Molecular and cellular insights into IKAP and Elongator functions

Faculty of Medicine

Department of Pharmacy
Laboratory of Medical Chemistry
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Thesis submitted by
Pierre Close
for the degree of
“Docteur en Sciences Pharmaceutiques”

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Abstract

As the first step in the complex process of gene expression, the transcription of genes from DNA to RNA by RNA polymerase II is subject to a multiplicity of controls and is thereby the endpoint of multiple cell regulatory pathways. We focused here on the molecular and cellular functions of IKAP and by extension of Elongator complex, initially found associated with the hyperphosphorylated RNA polymerase II during the elongation stage of transcription. IKAP is required for the assembly of Elongator subunits into a functional complex. Elongator has a histone acetyltransferase (HAT) activity associated with one of its subunits, named hELP3. In agreement with a potential role in transcript elongation, Elongator is associated with nascent RNA emanating from the elongating RNA polymerase II along the transcribed region of several yeast genes and chromatin immunoprecipitation experiments have also demonstrated an association of Elongator with genes in human cells. Different mutations in the human *IKBKAP* gene, encoding IKAP/hELP1, cause familial dysautonomia, a severe neurodevelopmental disease with complex clinical characteristics. Affected individuals are born with the disease and abnormally low numbers of neurons in peripheral nervous ganglions.

To gain insight into the role played by IKAP and the Elongator complex in the transcription of genes and concomitantly learn about the molecular defects underlying the FD, an RNA interference approach was used to deplete the IKAP protein in human cells. In yeast, disruption of ELP1 (yeast homolog of human IKAP) is known to destabilize the ELP3 catalytic subunit, which leads to loss of Elongator integrity. Our experiments performed in human cells revealed that the levels of hELP3 protein is also affected by IKAP depletion after RNAi.

We took advantage of this cellular loss-of-function model to identify genes whose transcription requires IKAP, by microarray experiments. Among the identified candidates, several were previously described to be involved in cell motility, or actin cytoskeleton remodelling. Because cell motility is of crucial importance for the developing nervous system, and therefore of obvious relevance to FD, the potential role of IKAP in cell motility was characterized at the cellular level. Several cell motility/migration assays demonstrated that the IKAP depletion has functional consequences so that IKAP-depleted cells showed defects in migration. Particularly, the reduced cell motility of neuronal-derived cell lines may be highly relevant to the neurodevelopmental disorder that affects FD patients.

Whether or not the defects in cell migration resulted of impaired transcriptional elongation of the IKAP-dependent genes was investigated by chromatin immuno-precipitation technique. These experiments indicated that IKAP depletion leads to a decreased histone H3 acetylation in the transcribed region of its target genes in the context of Elongator complex. These acetylation defects are correlated with a decrease of the RNA polymerase II recruitment through the transcribed region of target genes, whereas the recruitment on the promoter is mostly unaffected. These results indicate that Elongator affects transcript elongation *in vivo*, but not the recruitment of the RNA polymerase II to the promoter. These very specific effects of IKAP/hELP1 depletion on histone acetylation and RNA polymerase II density across target genes are consistent with a direct effect of Elongator on transcriptional elongation *in vivo* and point to a function for Elongator in histone acetylation during transcript elongation.

Université de Liège – Faculté de Médecine
Département de Pharmacie
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Caractérisation des rôles biologiques de la protéine IKAP et du complexe Elongator

Résumé

La transcription des gènes de l'ADN en ARN est fondamentale pour l'expression des protéines et la capacité de nos cellules à s'adapter à leur environnement. Ce processus finement régulé est catalysé par un enzyme, l'ARN polymérase II, vers lequel convergent une multitude de voies de signalisation. Dans le cadre de ce travail, nous nous sommes intéressés aux fonctions moléculaires et cellulaires de la protéine IKAP et du complexe Elongator. IKAP est la protéine qui assemble les sous unités d'Elongator en un complexe fonctionnel. Le complexe Elongator est associé à l'ARN polymérase II hyper-phosphorylée pendant l'étape d'élongation de la transcription et possède une activité histone acétyltransferase associée à une de ses sous unités, appelée ELP3. Chez la levure, Elongator est recruté an niveau des ARNs naissants, qui émanent directement de l'ARN polymérase II au niveau de la région transcrite des gènes étudiés. De plus, des expériences d'immunoprécipitation de la chromatine ont mis en évidence la présence du complexe Elongator au niveau de plusieurs gènes humains. Différentes mutations au niveau du gène *IKBKAP*, codant pour la protéine IKAP, sont responsables de la dysautonomie familiale, une maladie génétique qui affecte le développement du système nerveux périphérique. En effet, les individus affectés présentent une diminution de la densité de neurones au niveau des ganglions nerveux périphériques.

L'objectif de nos travaux est de comprendre davantage le rôle de la protéine IKAP et du complexe Elongator dans la transcription des gènes et ainsi, d'investiguer les mécanismes moléculaires responsables dans la physiopathologie de la dysautonomie familiale. Un modèle de perte de fonction pour la protéine IKAP a d'abord été généré par interférence d'ARN. Des travaux réalisés chez la levure indiquent que la protéine ELP1 (homologue de IKAP chez la levure) est essentielle pour la stabilité de la sous unité catalytique du complexe, la protéine ELP3. Les expériences réalisées sur notre modèle humain démontrent que le taux de la protéine ELP3 est également affecté par la déplétion d'IKAP causée par l'interférence d'ARN.

Ce modèle de perte de fonction a été utilisé afin d'établir la liste des gènes dont l'expression est contrôlée par la protéine IKAP, par des expériences de microarrays. Parmi les candidats identifiés, plusieurs ont été décrits comme impliqués dans la migration cellulaire et le remodelage du cytosquelette d'actine. Le processus de migration des cellules est fondamental au cours du développement du système nerveux et par conséquent particulièrement relevant dans le contexte de la dysautonomie familiale. L'implication d'IKAP dans la migration cellulaire a été investigué par différents tests de fonction qui montrent que la diminution d'IKAP dans différentes lignées cellulaires entraîne une réduction significative de leur capacité migratoire. Ces résultats suggèrent que la diminution du nombre de neurones observée dans les ganglions périphériques des patients atteints de la dysautonomie familiale pourrait résulter d'une altération de leur capacité à migrer au cours du développement.

Enfin, des expériences d'immunoprécipitation de la chromatine ont été menées en utilisant notre modèle afin de déterminer dans quelle mesure le déficit de migration observé en l'absence d'IKAP serait la conséquence d'un défaut de la fonction d'Elongator au niveau de l'élongation de la transcription des gènes. Les résultats nous ont montré que la diminution d'expression d'IKAP entraîne une réduction de l'acétylation des histones H3 dans la région transcrite de ses gènes cibles. De plus, ce déficit d'acétylation est directement corrélé avec un désengagement progressif de l'ARN polymérase II le long de la région transcrite de ces gènes. Par conséquent, ces résultats démontrent que le complexe Elongator affecte l'élongation des transcrits *in vivo*, mais pas le recrutement de l'ARN polymérase II au niveau du promoteur. Ces effets très spécifiques de l'absence d'IKAP sur l'acétylation des histones et l'engagement de la polymérase II dans la transcription des gènes cibles montrent qu'Elongator exerce un rôle direct au niveau de l'élongation de la transcription de ces gènes. De plus, ces résultats suggèrent que la fonction d'Elongator serait d'acétyler les histones au cours de l'élongation transcriptionnelle *in vivo*.

Au terme de la rédaction de ce mémoire, je voudrais remercier toutes les personnes qui ont contribués à l'aboutissement de ce travail.

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La réalisation de ce travail n'aurait pas été possible sans le soutien du comité F.N.R.S. Télévie qui m'a octroyé un mandat de recherche et à qui j'adresse toute ma reconnaissance.

Enfin, je voudrais remercier ma famille et mes proches pour leur soutien sans faille durant ces années.

Preface

The mechanisms that underlie the transcriptional control of gene expression are of fundamental importance to many areas of biomedical research, ranging from the understanding of basic issues to practical applications in industry and medicine.

The established link between defects in gene transcription and many human disorders has considerably stimulated activities in the biomedical area, particularly in the domain of cancer. This attention has uncovered numerous novel factors involved in gene transcription, but the mechanism(s) that effectively link(s) these proteins to transcription remains to be established in most cases.

In this work, we took advantage of a genetic disorder to further decipher the mechanism that involves a protein complex in gene transcription. Beside improving the basic information about the process of transcriptional elongation, our approach will also try to provide potential evidence that could explain the molecular mechanisms that may underlie the disease.

TABLE OF CONTENTS

Introduction

Part I: Gene transcription and its regulation

1. Transcription of genes

The genome is made of deoxyribonucleic acid (DNA) and, in mammalians, contains about 3.3 x10^9 base pairs which represents about two meters of nucleotides chains. The genome is divided into genes which are genetic functional units and commands the production of every proteins and RNA molecules of the organism. However, most of eukaryotic DNA remains silent and is never transcribed. For instance, the mammalian genome contains enough base pairs to form more than 300,000 genes but the total number of genes in these organisms is probably less than 30,000.

The need to compact two meters of DNA into the nucleus of a eukaryotic cell has severe consequences for processes that require access to DNA. This compaction is achieved by the binding of proteins that mediate successive orders of DNA folding: two copies each of histones H2A, H2B, H3 and H4 form a protein core that wraps 147 base pairs of DNA tightly on its surface to form a nucleosome (figure 1) (Kornberg and Lorch, 1999).

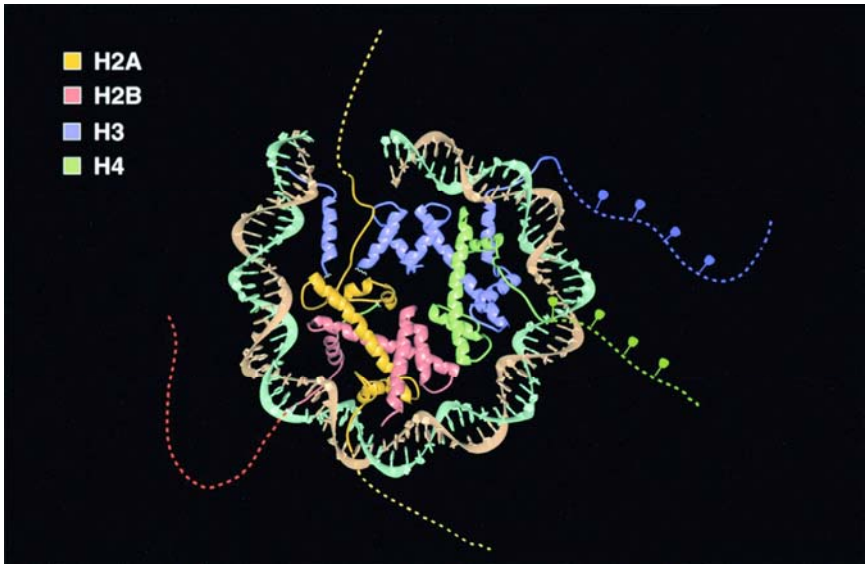

Figure 1. X-Ray structure of nucleosome core particle.

Half the core particle, with four histone core molecules and 73 DNA base pairs, is shown. The unstructured histone tails are drawn as dashed lines (Kornberg and Lorch, 1999).

With the aid of additional proteins, nucleosomes are compacted to form chromatin, a complex and highly ordered nucleoprotein assembly that is inaccessible to DNA-binding proteins (figure 2) (Hayes and Hansen, 2001; Luger and Hansen, 2005; Woodcock and Dimitrov, 2001). As a result, the signals that mediate rapid transcriptional responses have to overcome nucleosomal repression before they can influence the transcriptional machinery (Bannister et al., 2002). Decompaction of

chromatin to facilitate access to DNA has been most widely studied for RNA polymerase II (RNAPII)-mediated transcription of protein-coding genes, a process that requires rapid access to genes for the response to environmental signals and programmed cellular events. These underlying principles are equally applicable to any process requiring interaction with DNA (such as DNA replication, etc).

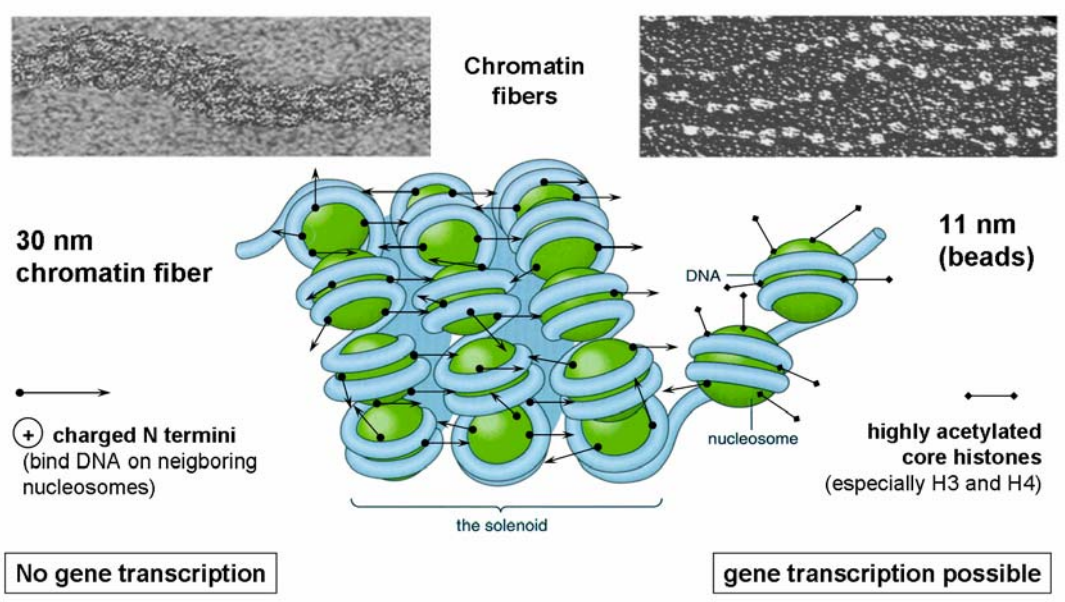

Figure 2. Structure of chromatin fiber.

Nucleosomes in an eukaryotic genome are spaced quite regularly (11 nm fiber) and further folding creates a spiral structure, a fiber of 30 nm in diameter, referred to as the chromatin higher order structure. The compaction of chromatin fibers is strongly correlated with gene activity. The two chromatin states are well-defined in electron micrographic images (J.H. Waterborg, UMKC).

The expression of protein-coding genes in eukaryotic organisms is regulated in a highly orchestrated and elaborated fashion to ensure that specific genes are turned on and off in a temporally and spatially appropriate manner according to genetic blueprints, cell cycle requirements, environmental factors, etc. Over the last two decades, intensive efforts have been directed to the unravelling of these regulatory pathways and the identification of the cellular components mediating regulation of mRNA synthesis in eukaryotes. Many of the polypeptides and protein complexes that make up the eukaryotic transcriptional machinery were initially isolated using biochemical purification strategies by tracking activities in reconstituted *in vitro* assays (Orphanides et al., 1996; Roeder, 1996). In eukaryotes, the entire length of the gene is transcribed into a large primary and nascent RNA, or pre-messenger RNA, which will be matured before becoming a messenger RNA and exported in the cytoplasm for translation into protein (figure 3).

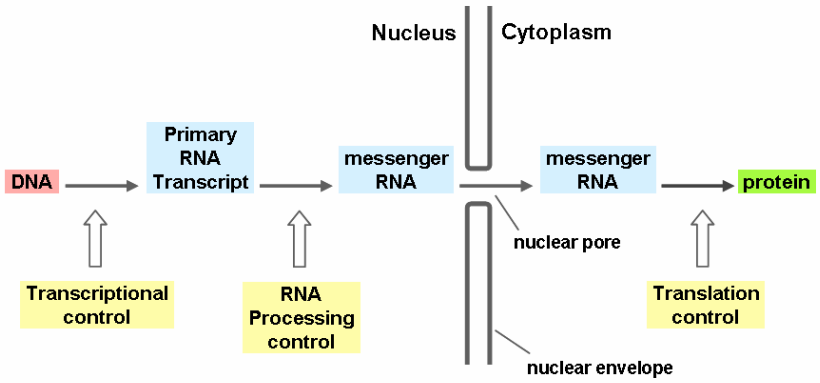

Figure 3. General process of gene expression from transcription to translation.

The transcription should be viewed as a progression of ordered events where transcription factor interactions and posttranslational modifications are required so that the cycle can progress correctly. The cycle of transcription can be divided into a number of different steps, including pre-initiation, initiation, promoter clearance, elongation and termination (figure 4). The machinery must contend with repressive chromatin structures in order to find its target DNA sequences (pre-initiation) or progress through the genes (promoter clearance and elongation) (Svejstrup, 2004).

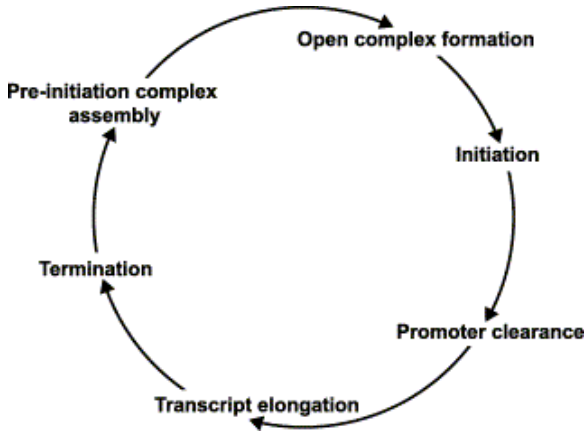

Figure 4. The transcription cycle (Svejstrup, 2004).

There are specific mechanisms at play for driving the transcription cycle in the context of chromatin during any point of the cycle. Regulation of transcription obviously occurs in the context of chromatin, adding yet another layer of processes that is prone to regulation. In order to cope with the fact that the DNA template is embedded in chromatin, RNAPII transcription entails recruitment of chromatin-remodelling complexes such as the ATP-dependent chromatin remodelling machines and the histone-modifying enzymes to facilitate the process (Orphanides and Reinberg, 2000; Workman and Kingston, 1998; Wu and Grunstein, 2000).

The transcription cycle starts with the pre-initiation complex (PIC) assembly at the promoter. The PIC includes the general transcription factors (GTFs) IID, IIB, IIA, IIE, IIF and IIH, and the RNA polymerase II (RNAPII), as well as several additional cofactors (Orphanides et al., 1996). Formation of an open complex between RNAPII and the DNA template is a prerequisite for transcription initiation. Melting of the double-stranded DNA into a single-stranded bubble is an ATP-dependent process which requires the action of two GTFs, i.e. IIE and IIH (Goodrich and Tjian, 1994; Holstege et al., 1996). As these events have proven to be a main target for the mechanisms and factors that regulate transcription, they have been thoroughly investigated (figure 5).

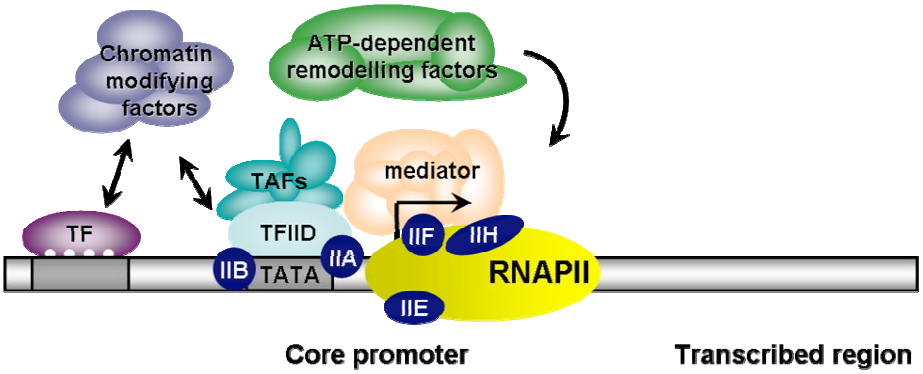

Figure 5. Preinitiation complex assembly.

The eukaryotic transcriptional apparatus can be subdivided into three broad classes of multi-subunit ensembles that include the RNA polymerase II core complex and associated general transcription factors (TFIIA, -B,-D,-E,-F and -H), multi-subunit cofactors (i.e. mediator complex) and various chromatin modifying or remodelling complexes (i.e. SAGA or SWI/SNF) (adapted from (Levine and Tjian, 2003)). TF, Transcription factor; TAFs, TATA box binding protein (TBP)-associated factors.

Following the chromatin remodelling of the promoter and the events leading to both the assembly of the pre-initiation complex and the open complex formation, RNAPII starts to initiate and synthesizes the first phosphodiester bond (initiation). Once the actual process of transcription has begun, a large number of chores await RNAPII and its co-factors.

First, the polymerase needs to escape the ties that bind it to the promoter and becomes engaged in processive mRNA production (promoter clearance). During this stage, the PIC is partially disassembled: a subset of GTFs remains at the promoter serving as scaffold for the formation of the next transcription initiation complex. An important but poorly understood aspect of the promoter clearance is that RNAPII now needs to start moving through chromatin, rather than merely be embedded in it at the promoter (Dvir, 2002; Eberharter and Becker, 2002; Thoma, 1991).

Subsequent efficient elongation thus requires that RNAPII does not pause or stall for prolonged periods of time because of unusual DNA structures or DNA bound proteins, such as nucleosomes, on the transcribed region (Sims et al., 2004; Svejstrup, 2002; Svejstrup, 2003). Entry of RNAPII into processive elongation is likely to be a stepwise or gradual process. For example, transcript elongation by RNAPII in early elongation complexes differs significantly from that seen by polymerases further away from the promoter, even on naked DNA template *in vitro* (Pal and Luse, 2003; Pal et al., 2001). Packaging of the DNA into chromatin introduces another complication. Factors such as Spt4/5, Spt6, FACT and Elongator play a key role (not yet completely defined) in dealing with chromatin during the transcript elongation (Svejstrup, 2002). Furthermore, the transcription machinery interacts with factors involved in downstream events, such as pre-messenger RNA processing and export (reviewed in (Maniatis and Reed, 2002; Proudfoot et al., 2002). Those events occur co-transcriptionally, as the nascent pre-messenger RNA is produced, introducing an additional level of complexity into the transcription process (for review see (Neugebauer, 2002). Recent studies suggest that the interactions between the transcriptional and processing machineries are reciprocal: not only does the transcription machinery facilitate the recruitment of processing factors to the pre-messenger RNA, but also some processing factors interact with the transcription machinery during elongation, regulating its activity (reviewed in (Jensen et al., 2003; Manley, 2002; Zorio and Bentley, 2004).

The final step in the cycle is transcript termination. At this stage, the messenger RNA is cleaved and the RNAPII is recycled for further rounds of transcription.

The phosphorylation state of the C-terminal repeat domain (CTD) of the largest subunit of RNAPII plays an important role for the progress of the transcription cycle. The CTD of the RNAPII consists of tandem repeats of a consensus heptapeptide sequence YSPTSPS (figure 6) (Prelich, 2002; Saunders et al., 2006).

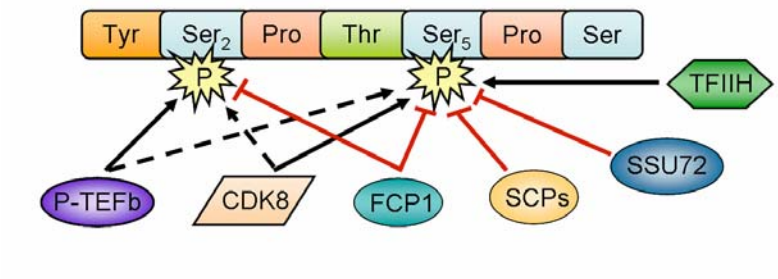

Figure 6. The C-terminal domain of RNA polymerase II.

The C-terminal domain (CTD) of the largest subunit of RNA polymerase II consists of tandem heptapeptide repeats. The CTD can be modified by phosphorylation at the serine 2 (Ser_2) and serine 5 (Ser_5). TFIIH and cyclin-dependent kinase 8 (CDK8) phosphorylate Ser_5. CDK8 also phosphorylates Ser_2. P-TEFb predominantly phophorylates the CTD at Ser_2. SSU72 is a Ser_5 phosphatase and FCP1 (TFIIF-associated CTD phosphatase-1) can dephosphorylate both Ser_2 and Ser_5. Finally, small CTD phosphatases SCPs preferentially dephosphorylates Ser_5 (Saunders et al., 2006).

Hypo-phosphorylated polymerase on the CTD is found on the promoter, whereas a hyper-phosphorylated form is responsible for elongation (Dahmus, 1996; Lin et al., 2002). Likely, this hyper-phosphorylated state of the CTD, acquired at the initiation-elongation transition needs to be maintained during the entire length of the run for efficient elongation, perhaps in order to keep the integrity of elongating RNAPII holoenzyme intact (Lee and Greenleaf, 1997). Interestingly, chromatin immunoprecipitation experiments have demonstrated that the pattern of CTD phosphorylation changes as RNAPII transcribes a given gene (Komarnitsky et al., 2000). They indicated that serine 5 phosphorylation is important at the promoter and decreases towards the 3'-end of the gene, while serine 2 phosphorylation increases towards the 3'-end of the gene (figure 7) (Cho et al., 2001).

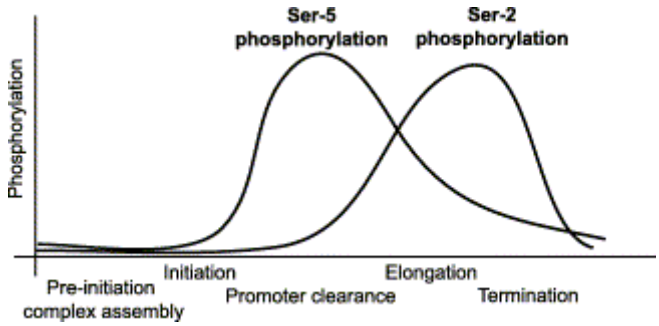

Figure 7. Phosphorylation of the CTD during the transcription cycle (Svejstrup, 2004).

Modification of the CTD markedly affects its conformation and ability to associate with factors that are involved in transcription elongation, RNA processing and termination (Saunders et al., 2006; Svejstrup et al., 1997). For example, serine 5 phosphorylation specifically facilitates the recruitment and activation of the mammalian capping enzymes (Ho and Shuman, 1999; McCracken et al., 1997). Consequently, the dynamic phosphorylation of the CTD and the preferential phosphorylation of serine 2 and serine 5 can be viewed as molecular switches that control the progression of RNAPII and the recruitment of factors involved in the synthesis and processing of the primary transcript. The extent and specificity of CTD phosphorylation are maintained by the opposing actions of CTD kinases and CTD phosphatase(s) (figure 6) (Cho et al., 2001; Palancade et al., 2001) (reviewed in (Kobor and Greenblatt, 2002; Saunders et al., 2006).

2. Chromatin structure and histone modifications

The eukaryotic genomic DNA in the nucleus is compacted more than 10,000-fold by highly basic proteins known as histones. The result is a highly structured entity termed chromatin whose fundamental unit is the nucleosome core particle, composed of histone octamer around which DNA is wrapped (Luger et al., 1997). Far from being a diverse set of molecules, the histones are among the most invariant proteins known. The reduction in DNA length produced by this histone-induced supercoiling is modest, but is an essential first step in the formation of higher-order chromatin structures. There are still much to learn about the molecular mechanisms required for the packaging of the genetic information and how the RNAPII machinery and its regulatory factors access DNA sequences and deal with this condensed structure to transcribe the genes. Several factors, including DNA methylation, histone modifications, and small nuclear RNAs, have been implicated in the regulation of transcription from chromatin (Bird, 2002; Kornberg and Lorch, 1999; Mattick and Makunin, 2005). Moreover, it has recently become clear that variants of the highly conserved core histones exist and have role in the regulation of transcription. Some variants have been associated with particular forms of chromatin, such as the highly compact forms found in centromeres or looser arrangements around active genes. Crucially, it appears that the variants can be swapped in and out of chromatin (histones are usually added when DNA is copied and were thought to be permanent fixtures). This provides a mechanism for dynamically controlling chromatin structure, for example when genes are activated. Although the full implications of this exchange are not clear, it adds yet another potential mechanism for controlling gene activity (reviewed in (Jin et al., 2005).

Those modes of regulation has been referred to as “epigenetics”, which defines all meiotically and mitotically heritable changes in gene expression that are not coded in the DNA sequence itself (Ahmad and Henikoff, 2002; Grewal and Moazed, 2003).

Consequently, in recent years, it has become clear that the nucleosome itself has an additional role than the packaging of DNA, perhaps equally important and conserved, namely the regulation of gene expression (Kornberg and Lorch, 1999). Particularly exciting is the growing probability that the nucleosome can transmit epigenetic information from one cell generation to the next and has the potential to act, in effect, as the cell’s memory bank (Turner, 2002). This information storage function resides primarily in the amino-terminal tails of the four core histones (H2A, H2B, H3 and H4). The tails are exposed on the nucleosome surface and are subject to a variety of enzyme-catalyzed, post-translational modifications of selected amino-

acids which include the following reactions (Margueron et al., 2005; Spotswood and Turner, 2002):

- lysine acetylation (Grunstein, 1997; Sterner and Berger, 2000);
- lysine and arginine methylation (Zhang and Reinberg, 2001);
- serine and threonine phosphorylation (Nowak and Corces, 2004);
- lysine ubiquitination (Davie and Murphy, 1990);
- sumoylation (Nathan et al., 2003);
- glycosylation (Liebich et al., 1993);
- ADP-ribosylation (Adamietz and Rudolph, 1984);
- biotinylation (Hymes et al., 1995).

The first four types of modifications have been extensively studied, in comparison to the others. Potential sites of post-translational modification on nucleosomal histones are shown in figure 8.

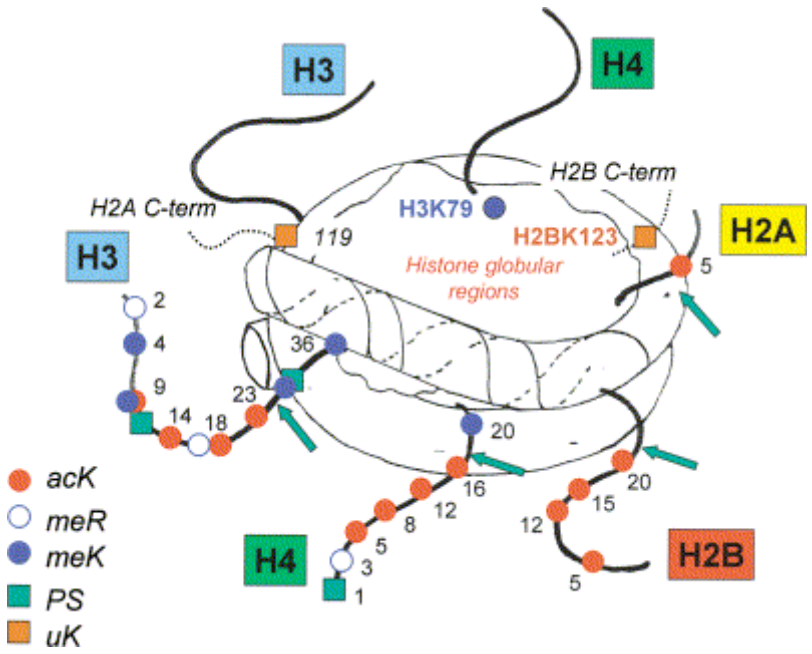

Figure 8. Principal histone modifications on the nucleosome core particle.

Sites of posttranslational modification are indicated by colored symbols that are defined in the key; acK, acetyl-lysine; meR, methylarginine; meK, methyl lysine; PS, phosphoryl serine; and uK, ubiquitinated lysine. Residue numbers are shown for each modification. Note that H3 lysine 9 can be either acetylated or methylated (Turner, 2002).

These modifications are not just a means of reorganizing nucleosome structure, but provide a rich source of epigenetic information. It has been suggested that specific tail modifications, or combinations thereof, constitute a code that defines actual or potential transcriptional states (Jenuwein and Allis, 2001; Richards and Elgin, 2002; Spotswood and Turner, 2002). It is now apparent that certain combinations of these modifications or 'marks' have profound impacts on

transcriptional regulation. The recognition of the dynamic interplay between histone modifications culminated in the **'histone code'** hypothesis (Jenuwein and Allis, 2001; Rice and Allis, 2001a). This hypothesis envisioned that a given modification on a specific histone residue is determinant for the subsequent modifications of the same or another histone molecule. It is evident that various histone modifications cooperate to regulate biological processes. Moreover, in a seemingly manner, a specific type of modification on a specific histone residue can provide the signal required for the printing or erasing of another mark, either on the same tail or on neighbouring tails, within the same or on a neighbouring nucleosome (Margueron et al., 2005). Moreover, individual types of modifications or their summation are 'read' by proteins that modulate chromatin structure and, consequently, regulate transcription. The code is set by histone modifying enzymes of defined specificity and read by non-histone proteins that bind in a modification-sensitive manner. This requires not only proteins that can read such combined modifications, but also mechanisms by which they can be put in place, maintained or removed. The encrypted information in the histones and their modifications is far from being fully deciphered.

As the outcome of histone modifications has been examined, two non-exclusive models emerged. The first suggests that histone modifications directly affect the chromatin structure. The second model proposes that histone modifications might affect transcription by serving as recognition sites for the recruitment of effector molecules. Accumulative body of evidence suggests that both models can simultaneously operate to regulate changes in the chromatin structure and thereby gene activity (Berger, 2002; Margueron et al., 2005).

2-1- Histone acetylation

The first association between a histone tail modification and a particular functional state of chromatin came with the demonstration that transcriptionally active chromatin fractions are enriched in acetylated histones (Hebbes et al., 1988; Pogo et al., 1966). Subsequently, region of transcriptionally silent heterochromatin (condensed and inactive state of chromatin) were found to be underacetylated (Jeppesen and Turner, 1993), consistent with the idea that acetylation of histone tails somehow caused chromatin to become more "open" (or less "condensed") and thereby more conductive to transcription. However, the first transcription-related histone acetyltransferase (HAT) activity was only demonstrated in 1996 (Brownell et al., 1996).

The primarily targets of HAT enzymes are the ε-amino groups of specific lysine residues on the amino-terminal tails of the histone proteins (figure 9). Each histone contains a globular carboxy-terminal domain important for the nucleosome assembly

and a highly charged amino-terminal tail domain which protrudes from the body of the nucleosome. This tail is the site of histone modifications.

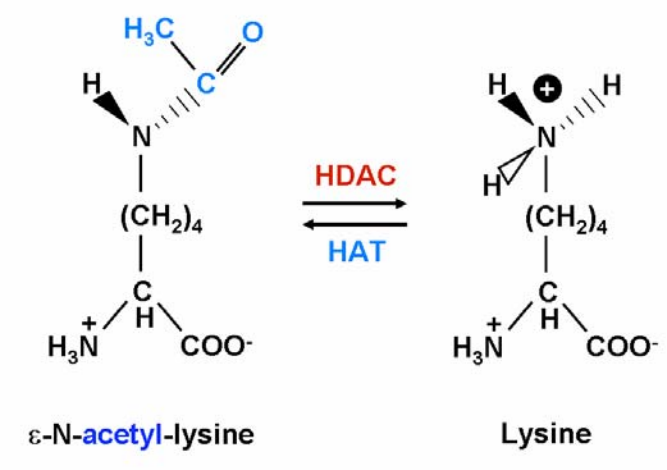

Figure 9. Mechanisms of lysine acetylation.

Acetyl substitution of the ε-amino group of lysine occurs once and results in positive charge neutralization of histones which affects their affinity for the negatively charged DNA (Rice and Allis, 2001b).

As these tails are highly basic, it has been postulated that acetylation of lysine leads to the reduction in its overall positive charge and decreases its affinity for the negatively charged DNA, facilitating the binding of proteins that regulate transcription to chromatin templates (Carrozza et al., 2003; Hong et al., 1993; Lee et al., 1993; Marmorstein and Roth, 2001; Norton et al., 1989). Indeed, acetylated N-terminal histones tails bind DNA with reduced affinity and are more mobile with respect to the DNA surface than unmodified histones (Cary et al., 1982). In addition to the weaken histone:DNA contact, acetylation can also alter the histone:histone interactions between neighbouring nucleosomes (Luger and Richmond, 1998; Tse et al., 1998; Wolffe and Hayes, 1999) as well as interactions between the histones and regulatory proteins (Carrozza et al., 2003; Edmondson et al., 1996; Hecht et al., 1995; Roth et al., 2001).

The level of charge neutralization required to facilitate the destabilization of chromatin higher order structure is so low that other structural features must amplify the consequences of acetylation. These might include alterations to secondary structure in the tail domains and/or changes in the association of the tails with non histone-proteins. Acetylation of histones probably serves to highlight particular nucleosomes and/or segments of chromatin for interaction with other chromatin remodelling factors (i.e. ATP-dependent nucleosome remodelling complexes) or components of the transcriptional machinery. The “regulated nucleosome mobility model” suggests a mechanism that explains how the histone code is implemented (Cosgrove et al., 2004). This model predicts that an important function of the histone N-termini tail modification may involve the recruitment of effector proteins and

nucleosome-remodelling activities that ultimately lead to changes in the nucleosome mobility. This model also supplies a plausible explanation to the fact that central ATPase subunit of various Swi/Snf family members contains histone modification recognition motifs, such as bromo- and chromo-domains (recognizing acetylated and methylated lysine, respectively) (Langst and Becker, 2004). It also explains why histone modification and ATP-dependent chromatin remodelling are functionally connected for gene regulation as their activities are required at the same promoter in yeast (Hassan et al., 2002; Syntichaki et al., 2000).

Any or all of these changes can affect the structure of individual nucleosomes as well as higher folding order, leading to a more open and permissive chromatin environment for transcription. The N-termini of the extensively studied histones H3 and H4 are among the most highly conserved sequences in eukaryotes. Although rather short (26 and 19 amino acids respectively), their documented and suspected interactions suggest central roles for these domains in both chromatin structure and function (Eberharter and Becker, 2002). Histone acetylation states are dynamic, with acetylated lysines of the hyperacetylated histones turning over rapidly with half-lives of minutes within transcriptionally active chromatin. These reactions are much less rapid for the hypoacetylated histones of the transcriptionally silent regions. The dynamic of histone acetylation provides an attractive mechanistic explanation for the reversible activation and repression of transcription (Eberharter and Becker, 2002; Roth et al., 2001).

Histone acetyltransferases (HAT)

If histone acetylation was discovered thirty-five years ago, the first HAT activity was only identified in 1996 (Brownell et al., 1996). The yeast version of this protein, Gcn5, was previously linked to transcriptional regulation via genetic and biochemical studies (Sterner and Berger, 2000) as a coactivator that bridged interactions between activator proteins and basal transcription factors. This discovery provided strong molecular evidence for a direct link between histone acetylation and transcriptional regulation. Upon recognition that Gcn5 houses HAT activity, these early studies immediately provided a general model for HAT recruitment to specific promoter by DNA-bound activating proteins (Brownell and Allis, 1996; Wolffe and Pruss, 1996).

A large number of coactivators are now recognized to have HAT activity (Sterner and Berger, 2000). Among these are PCAF, which is similar to Gcn5, nuclear hormone receptor cofactors such as SRC-1 and ACTR, and the multifunctional CBP/p300 proteins (Marmorstein and Roth, 2001). At least one sequence-specific DNA-binding transcriptional activator, ATF-2, also houses HAT activity (Kawasaki et al., 2000). Chromatin immunoprecipitation experiments using antibodies specific for acetylated histone isoforms indicate that these HATs remodel

chromatin in promoter regions, most likely facilitating the subsequent binding of other transcription factors (figure 10). At least two general transcription factors (GTFs) contain HAT activity, i.e. TAFII250 and Nut1. TAFII250 is part of the TFIID, a GTF that provides a critical first step in the transcription initiation mechanism (Mizzen et al., 1996). Nut1 is part of the yeast mediator complex, which is directly associated with RNA polymerase II (Lorch et al., 2000) (reviewed in (Marmorstein and Roth, 2001).

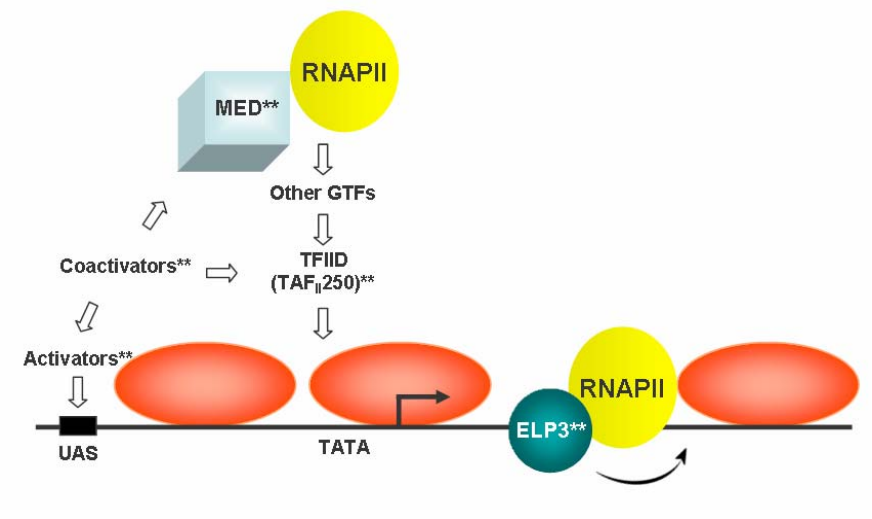

Figure 10. HATs are involved in multiple steps of RNAPII transcription.

HAT activities (indicated by double asterisks) are associated with activators (i.e. ATF2), several coactivators, GTFs, the mediator complex, and elongation complexes in RNA polymerase II transcription (Elongator-ELP3). These activities acetylate nucleosomes (red ovals) in upstream, promoter, and downstream transcribed regions of genes (Marmorstein and Roth, 2001).

Subsequent steps of transcription probably require histone acetylation, as illustrated by the finding that the yeast transcriptional elongation protein Elp3 (the catalytic subunit of Elongator complex) is a HAT (figure 10) (Wittschieben et al., 1999). HATs travelling with elongating RNAPII may provide a combination of recognition sites for chromatin-modifying complexes that assist the elongation process, such as FACT, Spt6 and additional HAT complexes (Orphanides and Reinberg, 2000; Svejstrup, 2002). The patterns of histone acetylation generated at different genes will undoubtedly reflect the combination of enzymes recruited at these different steps of transcription, perhaps contributing to the complexity of the 'histone code' proposed to govern gene regulation (Strahl and Allis, 2000). The fact that HATs are coactivators rather than DNA-binding activators underscores the need for flexibility, regulation and alternative strategies in regulating chromatin and the basal transcription machinery (Marmorstein and Roth, 2001).

Although localized changes in histone acetylation accompany HAT recruitment, additional proteins can also be important substrates. Other acetylated proteins include transcriptional regulators (p53, E2F1, GATA1, TCF, HNF4, TFIIEβ and TFIIF), structural proteins (such as tubulin) and nuclear-import proteins (the

importin-α family). The acetylation of such proteins may regulate the DNA binding ability (for transcription factors), protein-protein interactions, protein localization or protein stability (Minucci and Pelicci, 2006). Acetylation of one HAT, ACTR, by other acetylases hints at acetylation cascades, which may transduce signals as do phosphorylation cascades (reviewed in (Kouzarides, 2000).

The histone acetyltransferases use acetyl-coenzyme A as a cofactor; they are divided into five families (Roth et al., 2001):

1- Gcn5-related acetyltransferases (GNATs) (including Hat1, Elp3, Hpa2 and PCAF);
2- MYST-related HATs (for 'MOZ, Ybf2/Sas3, Sas2 and Tip60);
3- p300/CBP HATs;
4- general transcription factor HATs (which include TAFII250);
5- nuclear hormone-related HATs SRC1 and ACTR (SRC3).

The GNAT and MYST-type HATs are generally linked to histone function (figure 11).

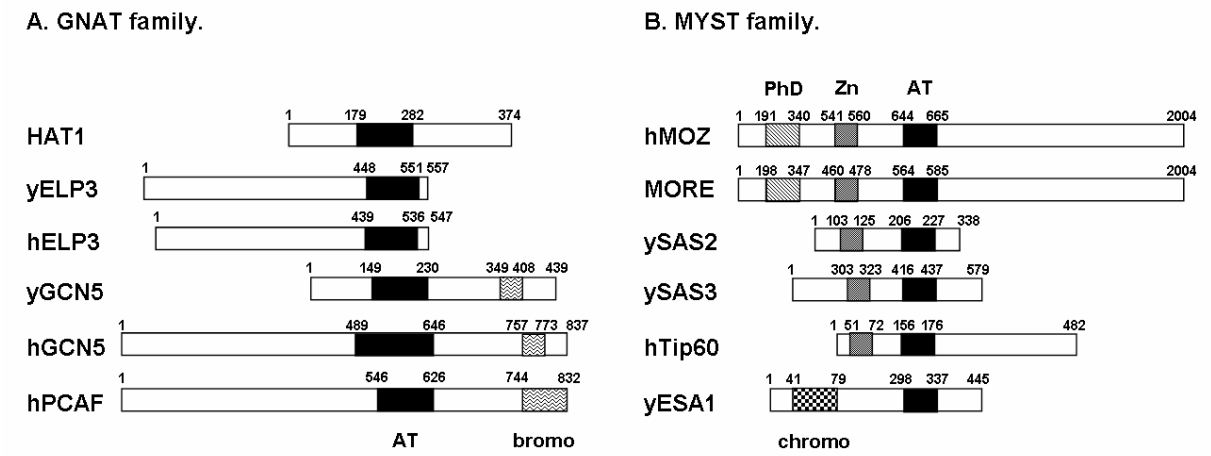

Figure 11. GNAT and MYST families of HATs.

Similarities of GNAT family members and MYST family members. The relative sizes and locations of conserved motifs for the GNAT and MYST families of HATs are indicated. AT, acetyltransferase domain; bromo, bromodomains; PhD, plant homeo domains; Zn, zinc finger domains; chromo, chromodomains (adapted from (Roth et al., 2001)).

Sequence analysis of HAT proteins reveals a high sequence similarity within families but poor or no similarities at all between families (Marmorstein and Roth, 2001). Moreover, each HAT family appears to have a distinct substrate preference, and different families tend to appear in different functional contexts. For example, the Gcn5/PCAF family interacts with a subset of transcriptional coactivators and preferentially acetylates lysine 14 in histone 3 in yeast (Kuo et al., 1996; Kuo et al., 1998; Marmorstein and Roth, 2001; Wang et al., 1998). These proteins mostly contain a carboxy-terminal bromodomain module that has been shown to be an

acetyl-lysine targeting motif (Dhalluin et al., 1999). In contrast, most MYST family HAT proteins "prefer" histone H4 as a substrate and contain a chromodomain shown to bind RNA (Akhtar et al., 2000) and N^{ε}-methylated lysine residues (Marmorstein and Roth, 2001; Nielsen et al., 2002).

Histone deacetylases (HDAC)

Simultaneously to the first HAT identification, the first histone deacetylase (HDAC) was also purified (Taunton et al., 1996). Remarkably, this mammalian HDAC was homologous to the well characterized yeast corepressor Rpd3p (Rundlett et al., 1996; Taunton et al., 1996). In the subsequent years, more corepressors were recognized to be HDACs, just as more coactivators were recognized as HATs, providing strong support for the idea that hyperacetylated chromatin is transcriptionally active whereas hypoacetylated chromatin is inactive (Roth et al., 2001). Indeed, some regulated activation events are presently known to involve the exchange of complexes containing HDAC functions for those containing HAT activities. For example, certain nuclear hormone receptors (such as the thyroid hormone receptor) interact with HDACs to repress transcription in the unbound state, but associate with HATs in presence of their ligand to activate their target genes (Freedman, 1999; Glass and Rosenfeld, 2000; Roth et al., 2001).

Phylogenic analyses have subdivided HDACs enzymes into four distinct classes (Gregoretti et al., 2004):

- class I (human HDACs 1, 2, 3 and 8), homologous to yeast Rpd3;
- class II (human HDACs 4, 5, 6, 7, 9 and 10), related to yeast Hda1;
- class III (human sirtuins 1-7), related to yeast sir2;
- class IV, related to the recently identified HDAC11.

Class I HDACs are widely expressed, whereas classes II and IV present various degrees of tissues specificity. In addition, class I and IV HDACs are mostly constitutively nuclear proteins, whereas class II HDACs shuttle between the nucleus and the cytoplasm in response to cellular signals (de Ruijter et al., 2003; Minucci and Pelicci, 2006). Class III enzymes are nicotinamide adenine dinucleotide (NAD) dependent (table 1) (Blander and Guarente, 2004; Finnin et al., 1999).

Table 1. HDAC family (Suzuki and Miyata, 2006).

HDAC	Localization	Function
Zinc-dependent HDACs		
Class I (Rpd3 homolog)		
HDAC1	nucleus	transcriptional repression
HDAC2	nucleus	transcriptional repression
HDAC3	nucleus, cytoplasm	transcriptional repression
HDAC8	nucleus	transcriptional repression
Class II (HDA1 homolog)		
HDAC4	nucleus, cytoplasm	transcriptional repression
HDAC5	nucleus, cytoplasm	transcriptional repression
HDAC6	cytoplasm	microtubule stability/function
HDAC7	nucleus, cytoplasm	transcriptional repression
HDAC9	nucleus, cytoplasm	unknown
HDAC10	cytoplasm	unknown
Class IV		
HDAC11	nucleus	unknown
NAD+-dependent HDACs		
Class III (Sir2 homolog)		
SIRT 1-7	nucleus	functional regulation of p53

2-2- Histone acetylation versus other histone modifications

Recent studies indicate that some histone modifications are closely correlated with each other and evidence for a certain level of crosstalk between different histone modifications does exist, although the generality of these observations remain unclear (Daujat et al., 2002; Lindroth et al., 2004; Mateescu et al., 2004; Shilatifard, 2006). The most studied modifications that can act together on transcriptional regulation are histone acetylation, methylation, phosphorylation and ubiquitination. Histone sumoylation also emerges in transcription regulation. Those modifications will be further defined and detailed, and some experimental evidence for their role in transcription will be described hereafter.

Histone methylation

Histone methylation was first discovered more than forty years ago (Murray, 1964). It occurs on arginine and lysine residues and is catalysed by histone methyltransferases (HMTs), belonging to three distinct families: the PRMT1, the SET-domain containing protein family, and the non-SET-domain proteins DOT1 and DOT1L (Bannister and Kouzarides, 2005; Margueron et al., 2005; Martin and Zhang, 2005). HMTs use S-adenosylmethionine (SAM) as a cofactor, just as HATs use acetyl-coenzyme A. *In vivo*, methylated lysines can be found either in a mono-, di- or trimethylated state (figure 12), whereas arginines can be either mono- or dimethylated. Both histone lysine (K) and arginine (R) methylation occurs on histone H3 and H4 (H3-K4, K9, K27 and H4-K20; H3-R2, R17, R26 and H4-R3).

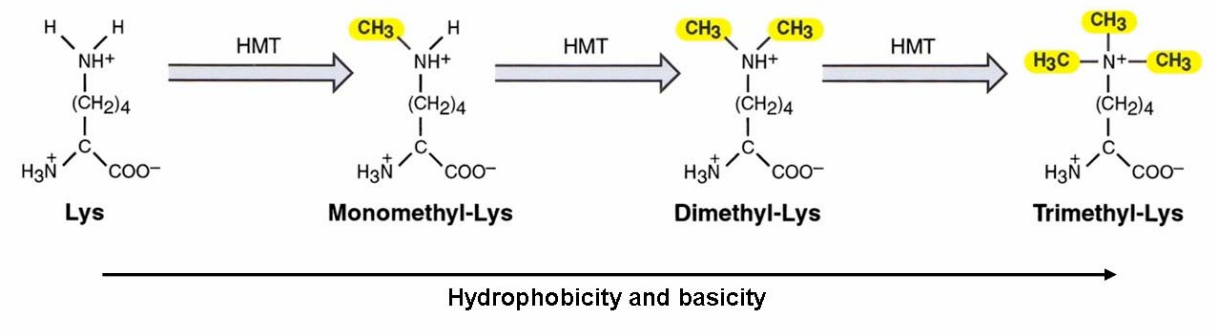

Figure 12. Molecular structure of lysine and mono-, di-, and tri-methyl-lysine (adapted from (Zhang and Reinberg, 2001).

HMT, histone methyltransferase; Lys, lysine.

Unlike acetylation, which generally correlates with transcriptional activation, histone lysine methylation can induce either activation or repression, according to the sites of methylation (Zhang and Reinberg, 2001). Furthermore, for certain processes, methylation on the same site can lead to different outcomes depending on the number of methyl groups added (Martin and Zhang, 2005). There is no evidence that lysine methylation directly affects chromatin dynamics as seen with histone acetylation, which neutralizes the positive charge of lysine. Since methylation of lysine does not alter their charge, any direct effect on chromatin folding would have to occur through a non-electrostatic mechanism, for example through hydrophobic interactions. Consistent with this hypothesis, recent studies on histone methylation identified at least three protein motifs, the chromodomain (Bannister et al., 2001; Lachner et al., 2001), the tudor domain (Huyen et al., 2004; Sanders et al., 2004) and the WD40-repeat domain (Wysocka et al., 2005), that are capable of specific interactions with metylated lysine residues (figure 13) (Martin and Zhang, 2005).

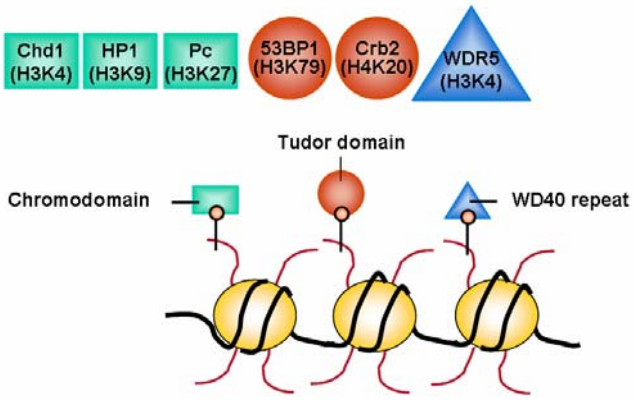

Figure 13. Methyl binding domains.

Methyl-lysine binding proteins contain one of three methyl-lysine binding domains: the chromodomain, the tudor domain or the WD40-repeat domain. These can not only interact with methyl-lysine, but also seem to discriminate between different methylated lysines. For example, the chromodomain of HP1 (heterochromatin protein 1) interacts specifically with methyl-H3-K9, whereas that of Polycomb (Pc) interacts specifically with methyl-H3-K27. This probably explains why different methylated lysines within histones H3 and H4 can have different biological outcomes (adapted from (Martin and Zhang, 2005).

The methylation on histone H3 lysine 9 (H3-K9) and histone H4 lysine 20 (H4-K20) is mostly associated with transcriptional repression. For example, it has been shown that pericentric heterochromatin is specifically enriched in tri-methyl-H3-K9 and H4-K20 (Rice et al., 2003; Schotta et al., 2004). By contrast, mono- and di-methyl H3-K9 and H4-K20 are found in non-heterochromatin regions referred to as euchromatin and seem to function in the silencing of individual genes (Tachibana et al., 2002). Histone H3 lysine 27 methylation has also been linked to several silencing phenomena and implies the Polycomb group of proteins (Cao and Zhang, 2004; Martin and Zhang, 2005).

The main sites of lysine methylation that have been associated with gene activity include lysines 4, 36 and 79 of histone H3 (H3-K4, H3-K36 and H3-K79). Interestingly, the methylation of all three sites seems to be directly coupled to the transcription process. In the case of H3-K4 and H3-K36 methylation, the enzymes responsible for both modifications (SET1 and SET2, respectively in yeast) have been shown to physically associate with RNAPII during elongation, resulting in histone methylation in the transcribed region. This association indicates that H3-K4 and H3-K36 methylation might be the result of the gene activity. This observation and the fact that methylated lysine within histones are relatively stable indicates that histone H3-K4, -K36 and -K79 methylation might mark active genes. The different methylated lysines could play the role of a molecular 'door stop' to maintain genes turned on (reviewed in (Martin and Zhang, 2005)).

Recently, enzymes responsible for demethylating lysine within histones were identified. The first one is LSD1 (lysine specific demethylase 1) which has been shown to catalyze the specific removal of methyl groups from mono- and dimethylated lysine 4 of histone H3 by an amine oxidase reaction (Shi et al., 2004). Subsequently, another family of proteins, the Jumonji C domain proteins, has been shown to demethylate histone lysine through a hydroxylation reaction. Histone demethylases reveal a site-specificity as well as a specificity in the methylation marks (mono-, di- or tri) at a particular site (Klose et al., 2006; Tsukada et al., 2006). The biological significance of histone demethylation as well as its role in transcription regulation remains unclear, requiring further investigation.

The methylation of arginine residues in histones has been correlated with the active state of transcription, much like acetylation. For example, the arginine methyltransferase PRMT4/CARM1 have been shown to be associated with the co-activators GRIP1 and CARM1/PRMT5 co-operates with the p300 HAT to stimulate transcription by nuclear receptor (Kouzarides, 2002). Furthermore, methylation on arginine, as observed with lysine acetylation, can use non-histone proteins as substrate. For example, a well studied substrate includes heterogeneous nuclear ribonucleoproteins (hnRNPs), which are involved in pre-mRNA splicing and RNA transport (Shen et al., 1998). It has been proposed that arginine methylation might serve as a maturation signal for certain hnRNP particles (Cote et al., 2003). Arginine methyltransferase activity has also been linked to signal transduction, with the finding that one arginine methyltransferase can bind the cytoplasmic region of the type I interferon receptor and that this binding constitutes a positive signal in the interferon pathway (Abramovich et al., 1997) (reviewed in (Bedford and Richard, 2005).

Histone phosphorylation

Histone phosphorylation involving serine 10 of histone H3 has also emerged as an important modification, both in transcriptional activation and in chromosome condensation during mitosis. As those two processes are expected to involve opposing physical alterations of chromatin (i.e. condensation of chromatin during mitosis and opening during transcription), the finding that the same modification is involved in both processes supports the "modifications-as-binding surface" hypothesis rather than direct alteration of chromatin (Berger, 2002; Nowak and Corces, 2004). Different kinases were identified having histones as substrate (histone kinases or HKs) (Lo et al., 2001; Sassone-Corsi et al., 1999). More recently, evidence has accumulated indicating that the phosphorylation of histone H3 at serine 10 has an important role in the transcriptional activation of eukaryotic genes (Nowak and Corces, 2004).

A relationship between acetylation and phosphorylation of histone H3 is largely supported by experimental data and the existence *in vivo* of the di-modified H3 isoform has been established (Cheung et al., 2000; Clayton et al., 2000). Moreover, *in vitro* experiments revealed that the yeast GCN5 histone acetyltransferase displayed a preference for binding to a portion of histone H3 tail that is pre-phosphorylated at the serine 10 position (Lo et al., 2000). This observation suggests that histone H3 has to be phosphorylated before being bound by GCN5 which acetylates the same histone H3 tail at the lysine 14 leading to the induction of transcription (Lo et al., 2001).

Histone ubiquitination and sumoylation

Although histone H2A was identified 31 years ago as the first protein to be ubiquitinated (Goldknopf et al., 1975), histone ubiquitination remains one of the least understood histone modifications (Zhang, 2003). Both histones H2A and H2B are subject to modification by ubiquitin at a specific residue (lysine 119 and lysine 120 in humans, respectively). The majority of ubiquitinated H2A and H2B is found in monoubiquitinated form and is not linked to degradation. In yeast, the only histone known to be ubiquitinated is H2B (on lysine 123) and consequently, H2B ubiquitination has been extensively studied in this organism. Recently, it has been shown that monoubiquitination of H2B-K123 is a prerequisite for methylation of K4 and K79 of histone H3 (Dover et al., 2002; Ng et al., 2002; Sun and Allis, 2002), is associated with elongating RNAPII and regulates its recruitment at the coding sequences of several active genes *in vivo*. This suggests that histone ubiquitination might be linked to transcription elongation (Pavri et al., 2006; Shukla et al., 2006; Xiao et al., 2005).

Ubiquitination of histones has been reported to be a highly dynamic reaction, turning over rapidly. Unlike other reversible histone modifications in which addition or removal of a group from a histone molecule results in opposing transcriptional effects, sequential ubiquitination and deubiquitination are both involved in transcriptional activation (Henry et al., 2003; Zhang, 2003). This suggests that histone ubiquitination has a unique role among histone modifications, possibly to orchestrate an ordered pathway of chromatin alterations. Enzymes involved in histone ubiquitination such as E_2-conjugating enzymes, E_3-ubiquitin ligases or histone deubiquitinases begin to be discovered (Emre et al., 2005; Henry et al., 2003; Robzyk et al., 2000; Wood et al., 2003) and their role in gene transcription regulation is still under investigation. Both ubiquitinated H2A and H2B were found to be concentrated in nucleosomes present at the 5'-untranslated region of highly transcribed genes, leading to the hypothesis that ubiquitinated histones might be required for transcriptional activation (Davie and Murphy, 1990; Nickel et al., 1989). Experimental evidence shows that histone ubiquitination actively participates to the "cross-talk" pathway for histone tail modifications active in transcriptional regulation (Shilatifard, 2006). For example, recent studies have demonstrated that mono-ubiquitinated histone H2B can be deubiquitinated by the enzyme Ubp8, a component of the SAGA histone acetyltransferase complex, suggesting that the mono-ubiquitination of H2B is followed by the recruitment of SAGA to the ubiquitinated nucleosome and subsequent deubiquitination of histone H2B (Daniel et al., 2004; Henry et al., 2003). Furthermore, as previously mentioned, histone H3 methylation by HMT enzymes such as COMPASS (H3-K4) or Dot1 (H3-K79) requires histone H2B mono-ubiquitination.

Ubiquitination on histone H3 and H4 has been also reported very recently; it seems to participate in the cellular response to DNA damage (Wang et al., 2006).

Finally, all four core histones are sumoylated at specific sites and it has been demonstrated that histone sumoylation sites are involved directly in transcriptional repression. A dynamic interplay between histone sumoylation and either acetylation or ubiquitination could be observed where sumoylation serves as a potential block to these activating modifications, suggesting that sumoylation may serve as a general dynamic mark to oppose transcription (Nathan et al., 2006; Shiio and Eisenman, 2003).

2-3- Histone modification and human disease/development

Dynamic programs of gene expression are required for the differentiation of pluripotent stem cells into specific tissue lineages during the development. Cell differentiation is largely a process of restricting specific gene expression patterns to particular types of cells. This restriction involves both activation and repression of tissue-specific genes. Histone modification affect both of these regulatory processes through effects on chromatin at individual genes and on large-scale chromatin domains. Recent studies indicate that global levels of histone acetylation and histone H3-K4 methylation, which are generally associated with gene activation, transiently decline once embryonic stem (ES) cells start to differentiate (Lee et al., 2004). Treatment of ES cells with histone deacetylase (HDAC) inibitor trichostatin A (TSA) blocks differentiation (Lee et al., 2004), confirming the functional importance of changes in histone acetylation patterns. Moreover, the re-establishment of pluripotency in somatic cells is associated with the re-establishment of active histone marks, including hyperacetylation and high levels of H3K4 methylation (Kimura et al., 2004; Lin and Dent, 2006).

Global alterations in histone modification sites and levels reflect not only large-scale shifts in heterochromatic domains but also locus-specific alterations in euchromatic structure. During neurogenesis for example, sequential activation of genes within the *Hox D4* locus is associated with a temporal and directional spreading of open chromatin marks. The direction of histone modifications colinears gene activation across *Hox* clusters, so these sequential changes in histone modifications might be crucial for proper anterior-posterior axis of the embryo. In this case, H3-K4 methylation first occurs, followed by the H3 and then the H4 acetylation (Rastegar et al., 2004).

At each stage of the development, gene expression is subject to a high degree of temporal and spatial regulation. Genetic studies in mice demonstrate that various chromatin-modifying activities are important throughout the process and that particular HATs, HDACs and HMTs have tissue-specific and dose-dependent functions in the developing embryos (Lin and Dent, 2006). Chromatin-modifying enzymes which are essential for normal mouse development are shown in figure 14. The diverse phenotypes caused by mutations in histone-modifying enzymes further indicate that particular developmental pathways require particular chromatin states (Lin and Dent, 2006). For example, loss of GCN5 HAT results in loss of paraxial mesoderm, whereas loss of p300 HAT causes cardiac and skeletal defects (Xu et al., 2000; Yao et al., 1998). Moreover, enzymes that modify the same residue in a given histone might have unique functions during development (Peters et al., 2001; Tachibana et al., 2002).

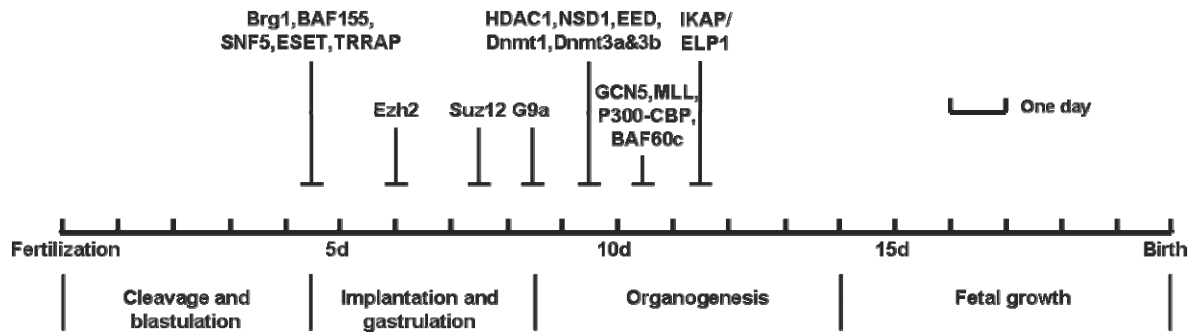

Figure 14. Chromatin-modifying enzymes or associated proteins are essential for normal mouse development.

The different stages of mouse development are depicted relative to days of gestation. The time of embryonic lethality caused by homozygous deletion of individual chromatin-modifying activities or associated proteins is shown. Abnormal phenotypes are usually observed somewhat earlier, reflecting developmental time-points and processes that require the deleted factors (adapted from (Lin and Dent, 2006).

Depletion of many chromatin-modifying proteins leads to early embryonic lethality, precluding assessment of their functions in later developmental stages. The use of conditional alleles of these genes that would enable deletion at later developmental stages or in specific tissues will greatly enhance the understanding of their functions during embryogenesis and in adult tissues. Moreover, future studies defining the developmental functions of enzymes that govern histone ubiquitination, sumoylation, and phosphorylation will nicely complement findings regarding the respective functions of HATs, HDACs and HMTs.

Additionally, deregulation of histone-modifying enzymes activity has been closely related to human disease, and most particularly to human cancer. Perturbation of HAT or HDAC activity may be evidenced in cancer. A balance between those two opposed activities must exist, and shift in this balance might have dramatic consequences on the cell phenotype. For example, monoallelic mutations of both p300 and CBP HATs are found in patients who are affected by the congenital Rubenstein-Taybi syndrome (RTS) (Gibbons, 2005; Petrij et al., 1995). Individuals with RTS present developmental defects and have a increased risk of developing cancer, usually childhood cancers of neural crest origin (Miller and Rubinstein, 1995). The HATs CBP, p300, MOZ and MORF are occasionally involved in fusion proteins that arise from chromosomal translocations associated with leukaemia. It appears that these fusion proteins represent gain of function mutants and that the leukaemogenic effect may be due to mistargeting of HATs, leading to aberrant acetylation and gene activation (Di Croce, 2005). Beside cancer, a neurodevelopmental genetic disease, the familial dysautonomia (FD), involves mutations in the *IKAP* gene, the scaffold protein of the Elongator HAT complex (see hereafter for more details).

As transcriptional repressors, HDACs can be targeted to specific genomic regions by interaction with sequence specific DNA binding factors but also by methyl-DNA binding proteins. For example, HDACs act as co-repressors for oncogenic translocation product fusion proteins such as PML-RAR and AML-ETO in specific forms of leukaemia and lymphoma. In contrast to the wild-type proteins, the fusion proteins actively suppress transcription by aberrant recruitment of HDAC containing co-repressors (Gibbons, 2005; Minucci and Pelicci, 2006; Zhang and Dent, 2005). Also, repression of the tumor suppressor *p16^{ink4a}* gene in melanomas and solid tumours is often associated with DNA methylation and with the recruitment of a multifactor repressor complex that contains a DNA methyltransferase as well as HDACs to reinforce the silent state (Zhang and Dent, 2005).

HDACs inhibitors are currently tested in clinical trials as anticancer drugs (Minucci and Pelicci, 2006). HDAC inhibitors induce, to variable extent, cell-cycle arrest, differentiation or apoptosis in tumour cells (Johnstone, 2002; Minucci and Pelicci, 2006). Given the known function of histone acetylation in transcription, it seems logical to postulate that inhibition of HDACs alone is unlikely to lead to a generalized increase in the transcription of all known genes. In fact, acetylation works together with other chromatin modifications, and blocking deacetylation might have very different outcomes depending on the chromatin state. Also, HDAC inhibitors can mediate acetylation of non-histone protein substrates, including tumor suppressors and transcription factors such as p53 or NF-κB (Yoo and Jones, 2006). This would lead to indirect transcriptional effects, or work through distinct, non-transcriptional mechanisms. A complex pattern of HDAC inhibitors target must therefore exist that is cell-type specific, cell-stage specific and dependent on the normal or pathological state of the cell. Nevertheless, there is great interest in using HDAC inhibitors alone or in combination with DNA demethylating agents to reactivate silenced tumour suppressor genes (see reviews (Gibbons, 2005; Minucci and Pelicci, 2006; Yoo and Jones, 2006).

The methylation of histone has also been extensively linked to cancer, and a growing number of the HMTs proteins have been shown to promote or inhibit tumorigenesis through their HMT activity. For example, SET1 methyltransferase family proteins have been related to acute leukemias (i.e. MLL proteins) or to lymphomas and prostate or breast cancers (i.e. EZH2 from the polycomb group). The *NSD1* gene, a human SET2 enzyme, is fused with the *NUP88* gene in a recurrent translocation found in acute myeloid leukemias (Zhang and Dent, 2005). Histone methylation is a more complex mechanism than acetylation. Indeed the specificity in the site as well as in the histone targeted and the level of methylation increases the potential of regulation. The methyl marks exist as both active and inactive markers. So, epigenetic therapy targeting histone methylation or demethylation has to be carefully evaluated in terms of enzyme specificity and benefit/risk balance (Gibbons, 2005; Yoo and Jones, 2006).

Epigenetic therapy is only in early stage of investigation and development. Progress in characterizing the role of all histone modifications and the interplay between them in transcriptional regulation are mandatory prerequisites. This will imply a better appreciation of the role of the enzymes responsible in establishing, removing or recognizing those modifications. Such advances will undoubtedly provide new insights into the processes that regulate human development and the molecular mechanisms that underlie many human diseases, particularly cancers.

Part II: IKAP and the Elongator complex

1- IKAP as a scaffold protein

IKAP or “IKK-associated protein” was first described as a scaffold protein of the IKK complex involved in NF-κB activation. It has been purified in a large interleukin-1 inducible IKK complex that contains the proteins NIK, IKK-α, IKK-β and NF-κB/RelA (Finnin et al., 1999). However, a role of IKAP protein in this pathway could not be experimentally confirmed and was later disproved (Krappmann et al., 2000). At this time, it was established that IKAP is not associated with IKKs and plays no specific role in the cytokine-induced NF-κB activation. Nevertheless, the mechanism by which IKAP acts as a scaffold protein essential for the assembly of cytoplasmic kinase complexes is supported by the presence of protein-protein interaction motifs in its sequence and by its sequence homology with another yeast scaffold protein, ELP1 (see below). A scaffold function of a protein might be defined as its ability to bring other proteins together to form a functional enzymatic complex.

IKAP has been proposed to play a role as a cytoplasmic scaffold protein of the MAP kinase signalling pathway, most particularly in the stress-induced JNK activation (Holmberg et al., 2002). The authors showed a direct interaction between IKAP and JNK *in vivo*, and they demonstrated that IKAP specifically enhanced JNK activation induced by UV light and by TNF-α (pro-inflammatory cytokine) or EGF (growth factor) (figure 15). Whether IKAP associates the MAP kinase JNK with its substrates or JNK with its upstream activators is still unclear. It remains possible that IKAP would be required for other kinase complex assembly, suggesting the potential role of IKAP in transcription factors activation, and consequently in gene expression.

Finally, the IKAP protein and its yeast homolog, ELP1, are structural components of highly conserved transcription elongation factor, the so called ‘Elongator’ complex (Hawkes et al., 2002; Kim et al., 2002) (figure 16). Elongator was first purified in the nucleus, associated with the transcriptionally active RNAPII (Otero et al., 1999). In this context, IKAP/ELP1 has a scaffold protein function, essential for the assembly of the complex. This suggests that IKAP would have a role in gene transcription in the context of Elongator complex, and consequently, might directly influence the gene expression.

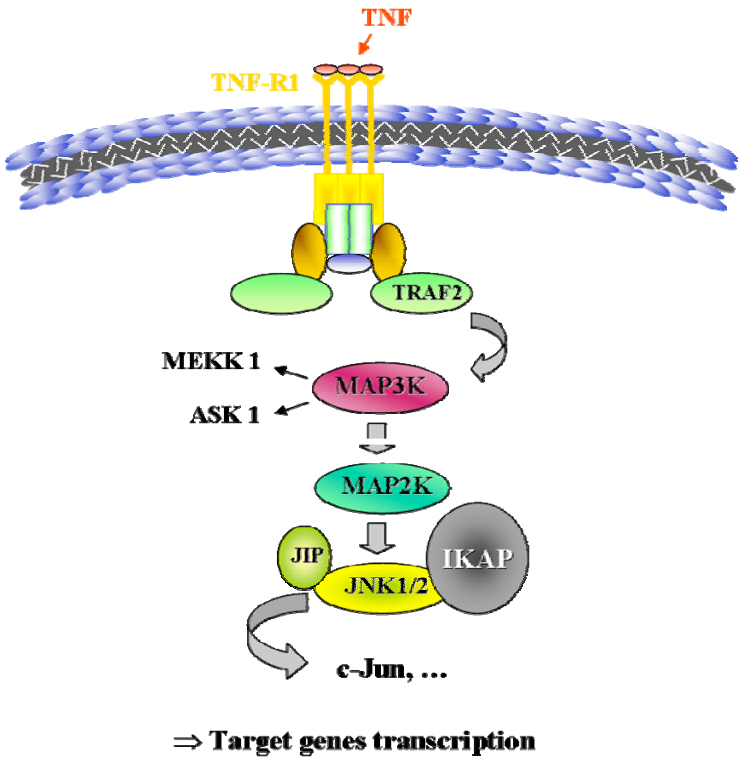

Figure 15. Proposed role of IKAP as cytoplasmic scaffold protein in the MAP Kinase JNK activation pathway.

TRAF, TNF receptor-associated factor; MAP3K, mitogen-activated protein Kinase Kinase Kinase; MAP2K, mitogen-activated protein Kinase Kinase; MEKK1, MAP3K MEK kinase 1; ASK1=MAP3K5; JIP, Janus interacting protein.

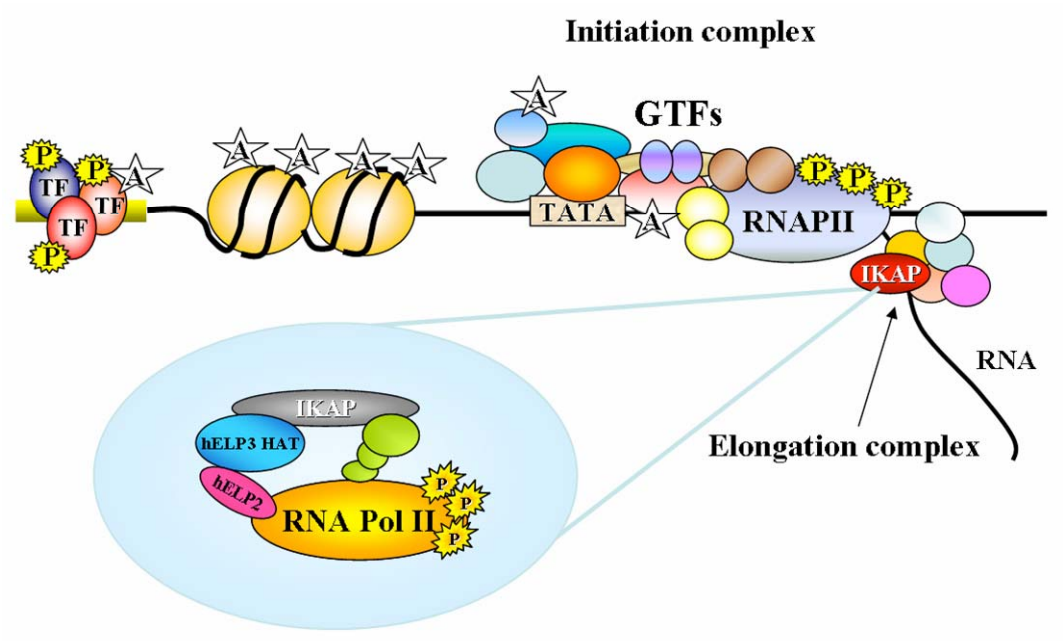

Figure 16. Elongator complex is involved in transcription elongation.

TF, transcription factor; P, phosphorylated; GTFs, general transcription factors; A, acetylated and RNAPII, RNA polymerase II.

2- IKAP in the Elongator complex

2-1- Purification and structure of the Elongator complex

Elongator was originally defined as a component of a hyperphosphorylated RNA polymerase II holoenzyme isolated from yeast chromatin (Otero et al., 1999). Although Elongator interacts weakly with hypophosphorylated RNAPII, stable Elongator-RNAPII interaction requires hyperphosphorylation of the carboxy-terminal domain (CTD) of the RNAPII which is found in the transcribed region of the genes during the transcription elongation. Elongator is composed of 6 different subunits (ELP1 to ELP6) divided into two non functional heterotrimers (figure 17): the core Elongator (ELP1, ELP2 and ELP3) and the HAP complex (ELP4, ELP5 and ELP6) (Li et al., 2001; Winkler et al., 2001). The catalytic subunit of the complex is ELP3 and contains motifs with homology to the GNAT family of histone acetyltransferases (HATs) (Wittschieben et al., 1999). The human homolog of Elongator was purified from HeLa cells nuclei and showed very similar composition (Hawkes et al., 2002; Kim et al., 2002).

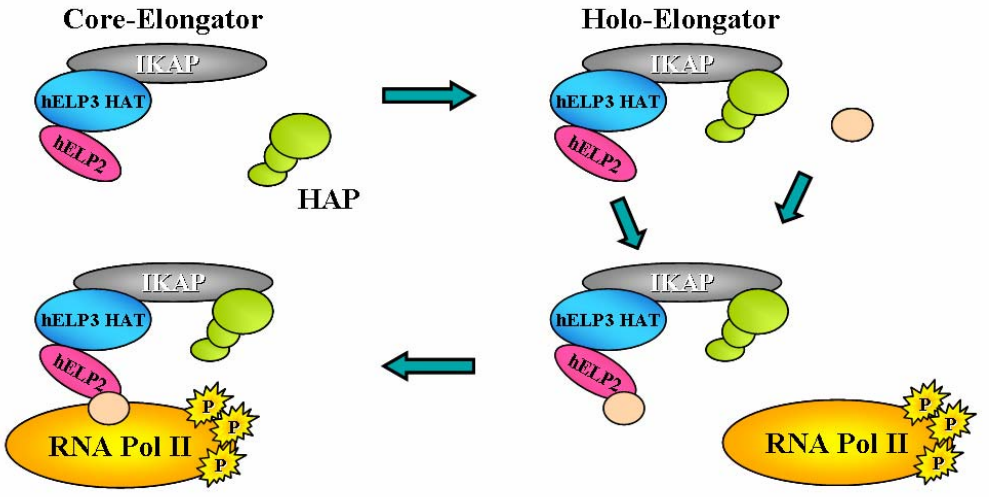

Figure 17. Model for Elongator complex structure.

The two non-functional heterotrimers associate and form functional Elongator or "holo-Elongator", which directly binds the phosphorylated form of RNAPII.

The closest homolog of yeast ELP1 in human cells is encoded by the *IKAP (IKBKAP)* gene (Finnin et al., 1999). In yeast, ELP1 is the largest subunit of the complex and has a scaffold function, essential for the interaction of the subunits forming the core-Elongator and for the assembly of the two heterotrimers to form a functional Elongator complex (figure 17) (Kim et al., 2002). ELP1 is indeed essential

for subunit communication within Elongator (Fichtner et al., 2002) and a deletion of *ELP1* results in substantial destabilization of the ELP3 protein, suggesting that ELP1 protein is required for the stability of ELP3, and consequently also for the Elongator integrity (Petrakis et al., 2004). In human, the IKAP/hELP1 sequence includes five WD40-repeat domains in the N-terminal part of the protein, which are referred as specific protein-protein interaction domains (figure 18) (Neer et al., 1994).

Figure 18. The human IKAP protein contains five WD40-repeat domains.

The structural ELP2 subunit contains eight WD40-repeat domains (Svejstrup et al., 1997). The human homolog of ELP2 is StIP1, identified through its interaction with the transcriptional activator "signal transducer and activator of transcription 3" (STAT3) (Collum et al., 2000). Interestingly, StIP1/hELP2 is localized in the cytoplasm, but upon IL-6 treatment, StIP1/hELP2 translocates to the nucleus (Kim et al., 2002). In yeast, the *ELP2* gene is the only ELP gene that can be deleted without significant loss of Elongator integrity. Surprisingly, an Elongator complex lacking ELP2 even retains the ability to acetylate histones *in vitro*.

HAP complex is composed of the ELP4, ELP5 and ELP6 subunits and may serve to regulate the Elongator-polymerase interaction.

2-2- Functions of Elongator complex

The findings that Elongator is associated with hyperphosphorylated RNAPII and that the ELP3 subunit harbours an HAT homology domain represented the first direct evidence that histone modification may be directly coupled to transcription elongation by RNAPII (Travers, 1999). The HAT activity of yeast ELP3 is essential for its function *in vivo*, as an ELP3-HAT mutant cannot rescue the ELP3-deletion phenotype (Wittschieben et al., 2000). Furthermore, both human and yeast ELP3 have HAT activity *in vitro*, primarily directed toward histone H3, and, at a much lesser extent, toward histone H4 (Hawkes et al., 2002; Kim et al., 2002; Winkler et al., 2002). In yeast, ELP3 mutation results in decreased histone H3 acetylation levels *in vivo* (Kristjuhan et al., 2002; Winkler et al., 2002).

In agreement with a role in the transcript elongation, human Elongator has been shown to facilitate transcription by RNAPII in a chromatin and acetyl-coenzyme A dependent manner *in vitro*, in a reconstituted system (Kim et al., 2002). Furthermore, Elongator is associated with the nascent RNA emanating from elongating RNAPII along the transcribed region of several yeast genes (Gilbert et al., 2004), and chromatin immunoprecipitation (ChIP) experiments have demonstrated an association of Elongator with genes in human cells (Kouskouti and Talianidis, 2005; Metivier et al., 2003). Microarray experiments in yeast and in plants have shown that Elongator affects the expression of a limited number of genes. More important, the gene expression profiles generated by strains (in yeast and in plant) harbouring deletion of genes encoding different Elongator subunits appeared very similar (Krogan and Greenblatt, 2001; Nelissen et al., 2005).

Surprisingly, a substantial fraction of IKAP/ELP1 and other Elongator subunits are cytoplasmic (Hawkes et al., 2002; Holmberg et al., 2002; Kim et al., 2002), suggesting that the Elongator complex may have additional cytoplasmic functions. For example, in yeast, genetic data have implicated the Elongator complex in processes as diverse as exocytosis (Rahl et al., 2005) and tRNA modification (Sun et al., 2005). The relationship between Elongator's role in transcription and these other processes remains poorly understood.

At a phenotypic point of view, yeast Elongator subunits mutants are slow at adapting to new growth conditions (Krogan and Greenblatt, 2001; Otero et al., 1999). Moreover, in plant, Elongator complex has a positive effect on the cell proliferation rate during organ growth (Nelissen et al., 2005). The *ELP1/IKAP* gene, as well as the *ELP3* gene, are essential in *Drosophila melanogaster*, and the mutants die with a remarkably similar terminal phenotype (Jane Walker and Jesper Svejstrup, unpublished data; James Gusella, personal communication), while deletion of *IKAP* gene in mice is embryonic lethal at day 11.5 (Alain Chariot and David Dombrowicz, unpublished data).

Part III: IKAP in Familial Dysautonomia

The crucial role of the protein IKAP in humans has been illustrated in a genetic disorder, the familial dysautonomia (FD). Indeed, different mutations in the gene *IKBKAP*, encoding the protein IKAP, have been shown to cause familial dysautonomia, also known as "Riley-Day syndrome", or "hereditary sensory neuropathy type III (HSAN III)". FD is an autosomal recessive disease that affects the development of sensory and autonomic nervous systems (see figure 19 for autosomal recessive disease transmission) (Axelrod, 2004). Affected individuals are born with abnormally low numbers of neurons in dorsal root peripheral ganglions and in sympathetic ganglions. FD is primarily confined to individuals of the Ashkenazi Jewish descent. Based on the birth incidence of FD, the predicted carrier frequency of the defective gene is ~1/30 in this population (Dong et al., 2002; Maayan et al., 1987).

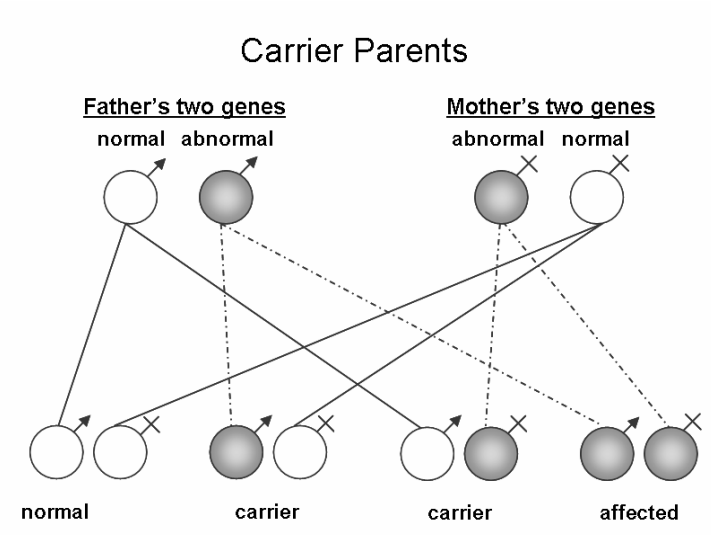

Figure 19. Autosomal recessive disease transmission.

1- Genetic aspects for FD

The *IKBKAP* gene contains 37 exons and encodes a 1332 amino acid protein named IKAP. The major mutation affecting this gene, found in 99.5% of FD patients, is a transition T→C in the 5' donor splice site of intron 20. This mutation results in skipping of exon 20 in the mRNA of patients with FD (figure 20).

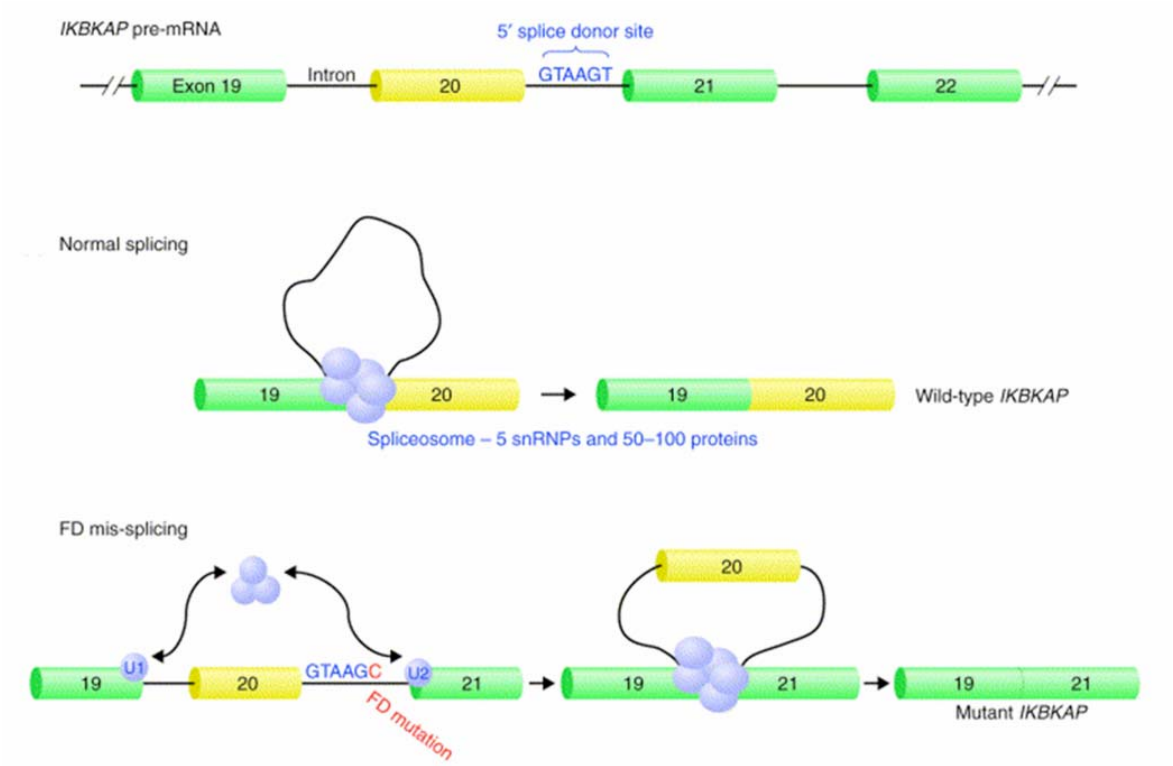

Figure 20. **Schematic representation of the aberrant splicing seen in FD.**

The wild-type splice site sequence (GTAAGT) is shown in the IKBKAP pre-mRNA. The major FD mutation at base pair 6 of intron 20 is shown in red. This mutation decreases the efficiency of splicing and sometimes results in the skipping of exon 20 (Slaugenhaupt and Gusella, 2002).

Translation of this aberrant mRNA results in a frameshift that leads to the apparition of a precocious stop codon and generates a truncated protein lacking all the amino acids encoded by exon 20 to 37; this protein is called IKAP-FD of 79 kD (figure 21).

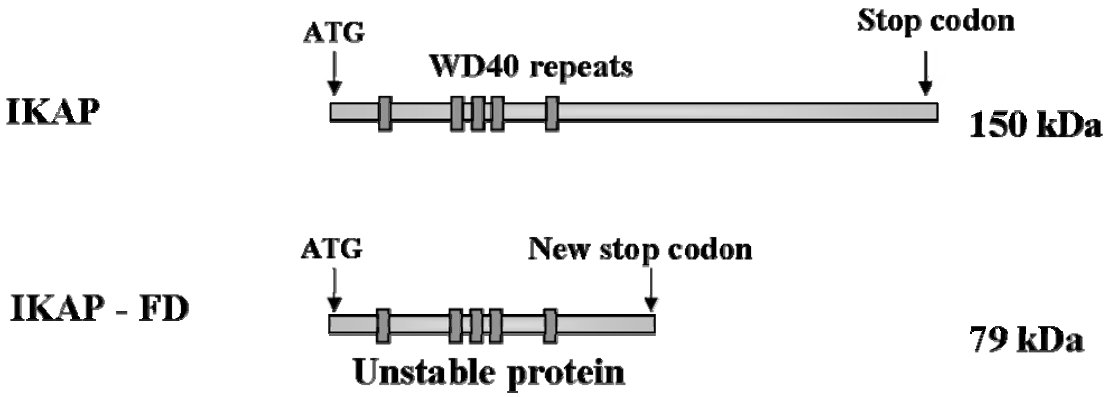

Figure 21. IKAP-FD is the truncated and unstable form of IKAP found in FD patient.

This mutation is not totally penetrant as wild-type IKAP mRNA can still be synthesized from the mutant allele, but at variable levels and in a tissue-specific manner (Slaugenhaupt et al., 2001). The nature of this major FD mutation makes it tempting to consider that the phenotypic variability might relate to the frequency of exon 20 skipping in specific tissues and at specific developmental stages, which may be governed by variations in many factors involved in RNA splicing. Indeed, Cuajungco and colleagues (Cuajungco et al., 2003b) have found more mutant transcript in various central nervous system (CNS) and peripheral nervous system (PNS) regions indicating that *IKBKAP*-splicing efficiency is distinctly affected by the mutation in these tissues while remaining mostly unaffected in lymphoblast cell lines, for example. It suggests that the identity and the relative amounts of cellular splicing factors involved in the correct splicing of *IKBKAP* may vary from tissue to tissue, with certain neuronal cells, including those in the sensory and autonomic nervous systems, particularly susceptible to miss-splicing (Cuajungco et al., 2003b). The new generated product, the IKAP-FD protein (figure 21), is almost unstable in FD cells so that the result of the decrease of wild-type *IKBKAP* mRNA in FD nervous tissue leads to either a complete absence of normal IKAP or to a reduction of the protein level to an almost undetectable level (Cuajungco et al., 2003a).

Two other minor mutations in the *IKBKAP* gene have been rarely associated with the FD phenotype. All FD patients tested carry at least the major mutation, with 99.5% being homozygous, and the remainder being heterozygous with either of the two minor mutations on the alternate allele. The first one is a missense mutation in exon 19 that leads to an arginine to proline substitution (R696P) in the IKAP protein, which is predicted to disrupt a potential serine/threonine phosphorylation site (Dong et al., 2002). The second minor mutation affects exon 26 and leads to a proline to leucine substitution (P914L). This last mutation is of particular significance because it was identified in a patient having no pure Ashkenazi Jewish ancestry, and consequently constitutes the first non-Jewish mutation in FD. Before this report, having Ashkenazi Jewish ancestry was considered as a major diagnostic criterion (Leyne et al., 2003).

2- Clinical symptoms

Homozygous individuals with FD are affected with a variety of symptoms caused by sensory and autonomic dysfunction which includes:

- decreased sensitivity to pain and temperature;
- cardiovascular instability (orthostatic hypotension, episodic hypertension)
- recurrent pneumonias;
- vomiting crises;
- gastrointestinal dysfunction;
- spinal deformity;
- lack of overflow tears;
- absence of fungiform papillae on the tongue (used for diagnosis: figure 22).

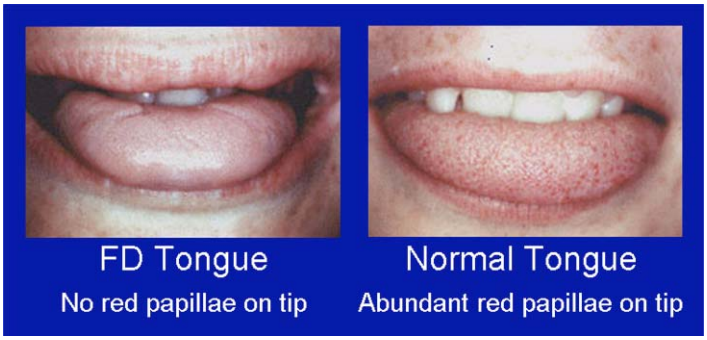

Figure 22. FD patients lack fungiform papilla on the tongue.

The genetic mutation probably affects the development, as well as maintenance of neurons because there is a neuropathological and clinical progression. Pathological alterations consist of decreased unmyelinated and small fiber neurons. Clinical features reflect widespread involvement of sensory and autonomic neurons. Sensory loss includes impaired pain and temperature appreciation. Autonomic features include dysphagia, vomiting crises, blood pressure lability, and sudomotor dysfunction. Central dysfunction includes emotional lability and ataxia. With supportive treatment, prognosis has improved greatly. About 40% of patients live over 20 years. The cause of death is usually related to pulmonary failure, unexplained sudden deaths, or renal failure (Axelrod, 2004).

3- Treatments of FD patients

Treatment of FD has largely reduced morbidity and improved survival. Although this is primarily a neurological disorder causing sensory and autonomic dysfunction, there are secondary systemic perturbations affecting ophtalmological, gastrointestinal, respiratory, cardiovascular, orthopaedic and renal functions. Preventive and supportive treatments have included (Axelrod, 2005):

- measure to maintain eye moisture;
- anti-reflux procedure with gastrostomy;
- the use of central agents such as benzodiazepines and clonidine to control vomiting and the dysautonomic crisis (Lahat et al., 2000; Marthol et al., 2003);
- fludrocortisone and midodrine to combat cardiovascular lability (Axelrod et al., 2005).

With the recent identification of the FD gene, it has been suggested that it may be possible to treat patients by modifying production and expression of the genetic product, namely the protein IKAP. Interestingly, the ability of tocotrienols, members of the vitamin E family, to increase transcription of full-length IKAP mRNA in FD-derived cells has been reported (Anderson et al., 2003b; Anderson and Rubin, 2005). Also, the (-)-epigallocatechin gallate (EGCG), a polyphenol, has been shown to increase the amount of wild-type IKBKAP-encoded transcript and functional protein by reducing the expression of hnRNP A2/B1, a trans-activating factor that favours the use of intron distal 5' splice sites (Anderson et al., 2003a). More recently, Slaugenhaupt and colleagues have demonstrate that kinetin, a plant cytokinin, alters splicing of *IKBKAP-FD* gene and significantly increases inclusion of exon 20, leading to an increased amount of the wild-type *IKBKAP* mRNA with a subsequent larger amount of IKAP protein in the FD cell lines (Slaugenhaupt et al., 2004).

Finally, for FD patients, a great expectation comes from the stem cells field for future cellular therapy. Indeed, Pomp and colleagues have produced normal human peripheral neurons from human embryonic stem cells. This opens the possibility to implement the peripheral ganglia with normal peripheral neurons produced *in vitro* (Pomp et al., 2005).

Aims of the work

The laboratory has been interested and involved for many years in deciphering the mechanisms underlying gene transcription. The IKAP protein has been recently cloned and its story is fairly questionable. Indeed, IKAP (IKK complex-associated protein) was initially identified as a scaffold protein associated with the IKK complex, involved in the NF-κB activation. This hypothesis has been refuted two years later and it has been demonstrated that IKAP has no role in the NF-κB activation. Similarly, another study suggested a potential role for IKAP as scaffold protein in the MAP kinases activation pathways, triggered by cytokines or growth factors.

Meanwhile, other studies performed in yeast and human cells identified Elongator as a transcriptional complex associated with hyper-phosphorylated RNA polymerase II through biochemical purification. The yeast subunit ELP1 has revealed sequence homology to the human IKAP protein. Subsequent studies demonstrated that the subunit ELP3 has a histone acetyltransferase activity.

Consequently, IKAP might regulate gene transcription through different mechanisms: as a scaffold protein in the cytoplasm by allowing the assembly of kinase complexes, and the subsequent activation of their downstream transcription factors, or directly in the nucleus through interaction with RNA polymerase II in the Elongator complex.

Moreover, a crucial role of IKAP in human development has been exemplified in familial dysautonomia, a genetic disease that affects neuronal development and maintenance. This genetic disorder affects the gene coding for IKAP and leads to a tissue-specific decreased IKAP expression.

The goal of this work is to determine the role played by IKAP and by extension by the Elongator complex, in the transcription of human genes. Concomitantly, further study its function at the cellular level will undoubtedly shed light on the potential mechanisms underlying familial dysautonomia.

The first part of this work is dedicated to the identification of the genes whose normal expression required IKAP by DNA microarray experiments after the generation of an IKAP loss-of-function model. We further examined whether transcriptional defects in the IKAP-depleted cells are correlated with changes in cell function.

In the second part, one would further study the implication of IKAP in human Elongator complex function and integrity.

Finally, a closer examination of the molecular mechanisms involving IKAP and the Elongator complex in transcriptional elongation of human genes will be investigated.

The corresponding publication is joined in the appendix 2 section:

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

Pierre Close, Nicola Hawkes, Isabelle Cornez, Catherine Creppe, Charles A. Lambert, Bernard Rogister, Ullrich Siebenlist, Marie-Paule Merville, Susan A. Slaugenhaupt, Vincent Bours, Jesper Svejstrup and Alain Chariot.

Molecular Cell, 22 (4), 521–531, May 19, 2006.

Techniques used in this work are described in the "experimental procedures" section of this article. Therefore, they will not be represented here.

Other related publications in collaboration:

Raloxifene-induced myeloma cell apoptosis: a study of NF-κB inhibition and gene expression signature.

Olivier Sabine, Close Pierre, Castermans Emilie, de Leval Laurence, Tabruyn Sebastien, Chariot Alain, Malaise Michel, Merville Marie-Paule, Bours Vincent, Franchimont Nathalie.

Molecular Pharmacology, 69 (5), 1615-23, May 2006.

TNF-α and IKKβ-mediated TANK/I-TRAF phosphorylation: implications for interaction with NEMO/IKKγ and NF-κB activation.

Bonif Marianne, Meuwis Marie-Alice, Close Pierre, Benoit Valérie, Heyninck Karen, Chapelle Jean-Paul, Bours Vincent, Merville Marie-Paule, Piette Jacques, Beyaert Rudi, Chariot Alain.

Biochemical Journal, 394 (Pt 3), 593-603, March 2006.

Results

Part I:
IKAP regulates the expression of genes involved in cell migration

1- Introduction

IKAP has been described as a protein involved in transcriptional elongation. Still, the genes whose expression is IKAP-dependent remain unknown. The first objective of our study was to identify these genes and by extension, to characterize the biological function of IKAP at a cellular and molecular level in human cells.

Our first goal was to generate a cellular loss-of-function model for IKAP by using the RNA interference approach. The RNA interference (RNAi) has been widely used as a tool to address protein function in many organisms (Sen and Blau, 2006). In this system, the expression of a gene of interest is specifically knocked-down by degrading its messenger RNA. The mediators of this post-transcriptional gene silencing mechanism are synthetic doubled-stranded RNAs of 21- to 23- nucleotides, called small interfering RNAs (siRNAs). Those siRNA molecules can be either transfected into cells (for a short-term effect) or produced from the genome of the cells after viral infection (for a long-term effect). In this work, we used those two approaches to deplete human cells from the IKAP protein.

Once the expression of IKAP was properly depleted in cells, one has analyzed their transcriptome and identified the genes whose normal expression is IKAP-dependent. Such loss-of-function models were also used to investigate the involvement of IKAP in the MAP kinase activation pathway. Finally, the function of IKAP at a cellular level has been characterized by using those IKAP-depleted cells.

2- Results

2-1- Loss of function models of IKAP

Firstly, siRNAs oligos that specifically target the IKAP transcript were designed according to the criteria described by Elbashir et al. (Elbashir et al., 2002). The target sequence and its location in IKAP transcript are detailed in figure 23A and the sequence of the corresponding siRNAs oligos are shown in figure 23B. In parallel, non-specific siRNAs, that target the transcript coding for the GFP protein absent from human cells, are used as negative control (see sequence in figure 23B).

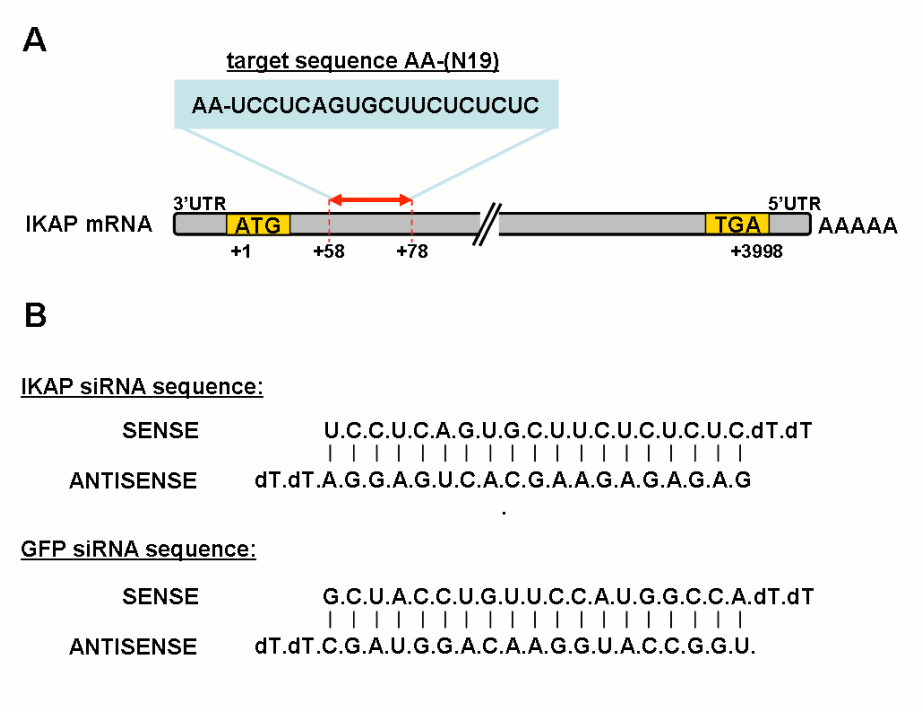

Figure 23. Design of siRNA oligos against the IKAP transcript.

(A). The target sequence on IKAP transcript is located between the bases 58 and 78 downstream the starting ATG (ATG). Stop codon (TGA), 3'- and 5'-untranslated regions (UTR) are also indicated. (B). The corresponding sequence for IKAP siRNA oligos is shown, as well as the sequence for non-specific control siRNAs targeting the protein GFP. The dT.dT 3' overhang is recommended for optimal stability of the siRNA duplex (dT = deoxythymidine).

HeLa cells were transfected with siRNA oligos that target either the IKAP transcript (IKAP siRNAs) or the GFP transcript as a negative control (GFP siRNAs). As shown in figure 24, specific decreased of the IKAP mRNA and protein expression were confirmed by quantitative RT-PCR and Western blot in HeLa cells transfected with IKAP siRNAs. It is important to note that the RNAi does not lead to a total loss of the protein expression, as a residual amount of IKAP is still detectable.

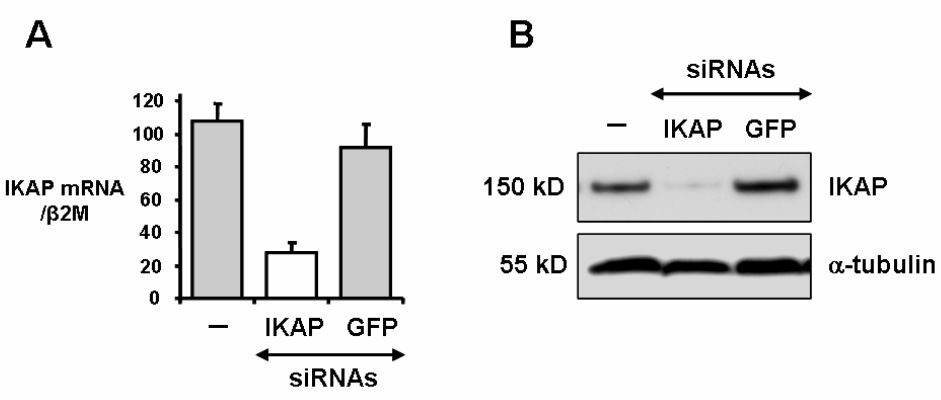

Figure 24. Decreased expression of IKAP in HeLa cells after siRNA oligos transfection.

Decreased expression of IKAP at mRNA (A) or protein level (B) in HeLa cells transfected with IKAP siRNAs, as compared to untransfected cells (–) or cells transfected with control GFP siRNAs, as judged by quantitative RT-PCR analysis (A) or by an anti-IKAP Western blot (B). An anti-α-tubulin Western blot is shown in (B) for normalization purposes.

As an additional experimental model, we sought to establish a stable loss of function model using a lentivirus system to deliver siRNAs into cells. The viral infection integrates a transgenic construct into the target cell genome which will express a short hairpin RNA (shRNA). The sequence of the generated shRNA can be divided into 3 parts:

1- the *sense* sequence which corresponds to the target mRNA sequence;

2- a loop of any 9 nucleotides;

3- the *antisense* sequence, corresponding to the reverse inverted sequence.

Once expressed, the sense and antisense sequences match each other and the shRNAs generated will be cleaved into siRNAs which can mediate the degradation of the target messenger. The lentiviral vector used is the pLentiLox 3.7 (pLL3.7) kindly provided by Dr Van Parijs (Rubinson et al., 2003). This vector was engineered to co-express enhanced green fluorescent protein (EGFP) as a reporter gene, permitting infected cells to be easily tracked by fluorescent microscopy (figure 25).

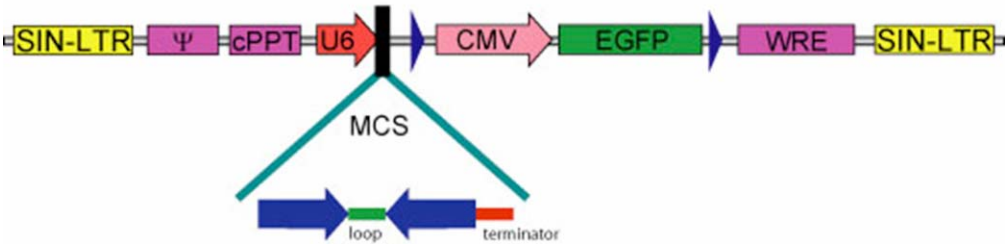

Figure 25. shRNA-expressing lentivirus vector pLL3.7.

The production of shRNAs depends on the RNA polymerase III U6 promoter. This vector also contains a CMV-EGFP expression cassette used as a reporter gene. SIN-LTR, self-inactivating long terminal repeat; Ψ, HIV packaging signal; cPPT, central polypurine track; MCS, multiple cloning site; CMV, cytomegalovirus promoter; WRE, woodchuck hepatitis virus response element. Sequences inserted are represented by blue arrows.

HeLa cells were infected with the pLL3.7 lentivirus expressing the specific shRNA which targets IKAP mRNA (IKAP shRNA) or the non-specific shRNA which targets GFP mRNA (GFP shRNA). The blots in figure 26 show a specific decrease of IKAP protein expression in IKAP shRNA cells, as compared to GFP shRNA cells. We took advantage of our IKAP shRNA construct to infect other cell lines, for example neuroblastoma-derived SK-N-BE cells or glioblastoma-derived U-373 cells. As seen in figure 26, in those two cell lines, the efficiency of the lentiviral infection is as good as the one observed in HeLa cells, as judged by the specific decrease of IKAP expression observed in IKAP shRNA SK-N-BE or U-373 cells compared to the IKAP levels in GFP shRNA control cells.

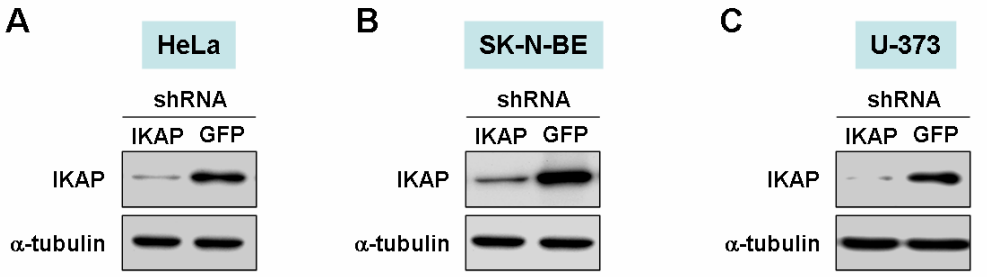

Figure 26. Depletion of IKAP expression in different cell lines after IKAP shRNA lentiviral infection.

The IKAP expression was specifically decreased in HeLa (A), SK-N-BE (B) or U-373 (C) cells infected with the lentivirus delivering IKAP shRNAs, as compared to those infected with the lentivirus delivering GFP shRNAs used as control, as judged by anti-IKAP Western blot analysis. An anti-α-tubulin Western blot is shown for normalization purposes.

Finally, we used fibroblasts derived from FD patients generously provided by Dr S. Slaugenhaupt, who supplied us two distinct cell lines from FD patients and the corresponding control fibroblasts. Because of the FD mutation, the FD cells express a lower amount of IKAP compared to the control cells, as shown in figure 27. The decreased expression of IKAP in FD cells is similar to that observed in IKAP RNAi cells.

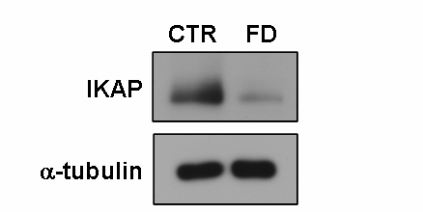

Figure 27. Decreased of IKAP expression in FD cells.

Expression of IKAP protein in FD cells as compared to the control fibroblasts examined by Western blot analysis. An anti-α-tubulin Western blot is shown as loading control.

2-2- IKAP as a scaffold protein for cytoplasmic signalling pathways

IKAP has been proposed to be implicated in assembling stress-induced and cytoplasmic kinase complexes. In this context, IKAP has been identified as a JNK-interacting protein in the cytoplasm. Furthermore, the authors have shown that IKAP overexpression enhances JNK activation in response to TNF-α, EGF and UV light (Holmberg et al., 2002).

We first used our loss-of-function models to address the potential implication of IKAP in stress-induced MAP kinase (MAPK) (Erk, p38 and JNK) activation. Our purpose was to determine whether IKAP downregulation alters MAPK activation in response to the growth factor EGF or to the pro-inflammatory cytokine TNF-α.

HeLa cells were infected with the lentivirus delivering small interfering RNA targeting either the IKAP transcript (IKAP shRNA) or the GFP transcript (GFP shRNA) and treated with either TNF-α or EGF for 5 to 60 minutes. The lower panels in figures 28A and 28B (on the left) show a dramatic decrease of IKAP expression after IKAP shRNA, but not GFP shRNA expression in HeLa cells. The activation of Erk and p38 was monitored by Western analysis using the relevant phospho-specific antibodies, whereas JNK activation was assessed by *in vitro* kinase assays, testing the ability of anti-JNK immunoprecipitates to phosphorylate a GST-c-Jun substrate. As shown in figures 28A and 28B (on the left), the decreased IKAP expression had no effect on TNF-α or EGF-mediated Erk, p38 or JNK activation. Likewise, the kinetics of IκBα degradation in response to a TNF-α stimulation, which reflects IKK and subsequent NF-κB activation (Hayden and Ghosh, 2004), was also unaffected by IKAP RNAi, as judged by an anti-IκBα Western blot (figure 28A, fifth panel from the top, on the left).

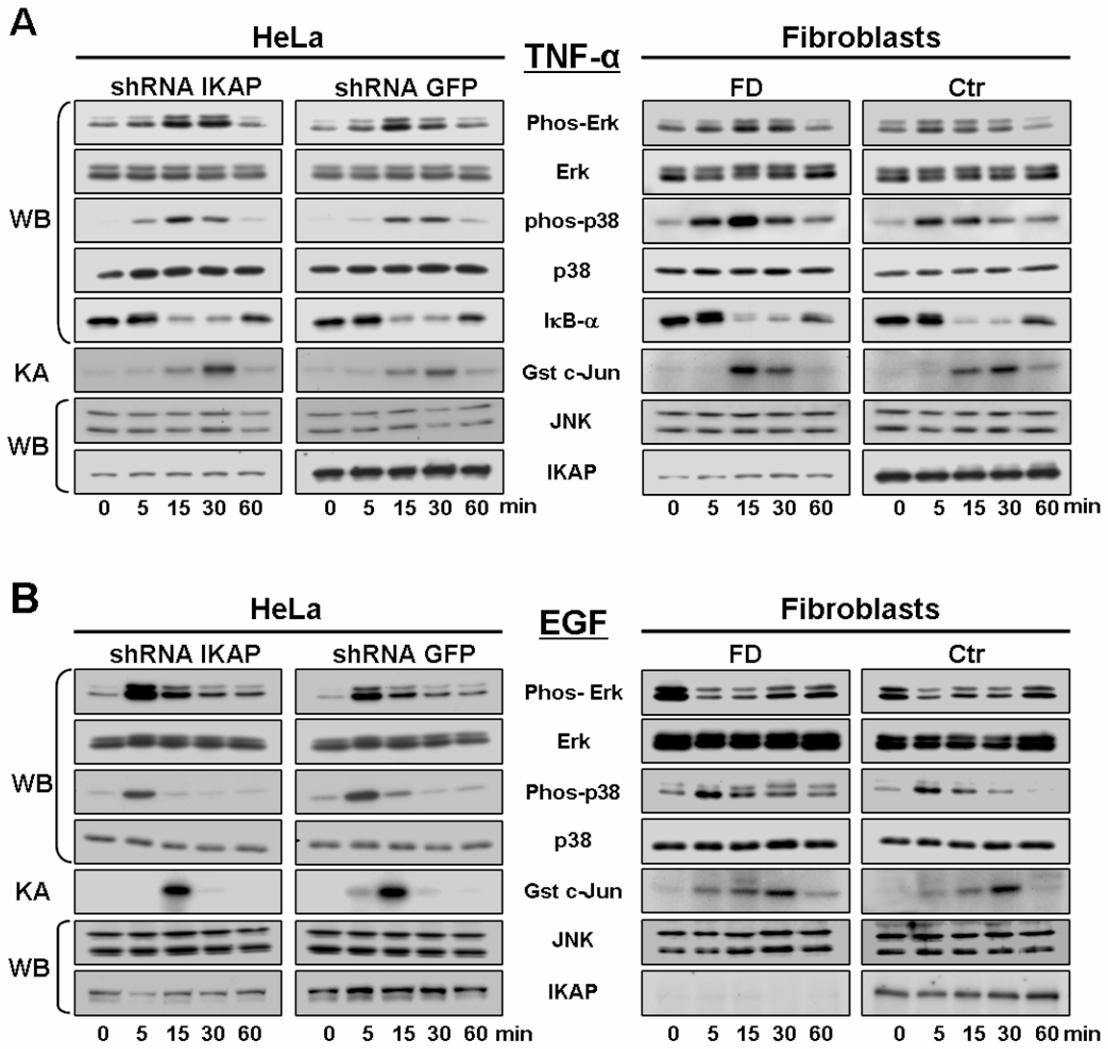

Figure 28. Decreased IKAP expression has no effect on TNF-α or EGF mediated Erk, p38 or JNK activation.

(A) TNF-α (1ng/ml) induced activation of Erk, p38 and JNK was assessed in IKAP-depleted cells, either in HeLa cells infected with IKAP shRNA (shRNA IKAP) or GFP shRNA (shRNA GFP) lentivirus or in FD (FD) and control (Ctr) fibroblasts. Cells were treated with TNF-α for 5 to 60 minutes and Erk and p38 activation was monitored by Western blot analysis using the relevant phospho-specific antibodies, whereas JNK activation was assessed by in vitro kinase assays testing the ability of anti-JNK inmmunoprecipitates to phosphorylate a GST-c-Jun substrate. The non-phosphorylated forms of Erk, p38 and JNK were simultaneously detected as normalization. Likewise, the kinetics of TNF-α-induced IκB-α degradation was monitored by Western blot analysis using an anti-IκB-α antibody.
(B) as in (A) treating the cells with EGF (50ng/ml). Cells were placed for 24 hours in a serum free medium before EGF stimulation. WB= Western blot; KA= kinase assay.

To determine whether TNF-α or EGF-induced MAPK activation might be impaired in FD patients, similar experiments were conducted in either normal fibroblasts (Ctr) or fibroblasts derived from FD patients (FD). Significantly, although FD fibroblasts indeed expressed lower amounts of IKAP (lower panels in figure 28A and 28B, on the right), they did not display altered Erk, p38 and JNK activation kinetics in response to TNF-α and EGF (figure 28A and 28B, on the right). Finally,

EGF-mediated Akt activation was unchanged in IKAP shRNA versus GFP shRNA HeLa cells as judged by an anti-phospho-serine 473 Akt Western blot (figure 29).

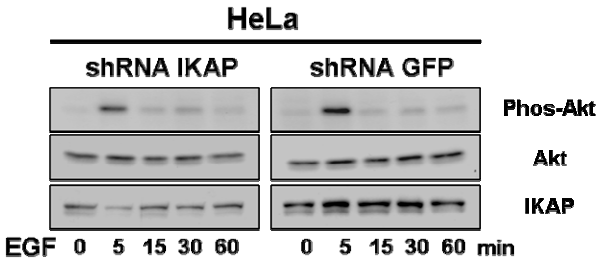

Figure 29. Decreased IKAP expression has no effect on EGF-induced Akt activation.

EGF (50ng/ml) induced phosphorylation of Akt was assessed in HeLa cells infected with IKAP shRNA (shRNA IKAP) or GFP shRNA (shRNA GFP) lentivirus. Cells were treated with EGF for 5 to 60 minutes and Akt phosphorylation on serine 473 was monitored by Western blot analysis using an anti-phosphoSer473-Akt antibody. The non-phosphorylated Akt was simultaneously detected as normalization. Cells were placed for 24 hours in a serum free medium before EGF stimulation.

Those results indicate that, in these cells, EGF and TNF-α–induced MAPK activation and TNF-α-induced NF-κB signalling pathways can proceed through IKAP-independent mechanisms.

2-3- Identification of genes regulated by IKAP

Previous experiments mostly performed in yeast strongly suggested a role for IKAP/Elongator in transcriptional elongation. In order to determine whether this conclusion can be extended to human cells, and also to identify the genes whose transcription requires IKAP, we took advantage of our cellular loss-of-function model for IKAP. We extracted total RNA from untransfected HeLa cells or transfected with siRNAs that target either the IKAP transcript or the GFP transcript (as shown in figure 24). The transcriptome of these cells was then analyzed by DNA microarray experiments. This technology is widely used for studying gene expression regulation and gives a rapid and dependable gene expression profile of the cells. Comparing the profiles observed in different conditions, one can easily identify the genes up- or down-regulated upon IKAP depletion.

Furthermore, to evaluate the implication of IKAP in the transcription of genes activated after TNF-α stimulation (through MAP Kinases activation for example) and to identify genes whose TNF-α-induced expression requires IKAP, untransfected HeLa cells or transfected with IKAP or GFP siRNAs were stimulated for 1 hour by TNF-α (1ng/ml). Total RNA was then extracted from those treated cells and also analyzed by DNA microarray experiments.

As a control for the relevance of our microarray results, the two untransfected conditions treated or not with TNF-α were first compared to see whether known TNF-α target genes were indeed induced upon TNF-α stimulation. The data represented in table 2 show some examples of genes strongly induced by TNF-α in our experiments. They include IL-8, IL-6, IL-1α, ICAM-1, TNFAIP-2, TNFAIP-3, TNFAIP-6 and TNF-α itself.

Table 2. Genes induced after one hour of TNF-α stimulation (1ng/ml) in HeLa cells.

Gene	Fold induction	p-value	Description
IL-8	42.2	$2\ 10^{-5}$	Interleukin 8
TNF-a	13.0	$2\ 10^{-5}$	Tumor necrosis factor alpha
IL-6	12.1	$2\ 10^{-5}$	Interleukin 6
TNFAIP-2	8.0	$2\ 10^{-5}$	TNF alpha induced protein 2
TNFAIP-6	4.2	$5\ 10^{-5}$	TNF alpha induced protein 6
IL-1a	4.0	$2\ 10^{-5}$	Interleukin 1 alpha
ICAM-1	2.8	$2\ 10^{-4}$	intracellular cell adhesion molecule 1
TNFAIP-3	2.5	$2\ 10^{-5}$	TNF-alpha induced protein 3

These genes were indeed previously reported as induced upon stimulation by this pro-inflammatory cytokine. Therefore, those results validate our microarray experiments.

Looking at the genes differentially expressed after IKAP depletion in comparison to the controls, the expression of about 100 genes was significantly affected as a result of IKAP siRNAs transfection in HeLa cells. Among them, 97 genes were down-regulated more than two fold (some of them are shown in the table 3; for full detailed list, see appendix 1) whereas some 15 genes were induced more than two fold (see table 4).

In the TNF-α stimulated conditions, we could not identify any TNF-α dependent gene whose expression was affected by knocking down IKAP. This suggests that the IKAP depletion has no direct consequence on the expression of genes induced in response to TNF-α.

Table 3. Examples of genes down-regulated more than two fold after IKAP depletion in HeLa cells (a full detailed list is shown in appendix 1).

Cell migration	**Cell proliferation**
CD61	thymidylate synthase
tenascin-C	p57KIP2
paxillin	
gelsolin	**Others**
laminin-β3	beclin-1
calreticulin	hMSH2
MMP-2	BHLHB2
swap-70	smad2
serpine-1	rab23
caveolin-1	rab31
CD151	COP9 homolog
S100A2	IL-7 receptor
synaptopodin	prohibitin-2
radixin	S100A3

Interestingly, among the genes down-regulated upon IKAP depletion, a significant proportion codes for proteins involved in cell motility, cytoskeleton remodelling as well as cell adhesion (table 3). For example, it concerns those coding for the integrin receptor CD61 (Wehrle-Haller and Imhof, 2002), the ligand tenascin-C (Midwood and Schwarzbauer, 2002), actin cytoskeleton modulators gelsolin (Cooper and Schafer, 2000), paxillin (Webb et al., 2003), caveolin-1 (Navarro et al., 2004) and SWAP-70 (Sivalenka and Jessberger, 2004). Genes coding for proteins involved in cell proliferation, such as thymidylate synthase (Derenzini et al., 2002) and the cyclin-dependent kinase inhibitor p57 Kip2 (Dyer and Cepko, 2001), 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 down-regulated as well (see table 3 and appendix 1).

Among the 15 genes induced by IKAP RNAi, many code for proteins involved in amino-acid metabolism and protein biosynthesis. This is the case for the phosphoserine aminotransferase, the asparagine synthase, the cystathionase, the methionine tRNA synthase, for example (see table 4).

Table 4. Genes induced more than two fold upon IKAP depletion in HeLa cells.

	Gene	Fold induction	p-value	Description
Metabolism	ASNS	4.0	$2\ 10^{-5}$	Asparagine synthase
	PSAT	2.6	$2\ 10^{-5}$	Phosphoserine aminotransferase 1
	ZPF-36	2.1	$5\ 10^{-5}$	Zinc finger protein 36, C3H type, homolog (mouse)
	CARS	2.1	$2\ 10^{-5}$	Cysteinyl tRNA synthase
	CTH	2.0	$2\ 10^{-4}$	Cystathionase (cystathionine gamma lyase)
	MARS	2.0	$2\ 10^{-5}$	Methionine-tRNA synthase
	NNMT	2.0	$2\ 10^{-5}$	Nicotinamide N-methyltransferase
	PHGDH	2.0	$2\ 10^{-5}$	Phosphoglycerate deshydrogenase
Transcription factor	EGR-2	2.1	$2\ 10^{-4}$	Early growth response 2
Apoptosis	COM-1	2.0	$2\ 10^{-5}$	p8 protein
Receptor	VLDLR	2.0	$8\ 10^{-5}$	Very low density lipoprotein receptor
Others	S100P	2.8	$2\ 10^{-5}$	S100 calcium binding protein P
	TRIB-3	2.3	$2\ 10^{-5}$	Tribbles homolog 3 (Drosophila)
	TMED-5	2.0	$2\ 10^{-5}$	Transmembrane emp24 protein transport domain containing 5
	HSPA4L	2.0	$2\ 10^{-5}$	Heat shock 70kDa protein 4-like

2-4- Confirmation of our microarray data by quantitative RT-PCR and Western blotting

We next examined the validity of our microarray results by performing quantitative RT-PCR with total mRNA extracted from untransfected HeLa cells or from cells transfected with either IKAP siRNAs or GFP siRNAs. Hereby, the decreased expression of most IKAP target genes identify by DNA microarray experiments was confirmed. In figure 30, graphs show the specific decrease of expression of tenascin-C, CD61, paxillin, gelsolin, swap-70, laminin β-3, calreticulin, CD151 and beclin-1 in IKAP RNAi cells as compared with the two control conditions (untransfected cells and GFP RNAi cells). The results are shown as a relative comparison, as the gene expression levels in untransfected condition were arbitrary

set to 100, and the values obtained from other conditions expressed relatively to that. The expression of the housekeeping gene β2-microglobulin was detected simultaneously and used for normalization. The expression of an unaffected gene, the β-glucuronidase (GUS), is also reported as control.

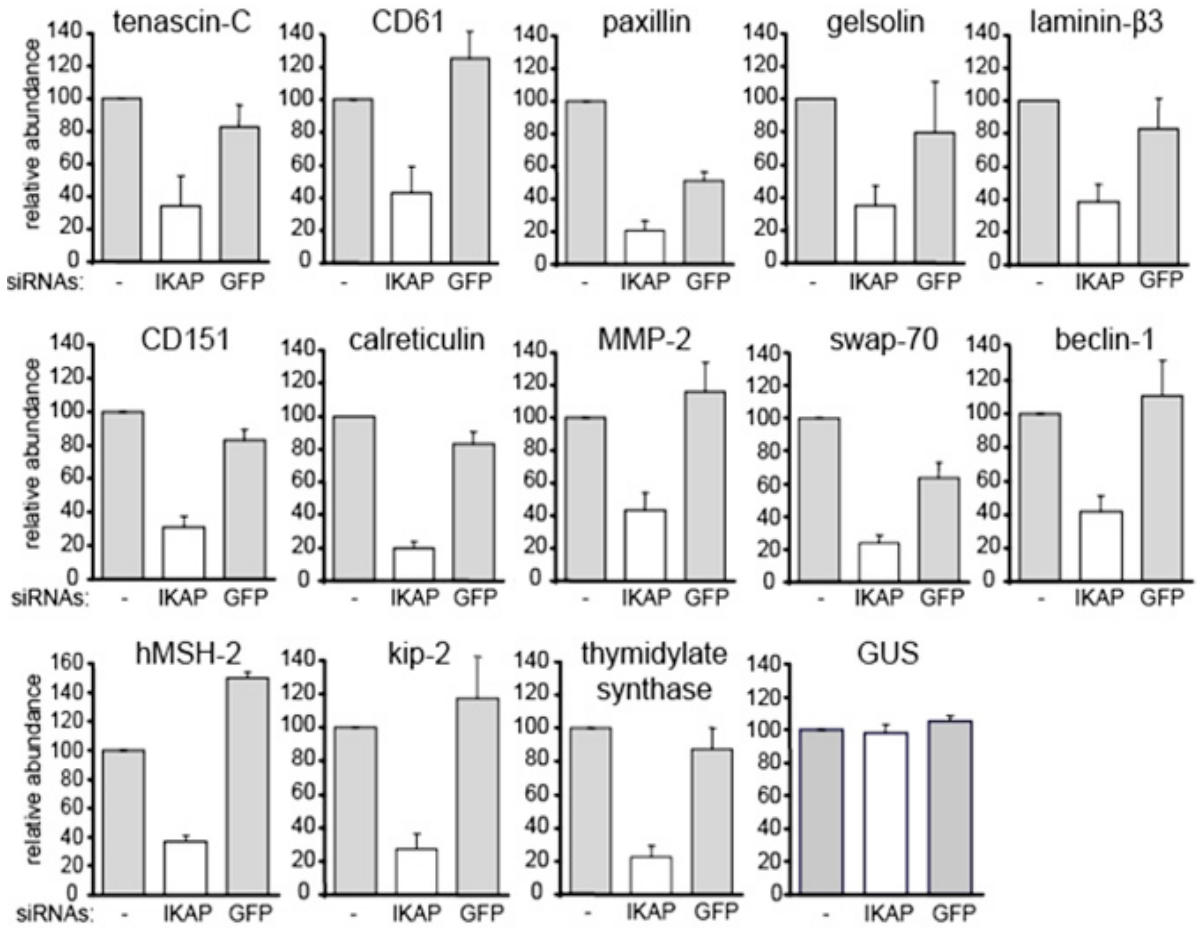

Figure 30. Decreased expression of a subset of genes in HeLa cells transfected with IKAP siRNAs.

Specific decrease of expression of a subset of genes observed by quantitative RT-PCR analysis after transfection of IKAP siRNAs in HeLa cells (IKAP), as compared with untransfected cells (-) or cells transfected with the control GFP siRNAs (GFP). Expression in untransfected cells was set to 100. β-glucuronidase (GUS) expression is shown as control.

Moreover, specific reduced expression of paxillin and calreticulin was also confirmed at the protein level in these cells, as shown in figure 31.

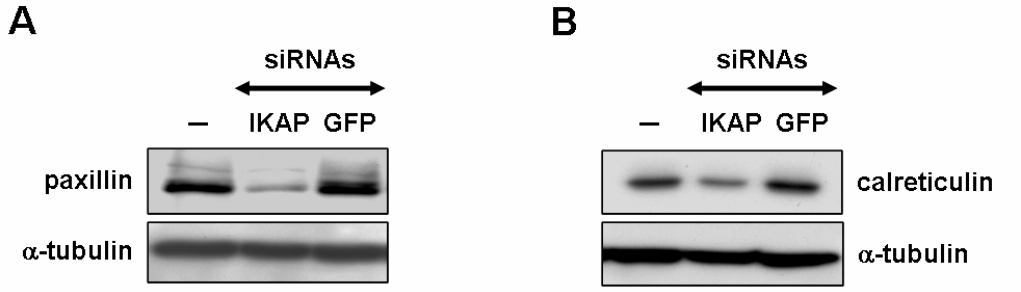

Figure 31. Decreased paxillin and calreticulin proteins expression in HeLa cells upon IKAP depletion.

Specific decrease of the expression of paxillin (A) and calreticulin (B) proteins observed by Western blot analysis after transfection of IKAP siRNAs in HeLa cells, as compared with untransfected cells or cells transfected with the control GFP siRNAs. An anti-α-tubulin Western blot is shown as loading control.

In order to investigate the physiological importance and possible disease relevance of the observed effect of IKAP RNAi on transcription, total RNA was also extracted from FD patient-derived fibroblasts and subjected to quantitative real-time PCR analysis. In support of the results obtained with RNAi-transfected cells, decreased expression of gelsolin, calreticulin, laminin-β3 and beclin-1 was also observed in FD fibroblasts in comparison with control fibroblasts (figure 32). Interestingly, some cell type specificity in the expression patterns might occur since the expression of several genes such as tenascin-C and MMP-2 did not appear to be significantly altered in FD fibroblasts.

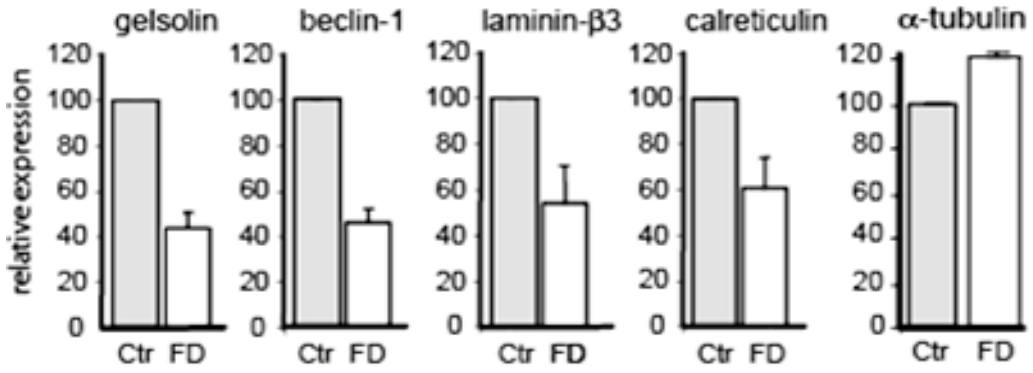

Figure 32. Expression of a subset of genes in FD fibroblasts as compared to control fibroblasts.

The expression of gelsolin, beclin-1, laminin-β3 and calreticulin mRNA in FD fibroblasts as compared to the corresponding control was measured by quantitative RT-PCR analysis. Expression in control fibroblasts was set to 100. α-tubulin expression is shown as control.

In fibroblasts, decreased expression of gelsolin 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 therefore investigated the RAC expression in FD fibroblasts versus control fibroblasts by Western blot. As shown in figure 33, we observed a slight increase in RAC expression in FD fibroblasts.

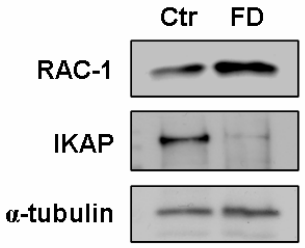

Figure 33. Expression of RAC-1 in FD fibroblasts.

Expression of RAC-1 in FD fibroblasts examined by Western blot analysis, probing with the antibodies is indicated on the left. An anti-α-tubulin Western blot is shown as loading control.

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

2-5- A role for IKAP in cell migration

The gene expression data described in previous sections suggest that IKAP depletion leads to lower levels of expression of numerous genes, including several genes implicated in cell motility (see table 3, page 66). Because normal cell motility is of crucial importance for the developing peripheral nervous system (reviewed by (Dent and Gertler, 2003; Gammill and Bronner-Fraser, 2003)) and therefore of obvious relevance to FD, we characterized the potential role of IKAP in cell motility.

IKAP shRNA and control GFP shRNA HeLa 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 in the gap left by physical disruption of cell monolayers (West et al., 2001). The figure 34 shows a significant delay in complete 'wound closure' in IKAP shRNA HeLa cells as compared to control cells. Whereas complete closure was observed after 22 hours with the control cells, gaps in the cell monolayers remained open in the IKAP shRNA cells.

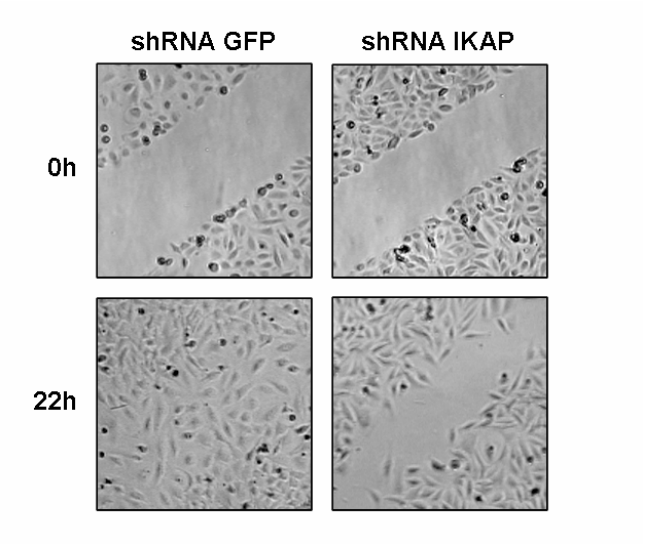

Figure 34. Cell migration defect in IKAP RNAi HeLa cells as seen by wound healing assay.

Wound Healing assay performed with IKAP shRNA (shRNA IKAP) or GFP shRNA (shRNA GFP) HeLa cells (right and left, respectively). Pictures were taken at the indicated times after wounding. Cell proliferation was inhibited by pre-treating the cells 2 hours with mitomycin C (1µg/µl).

Next, the ability of IKAP shRNA HeLa cells to migrate in response to a serum gradient was investigated with a chemotaxis assay using Boyden chambers (Riedy et al., 1999). A serum gradient has been obtained by filling up the insert with serum free medium and the well with medium supplemented with 10% of serum, the two compartments being separated by an 8 µm pore membrane. Cells having migrated through the membrane following the serum gradient were fixed, colored and counted (for principle, see figure 35).

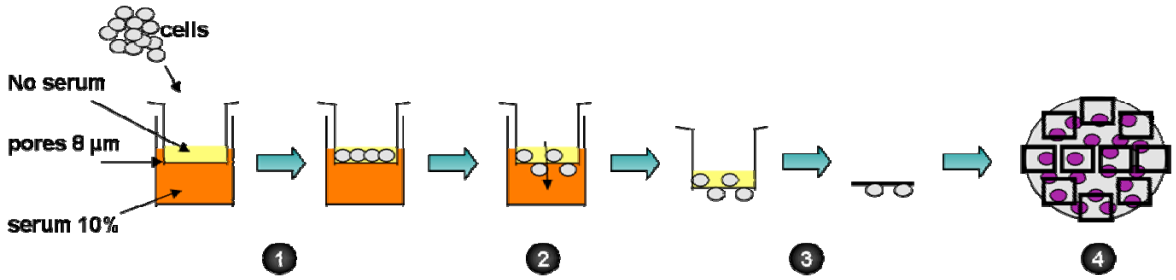

Figure 35. Principle of chemotaxis assay using boyden chamber.

***Step1:** cells are cultured in serum free medium and put in the insert. The wells are filled up with medium supplemented with 10% FBS. The two compartments are separated by an 8µm pores membrane. **Step2:** cells that migrate pass through the membrane following the serum gradient. **Step3 and 4:** cells that have migrated to the lower membrane are isolated, colored, systematically photographed (10 pictures/membranes) and counted.*

As shown in the graph of figure 36, a clear defect in cell migration through serum gradient was observed in IKAP shRNA HeLa cells compared to GFP shRNA HeLa cells.

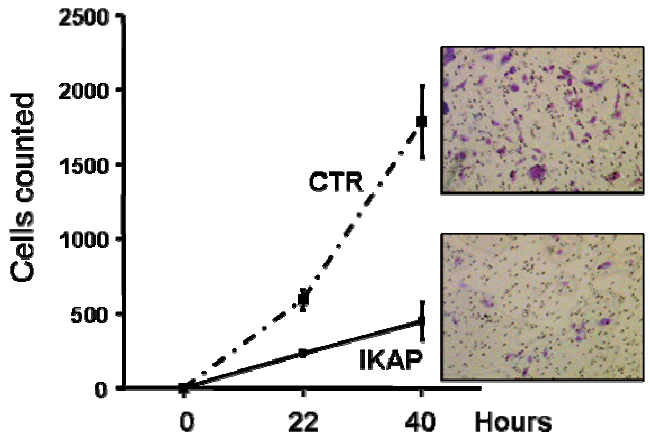

Figure 36. Cell migration defect in IKAP RNAi HeLa cells as seen by chemotaxis assay.

Chemotaxis assays using boyden chambers on serum gradient carried out with GFP shRNA (CTR) or IKAP shRNA (IKAP) HeLa cells. Cells migrating to the lower membrane were counted. The figure shows the total number of migrating cells counted after the indicated time. A representative picture is shown for illustration.

To further correlate the observed migration defects with decreased IKAP expression and FD, wound healing assays were also performed with two distinct cell lines from FD patients (FD), and the corresponding control fibroblasts (CTR). While significant closure of the gaps in cell monolayers had occurred after 24 hours with control fibroblasts, a clear defect in gap closure by cell migration was still observed with the FD fibroblasts (figure 37).

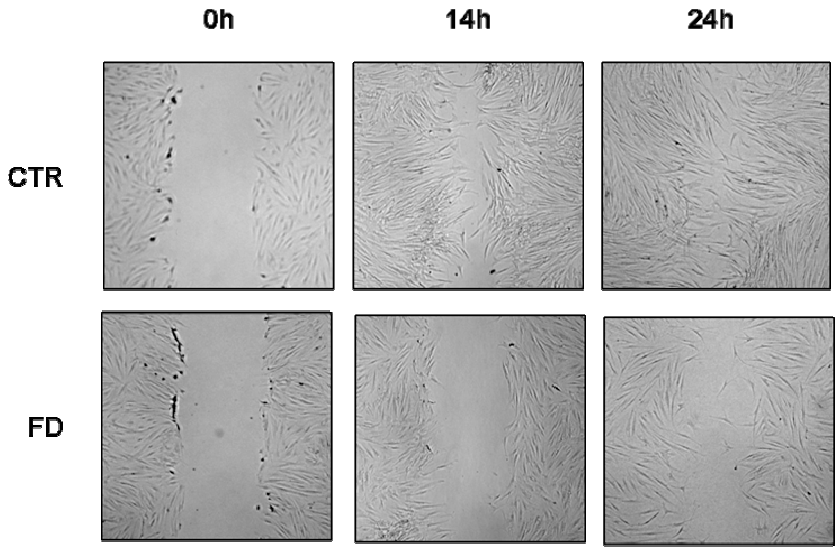

Figure 37. Cell migration defect in FD cells as seen by wound healing assay.

Wound Healing assay performed with FD (FD) or control (CTR) cells (down and up, respectively). Pictures were taken at the indicated times after wounding. Cell proliferation was inhibited by pre-treating the cells 2 hours with mitomycin C (1µg/µl). Experiments were done using 2 different FD cell lines and their corresponding control.

Fibroblast cell motility 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 performed of such experiments to further characterize the cell motility defects in FD fibroblasts. After a 10 day period, with 1 mg/ml collagen gels, we observed that control fibroblasts (CTR) were significantly more potent than their FD (FD) counterpart in contracting collagen gels (figure 38), indicating that the ability of the mutant cells to spread and elongate is significantly hampered. Indeed, to properly contract the collagen gel, the cytoskeleton of the cells has to be remodeled continuously, in order to rearrange the surrounding extracellular matrix.

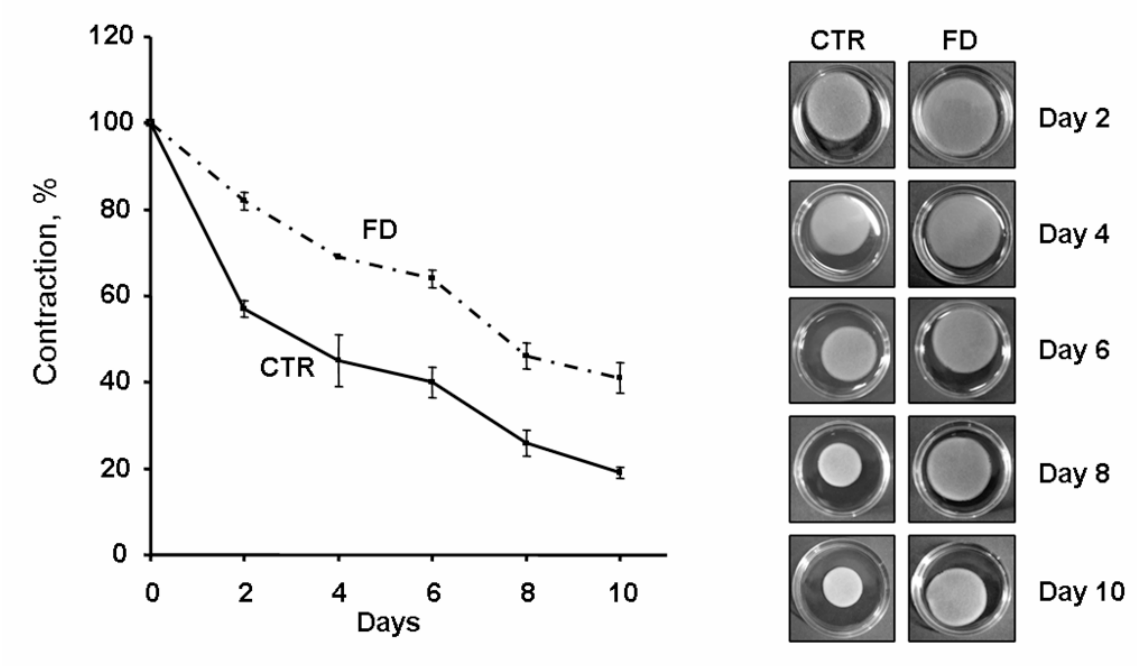

Figure 38. Collagen gel contraction assay using FD fibroblasts.

Contraction of free-floating collagen lattices (1mg/ml) seeded with either control (CTR) or FD (FD) fibroblasts. Photographs taken during the course of a representative experiment are shown on the right. Graphs represent the percentage of contraction during the time of the experiment.

Similar results were obtained with less concentrated collagen gel (0.3mg/ml) over period of 20 hours (figure 39). Therefore, decreased IKAP expression correlates with a cell migration defect in fibroblasts isolated from FD patients.

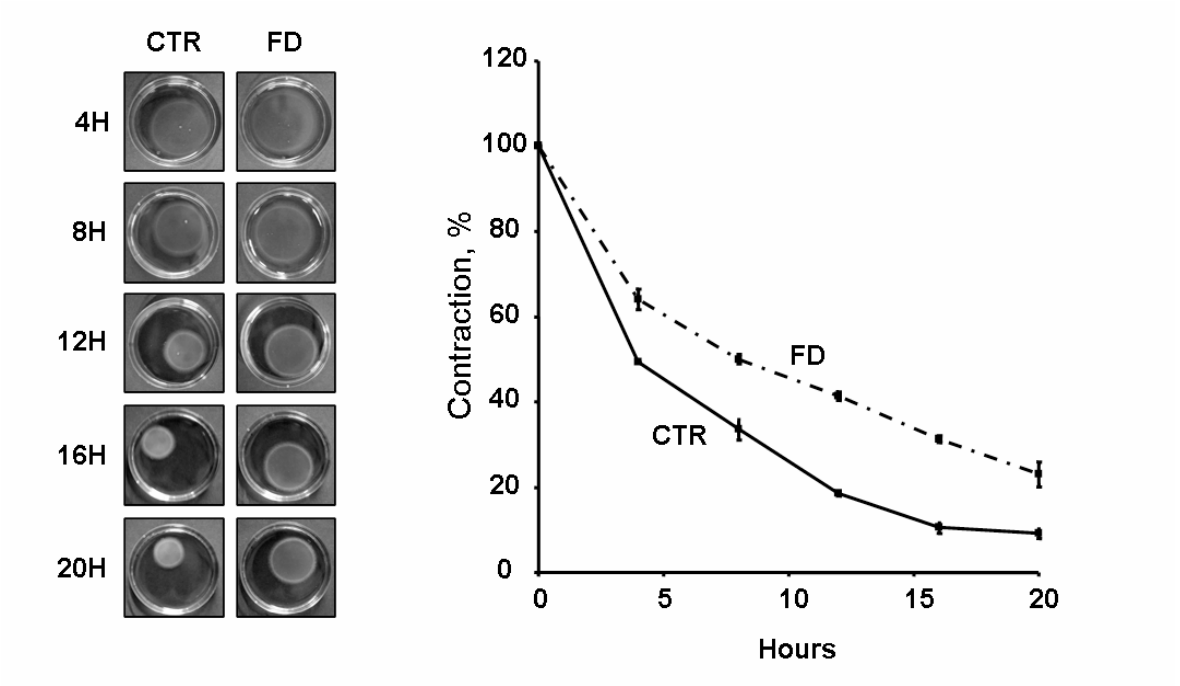

Figure 39. Collagen gel contraction assay using FD fibroblasts.

Contraction of free-floating collagen lattices (0.3mg/ml) seeded with either control (CTR) or FD (FD) fibroblasts. Photographs taken during the course of a representative experiment are shown on the right. Graphs represent the percentage of contraction during the time of the experiment.

Because FD mainly affects the population of neurons in autonomic and sensory ganglions, and because these neurons migrate from the neural crest to the ganglions during the development, we assessed cell migration in IKAP-depleted neuron-derived cell lines. Neuroblastoma-derived SK-N-BE cells and the glioblastoma-derived U-373 cells were infected with the IKAP or GFP shRNA viral constructs (Western blot, see figure 26, page 61). The infected cells were then plated on fibronectin-coated plates and subjected to wound healing assays. Control SK-N-BE cells had filled in the wound area after 36 hours, whereas the SK-N-BE expressing lower amounts of IKAP were significantly retarded in their ability to close the wound (figure 40, on the left). A similar cell migration delay was also obtained using IKAP-depleted glioblastoma-derived U-373 cells after 26 hours (figure 40, on the right).

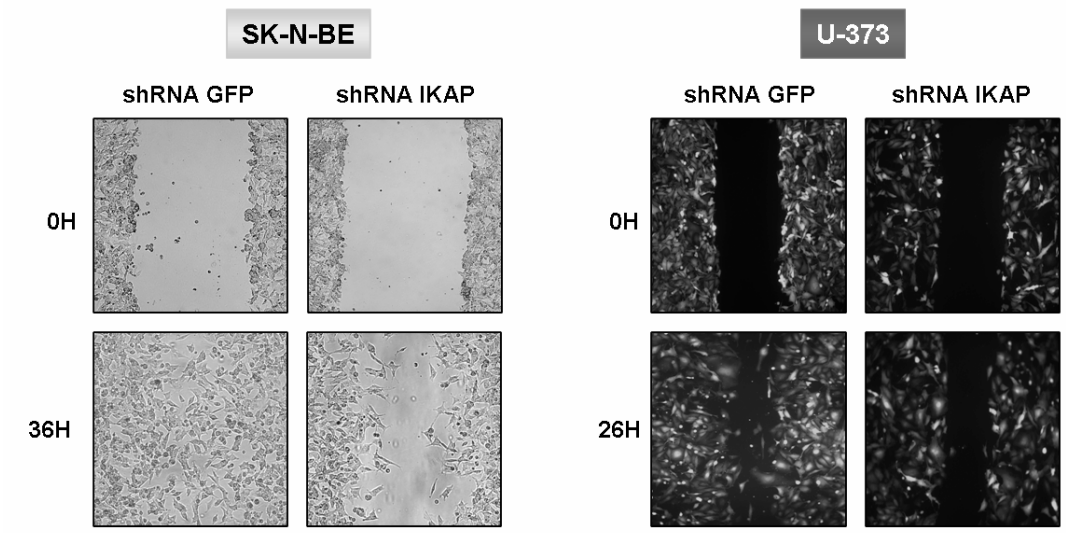

Figure 40. Cell migration defect in IKAP RNAi SK-N-BE and U-373 cells as seen by wound healing assay.

Wound healing assays performed with GFP shRNA (shRNA GFP) or IKAP shRNA (shRNA IKAP) SK-N-BE (on the left) or U-373 cells (on the right) plated on fibronectin coated plates. Pictures were taken at the indicated times after wounding. Cell proliferation was inhibited by pre-treating the cells 2 hours with mitomycin C (1µg/µl).

Moreover cell motility defects in serum gradient were observed in SK-N-BE and in U-373 IKAP-depleted cells as well, as judged by the data generated by the chemotaxis assays using boyden chambers (figure 41).

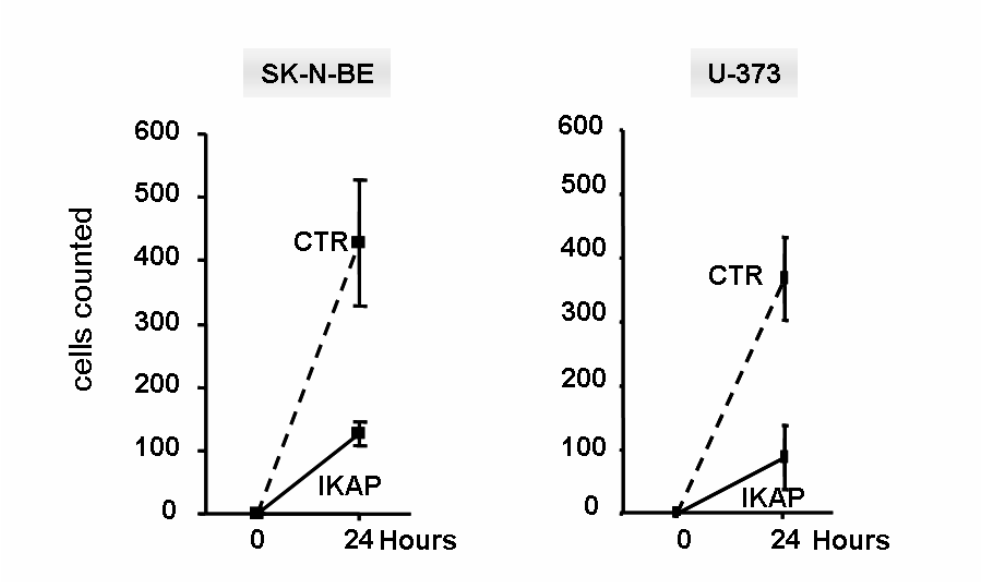

Figure 41. Cell migration defect in IKAP RNAi SK-N-BE and U-373 cells as seen by chemotaxis assay.

Chemotaxis assays using boyden chambers on serum gradient carried out with GFP shRNA (CTR) or IKAP shRNA (IKAP) SK-N-BE (on the left) or U-373 cells (on the right). Cells migrating to the lower membrane were counted. The figure shows the total number of migrating cells counted after the indicated time.

Taken together, these functional assays strongly suggest that the transcription defect in IKAP RNAi cells has cell functional consequences, as various cell types with lower levels of IKAP have significantly reduced cell motility. In particular, the reduced motility of neuron-derived cell lines may be highly relevant to the neurodevelopmental disorder of FD patients.

3- Discussion

In this first part, we investigated the role of IKAP in gene expression and its function at a cellular level by generating a cellular loss-of-function model for the protein IKAP by RNA interference, either by transiently transfecting specific siRNAs into cells or by infecting the cells with a lentiviral construct which expresses specific shRNAs.

As a first step in deciphering the role of IKAP in gene transcription, we identified the IKAP target genes by microarray experiments. We demonstrated that IKAP regulates the expression of several genes whose products are involved in various cellular functions. The most obviously affected cellular function was the cell motility/migration and the actin cytoskeleton remodelling, but our experiment also suggested that IKAP could also be involved in other processes such as cell proliferation, autophagy cell death, DNA repair and metabolism. It is worth pointing out that the gene expression profile is highly dependent upon the IKAP levels, in other words, upon the RNAi efficiency. The example of the thymidylate synthase (TS) illustrates nicely this issue as an efficient IKAP decrease leads to lower expression of TS mRNA, but this is not true if the RNAi efficiency is not optimal. Likewise, cell specificity might occur as some target genes were not confirmed in testing different cell types.

It is essential to emphasize that although RNAi and the FD mutation affect IKAP levels, there are still residual amounts of the protein in these cells. We believe this residual amount may be important for the transcription of a larger number of genes, as well as for the cellular viability. Indeed, the *ELP1/IKBKAP* gene as well as the *ELP3* gene are essential in *Drosophila melanogaster*, and loss-of-function mutants die with a remarkably similar terminal phenotype (Jane Walker and Jesper Svejstrup, unpublished data; James Gusella, personal communication).

We next confirmed that the transcription defects observed in IKAP-depleted cells have consequences at the cellular level. As judged by different assays, the migration of four different cell lines, including neuron-derived cell lines and FD fibroblasts, is considerably affected by the IKAP depletion. FD is a neurodevelopmental and neurodegenerative genetic disorder with severe

pathological consequences. IKAP mutation affects the development and maintenance of neurons in peripheral ganglions, resulting in neuropathological and clinical progression. All peripheral neurons arise from neural crest cells that migrate away from the neural tube and navigate to the location where ganglia will form (reviewed in (Graham, 2003; Young et al., 2004). Moreover, cytoskeleton remodelling factors also play a central role in the axon guidance during nerve development (Dent and Gertler, 2003). Our results suggest an intriguing model to explain FD at a molecular level: the mutation in the gene encoding IKAP results in neuronal-specific decrease in the ability of cells to migrate, which in turn leads to neurodevelopmental abnormalities and neuropathology of FD patients. Obviously, other genes that are down-regulated in cells upon IKAP depletion may contribute to the disease as well.

Among the genes that are up-regulated by the absence of IKAP, many of these are described to be involved in amino-acid and protein metabolism. Such an observation has already been done in yeast where the deletion of genes encoding Elongator subunits lead to the up-regulation of genes involved in amino-acid metabolism (Krogan and Greenblatt, 2001). A similar subset of genes has been reported to be co-induced under unfavourable conditions (Jelinsky and Samson, 1999; Natarajan et al., 2001). According to Krogan and Greenblatt, Elongator gene deletions seem to cause problems for the cells, resulting in the up-regulation of this particular group of stress-induced genes (Krogan and Greenblatt, 2001).

Several models for the function of IKAP have been proposed: Cohen et al. (1998) and Holmberg et al. (2002) suggested that IKAP might be involved in cytoplasmic signalling in the NF-κB and JNK pathways, respectively. If true, this means that a depletion of IKAP would affect the kinetics or the intensity of the activation of these pathways. Moreover, as the two pathways lead *in fine* to the activation of transcription factors and consequently to the transcription of their target genes, gene expression profiles after activation of these pathways should be altered after IKAP depletion. Our results from microarray experiments and from several phosphorylation assays as well as results from others (Krappmann et al., 2000) failed to support an involvement of IKAP in these cytoplasmic transduction pathways. This strongly suggests that IKAP acts mainly in transcription via its scaffold function in the Elongator complex. Furthermore, in yeast, diverse roles of IKAP/ELP1 have been described in the cytoplasm, independently of genes transcription but still in the context of the Elongator complex. For example, ELP1/Elongator has been described as a negative regulator of exocytosis by interaction with the protein Sec2p, a guanine nucleotide exchange factor (GEF) (Rahl et al., 2005). Also, ELP1 and ELP3 might participate in tRNA modification by directly interacting with tRNAs (Sun et al., 2005). In such context, phenotypes of *ELP1-ELP6* mutants could be a consequence of less efficient translation and/or mistranslation due to a lack of modified tRNAs. The name IKAP is therefore a misnomer, and we have suggested that the protein should be designated as human ELP1 (hELP1) instead of IKAP. In the following parts of this work, the IKAP abbreviation will be replaced by IKAP/hELP1.

Part II: Elongator integrity and function is affected by FD mutation

1- Introduction

As described in the first part of this work, cells with reduced levels of IKAP/hELP1 have a decreased expression of several genes and this has consequences on the cell function. Moreover, our results suggest that the role of IKAP/hELP1 in transcription is achieved in the context of Elongator complex. However, precisely how these defects relate to the Elongator complex and its cellular role remained unclear. In this part of the work, we will address this issue and investigate the role of IKAP/hELP1 in the Elongator complex integrity and function.

Elongator complex was initially identified as a component of a hyperphosphorylated RNA polymerase II holoenzyme isolated from budding yeast chromatin (Otero et al., 1999). It is associated with nascent RNA (Gilbert et al., 2004) and several reports have shown by chromatin immunoprecipitation experiments that Elongator is associated with human DNA at the genes (Kouskouti and Talianidis, 2005; Metivier et al., 2003).

2- Results

2-1- IKAP/hELP1 in the Elongator complex integrity and function

Previous experiments performed in the analogous yeast system showed that deletion of *ELP1* gene, the yeast homolog of IKAP/hELP1, leads to the loss of ELP3 at the protein level as a consequence of a substantial destabilization. This suggests that, in yeast, ELP1 protein is required for the integrity of ELP3 and therefore, for the integrity and function of Elongator (Petrakis et al., 2004).

If one extends this observation from yeast to human cells, one would predict that the amount of functional Elongator should also be altered in FD and in IKAP/hELP1 RNAi cells because of their decreased IKAP/hELP1 production. We therefore investigated the level of hELP3 in our IKAP/hELP1 loss-of-function models. As shown in Figure 42A, a very strong decrease of IKAP/hELP1 expression, as observed in IKAP/hELP1 shRNA infected HT29 cells, brings about a nearly total disappearance of the hELP3 subunit. Likewise, in FD cells, hELP3 is expressed at a lower level than in control fibroblasts (figure 42B).

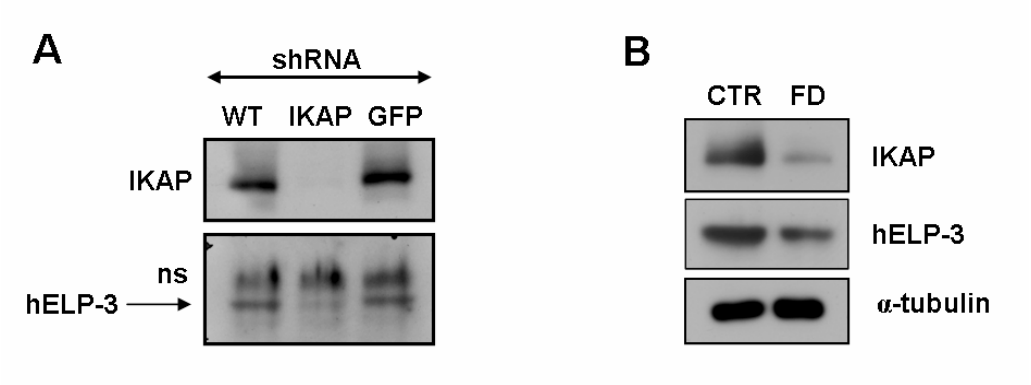

Figure 42. IKAP/hElp1 is required for the integrity of hELP3 protein.

Decreased hELP3 levels in human cells with low IKAP/hELP1 levels. Cells extracts from HT29 cells infected with the indicated shRNA lentivirus (A) or from control and FD cells were subjected to Western blot analysis using an anti-hELP3 antibody. "ns" indicate a non specific band obtained with the anti-ELP3 antibody in HT29 cells. This band serves as a loading control in this experiment. In (B), α-tubulin has been detected for normalization purposes.

This result indicates that, as expected from the yeast data, hELP3 levels are significantly affected by the removal of IKAP/hELP1, suggesting a direct link between IKAP depletion, Elongator loss of expression and transcription defects.

To more directly control this hypothesis, we generated a hELP3 cellular loss-of-function model by transfecting hELP3- or GFP- siRNAs into fibroblasts. Unfortunately, although the hELP3 mRNA level was clearly reduced (figure 43A, second lane), hELP3 RNAi did not lead to an efficient depletion at the protein level (figure 43B, middle).

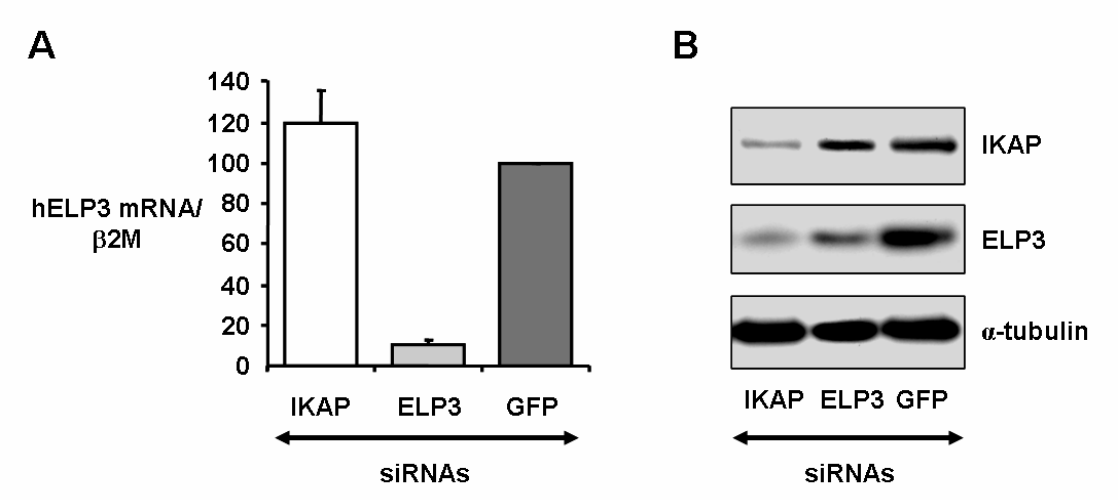

Figure 43. Expression of hELP3 after IKAP/hELP1-, hELP3- or GFP- siRNAs transfection in human fibroblasts.

Fibroblasts were transfected with IKAP/hELP1- (IKAP), hELP3- (ELP3) or GFP- (GFP) siRNAs as indicated and the expression of hELP3 was examined at mRNA level by quantitative RT-PCR (A) or at protein level by Western blot analysis using an anti-hELP3 antibody (B). In (B), α-tubulin has been detected as loading control.

Nevertheless, our quantitative RT-PCR experiments showed that gelsolin and beclin-1 (but not α-tubulin) expression was consistently decreased in hELP3 RNAi fibroblasts; although to a smaller extent than in IKAP RNAi cells (figure 44). This result might be compared to the hELP3 levels shown in figure 43B (middle, second lane), where we clearly observe that hELP3 levels are lower in IKAP RNAi cells than in hELP3 RNAi cells, indicating that the IKAP/hELP1 loss-of-function model is a efficient tool to monitor the Elongator function.

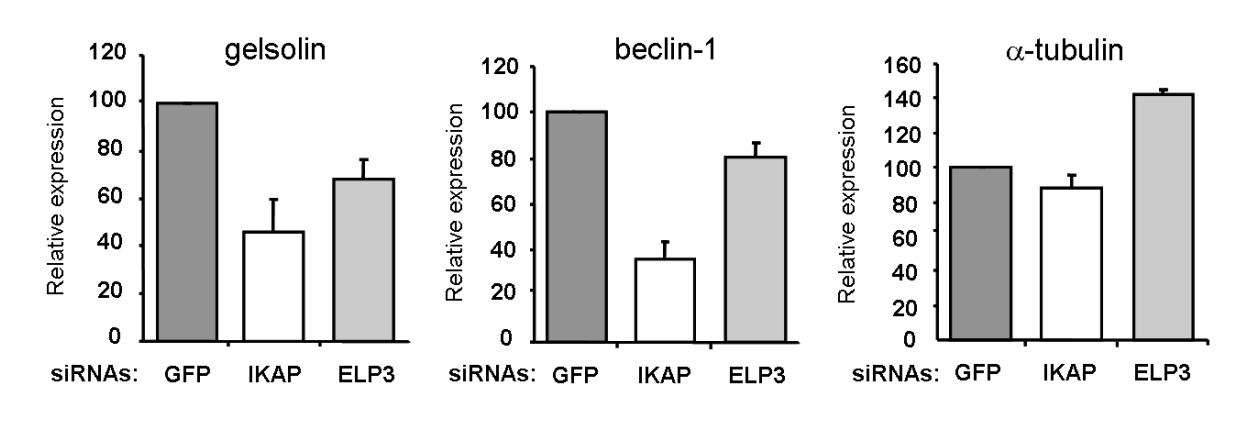

Figure 44. Expression of previously identified IKAP/hELP1-dependent genes in GFP-, IKAP/hELP1-, and hELP3 siRNAs fibroblasts.

Quantitative RT-PCR using primers to amplify the gelsolin, beclin-1, or α-tubulin transcripts was performed by using total RNAs extracted from fibroblasts transfected with GFP-(GFP), IKAP/hELP1-(IKAP) and hELP3- (ELP3) siRNAs.

In conclusion, these results demonstrate that IKAP/hELP1 is essential for the Elongator complex integrity and function through stabilization of its catalytic hELP3 subunit. They also suggest that cell expression deficiencies of two Elongator subunits, namely IKAP/hELP1 and hELP3, have similar consequences for the gene expression. Still, as some IKAP/hELP1-dependent genes were not dramatically affected by ELP3 depletion, both hELP1 and hELP3 might have some independent functions.

2-2- Elongator is recruited at the genes and is removed from DNA by FD mutation

Elongator is detected in both the cytoplasm and nucleus of human cells (Hawkes et al., 2002; Kim et al., 2002; Kouskouti and Talianidis, 2005). Analyzing nuclear and cytoplasmic extracts from normal (CTR) and FD (FD) fibroblasts, we found that, not surprisingly, the IKAP/hELP1 protein is depleted to a similar extent in both compartments in FD cells (figure 45).

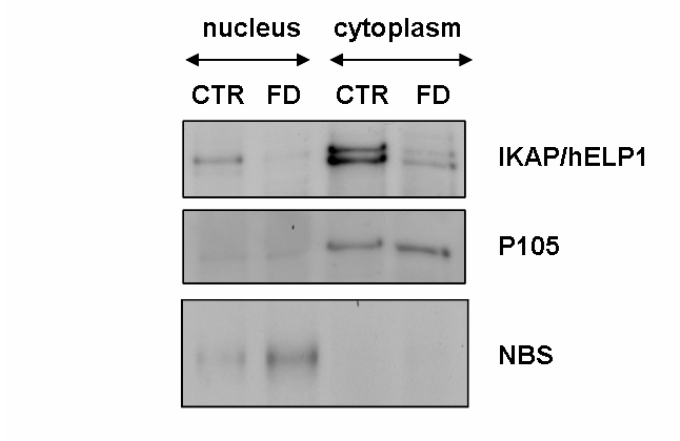

Figure 45. IKAP/hELP1 sub-cellular localization in normal and FD cells.

IKAP/hELP1 is mainly localized in the cytoplasm and is also present in the nucleus. Depletion of IKAP/hELP1 observed in FD cells does not specifically target a sub-cellular pool of IKAP/hELP1, but rather the entire pool. For normalization, antibodies against p105 and NBS (Nijmegen breakage syndrome protein) were used, respectively for cytoplasmic fraction and nuclear fraction.

In theory, the effect of IKAP/hELP1 depletion on gene expression could be either indirect or direct. If the effect is direct, one would expect that Elongator is present at the target genes, and that its absence has a specific effect on their transcription. If the effect is indirect, for example through signalling from the cytoplasm, such effects would not be expected. Previous studies have reported the association of human Elongator with the DNA at the genes. For example, hELP3 and IKAP/hELP1 have been shown to be recruited on three human genes, namely the albumin, HNF-1 and HNF-4 (hepatic nuclear factor) genes, at positions downstream of 2.5 and 5kb after the transcription start site (Kouskouti and Talianidis, 2005). Those two subunits of Elongator were also detected at the pS2 promoter (Metivier et al., 2003).

To address the possibility that the effect of reduced IKAP/hELP1 levels on transcription is direct, standard chromatin-immunoprecipitation (ChIP) technique, in conjunction with quantitative real-time PCR, was used to detect the Elongator complex at genes in normal and FD fibroblasts (for ChIP technique principle, see

figure 46A). The use of FD fibroblasts served as an excellent control for checking the specificity of the antibodies used. Indeed, lower levels of IKAP/hELP1 would be expected to result in significantly decreased IKAP/hELP1 and hELP3 ChIP signals in these cells. We looked at two Elongator's target genes, namely, the gelsolin and the beclin-1 genes, and at one control gene, the α-tubulin gene, whose expression is not affected by IKAP/hELP1 depletion. For those experiments, we designed for each gene, one primer pair in the promoter region (Prom) and other pairs across the transcribed region (TR), starting downstream of the transcription start sites (+500bp) until the end of the genes. The structure of the studied genes and the location of the primers pairs are represented in figure 46B.

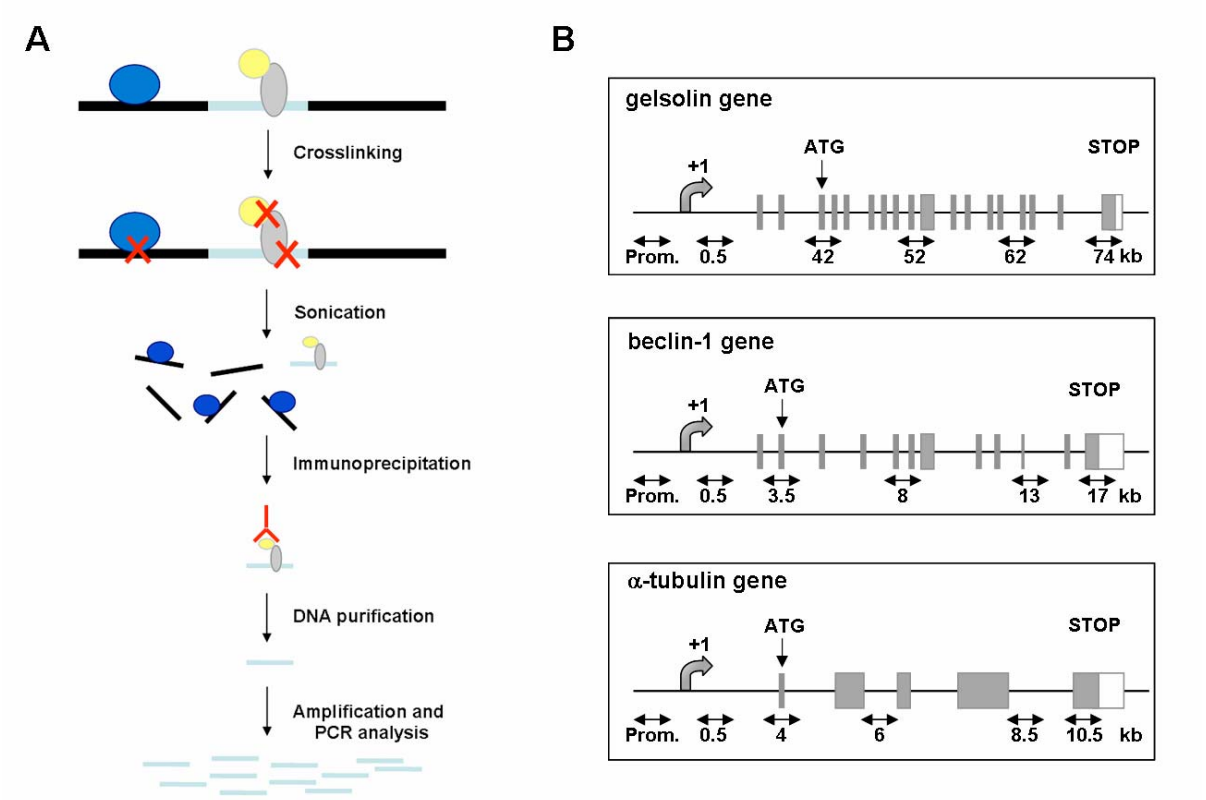

Figure 46. Chromatin immunoprecipitation experiments.

A. *Principle for Chromatin immunoprecipitation experiments. The interactions protein:DNA are crosslinked with paraformaldehyde and DNA is sonicated to generate fragments from 500 to 1000 bp. The protein of interest is immunoprecipitated using the relevant antibody and associated DNA is isolated, purified, and analyzed by PCR.* ***B.*** *Schematic representation of the genes investigated by ChIP experiments. Exons are depicted by boxes and transcription initiation sites (arrows with +1), ORF start codons (ATG) and stop codons (STOP) are also indicated. The localization of primers used for ChIP analysis is illustrated by arrows below. The numbers show the positions of these primers relative to the transcription initiation site.*

In the first ChIP experiment, we looked at the recruitment of IKAP/hELP1 on the gelsolin promoter and transcribed region (primer 62kb, see figure 46B, top panel). As illustrated in figure 47, IKAP/hELP1 was detected in the transcribed region of the gelsolin gene, but not at the promoter.

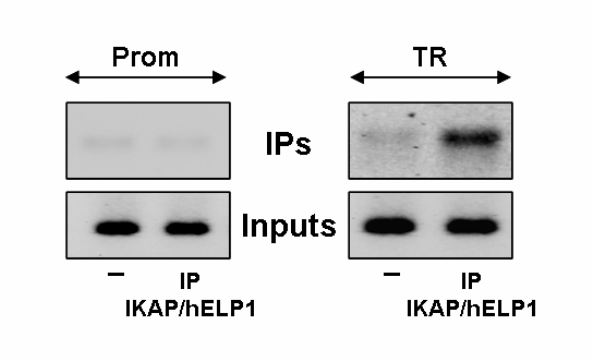

Figure 47. IKAP/hELP1 is recruited at the transcribed region of the gelsolin gene.

ChIP assay with an anti-IKAP/hELP1 antibody was performed with HeLa cells. Associated DNA was analyzed by classical PCR using primer derived from the promoter (Prom) or the transcribed region (TR) of the gelsolin gene (gelsolin TR primer, 62kb).

Interestingly, doing the same experiment on the beclin-1 and α-tubulin genes, we detected IKAP/hELP1 not only at the transcribed region (and to a smaller extent the promoter) of the beclin-1 target gene, but also at the α-tubulin gene although expression is not affected by IKAP/hELP1 depletion (figure 48, see the grey columns). This means that IKAP/hELP1 is present at many or possibly most genes without being required for their transcription.

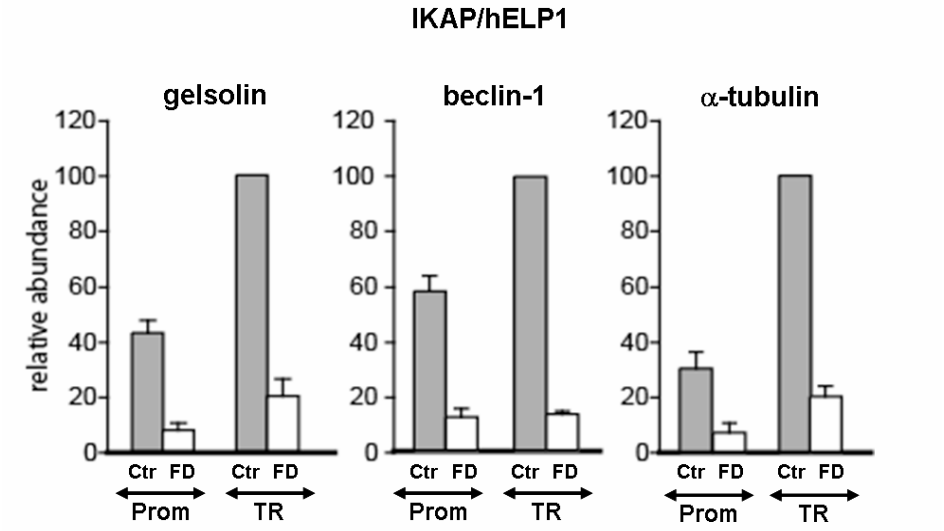

Figure 48. Recruitment of IKAP/hELP1 at target and non-target genes.

ChIP assay using an anti-IKAP/hELP1 antibody were performed with normal (Ctr) or FD (FD) fibroblasts. Associated DNA was analyzed by quantitative RT-PCR using primers derived from the promoter (Prom) and the transcribed region (TR) of the indicated genes (gelsolin TR primer, 62 kb; beclin-1 TR primer, 8kb; α-tubulin TR primer, 6kb). For ease of comparison, IKAP/hELP1 density at the transcribed region in normal fibroblasts (Ctr) was set to 100 and the other values expressed relative to that.

Experiments performed with fibroblasts derived from FD patients showed that the amount of IKAP/hELP1 recruited on the genes was as expected significantly decreased in these cells (figure 48, compare white and grey columns). In agreement

with the idea that IKAP is crucial for Elongator function through the stabilization of the catalytic hELP3 subunit, the recruitment of the hELP3 protein to the transcribed region of the genes was indeed dramatically affected by IKAP/hELP1 depletion (figure 49). Likewise, recruitment of other Elongator subunits, such as hELP4 and hELP5, was impaired as well (figure 49). These results further indicate that recruitment of the whole Elongator complex is affected by the FD mutation.

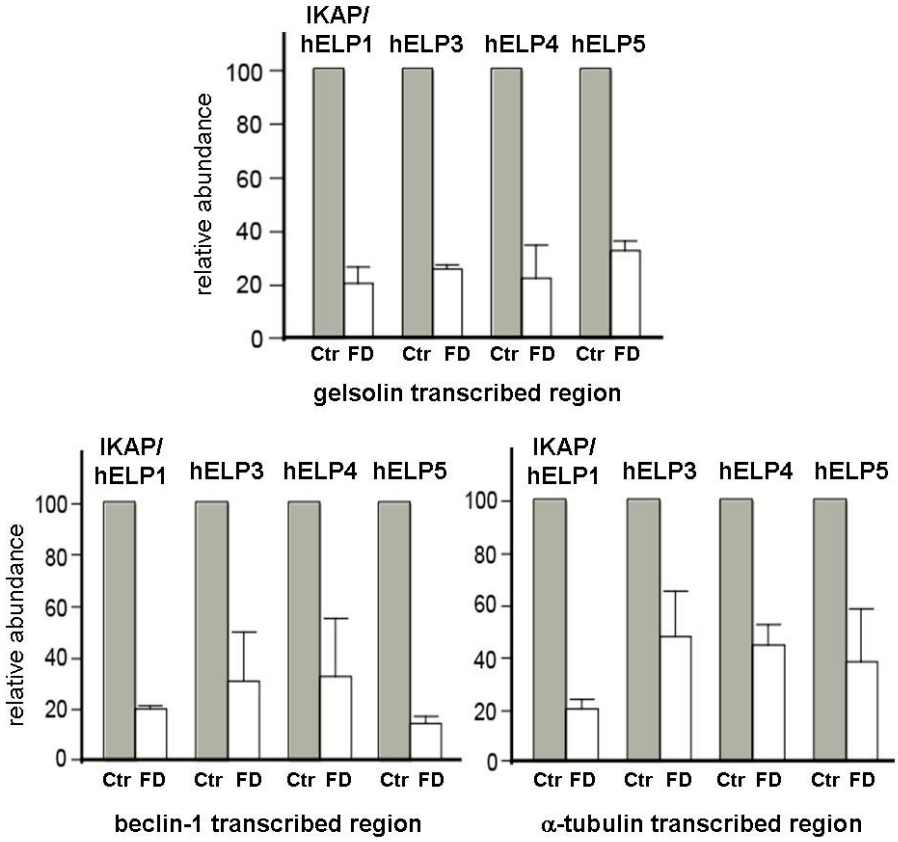

Figure 49. Recruitment of entire Elongator complex is impaired upon IKAP/hELP1 depletion.

ChIP assays with the Elongator antibodies indicated above the graphs were performed using normal (Ctr) or FD (FD) fibroblasts. Associated DNA was analyzed by quantitative RT-PCR using primers derived from the transcribed region of the indicated genes. For ease of comparison, density in normal fibroblasts was set to 100, and the values obtained in FD fibroblasts expressed relative to that.

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. The overall density profile of the two proteins on beclin-1 gene was remarkably similar as seen in figure 50. Elongator subunits are detected mostly in the transcribed region of the genes and, more weakly at the promoter. This further supports the idea that the cellular level and function of the entire Elongator complex is affected by a decreased IKAP/hELP1 expression.

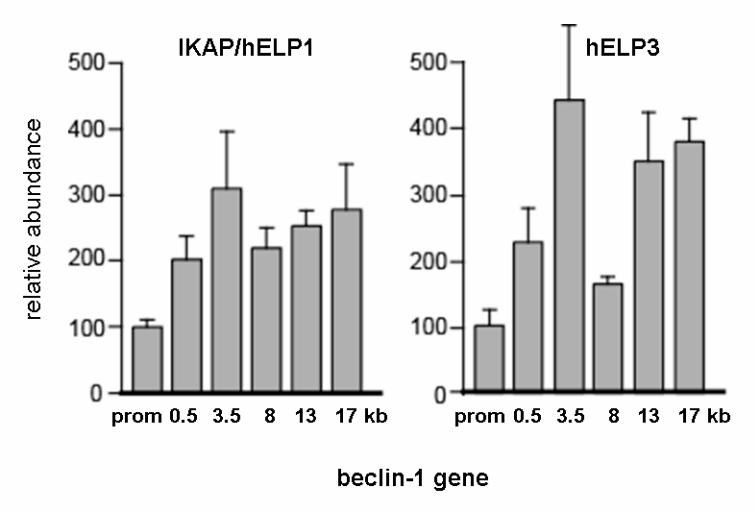

Figure 50. Recruitment of IKAP/hELP1 and hELP3 across the beclin-1 gene.

ChIP assays with the IKAP/hELP1 and hELP3 antibodies were performed by using primers derived from the regions of the beclin-1 gene indicated on the X axis. For ease of comparison, density at the promoter was set to 100 for primer derived from the promoter and the other values expressed relative to that.

3- Discussion

In yeast, ELP1 protein assembles the Elongator complex, which also includes the histone acetyltransferase ELP3 and four additional subunits. The crucial importance of ELP1 for Elongator integrity is underscored by the fact that deletion of *ELP1* gene results in almost undetectable ELP3 levels (Petrakis et al., 2004). Here, we show that IKAP/hELP1 depletion also results in lower hELP3 levels in human cells. Moreover, hELP3 depletion through RNAi also reduces mRNA levels of several IKAP/hELP1 dependent genes, suggesting that IKAP/hELP1 depletion effects were linked to Elongator complex loss-of-function. In yeast, similar conclusion has already been achieved as very similar expression profiles have been obtained after deletion of the various Elongator subunits (Krogan and Greenblatt, 2001).

Surprisingly, we detected Elongator not only on genes whose expression was affected by decreased IKAP/hELP1 levels, but also on the unaffected α-tubulin gene. Other groups have previously detected Elongator on three human genes, but factors such as FACT, CBP, PCAF and SNF2H were only associated with one or two of the studied genes (Kouskouti and Talianidis, 2005). Elongator was also detected at the oestrogen induced pS2 gene (Metivier et al., 2003). Elongator is thus present at many genes, although our expression data suggest that only relatively few are affected by IKAP/Elongator depletion. Interestingly, this finding fits well with recent

data on other chromatin modifying factors. For example, histone acetyltransferases, such as Gcn5 and Esa1, are both generally recruited to promoters of active genes in yeast but only affects a small percentage of them (Robert et al., 2004). Possibly, residual Elongator activity and/or other HATs/chromatin modifiers can probably allow the appropriate expression of IKAP/hELP1 independent genes.

The recruitment of Elongator subunits at human genes, as well as its impairment following IKAP/hELP1 depletion, strongly suggests that the effect of IKAP/hELP1 on gene transcription results from a direct effect of Elongator complex at the studied target genes.

Part III: IKAP/Elongator in transcriptional elongation

1- Introduction

Using FD cells as a cellular model, we have shown that IKAP/hELP1 depletion affects the integrity and function of the whole Elongator complex, leading to the destabilization of the catalytic hELP3 subunit and to the removal of Elongator subunits from the genes. IKAP/Elongator is indeed present at the human genes but in a non-specific manner since we have found Elongator at the α-tubulin control gene.

Elongator was initially identified in hyperphosphorylated RNA polymerase II holoenzyme, which is essentially found during the elongation step of the transcription (Otero et al., 1999). Previous results have shown that transcription is associated with increased acetylation of both histones H3 and H4 (for example, see (Kouskouti and Talianidis, 2005). The histone acetyltransferase activity of Elongator primarily targets histone H3 *in vitro*, and *in vivo* in yeast (Hawkes et al., 2002; Kim et al., 2002; Winkler et al., 2002; Wittschieben et al., 2000). Furthermore, yeast *ELP3* mutation results in decreased histone H3 acetylation levels in chromatin *in vivo* (Kristjuhan et al., 2002; Winkler et al., 2002).

The data showing a direct recruitment of Elongator at human genes suggest that Elongator directly affects genes transcription. In the last part of this work, we sought to investigate whether Elongator depletion would have an effect on the transcriptional elongation in human cells. Elongator being a histone acetyltransferase, we first looked at the acetylation levels in Elongator target genes after IKAP/hELP1 depletion. We further examined the consequences of removing Elongator in term of transcriptional elongation by the RNA polymerase II.

2- Results

2-1- Elongator affects histone H3 acetylation in the transcribed region of the genes

We analyzed the density of various histone modifications in the studied genes by ChIP analysis. In these experiments, the FD cells were used as a tool to investigate the effects of lower IKAP/hELP1 and lower Elongator levels on histone acetylation at the gelsolin, beclin-1 and α-tubulin genes. The structure of the studied

genes and the location of the primer pairs designed across these genes are represented in figure 46B (page 86). For each primer pairs used, the absolute signal obtained from control cells was set to 100 and the signal issued from FD cells is expressed in relative values. The experiments were performed with specific antibodies recognizing acetylated forms of histone H3 on lysine 9 (H3-acK9) or lysine 18 (H3-acK18) and tetra-acetylated form of histone H4 on lysine 5, 8, 12 and 16 (H4-tetraAc).

We first investigated the acetylation density on H3-K9 and H4 at the promoter (Prom) and at the transcribed region (TR) of the studied genes in both control and FD cells. Interestingly, histone H3-K9 acetylation (figure 51A), but not histone H4 acetylation (figure 51B), was strongly reduced in the transcribed region of the gelsolin and beclin-1 genes in FD cells as compared to control cells. Moreover, the histone H3-K9 acetylation at the promoter was barely unaffected by reduced Elongator levels (figure 51A).

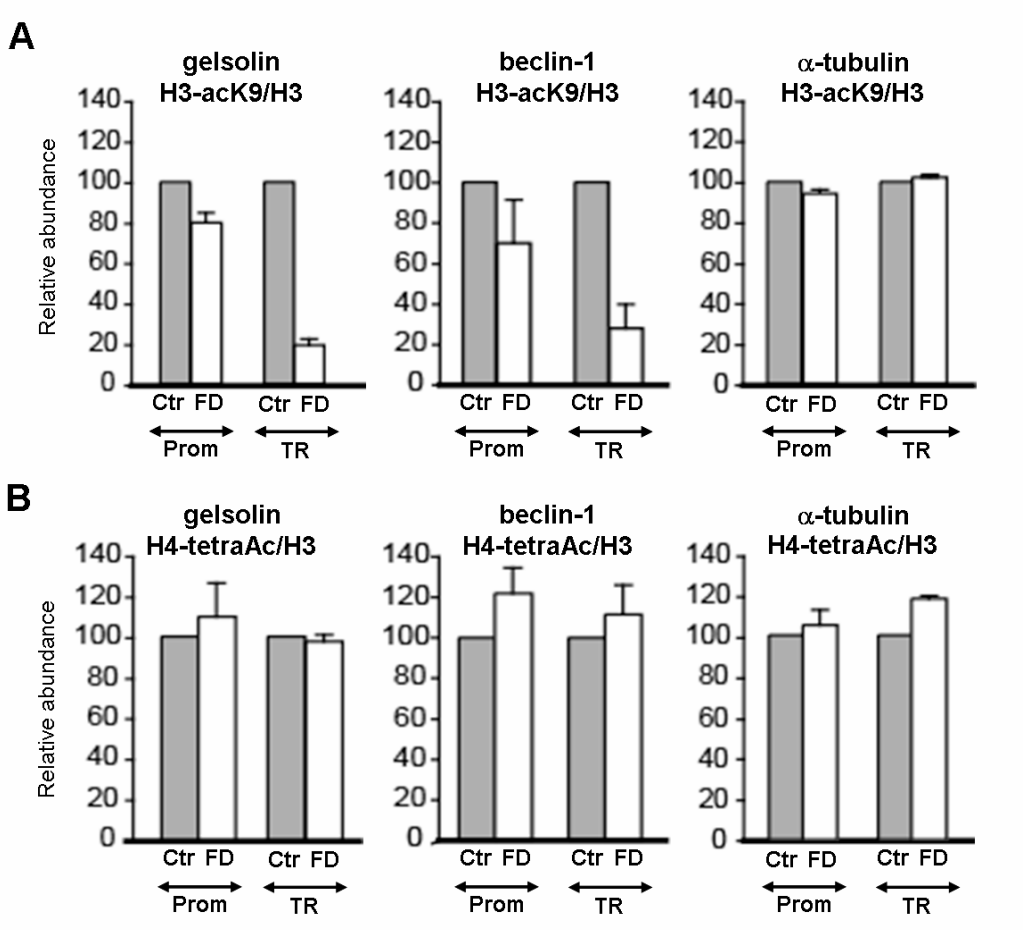

Figure 51. Elongator depletion results in histone H3 hypo-acetylation in the transcribed region of target genes.

(A) ChIP assays with an anti-histone H3K9ac-specific antibody were performed with normal (Ctr) and FD (FD) fibroblasts. The values were normalized for histone content by performing an anti-H3-C-terminus ChIP in parallel. For ease of comparison, signals in normal fibroblasts were set to 100 and those obtained from FD fibroblasts expressed relative to that. (B) as in (A), but using the anti-histone H4-tetraAc-specific antibody.

A similar reduction in acetylation was observed at histone H3-K18 in the gelsolin and beclin-1 transcribed regions (figure 52, on the top). Importantly, these effects were specific for the Elongator target genes, as no significant change in histone modification was observed at the α-tubulin control gene in FD fibroblasts (figure 51A and B, on the right and figure 52 on the bottom). It is important to mention that this reduced acetylation was not due to a loss of histone H3-DNA contact in the target genes, as histone acetylation levels were normalized for histone content after ChIP with an antibody specific for the histone H3 C-terminus.

Having established that Elongator affects the level of histone H3 acetylation of target genes, we next looked to define more precisely the spatial distribution of histone H3 acetylation across the genes. We used primer pairs all along the studied genes from the promoter to the termination site (for primer localization, see figure 46B, page 86). ChIP experiments were performed using H3-K18 and C-terminus-H3 antibodies in FD and normal fibroblasts. Respectively, for each primer pairs, the signal obtained from control cells was set to 100 and the signal from FD cells expressed relatively. Interestingly, histone H3 acetylation at the promoter and the beginning of the transcribed region was nearly unaffected by the absence of IKAP/Elongator. However, more dramatic decreases were observed further into the transcribed region (figure 52). Again, histone acetylation in the transcribed region correlated with the lower expression of IKAP/hELP1 target genes in FD cells; it was lowered in the beclin-1 and gelsolin genes but remained unchanged in the α-tubulin gene (figure 52). This strongly suggests that Elongator is involved in the transcriptional elongation of its target genes rather than in the initiation phase.

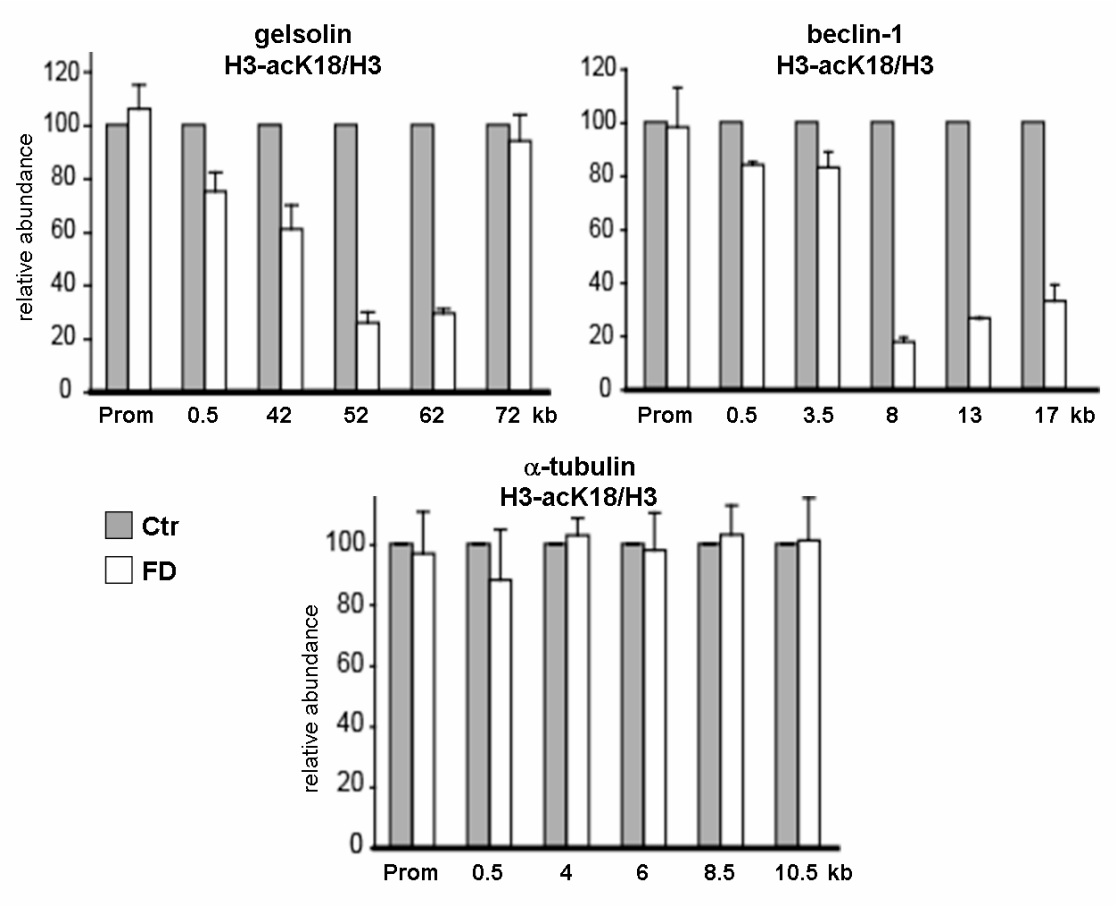

Figure 52. Low levels of Elongator results in histone H3 hypo-acetylation at lysine 18 across the transcribed region of target genes.

ChIP assays with an anti-histone H3K18ac-specific antibody were performed with normal (Ctr) and FD (FD) fibroblasts. The values were normalized for histone content by performing an anti-H3-C-terminus ChIP in parallel. For ease of comparison, signals in normal fibroblasts were set to 100 and those obtained from FD fibroblasts expressed relative to that. The reason for the apparently normal level of acetylation observed at the very end of the gelsolin gene in FD cells is unknown, but may be due to the next gene downstream being relatively near (~6kb).

2-2- Elongator affects the recruitment of the polymerase through its target genes

As histone acetylation is essential for an active gene transcription (for example, see (Kouskouti and Talianidis, 2005)), and in order to determine whether the histone acetylation defects in IKAP/hELP1 depleted cells are responsible for transcriptional defects, we finally looked at the occupancy of the RNA polymerase II across the tested genes. Indeed, if Elongator is involved in transcriptional elongation, then the density of the RNA polymerase II might be relatively lower in the 3'-end of the gene than at the promoter upon IKAP/hELP1 depletion. To address this issue, we performed ChIP experiments using anti-RNAPII antibody (4H8) and the primers covering the entire length of the genes (see figure 46B for primers localization, page

86). Remarkably, RNA polymerase II occupancy was indeed decreased in FD cells across both the gelsolin and the beclin-1 genes with about 30% density observed at the end of the gene (figure 53A and 53B). At the promoter, RNA polymerase II density measured in FD cells was nearly similar to the control cells.

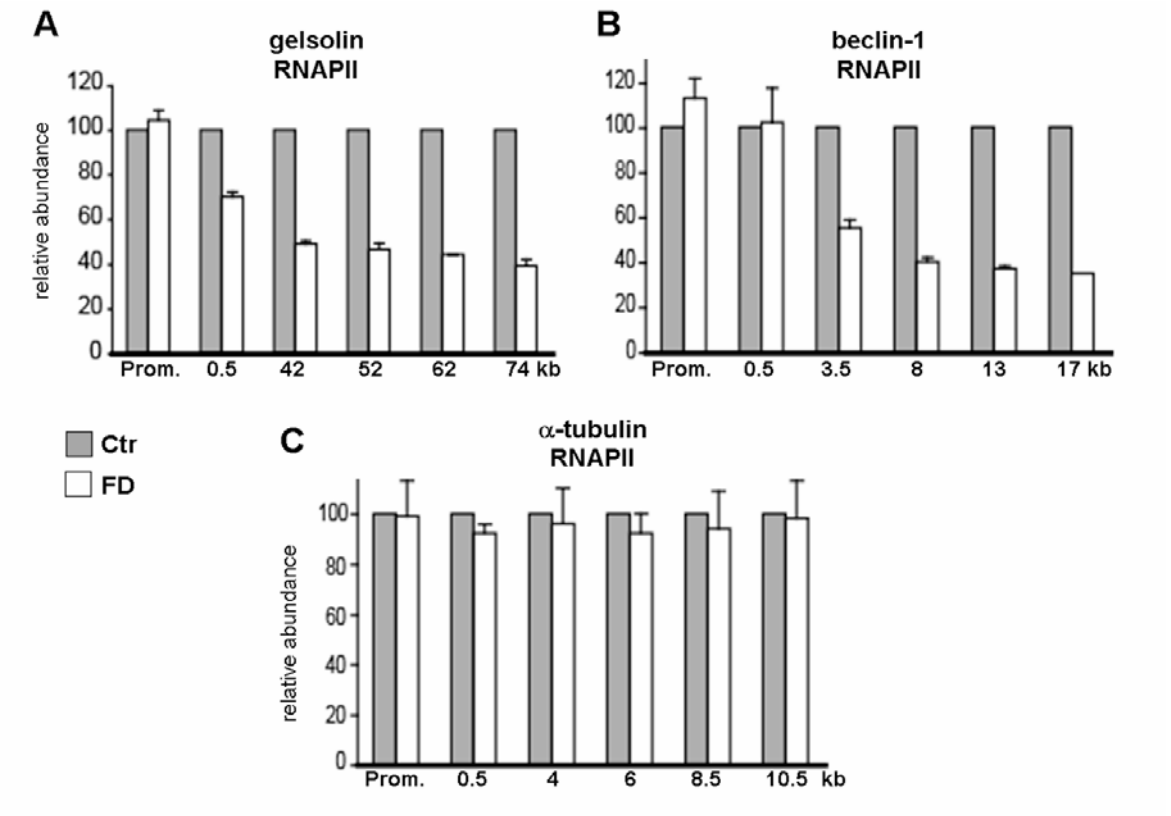

Figure 53. IKAP/Elongator depletion results in lower density of RNAPII through the transcribed region of target genes.

ChIP assays with an anti-RNAPII antibody (4H8) were performed with normal (Ctr) or FD (FD) fibroblasts. Primers were derived from the indicated region of the gelsolin (A), beclin-1 (B) or α-tubulin (C) genes. For ease of comparison, density in normal fibroblasts was set to 100 for each primer set and the FD values expressed relative to that. The signal obtained from an untranscribed region (downstream the albumin gene, see Kouskouti and Talianidis, 2005) was used to normalize the signal between the two cell lines.

In contrast to Elongator's target genes, the RNA polymerase II density across the α-tubulin control gene remained unaffected by the decrease in IKAP/Elongator levels, as expected (figure 53C). Together, these data indicate that Elongator affects transcript elongation, but not the recruitment of RNA polymerase II to the promoter, of genes whose expression is affected by decreased IKAP/hELP1 levels.

3- Discussion

The very specific effects of IKAP/Elongator depletion on histone acetylation and RNA polymerase II density across the target genes are consistent with a direct effect of Elongator on the transcriptional elongation. Elongator is present in the transcribed region of genes and histone H3 acetylation is significantly reduced in the transcribed region, but not at the promoter of the affected genes in its absence. IKAP/Elongator depletion leaves the RNA polymerase II recruitment to the promoter largely unaffected. The RNA polymerase II density in the first few hundred to several thousand nucleotides of the transcribed region is also normal but RNA polymerase II occupancy is progressively lowered through the transcribed region of the target genes. This supports the idea that Elongator assists RNA polymerase II during transcript elongation through chromatin as the polymerase moves further away from the 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 the genes.

Because data from ChIP experiments can only show correlation between the presence of a protein and effects at sites of action, our ChIP data from human cells do not by themselves prove that Elongator acetylates histones during transcriptional elongation. However recent results have shown that both histone H3 and H4 acetylation are measurably increased in the transcribed region of several genes as a consequence of an active transcription (Kouskouti and Talianidis, 2005) and that Elongator is primarily an histone H3 acetyltransferase *in vitro* (Hawkes et al., 2002; Kim et al., 2002; 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. Moreover, previous data from yeast showed that mutations in Elongator genes are 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 H3 is essential) (Wittschieben et al., 2000). Finally, yeast cells lacking both *ELP3* and *GCN5*, the gene encoding another histone H3 acetyltransferase, have severe growth defects which 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 H3-K9 and H3-K18 *in vivo*, this does not rule out the possibility that lysine H3-K14, which is a primarily target site of purified yeast Elongator *in vitro*, is targeted in human cells as well. Taken together with the data from the yeast model, our results obtained in human cells strongly point to a function for Elongator in histone acetylation during transcriptional elongation.

Conclusions and perspectives

In this work, we provided several insights into the IKAP and Elongator functions. First, we demonstrated a role for IKAP/hELP1 and Elongator in histone H3 acetylation and transcriptional elongation of human genes. Second, our results indicate that the mutation carried by individuals suffering from FD causes abrogation of Elongator function, not just IKAP/hELP1 expression. Third, normal levels of IKAP/Elongator are important for normal expression of several human genes, including some implicated in cell motility. Indeed, HeLa and neuron-derived IKAP/hELP1 depleted cells, as well as FD fibroblasts, exhibit defects in cell motility *in vitro*. Cell motility is crucial for the normal development of the peripheral nervous system, so our results also point to molecular defects that may underlie FD.

Transcription elongation and histone modifications

Transcription elongation through chromatin results in modification of histones in the transcribed regions. This includes histone acetylation, histone H3 methylation (on lysine 4 and 36), and histone H2 ubiquitination. The results showing that Elongator acetylates histones H3 in the transcribed region of the genes should be linked to other works that studied nucleosome displacement during transcription. RNAPII-associated histone acetyltransferases such as Elongator might acetylate the nucleosomes and thus facilitate histone displacement. Indeed, recent works have shown that the function of histone H3 methylation on lysine 36 (H3 methyl K36) in transcription is linked to gene activity and to histone deacetylation in transcribed region (Carrozza et al., 2005; Joshi and Struhl, 2005). A model has been recently proposed by Jerry Workman in which transcriptional elongation by RNAPII occurs firstly through acetylation of histones by the associated HAT activities and secondly, through histone methylation, a signal for subsequent histone deacetylation (figure 54). This model is in agreement with previous reports which demonstrated that both histone acetylation and histone deacetylation in the transcribed regions of genes are important for efficient transcription (Wang et al., 2002). Moreover, it sets up the chronology of the histone modifications during the passage of the RNAPII.

This model could also explain why an accumulation of acetylated nucleosomes is not systematically observed within the 3' part of the transcribed regions as a result of histone acetylation during transcriptional elongation. Indeed, high levels of histone H3 and H4 acetylation are generally detected at the promoter and in the 5' regions of active genes, and to a lesser extent in the 3' region (Saunders et al., 2006). Our results suggest that Elongator acetylates histones H3 in the 3' region of the target genes, after the typical HAT sphere found around the promoter, where there is no apparent increase in histone acetylation proportionally to the transcription rate. One explanation would be that RNAPII directly signals for subsequent nucleosome deacetylation as a result of an efficient transcription elongation.

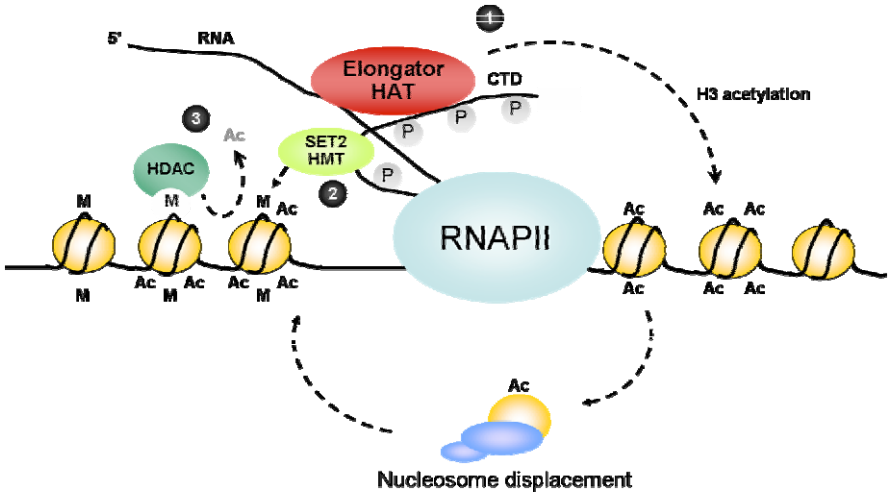

Figure 54. Model of dynamic interplay between histone acetylation, methylation and deacetylation during transcriptional elongation.

During transcriptional elongation, acetyltransferase complexes such as Elongator, associated with the elongating RNAPII, acetylate nucleosomes in front of the RNAPII (1). This facilitates their partial or complete disassembly, transferring the histones onto the nucleosome displacement machinery (i.e. FACT and SPT6 in yeast). The nucleosome is reassembled behind the RNAPII, and histone H3 is methylated on lysine 36 by a RNAPII-bound histone methyltransferases (SET2-domain HMTs) (2). Histone deacetylase complexes recognize the methylated histone H3 on lysine 36 and deacetylate those nucleosomes to restore their previous stability (3). The reassembled nucleosomes behind polymerase may also contain new histones from the soluble cellular pool. This model is adapted from (Workman, 2006).

This attractive model suggests a strong correlation between histone acetylation, histone H3 methylation on lysine 36 and histone deacetylation in the transcribed region of the genes. In the context of transcription involving Elongator, it would be of great interest to further identify all the enzymatic activities associated with Elongator during the transcriptional elongation of its target genes. This could be searched for example, by investigating whether a histone methyltransferase (HMT) activity is associated with Elongator. Preliminary results suggest that the histone acetylation defects observed in Elongator-depleted cells correlates with a specific histone methylation defect on lysine 36 in the transcribed region of Elongator target genes. Furthermore, methylation of histone H3 on lysine 36 has been associated with transcriptional elongation towards the 3'-ends of the genes (Krogan et al., 2003; Martin and Zhang, 2005) and several human proteins harbouring a HMT activity specific for histone H3 K36 (for example, the huntingtin interacting protein B (HYPB) (Sun et al., 2005) or nuclear receptor-binding SET domain-containing protein 1 (NSD1) (Rayasam et al., 2003)) have been firmly identified. Moreover, HYPB has been shown to interact with elongating hyper-phosphorylated RNAPII (Sun et al., 2005). Chromatin immunoprecipitation experiments could be performed to examine the recruitment of these H3-K36-specific HMTs at Elongator target genes in the presence or in the absence of Elongator. Likewise, co-immunoprecipitation experiments would give additional information on potential direct interactions between Elongator and HMT enzymes. Similarly, the same set of experiments could

be performed in order to study the co-recruitment of specific histone deacetylases on Elongator target genes in its presence or absence. Moreover, mono-ubiquitinated histone H2B has been linked to transcription elongation as it associates with elongating RNAPII. In this context, the histone H2 ubiquitination status on the Elongator target genes could be explored by performing ChIP experiments. Indeed, the yeast SAGA HAT complex, is recruited at the genes after histone H2B mono-ubiquitination and histone acetylation by SAGA is needed for subsequent deubiquitination (Daniel et al., 2004; Ingvarsdottir et al., 2005). An additional perspective would thus be to determine whether Elongator-dependent transcription is to some extent linked to histone H2 ubiquitination. Finally, once these links are established and potential partners identified, parallel loss-of-function studies will inform on possible correlations between those enzymatic reactions during the transcription elongation: it will be interesting to know the chronology of these events and therefore to define the cascade of reactions that are required for proper transcription elongation by Elongator.

Elongator interaction with the cytoskeleton

Another exciting model to explain the role of Elongator in transcriptional elongation comes from recent growing evidences that cytoskeleton components such as actin or myosin proteins have nuclear pool and are involved in the transcription regulation. *In vitro* studies suggest that actin is involved in transcription elongation (Percipalle and Visa, 2006). In this case, actin is bound to a specific subset of pre-messenger RNA binding proteins and these complexes may constitute a molecular platform for recruitment of histone-modifying enzymes, such as histone acetyltransferases (Percipalle and Visa, 2006; Sjolinder et al., 2005). Likewise, yeast Elongator has been shown to bind nascent and un-spliced pre-messenger RNA (Gilbert et al., 2004) and other yeast data show genetic interactions between Elongator subunits and actin or myosin cytoskeleton components (unpublished data from Jesper Svejstrup's laboratory). Moreover, our microarray data showed that Elongator controls the expression of genes whose products are related to actin cytoskeleton remodelling, for example actin filament-associated proteins such as gelsolin, swap-70, radixin or synaptopodin. We might speculate that Elongator could be recruited at the interface between DNA and pre-messenger RNA, through actin-associated hnRNP particles (figure 55). Indeed, its histone acetyltransferase activity might be essential for the transcription elongation of a subset of genes whose pre-messenger RNA binds actin. In this case, actin could serve as a specific anchor to recruit Elongator.

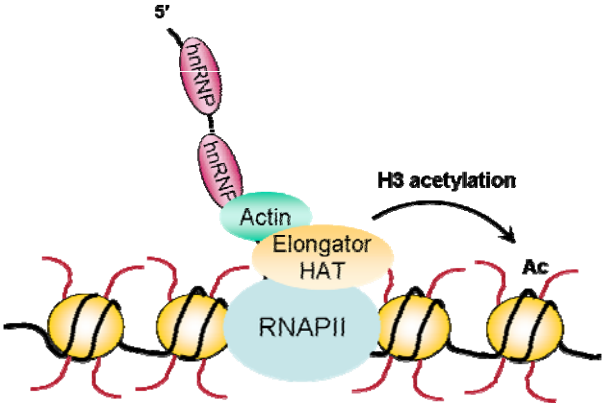

Figure 55. Co-transcriptional recruitment of actin and HAT activity to growing pre-mRNPs.

Putative model involving Elongator histone H3 HAT activity and actin in transcriptional elongation by RNAPII. hnRNPs are associated with nascent RNA emanating from the RNAPII and regulate its processing. This schema is adapted from (Grummt, 2006) and (Sjolinder et al., 2005).

In this context, it would be very interesting to study a possible Elongator association with the actin-myosin components, including the cellular compartment where it occurs, in order to understand the role of such an interaction in transcription elongation. In the case of a direct link, it would be very useful to look at the consequences of removing actin or myosin proteins in terms of Elongator function in transcription. In other words, are actin or myosin components essential for Elongator-dependent gene transcription? The opposite question would also be interesting to investigate.

Further studying Elongator-dependent transcription elongation as an integrative process involving different actors that act together will undoubtedly improve the global view of mechanisms that govern transcriptional elongation. This would also point to new potential therapeutic targets for human diseases that involve histone modifying enzymes, such as FD or cancer.

Other results presented in this work focused on the biological function of IKAP/hELP1 and the Elongator complex. We demonstrated that IKAP/hELP1 and Elongator have a role in cell motility/migration through the expression of several genes that regulate actin cytoskeleton remodelling and cell motility/migration. It is worth noting that although our data indicate that the cell migration defect is a consequence of lowered gene expression of genes, we cannot rule out the possibility that Elongator also plays a more direct role in cell motility, for example through a direct cytoplasmic interaction with cytoskeleton proteins/filaments or cytoskeleton regulating proteins. Further experiments are required to clarify this issue, for example by determining whether or not IKAP/Elongator is associated with cytoskeleton proteins or cytoskeleton regulating proteins in the cytoplasm.

Elongator and familial dysautonomia

FD is a neurodevelopmental genetic disorder characterized by a decreased number of neurons in peripheral ganglions. The cells situated in peripheral ganglia derive embryonically by migration from neural crest (figure 56). Our data thus strongly suggest that the depletion of neurons in peripheral ganglia observed in FD patients might be the result of an inefficient migration of neural crest cells during embryogenesis.

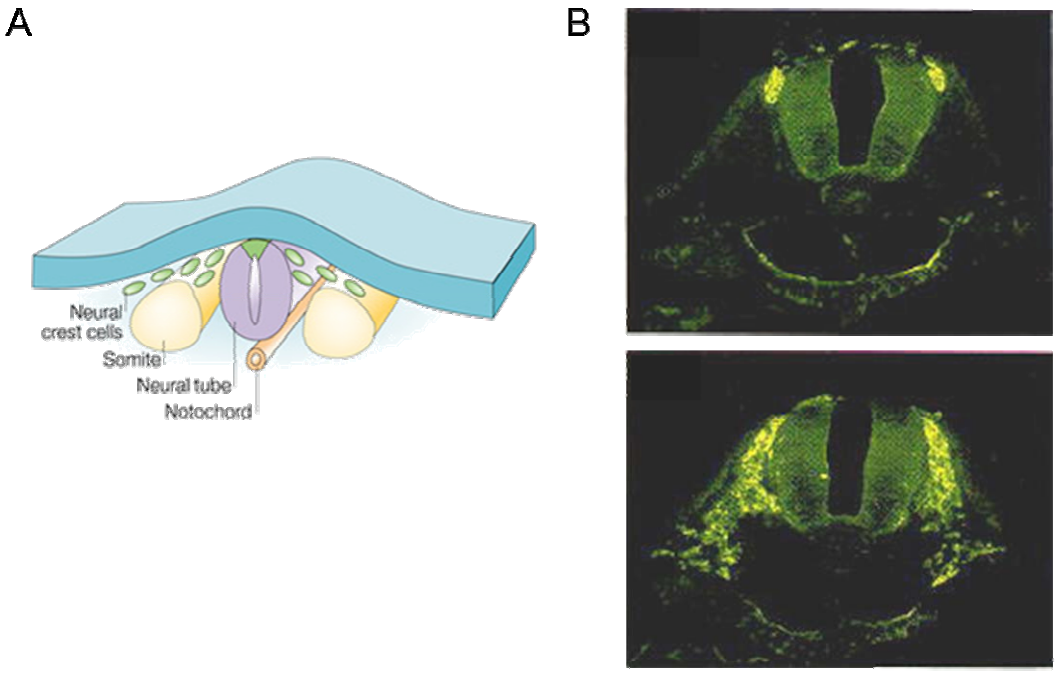

Figure 56. Migration of neural crest cells during development.

A. Neural crest cells initiate migration from the dorsal neural tube (represented in green) (adapted from (Gammill and Bronner-Fraser, 2003)). B. Neural crest cell migration along the neural tube of the chick embryo. (These pictures are from Developmental Biology 6th edition, Scott F. Gilbert, 2000. Sinaur Associates, Sunderland, MA, USA).

In this context, it would be interesting to further characterize the mechanisms underlying this cell motility defect. To migrate properly, cells must generate different forces that require different regulation mechanisms (Lauffenburger and Horwitz, 1996):

- the protrusive force that relies on actin polymerization and cytoskeleton/structural organization with the generation of membrane processes (initiation);
- the contraction force which depends on active myosin-based motors and moves the cell body forward (nucleokinesis).

Both phenomena occur independently of each other. Likewise, a rapid migration requires efficient mechanisms to release adhesions at the rear of the cell. As we have shown that Elongator-depleted cells have cell migration defects, further investigation will tell us which of the cell protrusion, cell contraction or cell adhesion is effectively perturbed. Our preliminary results suggest that Elongator affects actin cytoskeleton regulating pathways, suggesting that Elongator mostly influences the initiation phase of the migration process or cell spreading rather than contraction or adhesion. Upon activation, small GTPases (Rho, Rac and CDC42) promote the formation of filipodia, actin stress fibers and actin polymerization, suggesting that these factors could play key roles in guidance cues on neural crest migration. To which extent Rho, Rac and Cdc-42 phases of activation are altered upon IKAP depletion is currently under investigation and will undoubtedly provide a better understanding of the role of Elongator in the cell motility.

The generation of conditional knock-out models of the *IKBKAP* gene that would enable the deletion at later stages of development or in specific tissues will greatly enhance the understanding of Elongator functions during embryogenesis and in adult tissues. Such a transgenic mouse is actually under development in the laboratory. Moreover, cancer obviously implies cell migration for progression and dissemination. Therefore, studying the role of Elongator in cancer formation and development might give novel insights into the pathological mechanisms involved in cancer and point to novel potential therapeutic targets.

Finally, we demonstrated that Elongator integrity and function is altered in FD patients, suggesting that FD is the result of tissue-specific loss-of-function of the entire Elongator complex, rather than IKAP solely. In yeast, Elongator has been implicated in cellular reactions as diverse as tRNA modification (Huang et al., 2005) and exocytosis (Rahl et al., 2005). 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 would be possible that translational imprecision might contribute to FD as well.

It remains to be explained why Elongator is localized in the cytoplasm (as well as in the nucleus). In yeast, the C-terminus of ELP1 protein harbours a sequence motif that confers nuclear localization to green fluorescent protein (Fichtner et al., 2003). This ELP1 domain is dispensable for the integrity of the Elongator complex but is required for normal Elongator function *in vivo* (Fichtner et al., 2002), suggesting that nuclear localization of yeast Elongator is crucial. The cytoplasmic Elongator function remains unclear and needs to be elucidated.

Our data showing that Elongator acetylates histones raises the hypothesis that histone deacetylase inhibitors may be used as potential therapeutic drug for FD patient to counteract acetylation defects (HDAC inhibitors are currently in clinical trials as anticancer drugs). However, as FD is a developmental defect, these

compounds would have to be administrated during embryogenesis. Unfortunately, HDACs inhibitors are rather toxic for proliferating cells and induce cell cycle arrest, cell apoptosis or precocious cell differentiation (which is favourable for cancer therapy). Moreover, acetylation of histone works together with other chromatin modifications, and blocking deacetylation might have different outcomes according to the chromatin state. The development of novel drugs targeting histone-modifying enzymes thus required excellent knowledge of protein interactions and biochemical reactions controlling the chromatin remodelling.

Appendix 1

	accession number	description	symbol	fold	p-value
Cell adhesion	NM_000602	plasminogen activator inhibitor type 1 (Serpine-1)	PAI-1	-3,4	0,0001
	NM_000214	jagged 1	JAG1	-2,8	0,00002
	NM_014021	synovial sarcoma, X breakpoint 2 interacting protein	SSX2IP	-2,6	0,00002
	NM_014376	cytoplasmic FMR1 interacting protein 2	CYFIP2	-2,5	0,00002
	NM_001753	caveolin 1	CAV1	-2,0	0,00002
cytoskeleton	NM_006612	kinesin family member 1C	KIF1C	-6,5	0,00004
	NM_001175	Rho GDP dissociation inhibitor, beta	GDI	-4,3	0,00007
	NM_000177	Gelsolin	GSN	-2,8	0,0001
	NM_015055	SWAP-70 protein	SWAP70	-2,8	0,00002
	NM_007286	synaptopodin	SYNPO	-2,7	0,0003
	NM_003980	microtubule-associated protein 7	MAP7	-2,1	0,0001
	BE222709	microfibrillar-associated protein 3	MFAP3	-2,0	0,0006
	NM_002906	radixin	RDX	-2,0	0,00002
	NM_003289	tropomyosin 2 beta	TPM2	-2,0	0,00002
	NM_012121	CDC42 effector protein (Rho GTPase binding) 4	CDC42EP4	-2,0	0,00003
	NM_017491	WD repeat domain 1	WDR1	-2,0	0,00002
	XM_170658	EH domain binding protein 1-like 1	EHBP1L1	-2,0	0,000006
cell proliferation/ growth	NM_000076	cyclin-dependent kinase inhibitor 1C (p57, Kip2)	CDKN1C	-3,3	0,001
	NM_002388	minichromosome maintenance deficient 3	MCM3	-3,0	0,00002
	NM_001071	thymidylate synthetase	TYMS	-2,6	0,00002
	NM_014059	response gene to complement 32	RGC32	-2,5	0,0002
	NM_002315	rhombotin 1		-2,3	0,00002
	NM_001311	cysteine-rich protein 1 (intestinal)	CRIP1	-2,2	0,0001
	NM_002514	nephroblastoma overexpressed gene	NOV	-2,0	0,0008
kinases	NM_015000	serine/threonine kinase 38 like	STK38L	-3,4	0,00003
	NM_002738	protein kinase C beta-II type	PRKCB1	-3,1	0,001
	NM_006296	vaccinia related kinase 2	VRK2	-3,0	0,00003
	NM_014216	inositol 1,3,4-triphosphate 5/6 kinase	ITPK1	-2,0	0,002
phosphatases	NM_002849	protein tyrosine phosphatase, receptor type, R	PTPRR	-3,5	0,00008
	NM_002709	protein phosphatase 1, catalytic subunit, beta isoform	PPP1CB	-2,5	0,00002
receptors	NM_000212	CD61	CD61	-12,9	0,001
	NM_020311	chemokine orphan receptor 1	CMKOR1	-3,0	0,00002
	NM_003979	G protein-coupled receptor, family C, group 5, member A	GPRC5A	-2,3	0,0002
	NM_000877	interleukin 1 receptor, type I	IL1R1	-2,2	0,0002
	NM_002185	interleukin 7 receptor	IL7R	-2,0	0,00007
	NM_004357	CD151 antigen	CD151	-2,0	0,0002
extracellular matrix	NM_002160	tenascin C	TNC	-2,8	0,00001
	NM_000228	laminin, beta 3	LAMB3	-2,5	0,0001
	NM_006528	tissue factor pathway inhibitor 2	TFPI2	-2,1	0,0002
	NM_000088	collagen, type I, alpha 1	COL1A1	-2,0	0,00002
ER metabolism	NM_004343	calreticulin	CALR	-2,5	0,0001
	NM_004530	MMP-2	MMP2	-2,3	0,00001
	NM_006334	olfactomedin 1	OLFM1	-2,2	0,0002
	NM_014607	UBX domain-containing 2	UBXD2	-2,0	0,0002
autophagy	NM_003766	beclin 1	BCN1	-2,0	0,00002
endocytosis	NM_018993	Ras and Rab interactor 2	RIN2	-2,3	0,0002
	NM_006868	RAB31, member RAS oncogene family	RAB31	-2,0	0,00002
	NM_016277	RAB23, member RAS oncogene family	RAB23	-2,0	0,0007

	accession number	description	symbol	fold	p-value
transcription factors	NM_001429	E1A binding protein p300	EP300	-3,0	0,002
	NM_003670	basic helix-loop-helix domain containing, class B, 2	BHLHB2	-2,2	0,00002
	NM_020347	leucine zipper transcription factor-like 1	LZTFL1	-2,2	0,0001
	NM_005901	SMAD, mothers against DPP homolog 2	SMAD2	-2,1	0,00002
	NM_181552	cut-like 1, CCAAT displacement protein (Drosophila)	CUTL1	-2,0	0,001
DNA repair	NM_000251	mutS (E. coli) homolog 2 (colon cancer, nonpolyposis type 1)	hMSH2	-2,3	0,00002
metabolism	NM_005173	ATPase, Ca++ transporting	ATP2A3	-4,2	0,0002
	NM_005737	ADP-ribosylation factor-like 7	ARL7	-3,7	0,0004
	NM_000433	neutrophil cytosolic factor 2	NCF2	-2,6	0,00002
	NM_005978	S100 calcium-binding protein A2	S100A2	-2,5	0,00002
	NM_198098	aquaporin 1	AQP1	-2,5	0,0001
	NM_001034	ribonucleotide reductase M2 polypeptide	RRM2	-2,3	0,00002
	NM_001630	annexin A8	ANXA8	-2,3	0,00002
	NM_002960	S100 calcium-binding protein A3	S100A3	-2,3	0,00006
	NM_014765	translocase of outer mitochondrial membrane 20 (yeast) homolog	TOMM20	-2,3	0,0002
	NM_004613	transglutaminase 2	TGM2	-2,1	0,00002
	NM_006710	COP9 constitutive photomorphogenic homolog subunit 8 (Arabidopsis)	COPS8	-2,1	0,00002
	NM_020299	aldo-keto reductase family 1, member B10	AKR1B10	-2,1	0,00003
	NM_001976	enolase 3	ENO3	-2,0	0,001
	NM_001829	chloride channel 3	CLCN3	-2,0	0,00002
	NM_005063	stearoyl-CoA desaturase	SCD	-2,0	0,001
	NM_014280	DnaJ (Hsp40) homolog, subfamily C, member 8	DNAJC8	-2,0	0,001
	NM_015700	HIRA-interacting protein 5	HIRIP5	-2,0	0,00002
RNA export	NM_007362	nuclear cap binding protein subunit 2	NCBP2	-2,0	0,00002
scaffold proteins	NM_021255	pellino homolog 2 (Drosophila)	PELI2	-2,0	0,00002
	NM_002859	paxillin	PXN	-2,0	0,0001
others	NM_020199	HTGN29 protein	HTGN29	-2,6	0,001
	NM_000735	glycoprotein hormones, alpha polypeptide	CGA	-2,5	0,0001
	NM_001008529	transmembrane anchor protein 1	TAMP1	-2,5	0,00002
	NM_002569	furin	FURIN	-2,5	0,00007
	NM_015534	zinc finger, ZZ-type containing 3	ZZZ3	-2,5	0,00002
	NM_022736	major facilitator superfamily domain containing 1	MFSD1	-2,5	0,00002
	NM_006333	nuclear DNA-binding protein C1D	C1D	-2,4	0,0002
	BC005136	solute carrier family 35, member 3	SLC35A3	-2,3	0,0002
	NM_000346	SRY (sex determining region Y)-box 9	SOX9	-2,3	0,0004
	NM_004403	deafness, autosomal dominant 5	DFNA5	-2,3	0,0001
	NM_013372	gremlin 1 homolog, cysteine knot superfamily (Xenopus laevis)	GREM1	-2,3	0,001
	NM_020182	transmembrane, prostate androgen induced RNA	TMEPAI	-2,1	0,00003
	NM_021977	solute carrier family 22, member 3	SLC22A3	-2,1	0,0003
	NM_001280	cold inducible RNA binding protein	CIRBP	-2,0	0,0002
	NM_001831	clusterin	CLU	-2,0	0,0001
	NM_003204	nuclear factor (erythroid-derived 2)-like 1	NFE2L1	-2,0	0,001
	NM_003498	stannin	SNN	-2,0	0,0001
	NM_005418	suppression of tumorigenicity 5	ST5	-2,0	0,001
	NM_006459	SPFH domain family, member 1	SPFH1	-2,0	0,00002
	NM_007273	prohibitin 2	PHB2	-2,0	0,00002
	NM_015455	CCR4-NOT transcription complex, subunit 6	CNOT6	-2,0	0,00002
	NM_018105	THAP domain containing, apoptosis associated protein 1	THAP1	-2,0	0,0003
	NM_145728	desmuslin	DMN	-2,0	0,00006

Appendix 2

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

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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 nontarget genes, only target genes display histone H3 hypoacetylation 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

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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. The expression of about 100 genes was significantly downregulated as a result of IKAP/hELP1 RNAi (Figure 1C and Figure S2A), whereas some 15 genes were upregulated (Figure S2B). The expression of TNFα and NF-κB-regulated target genes such as IL-1β and IκBα was not altered in TNFα-stimulated IKAP/hELP1 RNAi cells (P.C. and A.C., unpublished data), further supporting the conclusion that these signaling pathways are insensitive to IKAP/hELP1 levels.

Interestingly, a significant proportion (15 out of ~100) of the downregulated genes encode proteins regulating cell motility, such as those coding for the integrin receptor CD61, the ligand tenascin-C, and the actin cytoskeleton modulators gelsolin, paxillin, and caveolin-1 (Figure 1C). Genes coding for proteins involved in cell proliferation, such as thymidylate synthetase and the cyclin-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 and Figure S2A).

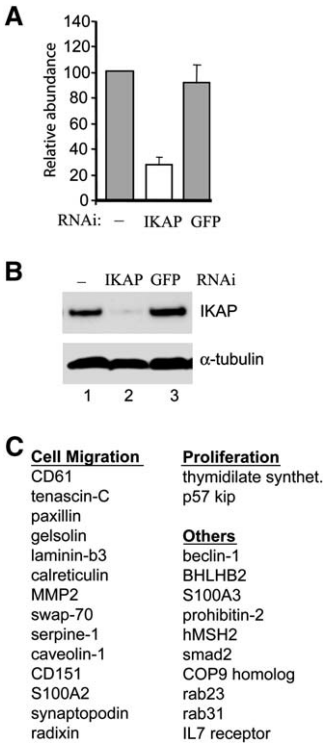

Figure 1. Identification of Genes Regulated by IKAP/Elongator

(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). An anti-α-tubulin Western blot is shown in (B) for normalization purposes.

(C) Identification of IKAP/hELP1-dependent genes. Total RNA was isolated from HeLa cells transfected with either IKAP/hELP1 or GFP RNAi, as well as from untransfected control cells. The RNA was then subjected to microarray analysis. Examples of affected genes are shown. The full list of affected genes is shown in Figure S2. 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 also 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, laminin β3, and beclin-1, but not α-tubulin, was also observed in FD fibroblasts (Figure 2C). Interestingly,

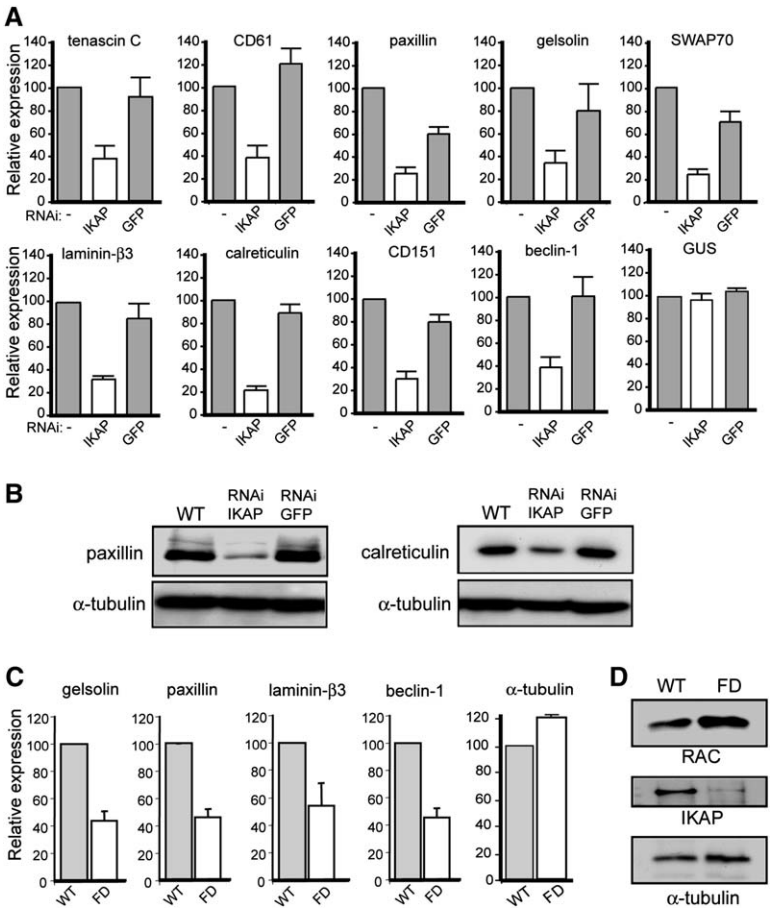

Figure 2. IKAP/Elongator Regulates the Expression of Genes Involved in Cell Migration

(A) Decreased expression of a subset of genes involved in cell migration in IKAP/hELP1 RNAi cells. RNA was extracted from untransfected HeLa cells (−) or HeLa cells transfected with IKAP/hELP1 RNAi (IKAP) or GFP RNAi (GFP), respectively, and gene expression was measured by quantitative real-time RT-PCR. Expression in the untransfected cells was set to 100. β-glucuronidase (GUS) expression is shown as a control.

(B) Levels of paxillin and calreticulin proteins in IKAP/hELP1 RNAi cells examined by Western blot analysis. An α-tubulin Western blot is shown as loading control.

(C) Decreased expression of a subset of genes in FD fibroblasts (FD) versus wild-type cells (wt) measured by quantitative real-time RT-PCR. α-tubulin expression is shown as a control. Expression in the wt cells was set to 100.

(D) Rac expression in FD fibroblasts examined by Western blot analysis, probing with the antibodies is indicated below the panels. Error bars in (A) and (C) denote standard deviation.

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

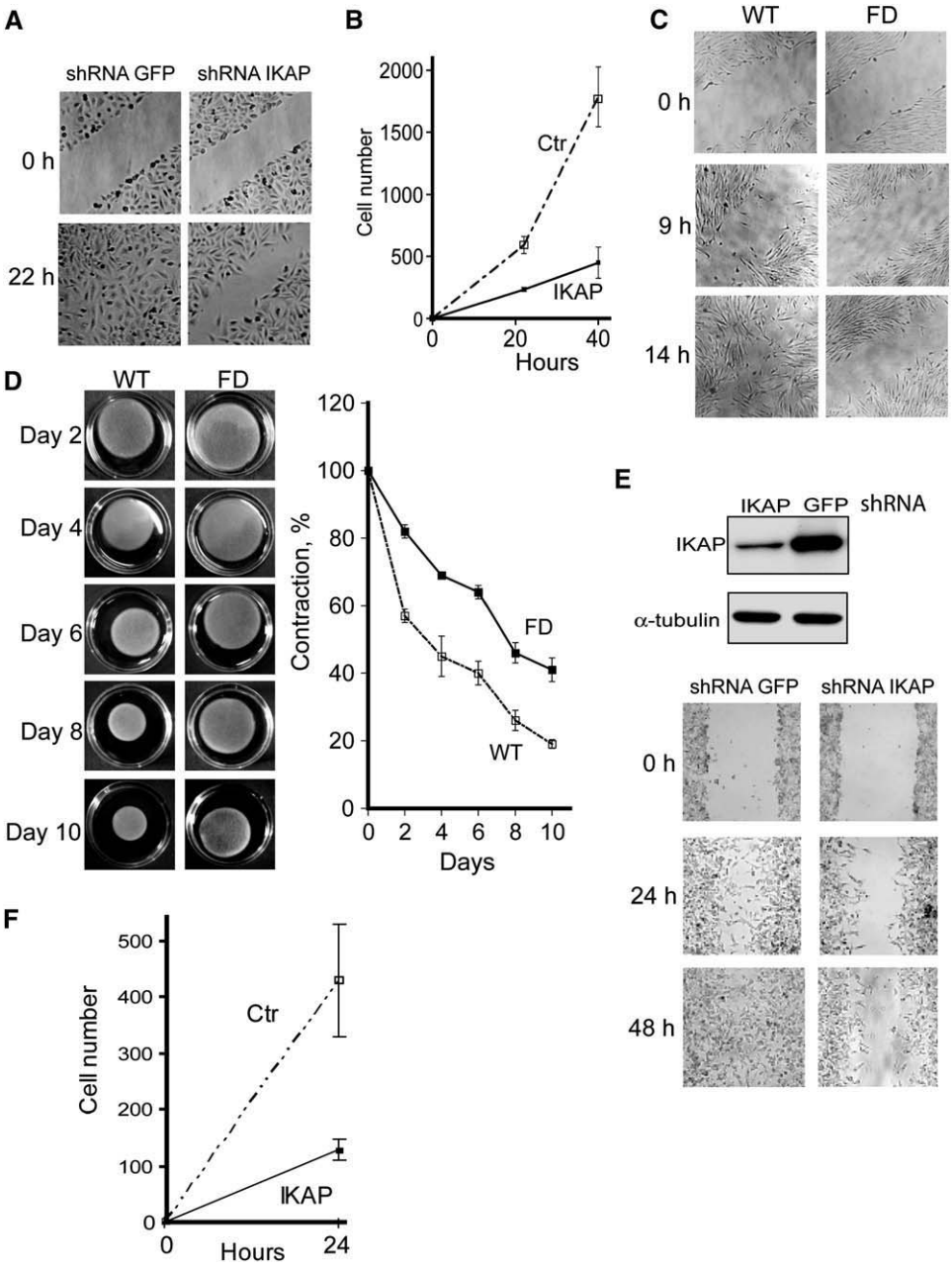

Figure 3. Cell Migration Defect in IKAP/hELP1 RNAi Cells, as Well as in FD Fibroblasts

(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.

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.

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 so, 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 still 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, not 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

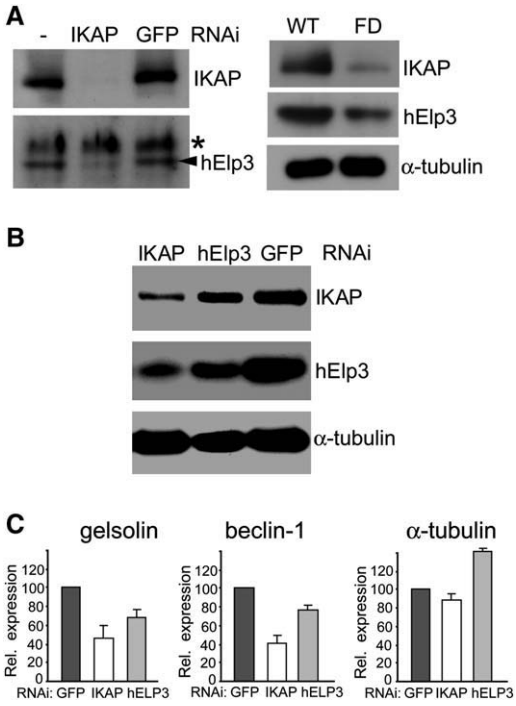

Figure 4. IKAP/hELP1 and ELP3 Depletion Have Similar Consequences for Gene Expression

(A) Decreased hELP3 levels in human cells with low IKAP/hELP1 levels. Cell extracts from HT29 cells infected with the indicated shRNA lentivirus (left panels) or from control or FD cells (right panels) were subjected to Western blot analysis. The asterisk indicates a nonspecific band obtained with the anti-ELP3 antibody in the RNAi cells. This band serves as a loading control in this experiment.

(B) hELP3 depletion after IKAP/hELP1 and hELP3 RNAi in human cells. Fibroblasts were transfected with GFP, IKAP/hELP1, or hELP3 RNAi as indicated, and protein extracts were subjected to anti-IKAP/hELP1, hELP3, and α-tubulin Western blot analysis.

(C) Gene expression of previously identified IKAP/hELP1-dependent genes in GFP, IKAP/hELP1, and hELP3 RNAi fibroblasts. Real-time PCR using primers to amplify the gelsolin, beclin-1, or α-tubulin transcripts was performed by using total RNAs extracted from the GFP, IKAP/hELP1, or ELP3 RNAi fibroblasts.

Error bars denote standard deviation.

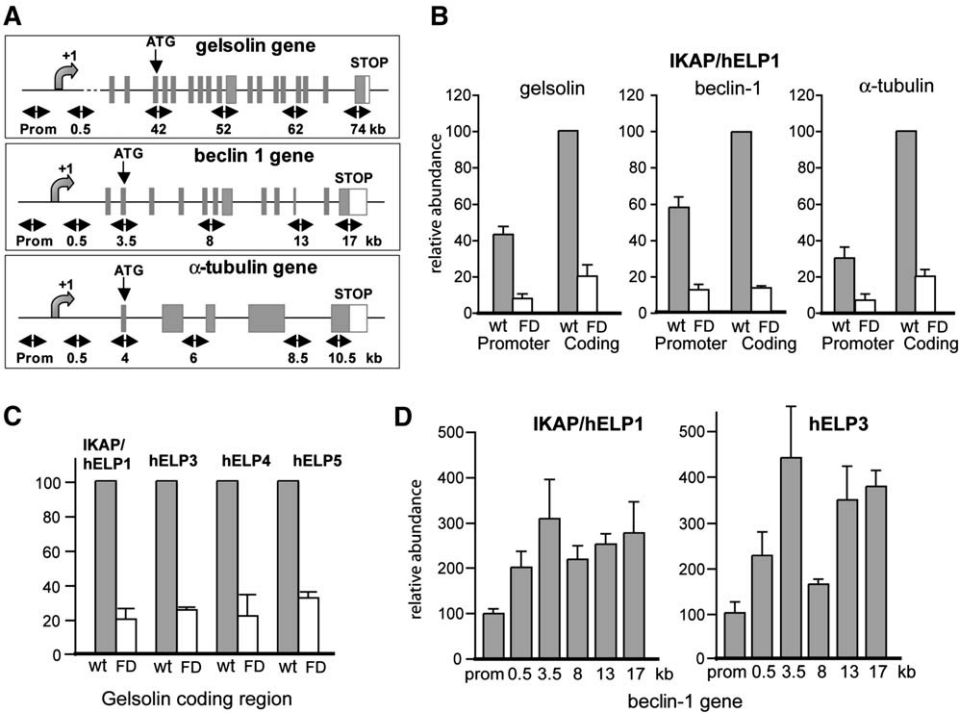

Figure 5. Elongator Is Present on the Coding Region of Target and Nontarget Genes and Its Recruitment Is Affected by IKAP/hELP1 Depletion

(A) Schematic representation of the genes investigated by ChIP. Exons are depicted by boxes and transcription initiation sites (arrow with +1); ORF start codons (ATG) and stop codons (STOP) are also indicated. The localization of primers used for ChIP analysis is illustrated by arrows below. Numbers show the positions of these primers relative to the transcription initiation site.

(B) ChIP assays with an anti-IKAP/hELP1 antibody (Figure S7) were performed with normal (wt) or FD (FD) fibroblasts. Associated DNA was analyzed by real-time PCR using primers derived from the promoter or the coding region of the indicated genes (gelsolin coding region primer, 62 kb; beclin-1, 8 kb; and α-tubulin, 6 kb). For ease of comparison, IKAP/hELP1 density in the coding region in normal fibroblasts was set to 100 and the other values expressed relative to that. See the Experimental Procedures for details.

(C) ChIP assays with the Elongator antibodies indicated above the graphs were performed as in (A), using primers derived from the coding region (62 kb) of the gelsolin gene. The fact that these antibodies coprecipitate less gelsolin DNA in FD cells indicates that recruitment of the whole Elongator complex is reduced and that the antibodies are specific.

(D) ChIP assays with the Elongator antibodies indicated above the graphs were performed by using primers derived from the regions of the beclin-1 gene indicated on the x axis. For ease of comparison, density at the promoter was set to 100 for each primer set and the other values expressed relative to that.

Error bars denote standard deviation.

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

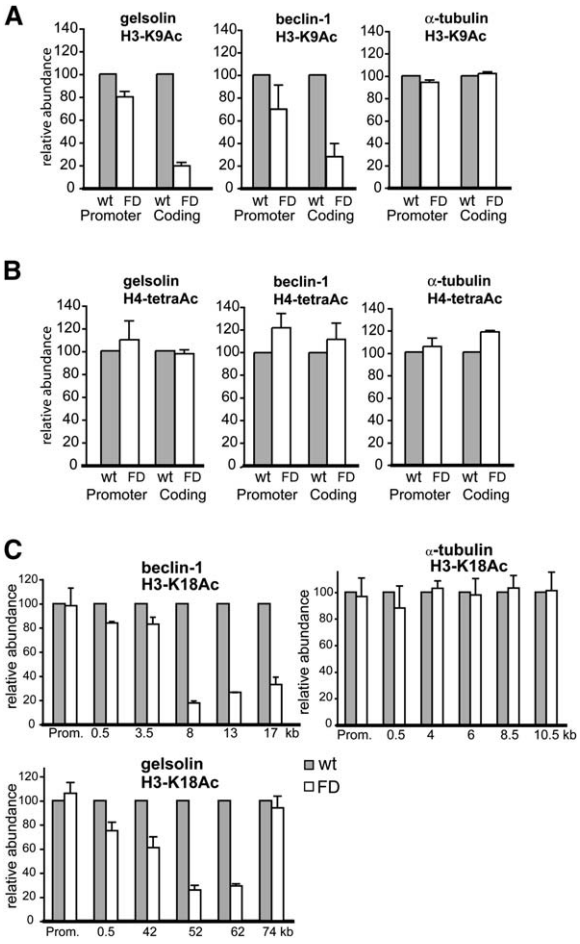

Figure 6. Low Levels of Elongator Result in Histone H3 Hypoacetylation through the Coding Region of Target Genes

(A) ChIP assays with an anti-histone H3K9ac-specific antibody were performed with normal (wt) or FD (FD) fibroblasts. For ease of comparison, density in normal fibroblasts was set to 100 for each primer set and the FD values expressed relative to that.

(B) As in (A) but using an anti-histone H4tetraAc-specific antibody.

(C) ChIP assays with an anti-histone H3K18ac-specific antibody were performed with normal (wt) or FD (FD) fibroblasts. Primers were derived from the indicated regions of the beclin-1, gelsolin, and α-tubulin genes (see Figure 5A). For ease of comparison, density in normal fibroblasts was set to 100 for each primer set and the FD values expressed relative to that. Error bars denote standard deviation. For a presentation of the data where the level of acetylation at the promoter in wt cells was set to 100, see Figure S5. The reason for the apparently normal level of acetylation observed at the very end of the gelsolin gene in FD cells is unknown but might be due to the next gene downstream being relatively near (~6 kb).

for the C terminus of histone H3) before tabulation. Importantly, these effects were specific for the Elongator target genes, as no significant change in histone modification was observed at the α-tubulin control gene in FD fibroblasts (Figures 6A, 6B, and 6C right). The lack of effect of Elongator on histone acetylation at α-tubulin is intriguing. Presumably, residual Elongator activity or other HATs fulfill the requirements for histone acetylation at this gene, but not at the target genes in FD cells (see Discussion).

Having established that Elongator affects the level of H3 acetylation of target genes, we next more precisely defined the spatial distribution of histone H3 acetylation across the genes. Interestingly, histone H3 acetylation at the promoter and the beginning of the coding region was more or less unaffected by the absence of IKAP/Elongator. However, further into the open reading frame, more dramatic decreases were observed (Figure 6C). Again, histone acetylation in the coding region correlated with the lower activity of IKAP/hELP1 target genes in FD cells; it was lowered in the beclin-1 and gelsolin genes but remained unchanged in the α-tubulin gene (Figure 6C).

Previous results have shown that transcription is associated with increased acetylation of both histones H3 and H4 (see, for example, Kouskouti and Talianidis [2005]), but Elongator HAT activity primarily targets histone H3 in vitro, and in vivo in yeast (Hawkes et al., 2002, Kim et al., 2002; Wittschieben et al., 2000; Winkler et al., 2002). The finding that histone H3, but not histone H4, acetylation is decreased by IKAP/hELP1 depletion is thus consistent with the idea that the observed change in H3 acetylation levels is a direct effect of Elongator depletion.

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.

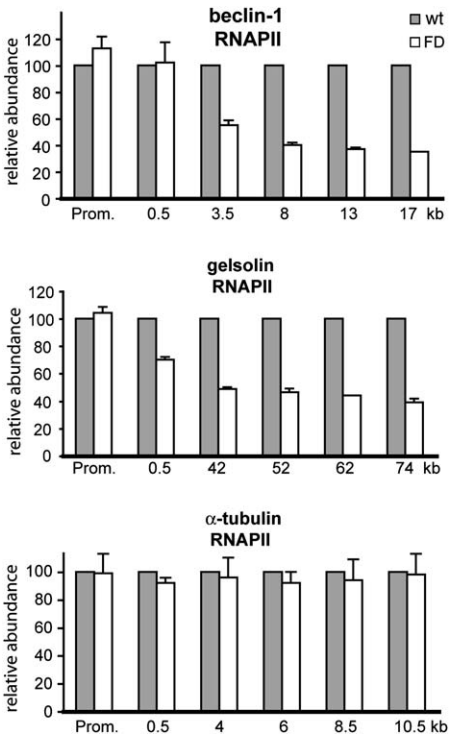

Figure 7. Low Levels of Elongator Result in Progressively Lower Density of RNAPII through the Coding Region of Target Genes

ChIP assays with an anti-RNAPII antibody (4H8) were performed with normal (wt) or FD (FD) fibroblasts. Primers were derived from the indicated regions of the gelsolin, beclin-1, and α-tubulin genes (see Figure 5A for localization of primers). For ease of comparison, density in normal fibroblasts was set to 100 for each primer set and the FD values expressed relative to that. Error bars denote standard deviation. For a presentation of the data where the level of acetylation at the promoter in normal cells was set to 100, see Figure S6.

IKAP/hELP1 Depletion Affects Elongator Integrity and Function

In yeast, the Elp1 protein assembles the Elongator complex, which also includes the histone acetyltransferase Elp3 and four additional subunits. The crucial importance of Elp1 for Elongator integrity is underscored by the fact that deletion of *ELP1* in yeast results in the catalytic Elp3 subunit becoming undetectable (Petrakis et al., 2004). Here, we show that IKAP/hELP1 depletion also results in lower hELP3 levels in human cells. Moreover, hELP3 depletion through RNAi also results in decreased mRNA levels of the tested IKAP/hELP1-dependent genes, supporting the idea that the uncovered effects are due to defects in the function of the Elongator complex. However, although we consider it unlikely, it cannot be ruled out that hELP1/IKAP also has roles distinct from that in the Elongator complex.

It is essential to emphasize that although RNAi and the FD mutation affect IKAP/hELP1 levels, there are still significant levels of the protein in these cells. We believe this residual amount may be important for transcription of a larger number of genes, as well as for cellular viability. Indeed, the *ELP1/IKBKAP* gene, as well as the *ELP3* gene, is essential in *Drosophila melanogaster,* and the mutants die with a remarkably similar terminal phenotype (Jane Walker and J.Q.S., unpublished data; James Gusella, personal communication).

It is worth pointing out that we detected Elongator not only on genes whose expression was affected by decreased IKAP/hELP1 levels but also on the unaffected α-tubulin gene. Other researchers have previously reported that Elongator was detected on three genes examined by ChIP but that factors such as FACT, CBP, PCAF, and SNF2H were only found at one or two of the genes studied (Kouskouti and Talianidis, 2005). Elongator was also detected at the estrogen-inducible pS2 gene (Metivier et al., 2003). Elongator is thus present at several genes, yet our expression data suggest that only relatively few are affected by IKAP/Elongator depletion. Interestingly, this finding fits well with recent data on other chromatin modifying factors. For example, histone acetyltransferases such as Gcn5 and Esa1 are both generally recruited to promoters of active genes in yeast yet only affect the expression of a small percentage of these genes (Robert et al. [2004] and references therein). So, although the mechanism underlying the lack of effects of Elongator depletion at, for example, the α-tubulin gene remains unknown, the finding is not unexpected. Possibly, histone acetylation may simply not be essential for the expression of this and other genes, or more likely, residual Elongator activity and/or other HATs/chromatin remodelers fulfill the requirements.

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;

Hawkes et al., 2002; Kim 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 *ELP3* 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 Kristjuhan 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 transcript 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 ELP1 (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 can 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 neuropathological 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 neuritogenesis (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 Biotechnologies; rabbit polyclonal anti-acetyl H3 K9, anti-acetyl H3 K18, and anti-acetyl H4 antibody, as well as 4H8 antibody used to precipitate RNAPII, were from Upstate Biotechnology; and rabbit polyclonal anti-H3 (C-terminus antibody) was from Abcam. Antibodies directed against Elp3, Elp4, and Elp5 have been described previously (Petrakis 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 lyzed 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, biotin-labeled 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 proliferation. 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 37ºC. Cells on the upper surface were removed, and membranes were fixed in ethanol at −20ºC 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 30ºC for 30 min with 1 μg of purified GST-c-Jun fusion protein and 10 μCi of [γ^{32}P] ATP in 20 μl kinase buffer (20 mM HEPES [pH 7.4], 10 mM $MgCl_2$, 25 mM β-glycerophosphate, 50 μM Na_3VO_4, 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, and immunoprecipitation was performed by incubating overnight at 4ºC with the relevant antibody, using HA antibody or preimmune serum as negative controls, and then 1 hr 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/.

Acknowledgments

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Accession Numbers

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

Supplemental Data

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

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Supplemental References

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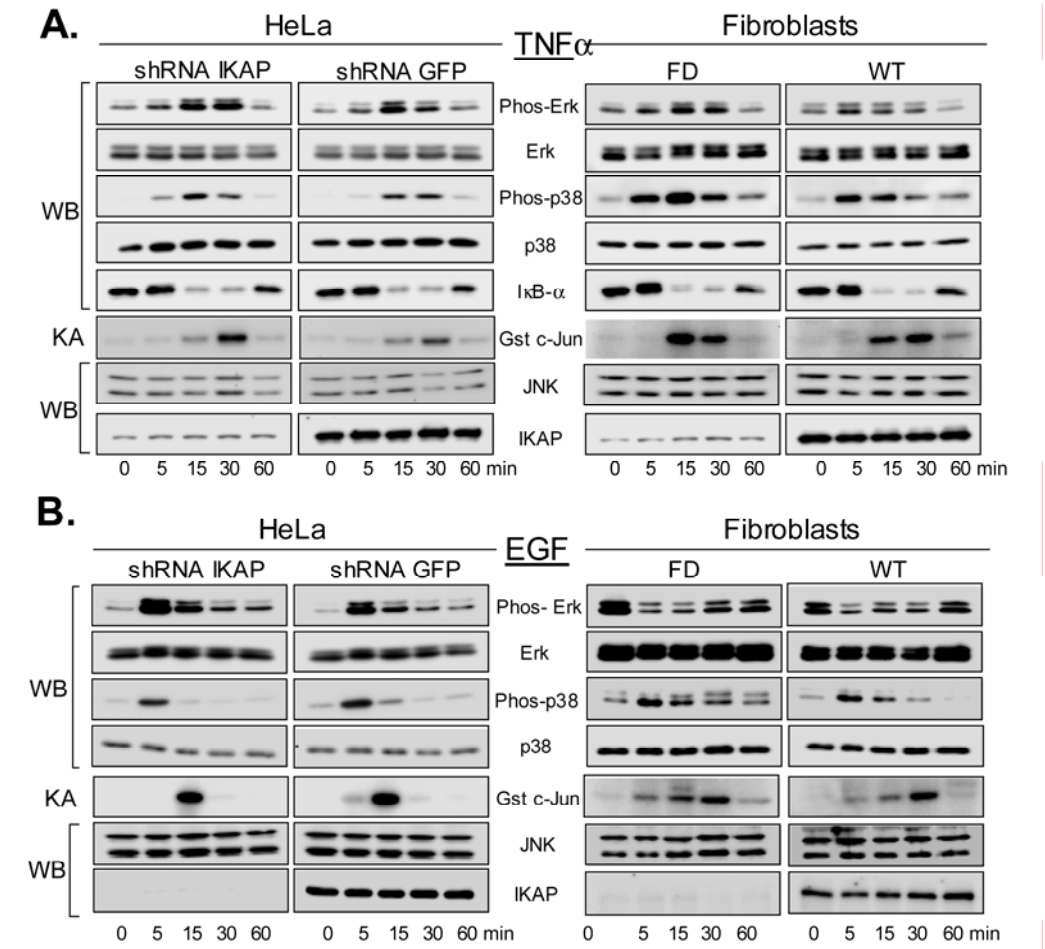


Supplementary Figure 1. Decreased IKAP expression has no effect on TNFα-, or EGF-mediated Erk1, p38 or JNK activation. To address the potential role of IKAP in stress-induced MAP Kinase (MAPK) activation, we determined whether IKAP down-regulation alters MAPK activation in response to growth factors and pro-inflammatory cytokines (A and B, panels on the left). Western blot analysis showed a dramatic decrease of IKAP expression in shRNA IKAP, but not in shRNA GFP-infected HeLa cells (A and B, top panels). Infected HeLa cells were treated with either TNFα or EGF for 5 to 60 minutes and Erk1 and p38 activation was monitored by western analysis using the relevant phospho-specific antibodies, whereas JNK activation was assessed by in vitro kinase assays, testing the ability of anti-JNK immunoprecipitates to phosphorylate a GST-c-Jun substrate. Likewise, the kinetics of IκBα degradation in response to TNFα, which reflects IKK and subsequent NF-kB activation (Hayden and Ghosh, 2004), was also unaffected by shRNA IKAP, as jugded by anti-IκBα (B, on the left, third panel from bottom). To determine whether TNFα and EGF induced-MAPK activation might be impaired in FD patients, similar experiments were conducted in either normal fibroblasts (WT), or fibroblasts derived from FD patients (FD) (A and B, panels on the right). Significantly, although FD fibroblasts indeed expressed lower amounts of IKAP (A and B, panels on the right, top panels), they did not display altered Erk1, p38 and JNK activation kinetics in response to TNFa and EGF (A and B, panels on the right). Finally, EGF-mediated Akt kinase activation was unchanged in FD fibroblasts versus control cells as well as in shRNA IKAP versus shRNA GFP HeLA cells (data not shown). WB= Western blot; KA= kinase assay.

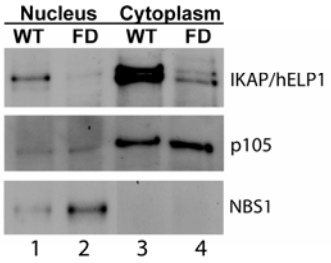
	accession number	description	symbol	fold	p-value
Cell adhesion	NM_000602	plasminogen activator inhibitor type 1 (Serpine-1)	PAI-1	-3,4	0,0001
	NM_000214	jagged 1	JAG1	-2,8	0,00002
	NM_014021	synovial sarcoma, X breakpoint 2 interacting protein	SSX2IP	-2,6	0,00002
	NM_014376	cytoplasmic FMR1 interacting protein 2	CYFIP2	-2,5	0,00002
	NM_001753	caveolin 1	CAV1	-2,0	0,00002
cytoskeleton	NM_006612	kinesin family member 1C	KIF1C	-6,5	0,00004
	NM_001175	Rho GDP dissociation inhibitor, beta	GDI	-4,3	0,00007
	NM_000177	Gelsolin	GSN	-2,8	0,0001
	NM_015055	SWAP-70 protein	SWAP70	-2,8	0,00002
	NM_007286	synaptopodin	SYNPO	-2,7	0,0003
	NM_003980	microtubule-associated protein 7	MAP7	-2,1	0,0001
	BE222709	microfibrillar-associated protein 3	MFAP3	-2,0	0,0006
	NM_002906	radixin	RDX	-2,0	0,00002
	NM_003289	tropomyosin 2 beta	TPM2	-2,0	0,00002
	NM_012121	CDC42 effector protein (Rho GTPase binding) 4	CDC42EP4	-2,0	0,00003
	NM_017491	WD repeat domain 1	WDR1	-2,0	0,00002
	XM_170658	EH domain binding protein 1-like 1	EHBP1L1	-2,0	0,000006
cell proliferation/ growth	NM_000076	cyclin-dependent kinase inhibitor 1C (p57, Kip2)	CDKN1C	-3,3	0,001
	NM_002388	minichromosome maintenance deficient 3	MCM3	-3,0	0,00002
	NM_001071	thymidylate synthetase	TYMS	-2,6	0,00002
	NM_014059	response gene to complement 32	RGC32	-2,5	0,0002
	NM_002315	rhombotin 1		-2,3	0,00002
	NM_001311	cysteine-rich protein 1 (intestinal)	CRIP1	-2,2	0,0001
	NM_002514	nephroblastoma overexpressed gene	NOV	-2,0	0,0008
kinases	NM_015000	serine/threonine kinase 38 like	STK38L	-3,4	0,00003
	NM_002738	protein kinase C beta-II type	PRKCB1	-3,1	0,001
	NM_006296	vaccinia related kinase 2	VRK2	-3,0	0,00003
	NM_014216	inositol 1,3,4-triphosphate 5/6 kinase	ITPK1	-2,0	0,002
phosphatases	NM_002849	protein tyrosine phosphatase, receptor type, R	PTPRR	-3,5	0,00008
	NM_002709	protein phosphatase 1, catalytic subunit, beta isoform	PPP1CB	-2,5	0,00002
receptors	NM_000212	CD51	CD51	-12,9	0,001
	NM_020311	chemokine orphan receptor 1	CMKOR1	-3,0	0,00002
	NM_003979	G protein-coupled receptor, family C, group 5, member A	GPRC5A	-2,3	0,0002
	NM_000877	interleukin 1 receptor, type I	IL1R1	-2,2	0,0002
	NM_002185	interleukin 7 receptor	IL7R	-2,0	0,00007
	NM_004367	CD151 antigen	CD151	-2,0	0,0002
extracellular matrix	NM_002160	tenascin C	TNC	-2,8	0,00001
	NM_000228	laminin, beta 3	LAMB3	-2,5	0,0001
	NM_006528	tissue factor pathway inhibitor 2	TFPI2	-2,1	0,0002
	NM_000088	collagen, type I, alpha 1	COL1A1	-2,0	0,00002
ER metabolism	NM_004343	calreticulin	CALR	-2,5	0,0001
	NM_004530	MMP-2	MMP2	-2,3	0,00001
	NM_006334	olfactomedin 1	OLFM1	-2,2	0,0002
	NM_014607	UBX domain-containing 2	UBXD2	-2,0	0,0002
autophagy	NM_003766	beclin 1	BCN1	-2,0	0,00002
endocytosis	NM_018993	Ras and Rab interactor 2	RIN2	-2,3	0,0002
	NM_006868	RAB31, member RAS oncogene family	RAB31	-2,0	0,00002
	NM_016277	RAB23, member RAS oncogene family	RAB23	-2,0	0,0007

	accession number	description	symbol	fold	p-value
transcription factors	NM_001429	E1A binding protein p300	EP300	-3,0	0,002
	NM_003670	basic helix-loop-helix domain containing, class B, 2	BHLHB2	-2,2	0,00002
	NM_020347	leucine zipper transcription factor-like 1	LZTFL1	-2,2	0,0001
	NM_005901	SMAD, mothers against DPP homolog 2	SMAD2	-2,1	0,00002
	NM_181552	cut-like 1, CCAAT displacement protein (Drosophila)	CUTL1	-2,0	0,001
DNA repair	NM_000251	mutS (E. coli) homolog 2 (colon cancer, nonpolyposis type 1)	hMSH2	-2,3	0,00002
metabolism	NM_005173	ATPase, Ca++ transporting	ATP2A3	-4,2	0,0002
	NM_005737	ADP-ribosylation factor-like 7	ARL7	-3,7	0,0004
	NM_000433	neutrophil cytosolic factor 2	NCF2	-2,6	0,00002
	NM_005978	S100 calcium-binding protein A2	S100A2	-2,5	0,00002
	NM_198098	aquaporin 1	AQP1	-2,5	0,0001
	NM_001034	ribonucleotide reductase M2 polypeptide	RRM2	-2,3	0,00002
	NM_001630	annexin A8	ANXA8	-2,3	0,00002
	NM_002960	S100 calcium-binding protein A3	S100A3	-2,3	0,00006
	NM_014765	translocase of outer mitochondrial membrane 20 (yeast) homolog	TOMM20	-2,3	0,0002
	NM_004613	transglutaminase 2	TGM2	-2,1	0,00002
	NM_006710	COP9 constitutive photomorphogenic homolog subunit 8 (Arabidopsis)	COPS8	-2,1	0,00002
	NM_020299	aldo-keto reductase family 1, member B10	AKR1B10	-2,1	0,00003
	NM_001976	enolase 3	ENO3	-2,0	0,001
	NM_001829	chloride channel 3	CLCN3	-2,0	0,00002
	NM_005063	stearoyl-CoA desaturase	SCD	-2,0	0,001
	NM_014280	DnaJ (Hsp40) homolog, subfamily C, member 8	DNAJC8	-2,0	0,001
	NM_015700	HIRA-interacting protein 5	HIRIP5	-2,0	0,00002
RNA export	NM_007362	nuclear cap binding protein subunit 2	NCBP2	-2,0	0,00002
scaffold proteins	NM_021255	pellino homolog 2 (Drosophila)	PELI2	-2,0	0,00002
	NM_002859	paxillin	PXN	-2,0	0,0001
others	NM_020199	HTGN29 protein	HTGN29	-2,6	0,001
	NM_000735	glycoprotein hormones, alpha polypeptide	CGA	-2,5	0,0001
	NM_001008529	transmembrane anchor protein 1	TAMP1	-2,5	0,00002
	NM_002569	furin	FURIN	-2,5	0,00007
	NM_015534	zinc finger, ZZ-type containing 3	ZZZ3	-2,5	0,00002
	NM_022736	major facilitator superfamily domain containing 1	MFSD1	-2,5	0,00002
	NM_006333	nuclear DNA-binding protein C1D	C1D	-2,4	0,0002
	BC005136	solute carrier family 35, member 3	SLC35A3	-2,3	0,0002
	NM_000346	SRY (sex determining region Y)-box 9	SOX9	-2,3	0,0004
	NM_004403	deafness, autosomal dominant 5	DFNA5	-2,3	0,0001
	NM_013372	gremlin 1 homolog, cysteine knot superfamily (Xenopus laevis)	GREM1	-2,3	0,001
	NM_020182	transmembrane, prostate androgen induced RNA	TMEPAI	-2,1	0,00003
	NM_021977	solute carrier family 22, member 3	SLC22A3	-2,1	0,0003
	NM_001280	cold inducible RNA binding protein	CIRBP	-2,0	0,0002
	NM_001831	clusterin	CLU	-2,0	0,0001
	NM_003204	nuclear factor (erythroid-derived 2)-like 1	NFE2L1	-2,0	0,001
	NM_003498	stannin	SNN	-2,0	0,0001
	NM_005418	suppression of tumorigenicity 5	ST5	-2,0	0,001
	NM_006459	SPFH domain family, member 1	SPFH1	-2,0	0,00002
	NM_007273	prohibitin 2	PHB2	-2,0	0,00002
	NM_015455	CCR4-NOT transcription complex, subunit 6	CNOT6	-2,0	0,00002
	NM_018105	THAP domain containing, apoptosis associated protein 1	THAP1	-2,0	0,0003
	NM_145728	desmuslin	DMN	-2,0	0,00006

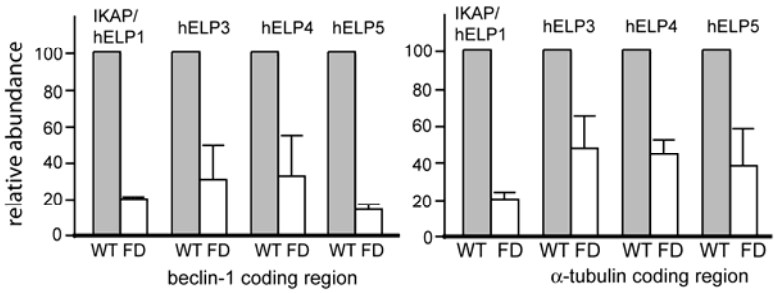
Supplementary Figure 2A. Genes down-regulated more than 2-fold by IKAP RNAi.

	accession number	description	symbol	fold	p-value
metabolism	NM_133436	asparagine synthetase	ASNS	4,0	0,00002
	NM_058179	phosphoserine aminotransferase 1	PSAT1	2,6	0,00002
	NM_003407	zinc finger protein 36, C3H type, homolog (mouse)	ZFP36	2,1	0,00005
	NM_139273	cysteinyl-tRNA synthetase	CARS	2,1	0,00002
	NM_001902	cystathionase (cystathionine gamma-lyase)	CTH	2,0	0,0002
	NM_004990	methionine-tRNA synthetase	MARS	2,0	0,00002
	NM_006169	nicotinamide N-methyltransferase	NNMT	2,0	0,00002
	NM_006623	phosphoglycerate dehydrogenase	PHGDH	2,0	0,00002
transcription factor	NM_000399	early growth response 2 (Krox-20 homolog, Drosophila)	EGR2	2,1	0,0002
apoptosis	NM_012385	p8 protein	COM1	2,0	0,00002
receptor	NM_003383	very low density lipoprotein receptor	VLDLR	2,0	0,00008
others	NM_005980	S100 calcium binding protein P	S100P	2,8	0,00002
	NM_021158	tribbles homolog 3 (Drosophila)	TRIB3	2,3	0,00002
	NM_016040	transmembrane emp24 protein transport domain containing 5	TMED5	2,0	0,00002
	NM_014278	heat shock 70kDa protein 4-like	HSPA4L	2,0	0,00002

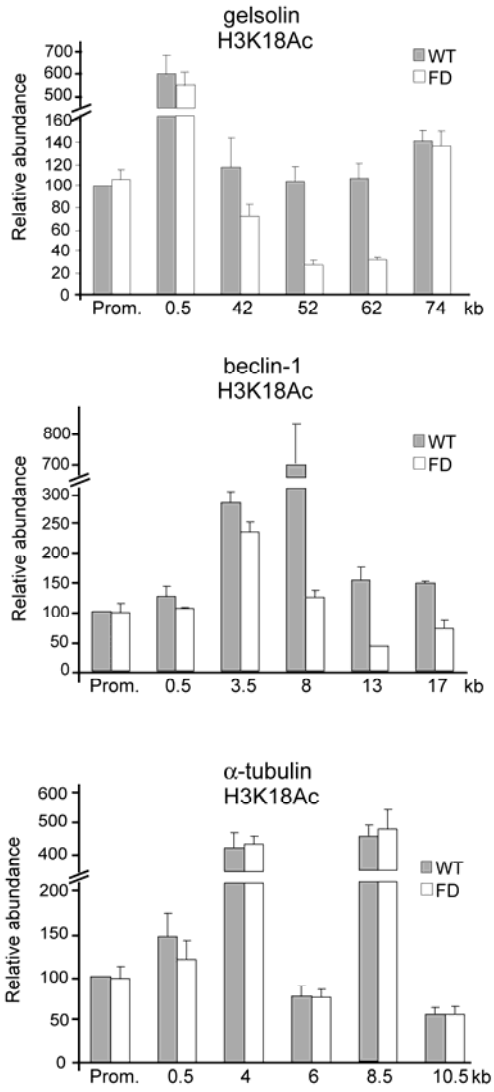
Supplementary Figure 2B. Genes up-regulated more than 2-fold by IKAP RNAi.



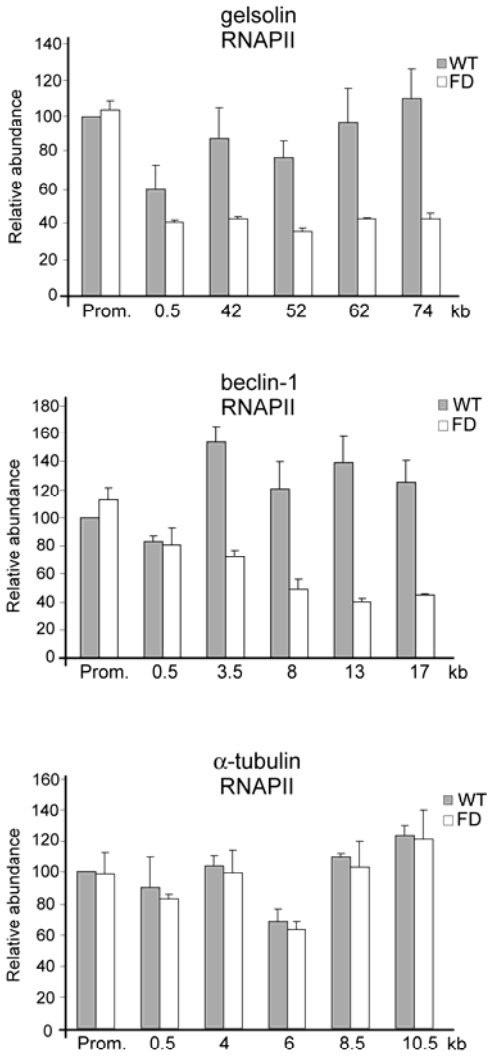
Supplementary Figure 3. The relative sub-cellular localization of IKAP/hELP1 is unchanged in FD cells. Subcellular fractionation was done by standard techniques. Briefly, after extensively washing with PBS, cells were resuspended/lyzed in cytoplasmic lysis buffer and incubated on ice for 5 minutes. After centrifugation (5 minutes at 2000 rpm), the supernatant was kept as the cytoplasmic fraction. The pellet was cautiously washed twice with wash buffer (10mM Hepes, pH7.9; 20 mM KCl, 2mM $MgCl_2$, 0.1 mM EDTA, pH 7.9) before lysis. The same proportions of the extracts (cytoplasmic and nuclear) were analysed by Western blot. p105, a member of the IκB family of proteins, which is primarily cytoplasmic, but also found in the nucleus, and Nijmegen Break Syndrome 1 (NBS1), which is nuclear, are shown as references. The relevance of the different modification status of IKAP/hELP1 in the nucleus and the cytoplasm is unknown (compare lane 1 with lanes 3 and 4).



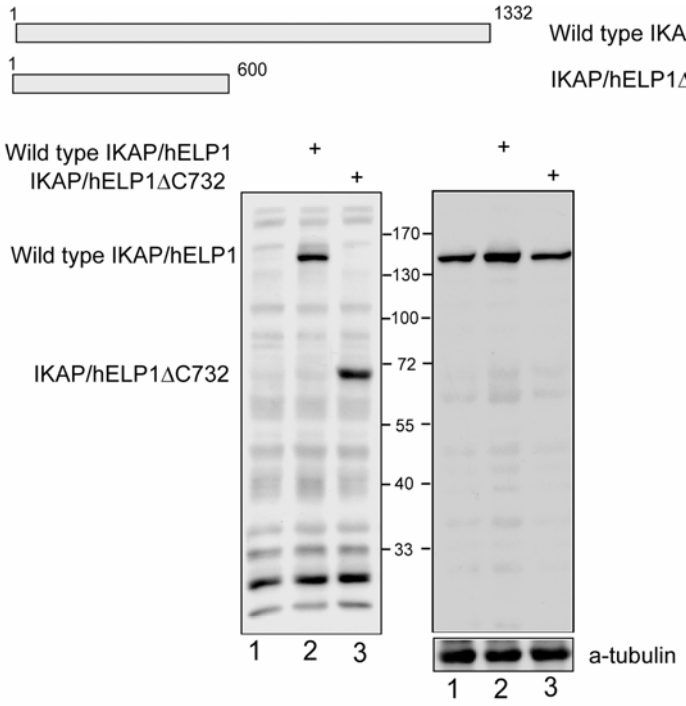
Supplementary Figure 4. Recruitment of the entire Elongator complex to genes is impaired upon IKAP/hELP1 depletion. Chromatin immunoprecipitation assays with the Elongator antibodies indicated above the graphs were performed using normal (WT) or FD (FD) fibroblasts. Associated DNA was analysed by real-time PCR using primers derived from the promoter or the coding region of the indicated genes. For ease of comparison, density in the coding region in normal fibroblasts was set to 100, and the other values expressed relative to that.



Supplementary Figure 5. Low levels of Elongator results in histone H3K18 hypo-acetylation in the coding region of target genes. Chromatin immunoprecipitation assays with an anti-histone H3K18ac-specific antibody were performed using normal (WT) or FD (FD) fibroblasts. Primers were derived from the indicated regions of the beclin-1, gelsolin and α-tubulin genes (see Figure 5A). Here, the level of acetylation at the promoter in normal cells was set to 100, and the other values expressed relative to that. The reason for the apparently normal level of acetylation observed at the very end of the gelsolin gene in FD cells is unknown, but may be due to the next gene downstream being relatively near (~6 kb).



Supplementary Figure 6. Low levels of Elongator results in progressively lower density of RNAPII through the coding region of target genes. Chromatin immunoprecipitation assays with anti-RNAPII antibody were performed using normal (WT) or FD (FD) fibroblasts. Primers were derived from the indicated regions of the beclin-1, gelsolin and α-tubulin genes (see Figure 5A). Here, the level of acetylation at the promoter in normal cells was set to 100, and the other values expressed relative to that.



Supplementary Figure 7: Specificity of the anti-IKAP/hELP1 antibody used in this study. Upper panel, schematic representation of the IKAP/hELP1 used to test the specificity of the anti-IKAP/hELP1 antibody. HeLa cells were transfected with the indicated expression constructs, and cell lysates were subjected to anti-FLAG antibody (left panel), -IKAP/hELP1 antibody (right panel) or -α-tubulin antibody (bottom small panel on the right) Western analysis. Whereas the anti-FLAG antibody recognizes both wild type and IKAP/hELP1 ΔC732 protein (left panel, lanes 2 and 3), the polyclonal anti-IKAP/hELP1 antibody, which was raised against a C-terminal hELP1 peptide, only detects endogenous (panel on the right, lanes 1-3, upper band) and overexpressed wild type IKAP/hELP1 (panel on the right, compare lane 2 with lanes 1 and 3), but fails to detect the expressed IKAP/hELP1ΔC732 protein (panel on the right, lane 3, lower band from left panel is not detected).

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