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Elk-1 phosphorylated at threonine-417 is present in diverse cancers and correlates with differentiation grade of colonic adenocarcinoma★

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Summary

Elk-1 is a member of the Ets family of transcription factors, which are identified by a conserved Ets DNA-binding domain that mediates transcriptional regulation at Ets sequence-containing promoters. The activation domain of Elk-1 is important for executing its physiologic functions and contains many phosphorylation sites targeted by various MAP kinases following exposure to cell stressors or mitogenic stimuli. The different combinations of phosphorylated sites allow specificity of cellular responses mediated through redundant signaling pathways activated by distinct stimuli. Through phosphorylation of S383, mitogen-activated protein kinase (MAPK)–activating stimuli have been shown to regulate various processes important in carcinogenesis through transcriptional regulation in various cell lines, including proliferation. Phosphorylation at the T417 site (pT417), but not the S383 site, is involved in neuronal apoptosis induced through dendritic signaling mechanisms and associates with neuronal lesions in many Lewy body diseases. This points to distinct roles for these different phosphorylation sites in pathophysiologic pathways. However, the S383 site remains the best characterized in the context of normal function and carcinogenesis in cell lines, and less is known about the biochemistry of other phosphorylation sites, particularly in more biochemically relevant models. Here, we show that Elk-1 pT417 is present in epithelial cell nuclei of various normal and cancer tissues and that the number of pT417-positive cells correlates with differentiation grade of colonic adenocarcinomas. This nuclear localization and correlation with tumor differentiation in adenocarcinoma suggests a potentially important transcriptional and biochemical role of this phosphorylation site in carcinogenesis of this tumor type.

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Keywords

Adenocarcinoma; Tumor grade; Elk-1; Phosphorylation; Colorectal cancer

1. Introduction

Elk-1 is a member of the Ets oncogene family of transcription factors, which are characterized by a conserved DNA-binding domain, or Ets domain. Phosphorylation of Elk-1 at S383 serves as an integration point within cells for redundant activation of upstream mitogen-activated protein kinase (MAPK) signaling cascades by distinct external stimuli. Growth factors and mitogens activate the Raf/mitogen-activated protein kinase kinase (MEK)/extracellular signal-regulated kinase (ERK) pathway, resulting in regulation of growth and differentiation, through transcriptional activation by Elk-1 pS383 [1,2]. Stress, inflammatory cytokines, and other growth factors activate the p38 MAPK and stress-activated protein kinase (SAPK)/Jun N-terminal kinase (JNK) pathways, resulting in regulation of inflammation, apoptosis, growth, and differentiation, also through transcriptional activation by Elk-1 at pS383 [1,2]. Elk-1 regulates transcription by interacting with serum response factor at serum response elements within the promoters of genes that subsequently execute the functions initiated by extracellular signals [3]. Elk-1 is most notably known for contributing to regulation of growth and proliferation through transcriptional activation of immediate early genes such as *c-fos* and *ZIF-268* [4,5].

The transcriptional activation of Elk-1 through phosphorylation at S383 has been extensively studied in many different cell lines. Yet, the fact that these cellular processes respond to different extracellular signaling molecules through common MAPK pathways that all culminate in activation of Elk-1 suggests the need for different phosphorylation patterns of Elk-1 to execute different cellular functions. For example, induction of ternary complex formation of Elk-1 with serum response factor following nerve growth factor (NGF) stimulation was shown to be dependent on phosphorylation at S383 and S389 by ERK1/2 in PC-12 cells [6]. Complex formation was enhanced by phosphorylation at S324 or S422 but inhibited by phosphorylation at T336 [6]. No phosphorylation was detected at T353, T363, T368, or T417 [6]. Mutation of S383 or S389 or both T363 and S422 or both T417 and S422 greatly reduced transcriptional activation of Elk-1 in response to epidermal growth factor (EGF) in NIH 3T3 fibroblasts [7]. 12-O-tetradecanoylphorbol-13-acetate (TPA) increased Elk-1 transcriptional activity in COS cells through phosphorylation of Elk-1 by ERK1/2 at S324, T336, S383, S389, or S422 [8]. Thus, although ERK1/2 was involved in phosphorylation of Elk-1 under all conditions, differentiation between stimuli was made possible through different phosphorylation patterns of Elk-1.

Transcriptional activation of Elk-1 through these signaling cascades results in regulation of a wide range of normal cellular functions, including cell-cell and cell-matrix adhesion, proliferation, and apoptosis [9,10]. Carcinogenesis involves dysregulation of these processes, and Elk-1 activation has been implicated in cancers of many tissues including breast, pancreas, and colon. Inhibition of Elk-1 in the breast cancer cell line MCF-7 enhanced the antiproliferative effects of breast cancer 1a/1b (BRCA1a/1b) in a MEK/ERK

pathway-dependent manner, presumably through interaction of its Ets DNA binding domain and subsequent inhibition of *c-fos* transcription [11]. Using the breast cancer cell lines MCF-7 and SK-BR-3, it was shown that EGF activation of human epidermal growth factor receptor 2 (HER-2) stimulated Elk-1 phosphorylation at S383 through the MEK/ERK pathway and promoted cell survival [12]. Knockdown of Elk-1 in an oncogenic RAS-expressing human pancreatic cancer cell line resulted in inhibition of proliferation of the cancer cells [13]. In the rat small intestine-derived RIE-1 cell line, Elk-1 phosphorylation at S383 by ERK1/2 resulted in increased transcription of cyclooxygenase-2 (COX-2), which caused loss of cell-matrix adhesion and decreased apoptosis [14].

Despite its nuclear site of action as a transcription factor, Elk-1 protein and messenger RNA (mRNA) have been found in the processes of cultured hippocampal neurons [4,15], and this localization was found to impact pathophysiologic responses. Elk-1 mRNA introduced specifically into neuronal dendrites, but not the soma, using a laser-based, site-directed transfection procedure known as phototransfection, initiated apoptosis [16]. This response was shown to be dependent on local translation of Elk-1 within the dendrite and on transcription [16]. In addition, this response was shown to be dependent on the phosphorylation state of Elk-1 [17]. The activation domain of Elk-1 contains many phosphorylation sites, which, excluding the S383 site, remain largely uncharacterized [6]. Mutation of these sites to create nonphosphorylatable mutants revealed that preventing phosphorylation at the T417 site, but not the S383 site, blocked the apoptotic response when the mutant mRNA were introduced into neuronal dendrites [17]. Phosphorylation at the T417 site was later shown to be an important marker for Lewy bodies in Parkinson disease, whereas other phosphoforms were not [17]. These studies suggest a role for this transcription factor outside the nucleus as well as providing impetus for characterizing the physiologic effects of other phosphorylation sites within the activation domain of Elk-1, particularly the T417 site.

Much of what is known about Elk-1 function in carcinogenic processes has been obtained from cell lines, which, although useful for approximating biochemical responses in cancer tissue, have a limited capacity to do so. This is especially important in colorectal cancer and medulloblastoma where sonic hedgehog signaling is important in promoting *in vivo* carcinogenesis [18,19], but signaling components are down-regulated in cultured tumor cells [19,20], limiting their utility in biochemical studies. In addition, Elk-1 activation in these studies is measured by phosphorylation of Elk-1 at S383, with no evaluation of the contribution of other phosphorylation sites.

Given these limitations in cell lines, the role of Elk-1 in many carcinogenic processes, its phosphosite specificity in pathophysiology, and the limited amount of information on the role of the T417 site in pathophysiology, the staining patterns for Elk-1 phosphorylated at T417 in various cancers and normal tissues was determined to identify its potential contribution to carcinogenesis. Elk-1 pT417 was found in many normal and cancer tissues screened in a tissue microarray. A screen of adenocarcinomas of the colon revealed higher Elk-1 pT417 staining in tumors compared with normal tissue. Quantification of the percentage of Elk-1 pT417-positive epithelial cells revealed a correlation with tumor differentiation grade, with well-differentiated tumors showing the highest and poorly

differentiated tumors showing the lowest percentage of positive cells, respectively. These results suggest a role for this particular phosphorylation site in the progression of colorectal adenocarcinoma.

2. Materials and methods

2.1. Tissue

Tissue microarrays (TMAs) were made-to-order from Imgenex (IMH-343). Two-mm-diameter tissue cores were punched from various normal and tumor tissues and arrayed in a 4×6 grid in a paraffin block. The TMAs were then cut in 4- μ m-thick serial sections and mounted on glass slides [21]. Thin serial sections allow detection of multiple antigens within the same tumor cells by performing immunohistochemistry for each antigen on distinct slides. The microarrays were supplied with information such as age and sex of patient and type of tumor. Some tumors were also supplied with patient survival data.

Five-micrometer sections of paraffin-embedded, formalin-fixed adenocarcinoma of the colon and rectum from patients were a generous gift from the Molecular Pathology and Imaging Core Facility of the Center for Molecular Studies in Digestive and Liver Disease at the University of Pennsylvania. All experiments on these tissues were carried out under the approval of the University of Pennsylvania IRB (protocol no. 812674). Samples were supplied with pathology reports, and an independent assessment by Dr Andres J. Klein-Szanto, pathologist at the Fox Chase Cancer Center, confirmed pathology of sections.

2.2. Immunofluorescence

Paraffin-embedded tissue sections were deparaffinized in 4 changes of fresh xylene for 5 minutes each. Sections were then rehydrated in graded alcohol washes, followed by 2 changes in Millipore water for 2.5 minutes each. Antigen retrieval was performed by immersing slides in 800 mL of 0.01-M citric acid, pH 6.0 in a 1-L beaker, and microwaving in a commercial microwave for 4 minutes at 100% power followed by 1.5 minutes at 10% power. The beaker was then removed and placed at room temperature for 20 minutes. Sections were quickly rinsed with Millipore water and washed with 0.01M Tris-buffered saline (TBS), pH 8.4, 2 times for 5 minutes each. The following steps, excluding washes, were performed in a humidified chamber. Slides were blocked in 0.01M TBS containing 10% normal goat serum, 10% bovine serum albumin (BSA), and 0.1% Triton X-100 (high block buffer) at 4°C overnight. Following blocking, the sections were incubated in rabbit anti-Elk-1 pT417 (1:500) diluted in 0.01M TBS containing 5% normal goat serum, 5% BSA, and 0.01% Triton X-100 (5/5 block buffer) at 4°C overnight. All Elk-1 antibodies were custom made for Dr. James H. Eberwine. The specificity of these antibodies has been demonstrated previously [17]. Slides were washed 3 times for 10 minutes in 0.1% TBST before incubating with the appropriate Alexa Fluor conjugated secondary antibodies (Invitrogen, 1:1000) for 1 hour at room temperature. Slides were washed again 3 times 10 minutes in 0.1% Tris-buffered saline/Tween 20 (TBST) then equilibrated in phosphate-buffered saline (PBS), pH 7.4, for 10 minutes followed by incubation with 4',6-diamidino-2-phenylindole (DAPI, 0.1 μ g/mL in PBS) for 10 minutes. Sections were rinsed in PBS followed by Millipore water and then mounted in fluoromount-g and sealed with nail polish.

2.3. Immunohistochemistry

Paraffin-embedded slides were deparaffinized in 4 changes of fresh xylene, 5 minutes each. For tissue microarrays, slides were first incubated vertically in a drying oven at 62°C for 1 hour to remove the protective layer of paraffin. Slides were hydrated in graded ethanol solutions followed by a 5-minute incubation in 0.15M phosphate buffer (PB), pH 7.4. For antigen retrieval, slides were laid flat on the bottom of a Tupperware container and covered with 0.01M citrate buffer, pH 6.0, and placed in a commercial microwave oven for 5 minutes each at 100%, 50%, and 10% power followed by a 20-minute incubation at room temperature. Slides were then rinsed in 0.15M PB. The following steps, excluding washes, were performed in a humidified chamber. Slides were blocked in high block buffer (10% normal goat serum, 10% BSA, and 0.1% Triton X-100 in 0.1M PB, pH 7.4) at 4°C overnight. Slides were washed 3 times for 5 minutes in 5/5 block (5% normal goat serum, 5% BSA, and 0.01% Triton X-100 in 0.1 M PB, pH 7.4) and incubated in one of the following primary antibodies: Elk-1 nonphosphoT417 (1:500), Elk-1 pT417 (1:500) at 4°C for 48 hours. All Elk-1 antibodies were custom made for Dr. James H. Eberwine. The specificity of these antibodies has been demonstrated previously [17]. The following steps were performed using the Vector Laboratories Vectastain Elite ABC kit (rabbit). Slides were washed again 3 times for 5 minutes in 5/5 block and incubated in biotinylated goat anti-rabbit IgG in 5/5 block for 1 hour at room temperature. Slides were washed 3 times for 5 minutes in 0.15M PB before incubation in ABC complex for 1 hour at room temperature. Slides were washed again 3 times for 5 minutes in 0.15M PB before incubating in SG substrate (Vector Laboratories) for 1.5 minutes. Slides were then dehydrated in sequential ethanol washes and cleared in xylenes 4 times, 5 minutes each. Slides were mounted with Permount.

2.4. Western blot

Seven fresh frozen 4- μ m sections of each human tissue sample were triturated by pipette in a gentle RIPA lysis buffer and spun down. Twenty microliters of the resulting supernatant was loaded and run under denaturing conditions on a 4% to 12% Bis-Tris gel. Protein bands were transferred to a polyvinylidene fluoride membrane, and the membrane was blocked overnight at 4°C with 10% BSA, 10% normal goat serum (NGS), 0.1% Triton X-100 in TBS followed by incubation overnight at 4°C with Elk-1 pT417 or Elk-1 nonphosphoT417 antibody diluted 1:2000 in 5% BSA, 5% NGS, and 0.01% Triton X-100 in TBS. For competition experiments, Elk-1 pT417 or nonphosphoT417 antibody was incubated with 5000 times molar excess of the corresponding competing peptide for 1 hour at room temperature before membrane incubation. The membrane was washed in 0.1% TBST before incubation in horseradish peroxidase conjugated antirabbit antibody diluted 1:10000 in 5% BSA, 5% NGS, and 0.01% Triton X-100 in TBS. The membrane was washed again in 0.1% TBST before development of the blot with Thermo Scientific SuperSignal West Femtomax Chemiluminescent Substrate (Rockford, IL, USA).

2.5. Imaging and analysis

2.5.1. Immunohistochemistry—Samples were imaged using an Axiovert 200M inverted light microscope (Carl Zeiss Microscopy, Thornwood, NY, USA) coupled to an Axiocam

ICc1 color camera (Carl Zeiss Microscopy). Intensity of staining in normal colon and adenocarcinoma tissue was scored by a pathologist using the following scale: 0, negative; 0.5, minimal or marginal stain, possibly negative; 1, mild to moderate stain intensity; 2, moderately intense staining; 3, very intense staining.

2.5.2. Immunofluorescence—Regions from each histologically unique area of the tumor or from normal tissue were acquired with a Zeiss confocal LSM 710 (Carl Zeiss Microscopy). The corresponding hematoxylin and eosin (H&E) slide was used to align the tumor areas with the DAPI fluorescence on the immunofluorescence slide. A threshold was applied to all images to remove the lowest 10% of signal intensities. The total number of Elk-1 pT417–positive epithelial cells in each region was counted and expressed as a percentage of the total number of epithelial cells derived from the DAPI image. Percentages from at least 3 regions were averaged for each tumor.

SigmaPlot (Systat Software, Inc., San Jose, CA, USA) was used for all statistical analyses.

3. Results

3.1. Elk-1 pT417 is present in normal epithelial cell nuclei

Tissue microarrays were processed for Elk-1 pT417 (pT417) or Elk-1 nonphosphoT417 (nonphosphoT417) immunohistochemistry, aligned to corresponding H&E slides for analysis, and qualitatively assessed based on relative staining characteristics across tissues. The pT417 antibody specifically recognizes Elk-1 phosphorylated at the T417 site, whereas the T417 antibody recognizes the T417 site when it is not phosphorylated. Tissue samples were organized into categories discussed below based on the intensity of staining and proportion of cells positive for pT417 and nonphosphoT417.

Interestingly, skin was the only tissue included on the TMA in which pT417 and nonphosphoT417 labeled the same cellular compartment (Fig. 1 and Table 1). Distinct populations of squamous epithelial cell nuclei of skin had strong staining for pT417 (Fig. 1A) or weak staining for nonphosphoT417 (Fig. 1E) such that all pT417-positive cells were nonphosphoT417 negative and vice versa. The remainder of tissues consistently contained pT417 in the nucleus and nonphosphoT417 in the cytoplasm.

A subset of tissues had strong staining for both pT417 and nonphosphoT417 in distinct cellular compartments (Fig. 1 and Table 1). A large percentage of epithelial cells within breast acini (Fig. 1B and F), stomach pits (Fig. 1C and G), and colonic crypts (Fig. 1D and H) showed strong staining for pT417 in the nucleus and nonphosphoT417 in the cytoplasm. Within the stomach, nonphosphoT417 showed stronger staining in the glandular region than the pits (Fig. 1G). Although basal cells of the prostate epithelium also showed strong staining for both pT417 in the nucleus and nonphosphoT417 in the cytoplasm (Table 1), a much lower proportion of cells were positive compared with these other tissues.

Another subset of tissues showed moderate to no staining for pT417 and varying levels for nonphosphoT417 (see Table 1). Elk-1 pT417 weakly or moderately stained a small percentage of epithelial cell nuclei in kidney. However, nonphosphoT417 showed very

strong staining in a majority of these cells. Elk-1 pT417 moderately labeled a subset of epithelial cell nuclei within normal lung epithelium, whereas nonphosphoT417 diffusely and moderately labeled the cytoplasm of these cells. Within the liver, pT417 moderately stained the nuclei of a subset of hepatocytes, whereas the nonphosphorylated form weakly stained the cytoplasm. The specificity of the Elk-1 pT417 and the nonphospho-T417 antibodies was confirmed by performing peptide competition experiments with detection by Western blot (Fig. 1, panel M).

The presence of both pT417 and nonphosphoT417 in epithelial cells from diverse tissues suggests a potential role for Elk-1 in epithelial homeostatic processes. Aside from skin, the exclusively nuclear localization of pT417 combined with negative nuclear staining for nonphosphoT417 suggests that all of the Elk-1 present in the nucleus is phosphorylated at T417. The specific localization of pT417 within epithelial cell nuclei may indicate a role in transcriptional regulation. In tissues such as the kidney where nonphosphoT417 is the predominant form of Elk-1, other posttranslational modifications may be more important in these processes. Conversely, in tissues such as liver where the pT417 levels predominate, this posttranslational modification of Elk-1 may be more important than others.

3.2. Elk-1 pT417 is present in diverse cancer types from various tissues

Cancers not supplied with normal adjacent tissue showed moderate to strong staining for pT417 and nonphosphoT417 but with different proportions of labeled cells (Table 1 and Fig. 2). Tumor cells of a urinary bladder transitional cell carcinoma (Table 1) and a rectal adenocarcinoma (Fig. 2A and D and Table 1) had high proportions of intensely pT417-positive nuclei and nonphosphoT417 cytoplasm, whereas tumor nests of a small bowel malignant stromal tumor (Table 1) had a medium proportion of cells with this staining pattern.

A subset of tissues had higher levels of either pT417 or nonphosphoT417 in cancer samples compared with normal tissue (Table 1). In the lung, levels of both antigens were higher in a squamous cell carcinoma compared with normal lung. Cells within tumor cell nests of the squamous cell carcinoma contained moderately intense pT417-positive nuclei with strongly intense nonphosphoT417-positive cytoplasm. A squamous cell carcinoma of the esophagus showed similar staining results to the squamous cell carcinoma of the lung but was not supplied with normal esophagus. Alternatively, similar to normal liver, pT417 moderately labeled a subset of hepatocyte nuclei in a hepatocellular carcinoma (Table 1). However, nonphosphoT417 strongly stained the cytoplasm, suggesting an increase in total Elk-1 compared with normal liver.

In breast and stomach tissues, the level of pT417 staining was lower in the corresponding cancer tissue compared with normal tissue, whereas nonphosphoT417 levels were comparable (Fig. 2, Table 1). An infiltrating ductal carcinoma of the breast showed weak staining for pT417 (Fig. 2B), with some moderately positive nuclei in well-differentiated glandular structures, a notable decrease compared with the strong staining of acini epithelial cells in normal breast (Fig. 1B). Some nuclei within tumor cell nests of a stomach adenocarcinoma were moderately positive for pT417 (Fig. 2C), which is lower compared with normal stomach (Fig. 1C). This suggests that the level of Elk-1 does not change, but a

smaller pool of Elk-1 is phosphorylated at T417 in these cancers compared with normal tissue.

3.3. Elk-1 pT417 is present at higher levels in colonic adenocarcinoma compared with normal colon

Because differences in levels of Elk-1 phosphorylation at T417 were seen between normal and adenocarcinoma samples of the stomach and because of strong staining in other gastrointestinal tissues and cancers, staining patterns of this phosphoform of Elk-1 were further investigated in archival adenocarcinoma of the colon and accompanying normal tissue.

A cohort of samples of adenocarcinoma of the colon and normal adjacent tissues were screened for Elk-1 pT417 and nonphosphoT417 immunoreactivity (Fig. 3). Staining for both antigens was most prominent among cells of the colonic crypts in normal tissue (Fig. 3A and D). Within tumors, staining was most prominent in the epithelial cells of abnormal glands (Fig. 3B and E). Again, pT417 was predominantly nuclear and the nonphosphorylated form cytoplasmic. This suggests that out of the available pool of Elk-1, the fraction of this pool that is phosphorylated at nonphosphoT417 is localized to the nucleus.

The intensity of staining was measured qualitatively by a pathologist and reported as intensity scores on a scale of 0 to 3. The median scores for pT417 were significantly higher in adenocarcinoma compared with normal colon (Mann-Whitney rank sum test, $P = .005$; Table 2), whereas those for nonphosphoT417 were not (Mann-Whitney rank sum test, $P = 1.000$; Table 2). Therefore, ratios of Elk-1 pT417 to nonphosphoT417 scores in the same tissue sample were calculated. The median ratio score for adenocarcinomas was significantly higher than that in normal tissue (Mann-Whitney rank sum test, $P = .011$; Table 2). This suggests that the pool of Elk-1 phosphorylated at nonphosphoT417 is higher in adenocarcinoma than normal colon. For visualization of these differences, data are displayed as the proportion of tissue samples assigned a specific score. These graphs clearly show that a higher proportion of tumor samples processed for pT417 immunohistochemistry were assigned higher values compared with normal colon samples (Fig. 3C), whereas scores for Elk-1 nonphosphoT417 did not differ between tissue samples (Fig. 3F), resulting in a higher proportion of tumor samples with higher ratio scores (Fig. 3I).

3.4. Elk-1 pT417 levels correlate with differentiation grade of colonic adenocarcinoma

Samples were categorized as the following: normal, tubulovillous adenoma (adenocarcinoma precursor), or adenocarcinoma. Adenocarcinomas were further divided into well, moderately, or poorly differentiated groups. Tumor cells in DAPI images were identified by aligning fluorescence images with H&E images. As in the various normal and cancer tissues screened above, ELK-1 pT417 was exclusively nuclear in all tissues (Fig. 4A-D).

Elk-1 staining was quantified by calculating the number of Elk-1 pT417-positive nuclei as a percentage of the total number of nuclei, as determined by DAPI. Analysis of percentages by differentiation grade revealed a significant main effect (1-way analysis of variance

[ANOVA], $P < .001$). A Tukey post hoc test revealed a significantly higher percentage of Elk-1 pT417 positive cells in well-differentiated adenocarcinoma compared with both normal colon ($P < .001$) or poorly differentiated adenocarcinoma ($P = .006$). Moderately differentiated adenocarcinoma showed the same pattern ($P < .001$ versus normal colon, $P = .011$ versus poorly differentiated adenocarcinoma). There was a trend for a higher percentage of Elk-1 pT417-positive cells in tubulovillous adenoma compared with normal colon, but this did not reach statistical significance.

To assess if these findings were due to differences in the metastatic characteristics of tumors between differentiation groups, the percentage of positive lymph nodes was used as a measure for this characteristic. The percentage of Elk-1 pT417-positive cells did not significantly correlate with the percentage of positive lymph nodes (Pearson correlation coefficient, 0.165; $P = .573$). This was further demonstrated by the fact that the average percentage of pT417-positive cells across different differentiation grades of tumors was not affected by the presence of positive or negative lymph nodes (2-way ANOVA, $P = .274$). Based on these results, it was determined that differences in Elk-1 pT417-positive cells between differentiation grades were not due to biased representation of metastatic tumors in one group versus another. This correlation of the percentage of Elk-1 pT417-positive nuclei with colonic adenocarcinoma differentiation suggests a role for this phosphorylation site in the progression of this particular cancer.

4. Discussion

Epithelia are self-renewing in that a population of stem cells constantly proliferate and differentiate to repopulate older cells of the epithelium that are discarded through anoikis. In the case of the colon, stem cells within the base of the colonic crypts divide such that at any given time a portion of the stem cell pool remains and a separate pool of progenitors continues to proliferate. The progenitor cells proliferate as they migrate up the crypt through the transit-amplifying region toward the surface, where they differentiate and are eventually replaced by newly produced progenitors. The proliferation of stem cells is in part controlled by a gradient of WNT/ β -catenin signaling that is highest at the base of the crypt and decreases toward the top. This creates an opposing gradient of EphB-ephrinB, the relative concentrations of which contribute to migration control of progenitor cells through regulation of cell-cell contacts [22,23].

Normally, an inhibitory protein complex that includes APC binds β -catenin and targets it for degradation [23]. Upon Wnt binding to its receptor, Frizzled, inhibition is released and β -catenin translocates to the nucleus to activate transcription of target genes [23]. In both sporadic and familial forms of colorectal cancer, a genetic mutation in the *APC* gene causes loss of ability to interact with β -catenin, preventing its degradation and allowing constitutive transcription of target genes and consequently uncontrolled proliferation [24]. Enhanced signaling through Jun N-terminal kinase (JNK) has been shown to exacerbate this pro-proliferative effect of *APC* mutations [25]. In addition, many members of the JNK signaling pathway have been shown to be dysregulated in colorectal cancers.

Levels of active, phosphorylated JNK are higher in well-differentiated colorectal cancer samples [26], similar to the pattern of Elk-1 pT417 staining that we have shown. JNK activates Elk-1 in response to cellular stress such as DNA damage [27,28] or growth inhibitory signals [29] through phosphorylation at the S383 site, resulting in transcription of c-FOS. JNK also phosphorylates and activates c-JUN, which complexes with c-FOS to form the transcription factor AP-1. The active phosphorylated form of c-JUN in epithelial cells of colon adenocarcinoma is significantly higher in well-differentiated tumors (ie, low in poorly differentiated cases), whereas c-FOS expression does not correlate with tumor differentiation [30]. The lack of similarity in staining of Elk-1 pT417 and c-FOS suggests that this phosphoform might not be responsible for transcriptional activation of this target gene in colon adenocarcinoma, suggesting distinct roles for this phospho-form from pS383. However, the similarity of staining patterns of JNK and its target pc-JUN to that of Elk-1 pT417, combined with the role of JNK signaling in carcinogenic processes, could point to potential for JNK regulation of Elk-1 phosphorylation at T417 in these tumors.

In addition to self-renewal, the existence of tight cell-cell and cell-matrix contacts mediated by interactions between cytoskeleton-anchored adhesion proteins such as selectins, integrins, or cadherins between adjacent cells is also an established characteristic of epithelia [31,32]. In the case of the colon, these contacts also play an integral role in migration of progenitor cells to the crypt surface. A number of integrin isoforms have been shown to be down-regulated in colorectal cancers, especially in poorly differentiated classifications [33]. Loss of cell adhesion and gain of the ability of cells to survive in this context can contribute to tumorigenesis. From our results, Elk-1 pT417 labels a higher proportion of epithelial cells until the colon adenocarcinoma progresses to lower grades of differentiation. This phosphoform is thus correlated with progressive down-regulation of cell-cell and cell-matrix contacts and could potentially be used as a marker for those cells that have lost establishment of appropriate contacts. Once the tumor reaches a poorly differentiated state, these cells could then gain the ability to metastasize and would explain the lower percentage of Elk-1 pT417-positive cells in poorly differentiated adenocarcinomas.

The maintenance of cellular adhesion is necessary for cell cycle progression of actively proliferating cells through G1/S, such as the progenitor cells of the transit amplifying region of the colon. In addition, activation of the ERK pathway, which culminates in phosphorylation of Elk-1, has been shown to be necessary for entry into S phase from G1 [34], and that this effect is adhesion dependent. In normal cells, activation of ERK by growth factors such as EGF results in Elk-1 phosphorylation at S383 only in the adherent state, whereas Elk-1 phosphorylation by p38 or JNK is unaffected by adhesion state [35]. In nonadherent conditions, activated ERK1/2 cannot translocate into the nucleus to phosphorylate Elk-1 [15,16]. JNK/SAPK has been shown to produce a phosphorylation pattern of Elk-1 distinct from that of ERK1/2 following stressful stimuli in NIH 3T3 fibroblasts [8]. In addition, JNK/SAPK and p38 retain the ability to translocate into the nucleus in nonadherent cells exposed to stressful cellular stimuli, such as DNA-damaging agents [35]. Combined with the loss of ERK1/2 nuclear translocation, this would result in phosphorylation of a combination of sites on Elk-1 different from that in the adherent state, potentially contributing to activation of a distinct cellular outcome. Thus, as a tumor progresses and more cells lose cellular contacts, more cells may demonstrate this altered

balance of phosphorylation of Elk-1. The T417 site could potentially be a part of that pattern in that increasing levels of Elk-1 pT417 across decreasingly differentiated cancers may indicate this signaling imbalance.

Here, we have shown that Elk-1 pT417 is located in the nuclei of normal epithelial cells in various glandular tissues, suggesting a role for ELK-1 phosphorylated at this site in transcriptional regulation. In addition, Elk-1 pT417 was shown to label nuclei of epithelial cells in tumors from various tissues. The overall trend of Elk-1 pT417 staining was different for different cancers compared with normal tissue, and this could be the result of differences in the balance of signaling components in different tissues as well as the differentiated state of the tumors. Finally, Elk-1 pT417 was shown to be elevated in colonic adenocarcinoma by qualitative assessment of intensity and quantitative assessment of positive cell numbers. Elk-1 pT417 labeled a higher proportion of epithelial cell nuclei in colonic adenocarcinoma compared with normal colon epithelium and this proportion correlated with tumor differentiation grade. Specifically, the proportion of Elk-1 pT417-positive nuclei was higher in less differentiated tumors up to, but not including, the poorest level of differentiation, at which point the proportion of Elk-1 pT417-positive cells did not significantly differ from normal colon. These results point to a role for this phosphoform of Elk-1 in the progression of this particular tumor and suggest the importance of carefully regulated signaling integration in normal cellular functions.

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Fig. 1.

Elk-1 nonphosphoT417 and Elk-1 pT417 immunohistochemistry in normal tissues. A tissue microarray composed of different normal and cancer tissues was processed for Elk-1 nonphosphoT417 or Elk-1 pT417 immunohistochemistry and aligned to the corresponding H&E slide (I, J, K, and L). Elk-1 pT417 (A) and nonphosphoT417 (E) are localized to distinct epithelial cell nuclei in skin. Elk-1 pT417 is localized to epithelial cell nuclei in breast (B), stomach (C), and colon (D), whereas nonphosphoT417 is localized to the cytoplasm in these cells (F, G, and H, respectively). Samples were imaged at original magnification $\times 200$, whereas insets represent original magnification $\times 400$ of the corresponding boxed area (red boxes) in the larger image. Specificity of the antibodies is shown in (M). The Elk-1 pT417 and nonphosphoT417 antibodies recognize a peptide of approximately 48 kD (asterisks), which is the published size of the Elk-1 protein in normal human colon lysates. Preincubation of each antibody with the corresponding blocking peptide prevented the antibody from binding to this band (\$). All samples shown were run on the same gel and blotted onto the same membrane.

Fig. 2.

Elk-1 nonphosphoT417 and Elk-1 pT417 immunohistochemistry in cancer tissues. A tissue microarray composed of different normal and cancer tissues was stained for Elk-1 nonphosphoT417 or Elk-1 pT417 and aligned to the corresponding H&E slide (G, H, and I). As in the normal tissues, Elk-1 pT417 is found in the nucleus of an adenocarcinoma of the rectum (A), an infiltrating ductal carcinoma of the breast (B), and an adenocarcinoma of the stomach (C), whereas nonphosphoT417 is localized to the cytoplasm (D, E, and F, respectively). Samples were imaged at original magnification $\times 200$.

Fig. 3.

Elk-1 pT417 levels are higher in adenocarcinoma compared with normal colon. Archival tissue, including adenocarcinoma of the colon and corresponding normal tissue, was processed for both Elk-1 nonphosphoT417 and Elk-1 pT417 immunohistochemistry and aligned to corresponding H&E images (G and H). Representative images of adjacent normal colon (A and D) and an adenocarcinoma (B and E) at original magnification $\times 200$ are shown along with the proportion of samples assigned a particular histology score by an independent pathologist for Elk-1 pT417 (C) and Elk-1 pT417 (F). Ratios of scores for pT417 to nonphosphoT417 staining for each sample were also calculated and displayed as the proportion of samples that have a particular score (I). See Table 2 for statistics. White bars, normal colon (n = 9-10); black bars, adenocarcinoma (n = 5-7).

Fig. 4.

Elk-1 pT417 levels correlate with differentiation grade of colonic adenocarcinoma. Immunofluorescence for Elk-1 pT417 (right panels) in normal colon (A), well-differentiated adenocarcinoma (B), moderately differentiated adenocarcinoma (C), and poorly differentiated adenocarcinoma (D) in which blue is DAPI, and red is pT417, are shown with corresponding H&E images (left panels). Black boxes selected on the H&E images indicate areas displayed in immunofluorescence images. Scale bars represent 100 μ m. E, Elk-1 pT417 immunofluorescence was quantified by calculating the number of pT417-positive

nuclei as the percentage of DAPI-positive cells and expressed as means and SEMs. A 1-way ANOVA revealed a significant main effect of differentiation grade on percentage of Elk-1 pT417-positive cells ($P < .001$). A Tukey post hoc analysis revealed a significant difference between well-differentiated adenocarcinoma ($n = 3$) and the following groups: normal colon ($P < .001$, $n = 15$), poorly differentiated adenocarcinoma ($P = .006$, $n = 5$), and between moderately differentiated adenocarcinoma ($n = 10$) and the following groups: normal colon ($P < .001$) and poorly differentiated adenocarcinoma ($P = .011$). The differences between tubulovillous adenoma ($n = 4$) and normal colon or poorly differentiated adenocarcinoma did not reach statistical significance.

Table 1

Qualitative assessment of immunohistochemical staining of TMAs

Tissue	Elk-1 pT417			Elk-1 nonphosphoT417	
	Stain intensity	Proportion positive cells	Location	Stain intensity	Location
Skin	Strong	High	Nucleus	Weak	Nucleus
Breast	Strong	High	Nucleus	Strong	Cytoplasm
Stomach	Strong	High	Nucleus	Strong	Cytoplasm
Colon	Strong	High	Nucleus	Strong	Cytoplasm
Prostate	Strong	Low	Nucleus	Strong	Cytoplasm
Kidney	Moderate	Medium	Nucleus	Strong	Cytoplasm
Lung	Moderate	Low	Nucleus	Moderate	Cytoplasm
Liver	Moderate	Medium	Nucleus	Weak	Cytoplasm
Urinary bladder, transitional cell carcinoma	Strong	High	Nucleus	Strong	Cytoplasm
Rectum, adenocarcinoma	Strong	High	Nucleus	Strong	Cytoplasm
Small bowel malignant stromal tumor	Moderate	Medium	Nucleus	Strong	Cytoplasm
Lung, squamous cell carcinoma	Moderate	High	Nucleus	Strong	Cytoplasm
Esophagus, squamous cell carcinoma	Moderate	High	Nucleus	Strong	Cytoplasm
Liver, hepatocellular carcinoma	Moderate	Medium	Nucleus	Strong	Cytoplasm
Breast, infiltrating duct carcinoma	Weak to Moderate	Low	Nucleus	Strong	Cytoplasm
Stomach, adenocarcinoma	Moderate	Medium	Nucleus	Strong	Cytoplasm

NOTE. Immunohistochemistry of each sample on the tissue microarray was assigned a qualitative score of negative, weak, moderate, or strong based on the intensity of staining in the most and least intensely stained samples. In addition, the proportion of cells stained by Elk-1 pT417 is given with designations of low, medium, or high compared with samples with the lowest and highest proportions of positive nuclei.

Table 2

Median histology scores for Elk-1 nonphosphoT417 and Elk-1 pT417 immunohistochemistry in normal colon and colonic adenocarcinoma

	Normal colon	Adenocarcinoma	<i>P</i>
Elk-1 nonphosphoT417	0.75	0.5	1.000
Elk-1 pT417	1	2	.005 ^a
Elk-1 pT417/T417	1	4	.011 ^a

^a A Mann-Whitney rank sum test performed on scores for each form of Elk-1 revealed that the median scores for Elk-1 pT417 and the median ratio scores for Elk-1 pT417 to Elk-1 nonphosphoT417 were significantly higher in adenocarcinoma (n = 5-7) compared to normal colon (n = 9-10).