



# Dual function of the epithelial specific ets transcription factor, ELF3, in modulating differentiation

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The ets family of transcription factors comprises many members which contribute to diverse cellular functions that vary depending upon the cell- and tissue-type context. Recently, different groups have identified a novel member of the ets family that is epithelial-specific. Variably called ESE-1, ERT, jen, ESX, this gene is designated currently as ELF3. In order to understand transcriptional regulatory mechanisms mediated by ELF3, we investigated its effect on the human keratin 4 gene promoter based upon the role of keratin 4 in early differentiation of the esophageal squamous epithelium. Interestingly, ELF3 suppressed basal keratin 4 promoter activity in both esophageal and cervical epithelial cancer cell lines, a novel result, while simultaneously activating the late-differentiation linked SPRR2A promoter. Furthermore, serial deletion constructs of the keratin 4 promoter continued to be suppressed by ELF3, a phenomenon that was only partially rescued by ELF3 ets domain mutants, but completely abrogated by deletion of the ELF3 pointed domain. These results suggest that ELF3 may have dual functions in the transcriptional regulation of genes involved in squamous epithelial differentiation. One of these functions may not be exclusively mediated through DNA binding in the context of transcriptional suppression of the keratin 4 promoter. *Oncogene* (2000) 19, 1941–1949.

**Keywords:** ets transcription factors; epithelial cell; differentiation

## Introduction

Ets transcription factors have been shown to be important in the transcriptional regulation of genes involved in diverse functions, including cell differentiation and proliferation, cell cycle progression, angiogenesis, and malignant transformation. The gene family consists of more than 35 members. The prototype member is the ets-1 oncogene, which is the progenitor of the viral oncogene v-ets (Wernert *et al.*, 1998). All members of the ets transcription factor/oncogene family share a highly conserved DNA binding domain, designated as the ets domain. This represents a winged helix-turn-helix domain that is required to bind the

major groove of specific DNA sequences centered at a conserved core 5'-GGAA/T-3' motif (Batchelor *et al.*, 1998). A subset of ets factors, e.g. ets-1, ets-2, fli-1, TEL, GABP $\alpha$ , and ELF3, share a homologous region at the N-terminus, referred to as the pointed domain. This domain appears to be important for the transcriptional regulatory mechanisms mediated by these ets factors and might be involved in protein-protein interactions (Slupsky *et al.*, 1998; Oettgen *et al.*, 1999).

A particularly important cell type for the manifestations of the biological functions of ets factors is the stratified squamous epithelium, which is composed of proliferating basal cells, early differentiated suprabasal, and terminally differentiated superficial squamous cells. Basal cells harbor a delicate equilibrium between proliferation and differentiation, ultimately proceeding through a complex differentiation program while migrating to the surface. Sites sharing the stratified squamous epithelium include the epidermis, the oropharynx and the esophagus. Since keratins are critically abundant genes in squamous epithelial cells, an understanding of their transcriptional regulation by ets factors may provide a useful paradigm for investigating the molecular mechanisms underlying differentiation. Our own work has elucidated how the suprabasal localized keratin 4 (K4), highly expressed in esophageal epithelial cells, is important in the switch from proliferation to early differentiation through K4 promoter studies and a K4 gene knockout mouse model (Jenkins *et al.*, 1998; Ness *et al.*, 1998; Opitz *et al.*, 1998).

Recent studies have revealed an essential role in epithelial cells for the ets transcription factor family. Several groups have cloned a novel ets factor family member whose expression appears to be epithelial specific. This epithelial specific ets factor, initially termed ESE-1, was first identified and characterized by Libermann and coworkers (Oettgen *et al.*, 1997a) who searched an expressed sequenced tag cDNA database for sequences homologous to known ets factors. Additionally, four other groups discovered the same gene. Jen was the term designated to this novel member that resulted from screening a human foreskin keratinocyte cDNA library with a conserved 3' region of the ets domain (Andreoli *et al.*, 1997). A search of expressed sequence tags with an 8 amino acid motif of the carboxyl terminal region of the ets domain led to the identification of the same factor, termed ESX for epithelial restricted with serine box (Chang *et al.*, 1997). The employment of a yeast two-hybrid system by screening a human placenta cDNA library again

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yielded the same ets factor, and was designated ERT for ets-related transcription factor (Choi *et al.*, 1998). Currently, ELF3 is the name applied to this gene (Tymms *et al.*, 1997; Oettgen *et al.*, 1999), based upon its belonging to the ELF (E74-like-factor) subfamily of the ets transcription factor family (Laudet *et al.*, 1999). Its genomic organization has been elucidated and the gene localizes to human chromosome 1 q32.1–q32.2, a region that is altered in several tumors (Tymms *et al.*, 1997; Oettgen *et al.*, 1997b, 1999) which may in part account for ELF3 overexpression in breast carcinogenesis (Chang *et al.*, 1997).

The full-length cDNA for ELF3 encodes two alternative splice products, one of which is 1846 bp or about 39 kDa (formerly called ESE-1a) and the other which is 1915 bp or about 41 kDa (formerly called ESE-1b) (Oettgen *et al.*, 1997a). The latter is the major product expressed *in vivo*. The predominant transcript is approximately 2.2 kilobases and found exclusively in epithelial cells under normal physiologic conditions, a finding unique among the ets factors. Furthermore, ELF3 is not expressed in lymphocytes, monocytes, fibroblasts or endothelial cells (Oettgen *et al.*, 1997a). ELF-3 is the prototype of a new subclass of ets factors. This transcription factor shows relatively low homology to other ets factors except in the ets domain and the pointed domain. Additionally, ELF3, in contrast to other ets factors, has a second putative DNA binding domain which is the A/T hook domain that is located 5' to the ets domain. The A/T hook domain has been described to bind the minor groove of AT rich tracts of double-stranded DNA (Reeves and Nissen, 1990) and is found in HMG proteins like TCF-1/LEF transcription factors, tamtrack factor, HRX/ALL-1/MLL-1 transcription factors, and retinoblastoma binding proteins RBP-1/2 (Aravind and Landsman, 1998).

Insights into the biological functions of ELF3 can be gained through an appreciation of target genes that are transcriptionally regulated by it. ELF3 transactivates the transglutaminase 3 (TGM3) promoter in HeLa, HaCaT and normal epidermal cells, but not in fibroblasts and neuroblastoma cells (Andreoli *et al.*, 1997). Of significance, ELF3 binds and transactivates enhancer elements within the SPRR2A and proflargin promoters, which along with the TGM3 promoter, are associated with terminal differentiation in stratified squamous epithelia (Andreoli *et al.*, 1997; Choi *et al.*, 1998; Oettgen *et al.*, 1997a). Moreover, ELF3 expression of ELF3 is induced *in vitro* during differentiation of primary human keratinocytes during Ca<sup>2+</sup> treatment (Andreoli *et al.*, 1997).

Keratin 4 is linked to early differentiation in the esophageal squamous epithelium. Given the epithelial specific expression pattern of ELF3 and its role in differentiation, we hypothesized that K4 might be a critical target of ELF3 especially in regulating the switch from proliferating basal cells to differentiating suprabasal cells. Surprisingly, the K4 promoter is repressed by ELF3 in esophageal squamous epithelial cells and in HeLa cells, suggesting dual functions of ELF3 in this cell type depending upon where in the differentiation cascade the target gene is involved.

## Results

### *ELF3 is expressed in esophageal squamous cancer cell lines*

To analyse the expression of ELF3 in different esophageal squamous epithelial cells, we performed reverse transcriptase-polymerase chain reaction (RT-PCR) analysis with total RNA derived from several different esophageal squamous cancer cell lines and from the HeLa cell line, the latter serving as a positive control for ELF3 expression. Two specific primers for ELF3 were used to amplify both splice forms of ELF3 (Table 1). As Figure 1 depicts, representative esophageal squamous cancer cell lines, TE-11 and TE-12, express only one of the forms of ELF3, namely the larger transcript formerly called ESE-1b. However, lower expression was observed in TE-12 cells. It should be noted that equal amounts of RNA templates were used and equal amounts of PCR products were loaded, which was confirmed by RT-PCR for the house-keeping gene  $\beta_2$ -microglobulin. Endogenous ELF3 protein was not detectable (data not shown), consistent with observations of other investigators (Oettgen *et al.*, 1997a). The TE-12 cell line was used to investigate further the functional consequences of ELF3 overexpression.

### *ELF3 regulates the differentiation-linked human keratin 4 promoter*

To investigate whether ELF3 transcriptionally regulates the differentiation-linked human keratin 4 promoter in TE-12 and HeLa cells, a tagged ELF3 expression vector was cotransfected with the full-length K4 promoter-luciferase construct, the latter was previously cloned and sequenced by our group (Opitz *et al.*, 1998). A *KpnI/EcoRI* fragment containing the entire open reading frame of ELF3 was subcloned in

**Table 1** Sequences of ELF3 specific primers

Primer	Nucleotide sequence (5' to 3')
ELF3 5'	CGG GGT ACC AAT GGC TGC AAC CTG TGA GAT T
ELF3 3'	CTG AAT TCT AGT TCC AAC CCT CAG TTC CG
ELF3-MT1 5'	GAA GCG GAA <b>AGC AGC AGC AGC</b> ACG AAA GCT GAG CAA A
ELF3-MT1 3'	TCA GCT TTC <b>GTG CTG CTG CTG</b> CTT TCC GCT TCC CGT
ELF3-MT2 5'	GGC CCA ACT <b>AGC AGG</b> CCA AAA GGC AAA GAA CAG CAA C
ELF3-MT2 3'	GCT GTT CTT TGC CTT TTG GCC TGC TAG TTG GGC CAC A
ELF3-MT3 5'	TGA GCC GGG CCA TGG <b>CAG CAG CAG</b> CAA AAC GGG AGA T
ELF3-MT3 3'	ATC TCC CGT TTT <b>GCT GCT GCT</b> GCC ATG GCC CGG CTC A
ELF3-INT 5'	ATC CCA CTG ATG GCA AGC TCT T

Nucleotides in bold represent the substituted nucleotides compared to the wild-type sequence

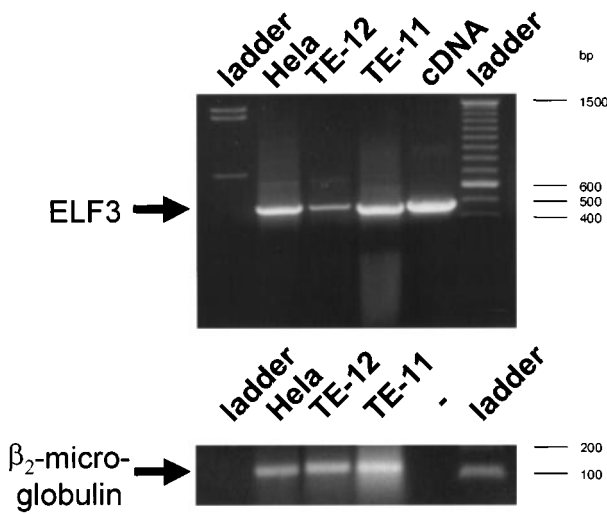
the mammalian expression vector pcDNA 3.1 His A. Overexpression of the tagged ELF3 with the expected

molecular mass of 48 kDa, which is a composite of the approximately 42 kDa ELF3 plus the 6 kDa Histidine tag, was confirmed using an anti-Xpress epitope antibody (not shown) and a ELF3 specific antibody in Western blot analysis (see later in Figure 5b).

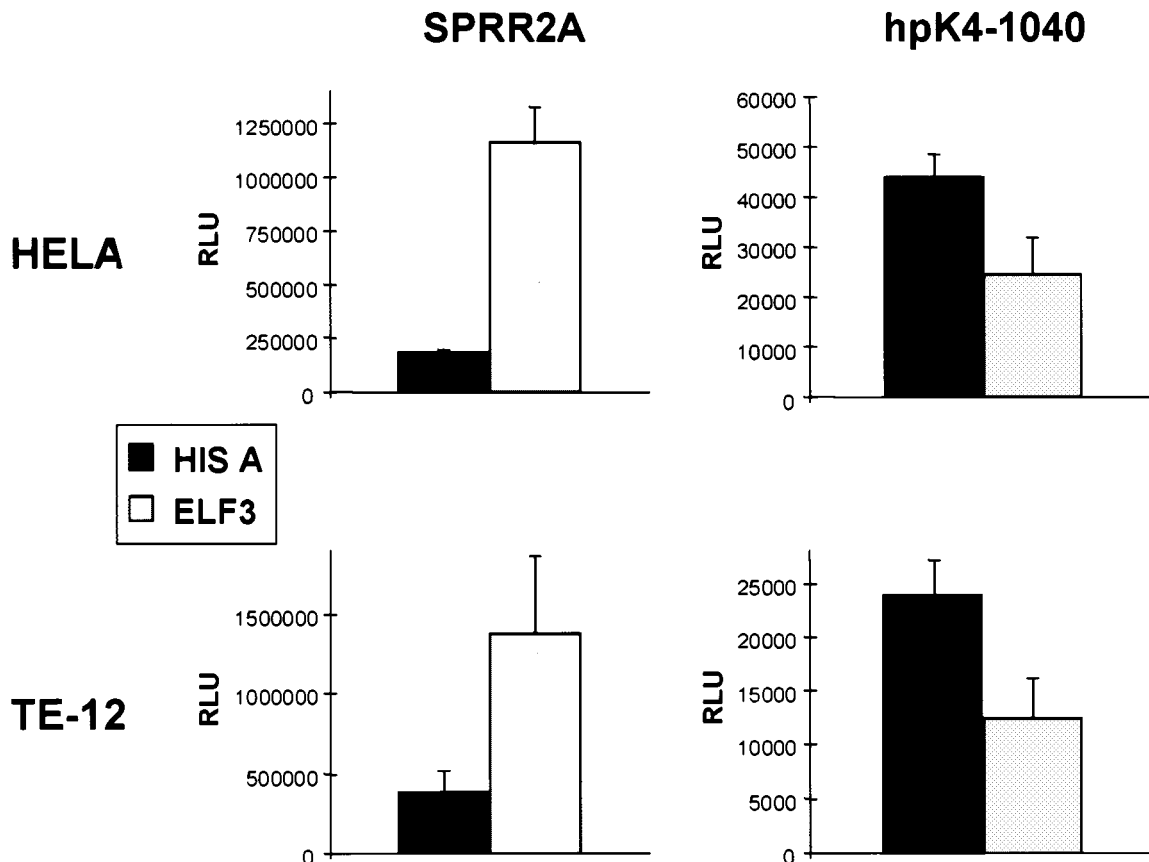
Using the -1040 human K4 promoter construct, we observed that ELF3 resulted in a 2–3-fold decrease of K4 promoter activity compared to cotransfection with an empty vector construct (Figure 2). It should be noted that basal K4 promoter activity is of levels similar to, if not identical to, that with co-transfection of the empty vector (Opitz *et al.*, 1998). A similar effect was evident in HeLa cells, namely repression of K4 promoter activity. In contrast, we found that the SPRR2A reporter construct, representing a late differentiation marker, is transactivated by ELF3 in both HeLa cells and TE-12 cells (Figure 2), corroborating the findings of Oettgen *et al.* (1997a) in HeLa cells.

*ELF3 expression negatively correlates with Keratin 4 protein levels*

We next investigated if endogenous protein levels of Keratin 4 correlate with the expression of ELF3 in these cells. Western blot analysis was performed using equal amounts of whole cell protein lysates. Western blots were probed with a monoclonal K4 specific antibody and visualized by chemiluminescence (Figure



**Figure 1** Reverse-transcription/PCR of ELF3. After reverse transcription of 1  $\mu$ g total RNA from different epithelial cell lines (HeLa, TE-12, TE-11), PCR was performed using an internal ELF3 specific 5'-primer and combined with the ELF3 3'-primer to yield an expected 450 bp fragment in the three different cell lines (upper panel). ELF3 cDNA served as a positive control. As an additional control for RT-PCR, reverse transcribed RNA for  $\beta_2$ -microglobulin was amplified with its own specific primers (lower panel)



**Figure 2** ELF3 suppresses the keratin 4 (K4) promoter reporter construct but transactivates the SPRR2A reporter construct. Three  $\mu$ g of the full-length K4 promoter luciferase reporter construct (K4-1040) was co-transfected with 1  $\mu$ g of either empty pcDNA 3.1 HisA or the pcDNA 3.1 HisA ELF3 expression vector into either HeLa or TE-12 cells, respectively. Transfections were performed in triplicate, and results of at least three independent experiments were calculated as the mean  $\pm$  s.e.m. values for relative luciferase activity (RLU). Parallel experiments were conducted with co-transfection of 2  $\mu$ g of the SPRR2A luciferase reporter construct and the same amounts of expression vectors

3). In TE-12 cells, which showed low ELF3 RNA levels, we detected abundant K4 protein expression. In contrast, HeLa cells which showed higher ELF3 expression by RT-PCR, demonstrated markedly lower endogenous K4 levels (Figure 3). After transient transfection of these cells with either empty expression vector (Figure 3, lane 2) or the ELF3 expression vector (Figure 3, lane 3), we found in both cell lines a slightly decreased expression of K4 upon overexpression of ELF3. Laser densitometry revealed an approximately 30% (HeLa) and 15% (TE-12) reduction in K4 expression compared to mock transfected controls (data not shown).

*Functional analysis of ELF3 mediated repression of the K4 promoter*

Recognizing the divergent effects of ELF3 on early-versus late-differentiation linked promoters, we next assessed the effect of ELF3 on serial deletions of the K4 promoter. Several putative ets protein DNA binding sites (-GGAA/T-) are located throughout this promoter both in the sense and antisense orientations. Constructs containing 800, 540, 340, 185, 163, and 140 bp of the flanking DNA sequence 5' to the putative transcription start site were used in a series of co-transfection experiments with ELF3. Upon co-transfection of ELF3, K4 promoter activity was repressed in all deletion constructs (Figure 4). A similar pattern was demonstrated in HeLa cells, suggesting that ELF3 mediated repression of the K4 promoter was similar in different cell lines (data not shown).

Basal K4 promoter activity was minimal with the -140 deletion construct and this could conceivably mask any potential effect mediated by ELF3. In an attempt to resolve this problem, we constructed further deletions of the K4 promoter (-102, -58) and co-transfected these constructs with the ELF3 expression vector. Although a trend towards ELF3 mediated suppression of K4 promoter activity was observed, we did not localize a putative DNA binding site for ELF 3

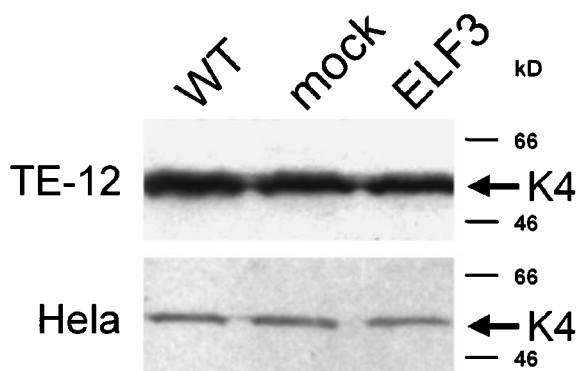
using those deletions (data not shown). This suggests that ELF3 mediated repression might not be linked to binding of ELF3 to DNA binding in the K4 promoter. To explore this further, we performed electromobility shift assays (EMSA) using nuclear extracts from mock transfected and ELF3 transfected TE-12 cells. We did not detect specific binding of ELF3 to an ets DNA binding site 116 bp (5'GGAA AGG ATG) of the K4 promoter where there is juxtaposition of ets and ELF3 sites (data not shown).

*An ELF3 ets domain mutant partially abrogates repression of the K4 promoter*

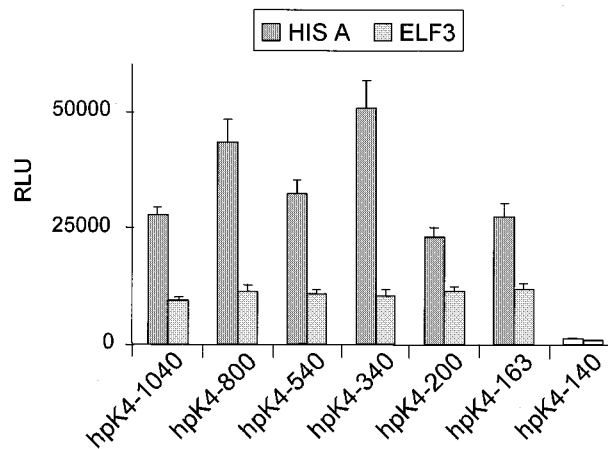
The repression of the K4 promoter activity by ELF3 raised the specter of whether this effect may not be mediated directly through DNA binding. In this context, we next investigated whether ELF3's effect on the K4 promoter may be mediated through its ets domain which is principally involved in DNA binding but also speculated to participate in protein-protein interactions with other transcription factors (Choi *et al.*, 1998; Basuyaux *et al.*, 1997). In addition, the A/T hook domain, uniquely found in ELF3 of all known ets factors, could potentially be involved through interaction with AT rich tracts of double-stranded DNA to stabilize binding of other transcription factors. This would suggest a role as co-activator or co-repressor as has been described for HMG I transcription factors containing an AT hook domain (John *et al.*, 1995; Arlotta *et al.*, 1997; Mantovani *et al.*, 1998).

We generated several ELF3 mutants using a PCR-based site-directed mutagenesis approach (Ho *et al.*, 1989), focusing on highly conserved amino acids in the ets domain of ets factors which have been shown to be important for DNA binding of the ets factor GABP $\alpha$  (Batchelor *et al.*, 1998) and likewise, conserved motifs in the A/T hook domain (Table 2).

An ELF3 A/T hook domain mutant, designated ELF3 MT1, and ELF3 ets domain mutants, designated



**Figure 3** ELF3 expression negatively correlates with Keratin 4 protein levels. Western blot analysis was performed using equal amounts of whole cell protein lysates (10  $\mu$ g per lane) of wild-type, mock and ELF3 transfected cells. Blots were probed with a monoclonal K4 specific antibody (Sigma) and visualized by chemiluminescence. Wild-type TE-12 cells express abundant K4 protein compared to HeLa cells. After transient transfection of these cells with the ELF3 expression vector, K4 expression was decreased 30% in HeLa cells and 15% in TE-12 cells compared to mock transfected cells



**Figure 4** ELF3 suppresses basal activity of K4 promoter-deletion constructs in TE-12 cells. Five  $\mu$ g of each K4 promoter deletion construct was transiently co-transfected with 1  $\mu$ g of either empty pcDNA 3.1 HisA or the pcDNA 3.1 HisA ELF3 expression vector into TE-12 cells. Transfections were performed in triplicate, and results of at least three independent experiments were calculated as the mean  $\pm$  s.e.m. values for relative luciferase activity (RLU)

ELF3 MT2 and MT3, were subcloned into the same pcDNA 3.1 HisA expression vector as wild-type ELF3. These ELF3 mutant expression vectors were then employed in further co-transfection experiments with the full-length K4 promoter. As Figure 5a depicts, the two ELF3 ets domain mutants (ELF3 MT2 and MR3) only partially abrogated the repressive effect of the wild-type ELF3 on the K4 promoter in TE-12 and HeLa cells, suggesting that functions beyond DNA binding may be relevant for the repressive effect of ELF3. It should be noted that there was equal expression of the wild-type and mutant ELF3 proteins with an expected size of approximately 48 kDa as confirmed by Western blot analysis using a polyclonal anti-ELF3 antibody (Figure 5b).

Our findings are consistent with the notion that the ets domain may have functions other than DNA binding since this region has been demonstrated to be important in protein–protein interactions and possibly have a dominant-negative effect as well (Choi *et al.*, 1998). Detailed structure-function mapping of the ets

domain has not been done to date, but ours is the first study to examine the biological consequences of ELF3 ets domain mutants. The A/T hook domain mutant ELF3 MT1 had the same repressive effect as wild-type ELF3 (Figure 5a). If the A/T hook domain is indeed involved in DNA binding by ELF3, then its function is not directly important for the effect of ELF3 on the K4 promoter.

#### *Deletion of the ELF3 pointed domain abolishes repression of the K4 promoter*

Apart from the ets and A/T hook domains, ELF3 contains the pointed domain in its N-terminus, which is found in several ets factors. It has been demonstrated that another ets factor, TEL, can act as a transcriptional repressor, in part through interaction with a co-repressor through its pointed domain (Fenrick *et al.*, 1999).

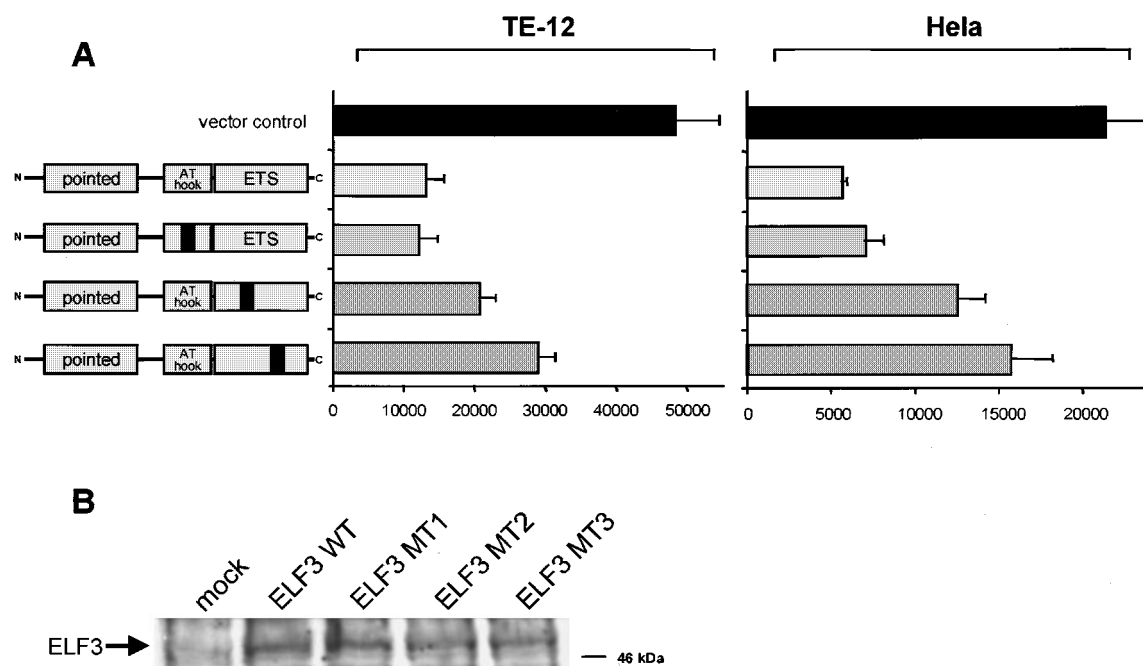
To further establish how the protein structure of ELF3 is linked to its function as a repressor of the K4 promoter, we used two deletion constructs of this protein in co-transfection studies. Using an untagged ELF3 expression vector as wild-type control, one construct bears a deletion of the ets domain and a second harbors a deletion of the pointed domain.

Co-transfection of the ELF3 $\Delta$ ets domain construct with the K4 promoter revealed a partial abrogation of the repression of the K4 promoter activity (Figure 6a) similar to the ets domain point mutants. In contrast, the repression of K4 promoter activity by ELF3 wild-type was completely abolished when co-transfection with the ELF3 $\Delta$ pointed domain construct was performed. Western blot analysis confirmed that all three

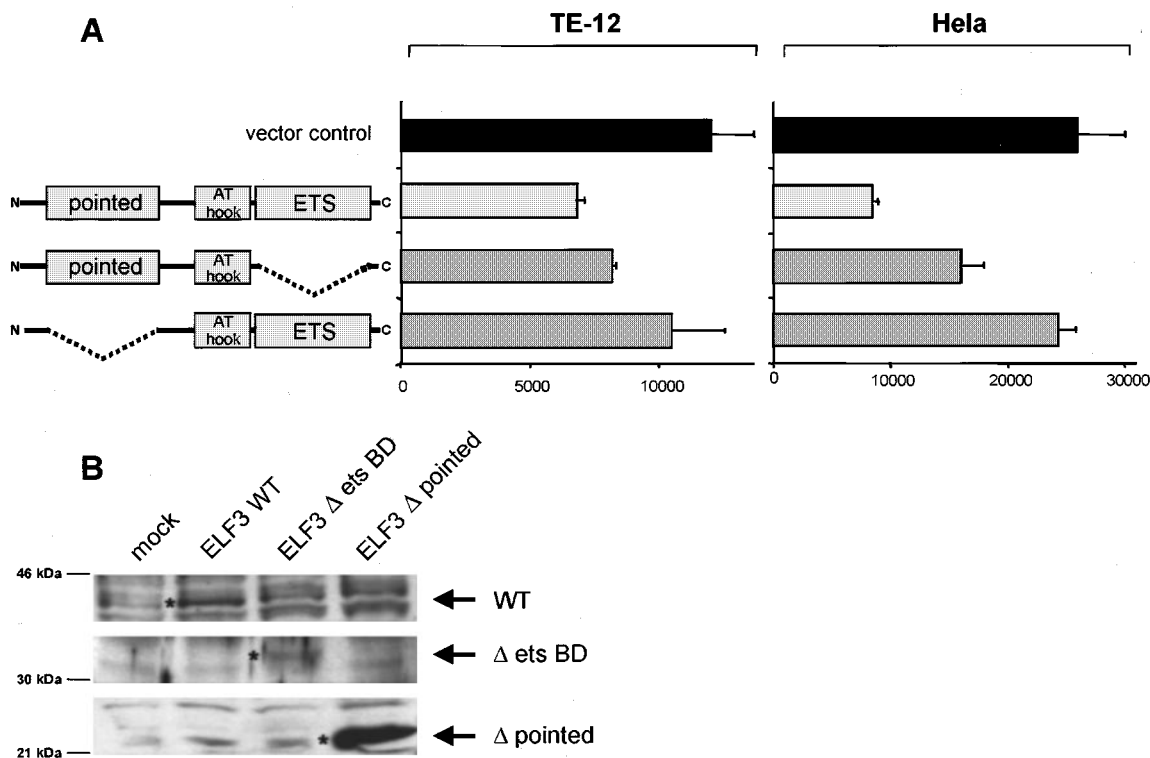
**Table 2** Amino acid sequences of ELF3 wild-type (WT) and ELF3 mutants within the AT Hook domain (MT1) and the ets domain (MT2, MT3)

AT HOOK	WT (240)	P K H G K R K R G R P R K L S K (255)
	MT1	P K H G K R K A A A A R K L S K
ETS	WT (311)	V A Q L W G Q K K K N S N (323)
	MT2	V A Q L A G Q K A K N S N
ETS	WT (330)	S R A M R Y Y K R E I (345)
	MT3	S R A M A A A A K R E I

The numbers in parentheses refer to the position in WT ELF3. Amino acids in bold represent the substituted amino acids compared to the wild-type sequence



**Figure 5** ELF3 ets domain mutants, but not the A/T hook domain mutant, partially reverse the suppression of the K4 promoter observed with wild-type ELF3. (a) 3  $\mu$ g of the full-length K4 promoter luciferase reporter gene was co-transfected with 1  $\mu$ g of either empty pcDNA 3.1 HisA (vector control) or the pcDNA 3.1 HisA ELF3 wild-type and compared to the activity of the ELF3 mutant expression vectors within the A/T hook domain (MT1) and ets-domain (MT2 and MT3; see Table 2). Transfections were performed in triplicate, and results of at least three independent experiments were calculated as the mean  $\pm$  s.e.m. values for relative luciferase activity (RLU). (b) Western blot analysis demonstrates expression of wild-type and mutant versions of ELF3 (at 48 kDa, as a composite of the approximately 42 kDa ELF3 plus the 6 kDa Histidine tag). Equal expression of all proteins was observed as detected by the anti-ELF3 antibody



**Figure 6** Deletion of the pointed domain of ELF3 almost completely abolishes the suppression of the K4 promoter by wild-type ELF3. (a) 3  $\mu$ g of the full-length K4 promoter construct was co-transfected with 1  $\mu$ g of either empty pCI (vector control), pCI-ELF3 wild-type or the ELF3 deletion constructs of the ets domain (ELF3 $\Delta$ ets domain) or of the pointed domain (ELF3 $\Delta$ pointed domain). Results of triplicate experiments were calculated as the mean  $\pm$  s.e.m. values for relative luciferase activity (RLU). (b) Western blot analysis of independent experiments to confirm expression of un-tagged wild-type ELF3 (at 42 kDa), ELF3 $\Delta$ ets domain (33 kDa) and ELF3 $\Delta$ pointed domain (22 kDa)

proteins (untagged wild-type ELF3 at 42 kDa, ELF3 $\Delta$ ets domain as a 33 kDa truncated protein and ELF3 $\Delta$ pointed domain at 22 kDa) are expressed using the ELF3 antibody (Figure 6b). However, the expression levels are different compared to the wild-type ELF3 protein which could be attributable to alterations in protein structure leading to variations in stability. Although there is a high level of the ELF3 $\Delta$ pointed domain protein, it nevertheless failed to repress K4 promoter activity in co-transfection studies.

## Discussion

The ets family of transcription factors is involved in diverse cellular functions. A recently described member, ELF3, has some distinguishing features, namely an epithelial-restricted expression pattern. Questions that emerge from this observation are what are the target genes for ELF3 and how the genes are transcriptionally regulated. A model system to address these questions is the stratified squamous epithelium, given the exquisite balance between proliferation and differentiation and the accompanying continuous cell renewal. Our previous work has demonstrated the importance of keratin 4 (K4) in mediating early differentiation in the esophageal squamous epithelium, through analysis of transcriptional regulation of basal K4 promoter activity and targeted disruption of the mouse K4 gene in embryonic stem cells (Jenkins *et al.*, 1998; Ness *et al.*, 1998; Opitz *et al.*, 1998). Thus, we postulated that the early-differentiation linked K4 gene might be a

target of ELF3 in squamous epithelial cells and compared this to a late-differentiation gene such as SPRR2A which is regulated by ELF3 (Oettgen *et al.*, 1997a).

To address this hypothesis, we transfected an epitope-tagged ELF3 and the K4 promoter into an esophageal cancer cell line (TE-12) with low endogenous ELF3 expression as well as into HeLa cells. Surprisingly, the basal K4 promoter activity was suppressed by ELF3, an effect that was retained even with serial deletions of the promoter. This repression was not mediated by DNA binding of ELF3. In contrast, ELF3 binds and transactivates the SPRR2A and Endo A (keratin 8) enhancers (Oettgen *et al.*, 1997a) and it also transactivates the TGM3 promoter in cell lines of keratinocyte origin (Andreoli *et al.*, 1997). Furthermore, ELF3 is a potent transactivator of the TGF- $\beta$  II receptor that is mediated through binding to a 5'-GGAAACAGGAA-3' motif in reverse orientation at +13 to +24 in the TGF- $\beta$  II receptor gene (Choi *et al.*, 1998). The HER2/neu oncogene contains a GGAA element in its proximal promoter which is bound with high affinity by ELF3 resulting again in transactivation in breast cancer cells (Chang *et al.*, 1997). However, in our current study we found that ELF3 acts as a transcriptional repressor in the context of the K4 promoter, an effect that appears to be independent of binding to a classical ets site.

We postulate that ELF3 might specifically suppress genes, such as K4, which are involved in early differentiation in squamous epithelial cells, but activate genes linked to terminal differentiation in the same cell type. This is further reflected by the fact that ELF3

itself is expressed in the terminally differentiated layer of the epidermis (Andreoli *et al.*, 1997). The latter is consistent with observations that report induction of ELF3 when NEHK cells are switched to media with higher concentration of calcium and the addition of TPA, both of which favor keratinocyte differentiation and expression of late differentiation markers such as TGM3 and profillagrin (Andreoli *et al.*, 1997). In addition, ELF3 cooperates with Sp1 and AP1 to transactivate the TGM3 and profillagrin promoters (Andreoli *et al.*, 1997).

How does ELF3 mediate suppression of K4 transcription? Suppression of K4 transcription was partially reversed by ELF3 mutants that harbored a mutation or deletion in the ets domain. Interestingly enough, when only the ELF3 ets domain is expressed as a fusion protein, there is suppression of the TGF- $\beta$  II receptor promoter basal activity, perhaps indicating that this region may act as a dominant negative mutant (Choi *et al.*, 1998). It is conceivable that the ELF3 ets domain may display this function in the suppression of K4 promoter basal activity. It is also important to note that a mutated A/T hook domain within ELF3 did not affect suppression of K4 promoter activity.

It is also possible that ELF3 may interact with proteins and such protein-protein interactions suppress K4 promoter activity through DNA binding or non-DNA binding mechanisms. It is known that ets-1 and ets-2 cooperate with AP-1 (Logan *et al.*, 1996) or Pax5 (Fitzsimmons *et al.*, 1996), ELK-1 interacts with SRF to modulate gene activation in response to serum (Janknecht and Nordheim, 1992), PU.1 interacts with NF-EM5 (Pongubala *et al.*, 1993). Ets2 interacts with *c-fos/c-jun* (Basuyaux *et al.*, 1997), and GABP $\alpha$  binds GABP $\beta$  (Brown and McKnight, 1992; Batchelor *et al.*, 1998). ERG, an ets factor, does not bind to the collagenase-1 gene ets DNA binding site unless it complexes directly with the *c-fos/c-jun* complex that binds a contiguous AP-1 site (Buttice *et al.*, 1996). In contrast, ERG binds to two linked ets DNA binding sites of the stromelysin-1 gene promoter but does not transactivate the promoter (Buttice *et al.*, 1996). However, when ERG is coexpressed with ets2, then ERG inhibits stromelysin-1 activation by ets-2, suggesting interaction between ets2 and ERG. Indeed, binding between ets2 and ERG occurs through several domains, one of which is the DNA binding domain (Basuyaux *et al.*, 1997).

Perhaps, most importantly from our findings, is that a deletion in the pointed domain completely rescues ELF3 mediated suppression of K4 promoter activity. It is known that TEL, another ets factor, acts as a transcriptional repressor. This repression is mediated by interaction with mSin3A through the pointed domain (Fenrick *et al.*, 1999). TEL also interacts with Fli-1 and inhibits Fli-1 mediated transactivation on the GPIX promoter (Kwiatkowski *et al.*, 1998). To date, no investigators have demonstrated that TEL has DNA binding activity (Kwiatkowski *et al.*, 1998). Our studies suggest that ELF3 in the context of the K4 promoter is not acting as a DNA binding protein. One model that emerges from our study is that the pointed domain of ELF3 interacts with a co-repressor or another ets factor that mediates K4 promoter repression. Recognizing that our results suggest complex regulatory mechanisms involving the ets and

pointed domains of ELF3, it is also possible that autoregulatory mechanisms may inhibit DNA binding activity as described in Ets-1 where two  $\alpha$ -helices located N-terminally to the ets domain and a single C-terminal  $\alpha$ -helix act cooperatively to alter DNA binding (Sharrocks *et al.*, 1997). To that end, protein-protein interactions may modulate autoregulatory mechanisms. Apart from TEL, other ets factors that have been reported to act as transcriptional repressors include ERF, LIN-1, and Yan/Pok (Sharrocks *et al.*, 1997).

Conceivably, there may be direct binding of ELF3 with a member of the general transcriptional factor machinery to modulate transcription as illustrated by another ETS factor, namely PU.1 binding to TFIID (Hagemeier *et al.*, 1993). Benz and coworkers have found that exon 4 of ELF3, which harbors the acidic or transactivation domain, interacts with the TATA-binding protein (TBP) (Chang *et al.*, 1999). However, our own work does not demonstrate an interaction between ELF3 and TFIID (unpublished observations).

In aggregate, our novel findings of keratin 4 transcriptional repression by ELF3 suggest a model in which repression by ELF3 of early-differentiation genes is balanced by activation of late-differentiation markers by ELF3 in squamous epithelial cells, perhaps contributing to the acceleration of the temporal programs of terminal differentiation.

## Materials and methods

### Cell culture and transient transfections studies

The human esophageal squamous carcinoma cell lines TE-11 and TE-12 and the human cervix cancer cell line HeLa (ATCC) were grown in 5% CO<sub>2</sub> at 37°C as subconfluent monolayers in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% v/v fetal calf serum (FCS), 100 U/ml penicillin, 100  $\mu$ g/ml streptomycin and 1% L-glutamine (all reagents obtained from Sigma). Experiments were carried out in the log phase of growth after the cells were seeded for 24 h.

For reporter gene activity assays, transient transfection of cells with the DNA constructs were performed using the calcium phosphate precipitation technique (5'-3', Inc.). Cells were plated at a density of  $1 \times 10^6$  cells/35-mm well and transfected 24 h later. Per 35 mm well, 3  $\mu$ g of the respective K4 promoter-luciferase reporter constructs, 2  $\mu$ g of the SPRR2A enhancer-luciferase reporter construct, and 1  $\mu$ g of the ELF3 expression vector were co-transfected. The transfectant mixture consisted of a 250  $\mu$ l solution of 125 mM CaCl<sub>2</sub>, 25 mM HEPES (pH 7.05), 0.75 mM Na<sub>2</sub>HPO<sub>4</sub>, 5 mM KCl, 140 mM NaCl, and 6 mM glucose. After 12 h incubation, cells were washed twice with phosphate-buffered saline (PBS) followed by exchange with fresh DMEM supplemented as above. Cells were washed twice with PBS 24 h later and lysed in 250  $\mu$ l of  $1 \times$  cell culture lysis reagent (Promega). Luciferase activity of 100  $\mu$ l cell lysate was determined using 100  $\mu$ l luciferase reagent (Promega) by measurement of relative luciferase units (RLU) in a luminometer (MLX Microtiter Plate Luminometer, Dynex Tech.).

Transfections were performed in triplicate, and results of at least three independent experiments were calculated as the mean  $\pm$  s.e.m. values for luciferase activity. Transfection efficiency was routinely monitored by cotransfections with 1  $\mu$ g of pGreen Lantern-1 (Life Technologies). Expression of green fluorescent protein was examined under a fluorescence

microscopy (475 nm excitation peak, 490 nm emission peak) and the percentage of successfully transfected cells determined in each well to ensure consistent transfection efficiency in independent experiments. The percentage of fluorescing cells was determined in each well and found not to vary within a given transfection experiment, indicating that transfection efficiency was uniform.

#### Reverse-transcriptase polymerase chain reaction (RT-PCR) analysis

Total RNA from HeLa, TE-12 and TE-11 cells was extracted as previously described (Nakagawa *et al.*, 1995). RT of RNA was performed using 1  $\mu$ g total RNA, 100 pM random hexamer primer, 1 mM dithiothreitol, 6 mM Mg<sup>2+</sup>, 500  $\mu$ M of each dNTP, 20 U RNasin and 200 units Maloney murine leukemia virus reverse transcriptase (MMLV-RT) (all components from Life Technologies). After 10 min annealing at 23°C, RT was performed for 45 min at 42°C followed by a 5 min inactivation of MMLV-RT at 95°C. This RT reaction mixture was promptly used as template for PCR at a 1:20 dilution. For amplification, an internal ELF3 specific 5'-primer was combined with the ELF3 3'-primer (Table 1) to yield an expected 450 bp fragment. The PCR reaction was performed using 2.5  $\mu$ l RT reaction mixture, 200  $\mu$ M of each dNTP, 0.5  $\mu$ M of each primer and 2.5 U Taq DNA polymerase (Fisher) in a 1 $\times$ -reaction buffer provided by the manufacturer. PCR conditions were as follows: denaturation 2 min at 94°C; 30 cycles of amplification with denaturation 60 s at 94°C, annealing 60 s at 55°C, extension 1 min at 72°C; final extension at 72°C for 10 min. As control, amplification of a 120 bp  $\beta_2$ -microglobulin specific product was amplified under the same conditions using the  $\beta_2$ -microglobulin 5' and 3' primers (Nakagawa *et al.*, 1995). As an internal negative control, one RNA aliquot was amplified without prior RT to ensure that the amplified PCR product did not result from amplification of contaminating genomic DNA.

#### DNA plasmid constructs

**K4 promoter-luciferase reporter deletion constructs and SPRR2A enhancer-luciferase reporter construct** The construction of the luciferase reporter vectors containing the full-length human K4 promoter (K4-1040) and the serial deletions, designated K4-800, K4-540, K4-340, K4-200, K4-163, and K4-140, has been described previously (Opitz *et al.*, 1998). The SPRR2A luciferase reporter construct has been described previously (Oettgen *et al.*, 1997a).

**Wild-type ELF3 constructs** The full-length ELF3 cDNA was inserted into the mammalian expression vector pCI as previously described (Oettgen *et al.*, 1997a). A Histidine-tagged wild-type ELF3 expression vector was constructed by PCR amplification of the open reading frame (ORF) of the cDNA using the pCI-ELF3 plasmid as template. For directional subcloning of the ELF3 PCR product into the mammalian expression vector pcDNA 3.1 His A (Invitrogen), *KpnI* and *EcoRI* restriction enzyme sites were generated using the ELF3 5'-primer and ELF3 3'-primer, respectively (Table 1). The PCR reaction was performed using 20 ng plasmid DNA, 200  $\mu$ M of each dNTP, 0.5  $\mu$ M of each primer and 2.5 U Pfu DNA polymerase (Stratagene) in a 1 $\times$ -reaction buffer provided by the manufacturer. After 3 min denaturation at 94°C, five initial cycles of amplification were done: denaturation 60 s/94°C, annealing 60 s/55°C, extension 60 s/72°C, followed by 25 cycles with annealing temperature of 60°C and a final extension at 72°C for 10 min. A single PCR product of the 1150 bp expected size was generated. The ELF3 PCR product and pcDNA 3.1 His A (providing in-frame insertion of the ELF3 cDNA with the His-Tag) were digested with *EcoRI* and *KpnI* restriction enzymes (New

England Biolabs), agarose gel-purified (Qiagen Gel extraction kit) and ligated with T4 DNA Ligase (New England Biolabs). The resulting construct was sequenced by the dideoxy method (US Biochemicals) to ensure correct insertion of the ELF3 cDNA. The plasmids were purified by a modified alkaline lysis method (Qiagen).

**Mutant ELF3 constructs** One mutant of ELF3 within the AT hook domain (ELF3 MT1) and two mutants of ELF3 within the ets-domain (ELF3 MT2 and ELF3 MT3) were generated by site-directed mutagenesis using overlap extension PCR (Ho *et al.*, 1989). Specific alterations in the ELF3 nucleotide sequence were introduced resulting in substitution of conserved amino acids to alanine (Table 2).

Complementary oligonucleotide primers (all in Table 1) containing the mutated nucleotide sequence were designed to generate two PCR fragments having overlapping ends in a primary amplification reaction. In this first step, wild-type ELF3 cDNA was used as template. The first PCR amplification step of ELF3 MT1 required the outer ELF3 5' primer and the internal ELF3 MT1 3' primer to generate a 760 bp fragment. This was accompanied by a PCR reaction with the internal ELF3 MT1 5' primer and the outer ELF3 3' primer to yield a 399 bp fragment. Construction of ELF3 MT2 required the outer ELF3 5' primer and the internal ELF3 MT2 3' primer to yield a 966 bp fragment. This was accompanied by a PCR reaction with the internal ELF3 MT2 5' primer and the outer ELF3 3' primer generating a 195 bp PCR product. The ELF3 MT3 construct required the outer ELF3 5' primer and the internal ELF3 MT3 3' primer to yield a 1022 bp fragment. This was accompanied by a PCR reaction with the internal ELF3 MT3 5' primer and the outer ELF3 3' primer to generate a 142-bp PCR product. The PCR products for each ELF3 mutant were gel purified (Qiagen) and combined in a subsequent 'fusion' PCR reaction. The overlapping ends of these fragments anneal and serve as template and as primer for the second amplification reaction. Using the outer 5' and 3' ELF3 primer, three different mutated full-length ELF3 cDNAs (1150 bp) were generated.

PCR conditions for first and second amplification steps were as follows: denaturation 45 s at 94°C; 30 cycles of amplification with denaturation 45 s at 94°C, annealing 45 s at 60°C, extension 2 min at 72°C; final extension at 72°C for 10 min. The ELF3 mutants were then subcloned into the pcDNA3.1 His A vector in an identical fashion as for wild-type ELF3. All constructs were verified by DNA sequencing by the dye terminator cycle sequencing method (ABI) in the automated DNA sequencing facility of the University of Pennsylvania.

**ELF3 deletion constructs** Using the untagged pCI-ELF3 wild-type plasmid (371 amino acids with predicted molecular mass of approximately 42 kDa size), two ELF3 deletions were generated. By *TflI/Xba* digestion of wild-type ELF3, the ets domain (74 amino acids) was removed, resulting in a protein of 297 amino acids with a predicted molecular mass of 33 kDa. The ELF3 $\Delta$ pointed domain construct represents a deletion of 173 amino acids from the pointed domain. The encoded protein product contains 198 amino acids with a predicted molecular mass of 22 kDa.

#### Western blot analysis

Endogenous Keratin 4 protein levels were determined using protein lysates from wild-type, mock and ELF3 transfected cells. For the preparation of total cell lysates, subconfluent monolayer cell cultures were washed twice with cold PBS, detached from culture dishes with a plastic cell scraper and resuspended in a lysis buffer containing 50 mM HEPES (N-[2-Hydroxyethyl]Piperazine-N'-[2-ethane sulfonic acid] pH 7.0, 250 mM sodium chloride, 5 mM EDTA and 0.1% NP-40 (Gibco BRL) supplemented with 1 mM phenylmethylsulfonyl

fluoride, 1 mM dithiothreitol and 0.1% aprotinin (Sigma). 10  $\mu$ g of total protein from each sample was separated on a 10% SDS-polyacrylamide gel. Following electrophoresis, the protein was transferred to an Immobilon membrane (Millipore Corp.). The membrane was blocked with 5% milk, 10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.2% Tween 20 for 1 h. The primary antibody (Anti-Keratin 4, Sigma) was used at a 1:4000 dilution, and the secondary antibody, horseradish peroxidase-conjugated sheep anti-mouse (Amersham Pharmacia Biotech) antibody, was used at a 1:5000 dilution. Horseradish peroxidase activity was detected with a chemiluminescence system (ECL system, Amersham Pharmacia Biotech).

Nuclear extracts were used for detection of ELF3 in Western blot analysis. Approximately  $5 \times 10^6$  cells were plated in 10-cm culture dishes. After an attachment period of 12 h, cells were incubated with 8 ml of the transfection mixture containing 16  $\mu$ g plasmid DNA and 200  $\mu$ l Lipofectamine reagents (Life Technologies) in serum-free DMEM medium. Cells were incubated for 5 h, followed by exchange with fresh DMEM containing 10% FCS, and then harvested after 72 h. Nuclear extracts from transiently transfected cells were

prepared as described previously (Jenkins *et al.*, 1998). The protein concentration was determined by a colorimetric method (Bio-Rad protein assay). Thirty  $\mu$ g of total protein from each sample was separated on a 12–15% SDS-polyacrylamide gel. Immunoblots were done with the primary anti-ELF3 antibody at a 1:1000 dilution, and the secondary horseradish peroxidase-conjugated donkey anti-rabbit (Amersham Pharmacia Biotech) antibody was used at a 1:1000 dilution.

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