD fibroblast cells where a subgroup of genes associated with cell migration and actin cytoskeleton regulation was shown to be downregulated in a microarray assay (9). It was suggested that the Elongator complex plays a role in transcription of these genes presumably by its component Elp3 that displays histone acetylation activity (9–11). In accordance, cell migration defects were observed in IKAP/Elp1-depleted HeLa, SK-N-BE neuroblastoma, glioblastoma-derived U373 and FD fibroblast cells (9). However, in recent studies, down-regulation of these genes was not observed in the cerebrum of FD patients (12) and in other cells (13,14), leaving open the question of the observed effect on migration. It was also suggested that the IKAP/Elp1 protein is involved in cell adhesion and migration through cytosolic interactions. This was based on experiments in which the depletion of IKAP/Elp1 resulted in decreased staining of filamin A in membrane ruffles and in disorganized actin cytoskeleton (13). IKAP/Elp1 was also demonstrated to be colocalized with keratin but not with microtubules (MTs), actin and vimentin in the cytoplasm of HeLa cells (15).

It has recently been shown that MTs, the major dynamic structural component of the cytoskeleton, could be the target of IKAP/Elp1. Contrary to Gardiner *et al.*'s (15) observation in HeLa cells, Creppe *et al.* (14) demonstrated that IKAP/Elp1 and Elp3, in fact, do interact with MTs in cortical neurons. Moreover, Creppe *et al.* (14) provided evidence suggesting that the Elongator regulates radial migration and branching of cortical projection neurons through acetylation of the MT monomer α -tubulin. While α -tubulin deacetylases are well characterized (16–18), the enzyme that acetylates α -tubulin was proposed to be Elp3 (14). Elp3 was shown to acetylate MT *in vitro* and IKAP/Elp1 and/or Elp3-deficient HT29 cells, and cortical neurons showed a significant decrease in acetylation of α -tubulin. Similar results were observed in *C. elegans* where the Elongator was shown to be critical for MT acetylation (19). Furthermore, the amount of Elp3 was found to be at a significantly decreased level in cells where IKAP/Elp1 was at lowered levels either by RNAi downregulation in HT29 cells or in FD-derived fibroblasts (9,14).

MTs are composed of α/β -tubulin dimers as building blocks and involved in several vital processes occurring within the cell: development and maintenance of cell shape, cell reproduction, cell signaling, intracellular transport, neurite extension and cell migration (20). MTs are particularly abundant in neurons (21) where they accomplish neurite outgrowth by reorganization. MTs show a highly dynamic instability, in which they are either growing (rescue) or shrinking (catastrophe) (22). The polymerization of MTs is modulated by MT-associated proteins on the

one hand and destabilizing proteins (stathmins) on the other hand. Among the stathmin proteins, superior cervical ganglia neural-specific membrane-associated protein [superior cervical ganglion-10 protein (SCG10) also known as STMN2] (23) attracted our attention since we found it to be upregulated in IKAP/Elp1-deficient cells. SCG10 is a neural-specific phosphoprotein that plays a critical role in neuronal development through its MT destabilizing activity. SCG10 is found in the Golgi area, along neurites, and enriched in growth cones (23) and plays a role in neurite outgrowth (24).

SCG10 is known to increase the rate of MT catastrophe, either through tubulin sequestration by forming a ternary complex or by direct stimulation at the plus end of MTs (reviewed in 24,25). Overexpression of *SCG10* in COS-7 cells, for example, had been shown to cause MT destabilization and disruption of MT networks (26). The activity of SCG10 is known to be affected by phosphorylation (27). The MT destabilization function of SCG10 is inhibited upon its phosphorylation (28). Interestingly, SCG10 was demonstrated to be phosphorylated by cJun N-terminal kinase (JNK1), which was shown earlier to be activated by IKAP/Elp1 (5). In addition, phosphorylation of SCG10 at serine residues in positions S62 and S73 is reduced in the brain of mice lacking JNK1. It was shown that the expression of SCG10 that could not be phosphorylated at these two positions by JNK1 significantly inhibited the MTs' turnover (27). It is noteworthy that the expression of SCG10 is restricted to neural cells by the RE-1-silencing transcription factor (REST) protein that is known to inhibit neuronal genes in non-neuronal cells (29).

In our current work, we demonstrate the function of IKAP/Elp1 in MT organization mediated by SCG10. We show that IKAP/Elp1 deficiency upregulates the MT destabilizing protein SCG10 and, in parallel, disorganizes the cytoskeleton in SH-SY5Y human neuroblastoma and FD fibroblast cells. The upregulation of *SCG10* was also observed in the IKAP/Elp1-deficient cerebrum. The *SCG10* elevation in cerebrum and in neuroblastoma IKAP/Elp1-deficient cells appears to be associated with downregulation of *REST* that binds to the *SCG10* promoter and acts as a specific repressor (29). In fibroblasts of FD patients and in IKAP/Elp1-deficient mouse embryonic fibroblast (MEF) cells, the *SCG10* gene is activated as well, but in a *REST*-independent mechanism.

These data provide the first evidence that IKAP/Elp1 is involved in regulating the expression of the MT destabilizing protein SCG10, lending support to the concept that *SCG10* elevation observed in IKAP/Elp1-deficient cells can alter the MT organization and dynamics which could play a pivotal role in FD.

normal (N), IKAP/Elp1 deficient (FD) and rescued FD fibroblasts (FD+Sh SCG10). Results represent percentage of cells with disorganized cytoskeleton out of total counted cells. (E) SH-SY5Y human neuroblastoma cells transfected with Sh empty vector (Sh Ctrl) and (F) transfected with Sh IKAP/Elp1 plasmid (see Materials and Methods) were stained with anti-tyrosinated α -tubulin (green) and visualized by confocal microscopy. (G) Effect of IKAP/Elp1 deficiency on neurites production. Result represent the percent of cells with abnormal neurites out of total counted cells. (H) MEF cells transfected with Sh empty vector (Sh Ctrl), and (I) MEF cells transfected with Sh IKAP/Elp1 plasmid were stained with anti-tyrosinated α -tubulin (red) and visualized by fluorescent microscopy at $\times 40$ magnification. (J) Effect of IKAP/Elp1 deficiency on production of processes in MEF cells. Results represent the percentage of multipolar cells out of total counted cells. (K) Oli-neu cells transfected with empty vector (Sh Ctrl). (L) Oli-neu cells transfected with Sh IKAP/Elp1 stained with anti-tyrosinated α -tubulin (green) and visualized by fluorescent microscopy at $\times 40$ magnification. (M) Effect of IKAP/Elp1 deficiency on production of processes in Oli-neu cells. Results represent the percent of multipolar cells out of total counted cells.

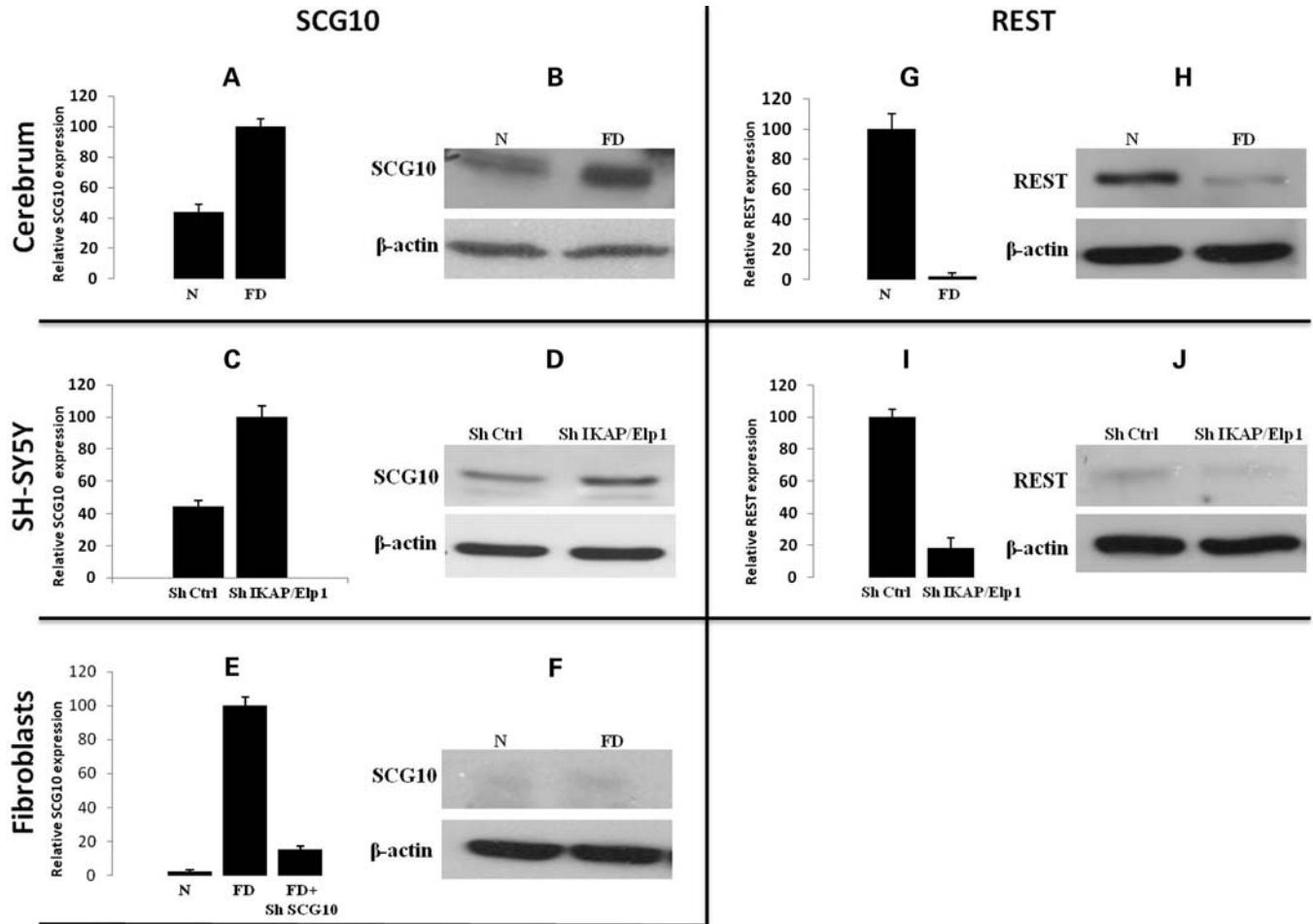


Figure 2. *SCG10* upregulation in IKAP/Elp1-deficient cells. (A) Expression of *SCG10* in the cerebrum of normal individual (N) and FD patient (FD) assayed by real-time PCR (see Materials and Methods). Results are presented as percent of relative expression, average of three experiments. (B) Western blot analysis of *SCG10* in the cerebrum of the normal (N) and of the FD patient (FD). β -Actin was used as control. (C) Expression of *SCG10* assayed by real-time PCR in the SH-SY5Y neuroblastoma cell line transfected with empty vector (Sh Ctrl) and with IKAP/Elp1 Sh RNA (Sh IKAP/Elp1). Values are average of two independent analyses (see Materials and Methods). (D) Western blot analysis of *SCG10* in Sh Ctrl control and IKAP/Elp1 downregulated SH-SY5Y cells (Sh IKAP/Elp1). β -Actin was used as control. (E) Expression of *SCG10* assayed by real-time PCR in human normal (N), FD fibroblasts (FD) and *SCG10* Sh RNA-treated FD fibroblasts (FD + Sh *SCG10*). (F) Western blot analysis of *SCG10* in normal (N) and (FD) fibroblasts β -actin used as control. (G) Expression of *REST* in cerebrum of FD patient (FD) and cerebrum of normal individual (N) assayed by real-time PCR and presented as percent of relative expression, average of three experiments. (H) Western blot analysis of *REST* in the cerebrum of the FD patient (FD) and normal individual (N). β -Actin used as control. (I) Expression of *REST* assayed by real-time PCR in Sh Ctrl cells and IKAP/Elp1 downregulated SH-SY5Y cells (Sh IKAP/Elp1). (J) Western blot analysis of *REST* in Sh Ctrl and IKAP/Elp1 downregulated SH-SY5Y cells (Sh IKAP/Elp1).

RESULTS

Recent reports suggested that IKAP/Elp1 is involved in cytoskeleton regulation. Depletion of IKAP/Elp1 resulted in decreased staining of filamin A in membrane ruffles and in disorganized actin cytoskeleton in MEF cells. IKAP/Elp1 was also suggested to be involved in cell adhesion and migration via cytosolic interactions (13). Another report suggested that the six-subunit RNA polymerase II Elongator complex controls the migration of cortical neurons via acetylation of α -tubulin by the Elp3 acetylase component of the Elongator (14).

We show here that IKAP/Elp1-deficient fibroblasts of FD patients stained with α -tubulin antibody revealed morphological aberrations reflected in MT disorganization when

compared with normal fibroblasts (compare Fig. 1A and B, see arrows). These results were validated by quantitative analysis that revealed a significant increase (>2-fold) in the number of cells with disorganized cytoskeleton in FD fibroblasts when compared with normal fibroblasts (N and FD in Fig. 1D). Cytoskeleton disorganization was also observed in IKAP/Elp1 downregulated neuroblastoma cells (Fig. 1F). In the control cells (Fig. 1E), tubulin is restricted mainly to long-extended neurites, while in α -tubulin-stained IKAP/Elp1 downregulated cells the tubulin network is concentrated mostly in one pole of the cells (Fig. 1F, see upper arrow), providing them with a comet-like structure and occasionally stretches to some of the neurites (Fig. 1F, see lower arrow). In addition, inter-tissue between the cells of the IKAP/Elp1 downregulated cells is much wider,

causing enhanced cell-to-cell adhesion (30). This is in contrast to what is seen in the control cells. In conclusion, the IKAP/Elp1 deficiency in SH-SY5Y cells caused a significant elevation of the number of cells with aberrant neurites in comparison with normal neuroblastoma cells (Fig. 1G).

IKAP/Elp1 deficiency causes disorganization of cytoskeleton in IKAP/Elp1-deficient MEF cells, as well (compare Figs. 1H and I). This is manifested in more condensed tubulin (Fig. 1I, see arrows) and in abnormal production of processes (Fig. 1J).

IKAP/Elp1-deficient Oli-neu cells (a mouse oligodendrocyte precursor cell line) showed abnormal staining with α -tubulin antibody. The IKAP/Elp1-depleted Oli-neu cells displayed abnormal rounded shapes and aberrant processes (Fig. 1L, see arrows). This is in contrast to what is observed in Oli-neu controls (Fig. 1K). In addition, a significant increase in the number of processes was observed (Fig. 1M) similar to what we observed in MEFs (Fig. 1J).

In conclusion, the results presented in Figure 1 show a clear correlation between IKAP/Elp1 deficiency and aberrant cytoskeleton organization which is in accord with a previous report (13).

IKAP/Elp1 deficiency enhances the expression of the MT destabilizing gene *SCG10*

We have previously performed a microarray expression analysis using RNA extracted from cerebrum of an 11-year-old FD male patient and a 47-year-old female FD patient and RNA extracted from the cerebrum of sex- and age-matched unaffected individuals and identified genes that changed their expression level (12). One of the upregulated genes in the above microarray analysis that attracted our attention was *SCG10*, also known as *STMN2*. *SCG10* is a neuronal growth-associated protein and also known to be a MT destabilizing protein. More specifically, *SCG10* is known to increase the rate of MT 'catastrophe', which represents the conversion from a polymerization (growth) to a depolymerization (shrinkage) state of MTs (reviewed in 23,31). Expression of *SCG10* is known to be restricted to neurons (32,33). The upregulation of *SCG10* was verified in the IKAP/Elp1-deficient FD cerebrum on the mRNA level by real-time polymerase chain reaction (PCR) (Fig. 2A) and on the protein level by western blot analysis (Fig. 2B). The upregulation of *SCG10* was also observed in IKAP/Elp1 Sh RNA-depleted SH-SY5Y human neuroblastoma cells on the mRNA level (Fig. 2C) and protein level (Fig. 2D). Interestingly, IKAP/Elp1 deficiency in FD fibroblasts was accompanied by activation of the *SCG10* gene which is practically inactive in the normal fibroblast cells (Fig. 2E). We are intrigued by the fact that the protein is practically not observed even in the IKAP/Elp1-deficient fibroblasts where *SCG10* mRNA is high (Fig. 2F). It is possible that the relative low level of *SCG10* mRNA, which was at least 10-fold lower in fibroblasts than the cerebrum, was too low to produce enough protein to be detected by western blotting.

Rescue of FD fibroblast phenotype by RNAi-mediated *SCG10* downregulation

To further support the contribution of *SCG10* upregulation (Fig. 2E) in the above-presented cytoskeletal disorganization (Fig. 1B), we performed *SCG10* Sh RNA downregulation of *SCG10* in FD fibroblast cells (FD+Sh *SCG10* in Fig. 2E). Indeed, this treatment caused the significant downregulation to approximately the basal *SCG10* level observed in normal fibroblasts (see N in Fig. 2E). The *SCG10* downregulation resulted in a rescue of the observed defect in cytoskeleton organization (Fig. 1C and D). These results indicate that *SCG10* upregulation is, in fact, involved in MT disorganization in IKAP/Elp1-deficient FD fibroblasts and possibly in other IKAP/Elp1-deficient cells.

Testing a possible mechanism for *SCG10* upregulation in IKAP/Elp1-deficient cells

The zinc finger protein *REST* is a transcriptional repressor that is known to repress neuronal genes in non-neuronal tissues. One of the *REST* target genes is *SCG10* (29). Since the upregulation of the *SCG10* gene in the IKAP/Elp1-deficient cerebrum appears to be on the transcriptional level (Fig. 2A) and reflected on the protein level (Fig. 2B), we asked whether a decrease in *REST* may be the reason for the upregulation of *SCG10* in IKAP/Elp1-deficient cells. To address this question, we performed real-time PCR and western blot analysis of *REST* expression in the IKAP/Elp1-deficient cerebrum. Indeed, *REST* was found to be significantly downregulated on both the RNA (Fig. 2G) and protein levels (Fig. 2H) in the cerebrum of FD patient. This result is consistent with the upregulation of *SCG10* observed in the IKAP/Elp1 cerebrum suggesting that the *SCG10* gene may be regulated by *REST* and that *SCG10* may be upregulated in IKAP/Elp1-deficient cells as a result of *REST* downregulation. Similar results were obtained with IKAP/Elp1 downregulated neuroblastoma cells. *SCG10* was upregulated on the RNA (Fig. 2C) and protein levels (Fig. 2D), and we were able to demonstrate a significant downregulation of the *REST* gene on the RNA level (Fig. 2I) and on the protein level (Fig. 2J). In human FD fibroblasts, although the *SCG10* gene was activated (Fig. 2E), no significant changes were observed in the *REST* level between normal and FD fibroblasts (data not shown), suggesting that the activation of *SCG10* in this case is independent of the *REST* level.

IKAP/Elp1 and Elp3 are not involved in acetylation of α -tubulin

It was recently reported (14) that in IKAP/Elp1-deficient HT29 and mouse cortical neuronal cells, α -tubulin acetylation is diminished. The decrease in acetylation was in accord with a decrease in the expression of *Elp3*, the acetyltransferase subunit of the Elongator complex. It should be mentioned that depolarization of microtubuli is associated with deacetylation of α/β -tubulin dimers (reviewed in 34). Since the deacetylation of α -tubulin could have been the reason for the disorganization of MTs that we observed in IKAP/Elp1-deficient FD fibroblast cells, SH-SY5Y human neuroblastoma,

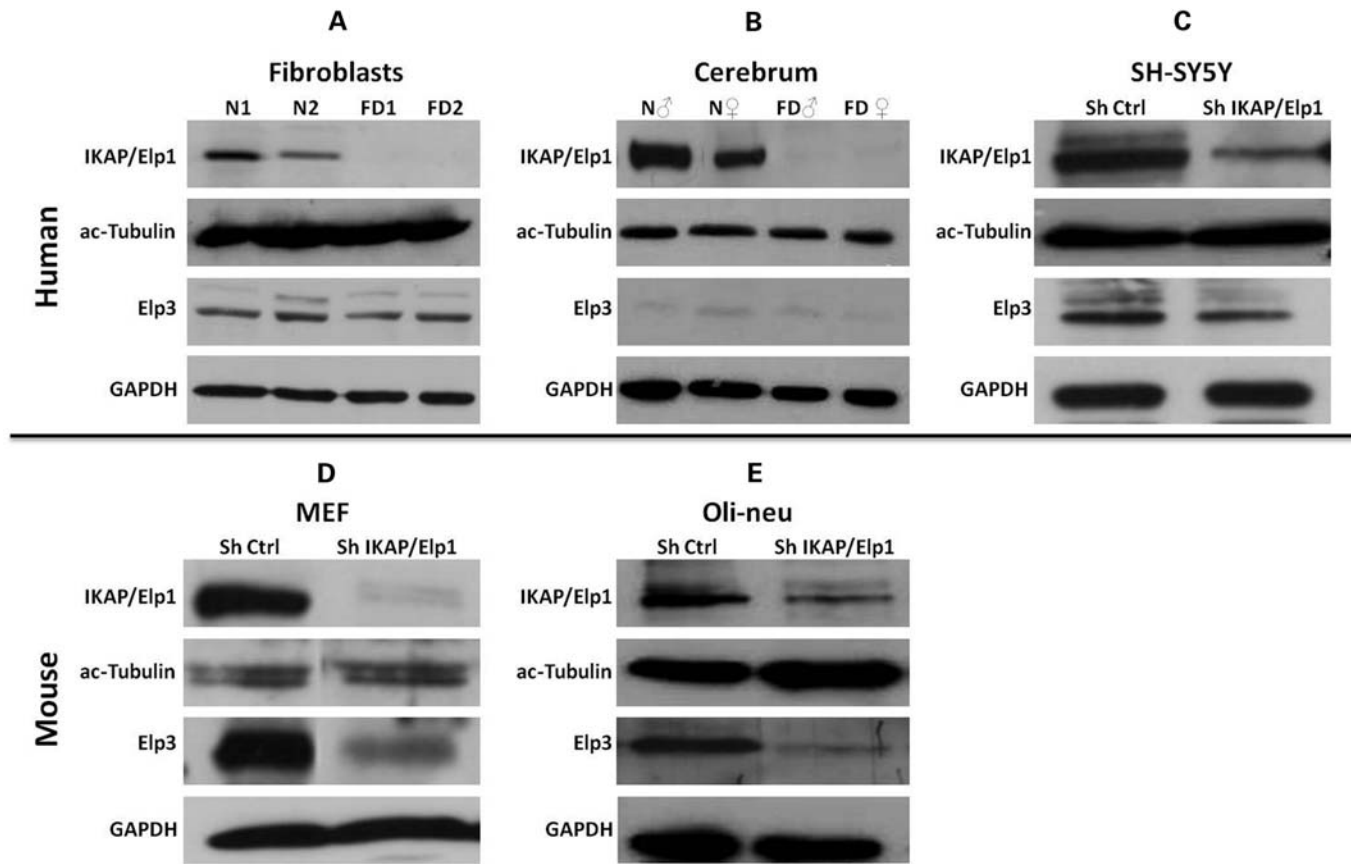


Figure 3. Effect of IKAP/Elp1 deficiency on the level of Elp3 and α -tubulin acetylation. Western blot analysis of IKAP/Elp1, Elp3 and acetylated tubulin (ac-tubulin) was performed in (A). Fibroblasts of normal individuals (N1, N2) and FD patients (FD1, FD2) in (B). Cerebrum of normal male (N♂), normal female (N♀), FD male (FD♂) and FD female (FD♀) in (C). SH-SY5Y neuroblastoma control (Sh Ctrl) and IKAP/Elp1-deficient (Sh IKAP/Elp1) cells in (D). MEF control-Sh Ctrl and IKAP/Elp1-deficient (Sh IKAP/Elp1) cells in (E). Mouse oligodendrocyte progenitor (Oli-neu) control (Sh Ctrl) and IKAP/Elp1-deficient (Sh IKAP/Elp1) cells; GAPDH served as control.

MEF and Oli-neu cells, stained with α -tubulin antibody (Fig. 1), we performed western blot analyses of acetylated α -tubulin in the IKAP/Elp1-deficient cells (Fig. 3). Surprisingly, in contrast to the reported findings in HT29 and in mouse cortical neuronal cells (14), we failed to detect any reduction in α -tubulin acetylation in FD fibroblasts (Fig. 3A), FD human cerebrum (Fig. 3B), human neuroblastoma SH-SY5Y cells (Fig. 3C), MEF cells (Fig. 3D) and mouse Oli-neu cells (Fig. 3E). In spite of a substantial decrease in the acetyltransferase (Elp3) level, in MEF and Oli-neu cells, no deacetylation of α -tubulin was observed (Fig. 3D and E). These results demonstrate that the Elp3 level does not affect the level of α -tubulin acetylation. It should be noted that even in HT29 cells that were used by Creppe *et al.* (14), no decrease in acetylated tubulin was detected (data not shown).

IKAP/Elp1 is involved in adhesion capacity of Oli-neu cells and FD fibroblasts

As mentioned above, IKAP/Elp1-depleted Oli-neu cells showed a disorganized cytoskeleton and abnormal rounded shape (Fig. 1L). Such disorganization can affect the adhesion capacity of the cells. To test this possibility, we carried out a cell

adhesion-to matrix assay. This assay revealed that in IKAP/Elp1-deficient Oli-neu cells, the adhesion capacity to fibronectin is reduced by 50% (Fig. 4A). The observed reduced adhesion capacity of Oli-neu cells can affect the recognition process of neurons by glial cells, which represents one of the main steps during myelination. Next, we asked whether the capacity of adhesion is also affected in other IKAP/Elp1-deficient cells. For this purpose, we tested the adhesion of FD fibroblasts. A significant decrease ($\sim 50\%$) in adhesion capacity of these cells had been observed as well (Fig. 4B).

These results are in accord with previous publications, where IKAP/Elp1 was shown to be involved in adhesion of MEF cells (13) and where IKAP/Elp1-deficient SH-SY5Y cells showed reduced adhesion to lamina (30). We suggest that the deterioration of the cytoskeleton shown above (Fig. 1) and of actin cytoskeleton that was shown by Johansen *et al.* (13) can explain the effect of IKAP/Elp1 deficiency on cell adhesion to matrix.

DISCUSSION

Although IKAP/Elp1 was found to be a multifunctional protein, the functions that are carried out by IKAP/Elp1 in the cell are not completely understood. At first, localization

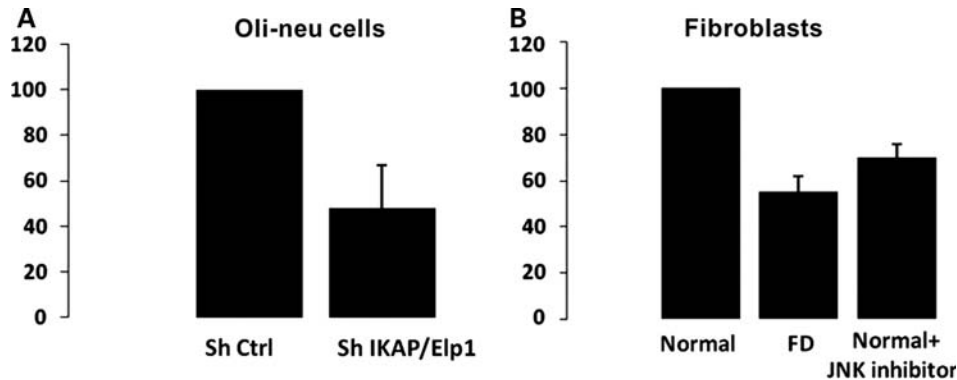


Figure 4. Effect of IKAP/Elp1 downregulation on adhesion of cells to matrix. (A) Adhesion capacity of Oli-neu cells transfected with Sh IKAP/Elp1 vector or empty vector (Sh Ctrl). (B) Adhesion to matrix of normal human fibroblasts (Normal), FD fibroblasts (FD) and normal fibroblasts treated with JNK inhibitor (SP600125). The cells in suspension were dispensed in triplicate wells of plates coated with BSA (control) and fibronectin, after which the adhesion assay (see Materials and Methods) was performed. Values are means of triplicates from one representative experiment of three performed and presented as percent of the control in Oli-neu cells (Sh Ctrl 100%) and percent of normal fibroblasts (Normal 100%).

of IKAP/Elp1 within the cell was expected to shed light on its possible roles, but this caused some confusion. Studies in the past suggested that IKAP/Elp1 is present predominantly in the nucleus (35). Later, it was suggested that IKAP/Elp1 is present primarily in the cytoplasm (5). Since IKAP/Elp1 had been suggested to be a component of RNA polymerase II Elongator complex (36), the possibility that the protein shuttles between the nucleus and the cytoplasm (35) was proposed. However, such a shuttle had not been shown. This and the fact that IKAP/Elp1 is expressed in various tissues and cell lines (5) lead to the conclusion that IKAP/Elp1 is an important basic cellular component. This is in accord with the observations described here that IKAP/Elp1-deficient cells display aberrations in MT organization.

The MTs, which are the major dynamic structural component of the cytoskeleton, are known to be involved in many cellular features (reviewed in 20,37). All these cellular features are expected to be affected in IKAP/Elp1-deficient cells and are therefore relevant to the FD phenotype. It was suggested that the regulation of MTs stability in the early stages of neurogenesis is important for the normal development of neurons (38). Therefore, the deterioration of MT organization can be one of the factors affecting tissues of FD especially in the neuronal system.

SCG10 is a neuron-specific MT destabilizing protein primarily localized in leading edges of neuron growth cones playing a role in neurite outgrowth (24,39). SCG10 is expressed during early development and its expression is diminished within 3 weeks postnatally.

We describe here experiments showing that IKAP/Elp1 deficiency increases the expression of the *SCG10* gene and as a result, elevate the level of the SCG10 protein (Fig. 5C). We show that the upregulation of SCG10 in the cerebrum of an FD patient and IKAP/Elp1-deficient neuroblastoma cells is accompanied by a significant downregulation of the *REST* gene (Fig. 5C). The SCG10 promoter harbors RE1 elements that bind specifically the SCG10 repressor, REST (29). It stands to reason, therefore, that the elevation of SCG10 in IKAP/Elp1-deficient cells may result from the observed

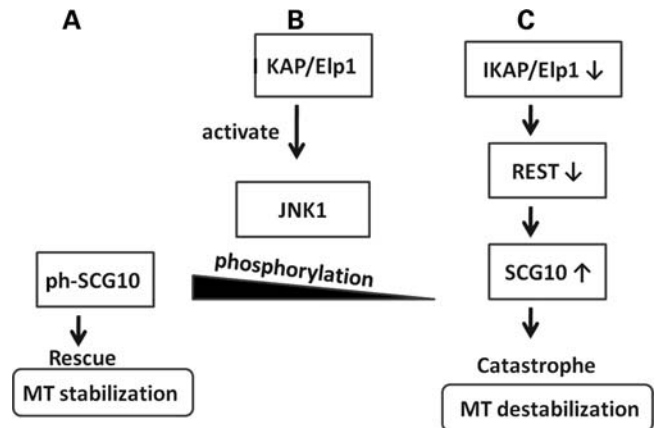


Figure 5. IKAP/Elp1 cellular level regulates the stability of the MTs. (A) Phosphorylated SCG10 has a stabilizing effect on microtubuli (Rescue). (B) IKAP/Elp1 C-terminus binds and activates JNK1 that phosphorylates SCG10. (C) Depletion of IKAP/Elp1 causes downregulation of the *REST* repressor of SCG10 and, as a result, the SCG10 level is increased that causes destabilization of the MTs (catastrophe).

REST depletion in these cells. It should be noted, however, that the expression of the *REST* gene was not downregulated in fibroblast cells derived from FD patients (data not shown), suggesting that another mechanism may be responsible for SCG10 activation in FD fibroblasts and that this SCG10 elevation may still affect stability of MT in these cells as has been shown in Figure 1B. In this regard, it should be mentioned that SCG10 overexpression in COS-7 cells (African Green Monkey Kidney Fibroblasts), which represent non-neuronal cells, showed disruption of the MTs, as well (40) and reviewed by Grenningloh *et al.* (23).

Despite the elevation of SCG10 in FD fibroblasts on the mRNA level, we failed to confirm SCG10 upregulation on the protein level in human FD-derived fibroblasts (Fig. 2F) and IKAP/Elp1 downregulated MEFs (data not shown). Yet, the fact that SCG10 is a potent element that destabilizes MTs (Fig. 5C) and that the stabilizing activity of MAP1B is not sufficient to completely counteract the destabilizing

effect of SCG10 (40) suggest that a relatively low level of SCG10 may be sufficient to destabilize MTs.

MT destabilizing activity displayed by SCG10 is regulated by phosphorylation (reviewed in 23) (Fig. 5B). Interestingly, the serine residues S62 and S73 that are phosphorylated by JNK1 (27) had previously been suggested to be potentially activated by IKAP/Elp1 (5). It has also been proposed that IKAP/Elp1 may be important as a physiological regulator of the stress-associated JNK signaling pathway (5). Interestingly, inhibition of JNK by the specific JNK inhibitor SP600125 (A.G. Scientific, Inc.) in normal fibroblast cells also caused significant adhesion impairment (Fig. 4B). These data indicate that JNK inhibition results in a decrease of adhesion similar to the effect of IKAP/Elp1 deficiency, suggesting that IKAP/Elp1 affects adhesion through JNK. It should be noted that the possible interaction between IKAP/Elp1, JNK and SCG10 had been proposed by Gardiner *et al.* (15).

One of the genes that was found to be downregulated in a microarray assay performed with cerebrum RNA of FD patients was the axonal guidance protein *Fez1* (12). *Fez 1* together with the JNK-interacting protein 1 bind to the Kinesin-1 motors and activate MT-based motility (reviewed in 41). Thus, IKAP/Elp1 deficiency may affect not only the MT stability via the MT destabilizing protein SCG10 and JNK, but also the MT-dependent cell motility by the downregulation of *Fez1*.

Since the level of SCG10 is elevated in IKAP/Elp1-deficient cells, it can shift the equilibrium of MTs stability toward catastrophe and MT disruption. This in turn can reduce brain-derived neurotrophic factor (BDNF) transport through MTs. BDNF is a neurotrophic factor which is required in neuronal developmental processes such as the development of taste papillae in the fetus. This is interesting since FD patients are known to lack taste papillae (42).

Intriguingly, SCG10 was recently demonstrated to be on the list of downregulated genes during differentiation of FD iPSC cells toward neural crest lineages (43). These results, which are contradictory to ours, may be attributed to the different experimental systems used by Lee *et al.* (43) and by us. In any event, it demonstrates the importance of this protein in the FD phenotype.

SCG10 is known to be upregulated during regeneration of injured postganglionic sympathetic nerve (44). Thus, the upregulation of SCG10 which we observed in IKAP/Elp1-deficient cells could reflect compensatory mechanisms that try to activate restoration of the nervous system which may be relevant to the FD etiology.

The reduced adhesion abilities that we observed in IKAP/Elp1-deficient cells (Fig. 4) may be a result of our observed MTs destabilization mediated by SCG10 upregulation. Obviously, this requires further investigation. Interestingly, the adhesion defect was shown to be rescued by reintroduction of the IKAP/Elp1 expression vector to IKAP/Elp1 downregulated MEF cells (13). The migration capacity of IKAP/Elp1-deficient cells was also reported to be reduced (9,13,14). We propose that both effects on adhesion and migration are a result of the cytoskeleton aberrations affected by IKAP/Elp1 deficiency.

SCG10 regulates the cytoskeleton by destabilizing the MT, through altering dendrites as well as cell shape, size and polarity. Alterations in cellular architecture could explain some of the morphological changes reported by us here and by others (13,14).

MATERIALS AND METHODS

Tissues and cells

Oli-neu cells (gift from J. Trotter, University of Mainz) (45) were grown on poly-L-lysine-coated culture dishes in Sato medium consisting of Dulbecco modified Eagle medium (DMEM) supplemented with 2 mg/ml NaHCO₃, 2 mM L-glutamine, 10 µg/ml apotransferine, 10 µg/ml insulin, 100 µM putrescine, 200 nM progesterone, 500 nM triiodo-L-thyronine, 220 nM sodium selenite, 520 nM L-thyroxine, 1% horse serum, gentamicin. Fibroblast cell lines (either cloned by us or obtained from Coriell Cell Repositories), 293T human embryo kidney cells and MEF cells were grown in DMEM in the presence of 10% fetal bovine serum, 5% glutamine and 1% penicillin–streptomycin.

SH-SY5Y human neuroblastoma cells were grown in medium containing DMEM with 5% glutamine and 10% heat inactivated FCS, 2 mM sodium pyruvate (Invitrogen) and antibiotics (50 U/ml of penicillin, streptomycin and nystatin).

All reagents were purchased from Biological Industries, Israel.

All cells were maintained at 37°C with 5% CO₂ in a humid atmosphere.

Brain tissues were obtained from the NICHD Brain and Tissue Bank for Developmental Disorders at the University of Maryland, Baltimore, MA, USA. 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).

RNA interference treatment

To suppress *IKBKAP* gene expression lentiviral (pLKO) vector was used. Five different double-stranded *IKBKAP* shRNA, accession number NM_026079 (for mouse cells) and NM_003640 (for human cells) were purchased from Sigma-Aldrich (Israel). Plasmids were transformed by FuGene6 (Roche) into 293T cells according to the manufacturer's protocol. Lentiviruses were assembled using the following three vectors: pLKO (transfer vector)—includes the insert (Sigma); PHR—lentivirus polymerase; VSVG—virus envelope. The day before transfection, 1.2×10^6 HEK293T cells were plated in a 10 cm dish (20–30% confluence). Next day, three vectors were transfected at the following ratio PHR:VSVG:LVTR1 = 9:1:10. Cells were incubated for 48 h followed by collection of the medium containing the virus filtered and used to infect the target cells. Selection with the 1 µg/ml puromycin (Sigma) was started after 48 h post-infection. SCG10 Sh RNA was kindly provided by Elena Aimbinder (Prof. Michel Revel's laboratory).

Adhesion assay

The adhesion assay was carried out as described earlier (46). Cells were detached with minimal trypsinization, washed and resuspended in serum-free DMEM medium supplemented with 1 mg/ml bovine serum albumin (BSA). Cell suspension (100 µl containing 1×10^5 cells) was added to the wells of

96-well titer plates precoated with fibronectin and BSA and was allowed to adhere for 1 h at 37°C. Non-adherent cells were rinsed off with PBS containing 2 mM CaCl₂, 1 mM MgCl₂ (warmed to 37°C), and adherent cells were fixed and stained in 2% toluidine blue/4% paraformaldehyde (PFA) (30 min at 37°C). Wells were then rinsed in tap water. Stained cells were solubilized in 100 ml 1% sodium dodecyl sulfate (SDS), and absorbance of the solution was measured at 595 nm in a microplate reader. Experiments were performed in triplicates and repeated at least three times.

Antibodies for western blotting and immunostaining

Antibodies for western blotting and immunostaining were purchased from the following companies:

Anti IKAP Rabbit polyclonal IgG (Anaspec) Cat no. 54494;
 Anti glyceraldehyde-3-phosphate dehydrogenase (GAPDH) monoclonal peroxidase (Sigma) Cat no. G9295;
 Anti β -actin monoclonal (Sigma) Cat no. A5411;
 Anti α -tubulin clone YL1/2 (Tyr-tubulin) Rat monoclonal (Serotec) Cat no. MCA77G;
 Anti acetylated Mouse monoclonal (Clone [6-11B-1] α -tubulin (Santa Cruz) Cat no. sc-23950;
 Anti REST Rabbit polyclonal (Milipore) Cat no. 07-579;
 Anti SCG10 and anti Elp3 were kindly obtained from Dr Andre Sobel and Dr Jesper Svejstrup, respectively.

All secondary antibodies including Donkey anti Rat Cy2 conjugated and Donkey anti-Rabbit Cy3 conjugated were purchased at Jackson IR.

Western immunoblotting

Cells were lysed in radioimmunoprecipitation assay buffer (150 mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate, 50 mM Tris-HCl pH 7.5, 1% NP-40, 1 mM phenylmethylsulfonyl fluoride). The protein concentration of the soluble fraction was measured by the Bradford assay. Total cell lysates were resolved by 8–12% SDS-polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane. After staining the membranes with reversible Ponceau red solution to confirm equal protein loading, the membranes were blocked with 5% Skim Milk in PBS (0.1% Tween-20) for 1 h at room temperature. The membranes were subsequently incubated in PBS with 0.1% Tween-20 at room temperature for 1 h with the relevant primary 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).

Real-time PCR

Total RNA was prepared from cells in culture using the Tri-reagent (Sigma) according to the manufacturer's protocol. Approximately 1 mg total RNA of each sample was reverse transcribed using the M-MLV Reverse Transcriptase (Promega) and random primers in a 20 μ l 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 μ l of cDNA, primers (sequences

available upon request) at a concentration of 0.3 μ M. The SYBR Green (Applied Biosystems) was added to the 20 μ l 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. To control for the amount of RNA, amplification of TATA box binding protein in mouse and 18S in human were performed and corrected for each cDNA sample.

PCR analysis of *IKBKAP* expression

PCR analysis was carried out as described earlier (12).

Immunostaining

For staining, the cells were fixed in 4% PFA at room temperature for 10 min, washed once in PBS. After permeabilization in 1% Triton X-100 and 1% BSA in PBS for 5 min at room temperature, sections were washed twice for 15 min in PBS and blocked in 1% BSA in PBS for 20 min at room temperature and incubated with primary antibody in blocking solution at 4°C overnight. The sections were then each washed twice with PBS for 5 min and incubated with appropriate secondary antibody for 45 min in blocking solution, washed twice with PBS for 5 min and mounted in Galvenol.

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Conflict of Interest statement. None declared.

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