INVESTIGATING THE REGULATION OF c-FES NON-RECEPTOR TYROSINE KINASE ACTIVATION AND GENE EXPRESSION

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The human c-*fes* locus encodes a non-receptor tyrosine kinase (c-Fes) that is structurally and functionally unique. Originally, c-*fes* was isolated as the normal cellular homolog of sarcoma-inducing avian and feline retroviruses. However, unlike its viral oncoprotein counterparts that display constitutive tyrosine kinase activity, c-Fes exhibits restrained activity that is regulated by an undefined mechanism. Adding to its unique nature, recent studies have implicated c-Fes as a colorectal cancer-associated tumor suppressor despite its status as a proto-oncogene and tyrosine kinase.

Previous work from our group has demonstrated that c-Fes forms high molecular weight oligomers *in vitro*, suggesting that c-Fes catalytic activity is governed by the interconversion of c-Fes between inactive monomeric and active oligomeric forms. However, this model was based largely on *in vitro* data and has not been assessed in living cells. To assess the involvement of oligomerization in regulating c-Fes activity *in vivo*, I employed a yellow fluorescence protein (YFP)-based bimolecular fluorescence complementation (BiFC) assay. Using BiFC, I demonstrated for the first time that c-Fes forms constitutive oligomers *in vivo*, regardless of its activation status. In addition, I determined that both coiled-coil domains mediate the oligomerization of c-Fes. Moreover, I established that c-Fes forms coiled-coil dependent oligomers in physiologically relevant cellular contexts, suggesting a new model for c-Fes

regulation where conformational changes rather than oligomerization govern c-Fes kinase activity in cells.

In colorectal cancers, loss of c-Fes expression is a common occurrence. This is not unusual, as tumorigenesis proceeds as oncogenes are activated and tumor suppressors are inactivated. To date, however, the mechanism responsible for c-*fes* gene repression has not been characterized. Upon determining that the absence of c-*fes* gene transcription was common among colorectal cancer cell lines, I used methylation inhibitor, bisulfite sequencing, and *in vitro* methylation analyses to establish that promoter methylation governs Fes gene and protein expression in colorectal cancers. Preliminary studies also suggest that promoter methylation governs c-Fes expression in human colon cancer surgical specimens. Taken together, the studies outlined in this thesis advance the field of c-Fes research by defining previously unknown regulatory mechanisms of both kinase activity and gene expression.

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I replied, “No, neither.” Ashok finished, “Well, you had better pick one, because you’ll need it.” I chose coffee.

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This thesis is dedicated to my grandparents

(Henry & Marjorie Kemp and Jim & Madelon Ramey)

who fought courageously against diseases we as scientists research everyday

1.0 INTRODUCTION

1.1 CHARACTERIZATION OF THE C-FES TYROSINE KINASE

The human c-*fes* locus (15.q26.1) encodes the 93 kDa c-Fes non-receptor protein-tyrosine kinase (1-4). Only one other protein is part of the c-Fes subfamily of non-receptor tyrosine kinases, the ubiquitously expressed Fer tyrosine kinase (5). Initially, *fes* was isolated as a transforming oncogene from avian [v-*fps*: Fujinami sarcoma virus (FSV); the PRC viruses (PRCIV, PRCIIp, PRCII); URI virus; and 16L virus] and feline [v-*fes*: Gardner-Arnstein (GA); Snyder-Theilen (ST); and Hardy-Zuckerman 1 (HZ1)] sarcoma retroviruses (6-11). However, unlike these transforming v-Fps and v-Fes viral counterparts that exhibit constitutive protein-tyrosine kinase activity [reviewed in (12,13)], the human c-Fes non-receptor protein kinase is non-transforming and exhibits restrained tyrosine kinase activity (14-17). Structurally, v-Fps and v-Fes consist of an amino-terminal viral gag sequence fused to either normal cellular Fps or Fes-derived sequences (Figure 1) (18-23). The structural motifs of c-Fes are detailed in Section 1.1.2.

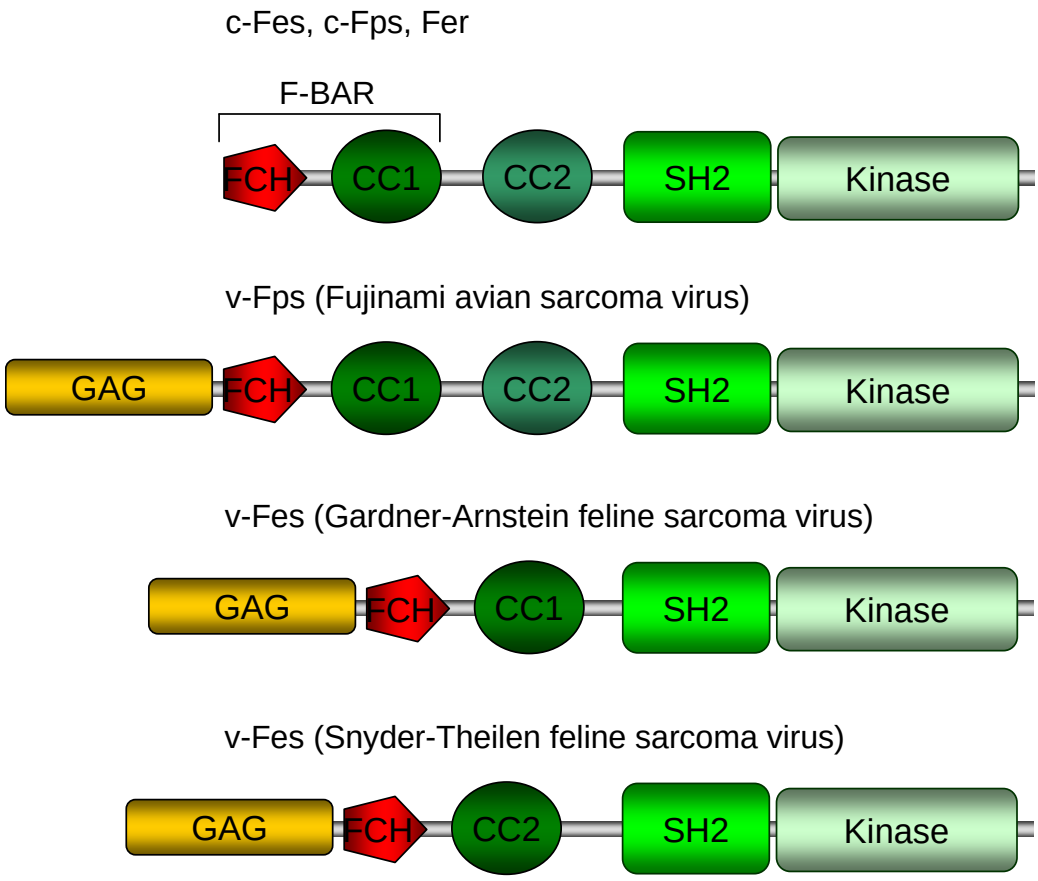

Figure 1: Structural Diagrams of c-Fes and Representative Avian and Feline Viral Homologs.

c-Fes is composed of an amino-terminal F-BAR domain [a Fes/Fer/CIP4 (FCH) domain and a coiled-coil (CC1) domain], a second coiled-coil (CC2) domain, a central Src-homology 2 (SH2) domain, and a carboxyl-terminal tyrosine kinase domain. Viral oncoprotein homologs of c-Fes (v-Fps and v-Fes) are the result of viral gag sequences being fused to sequences derived from the normal c-Fps or c-Fes proteins.

1.1.1 Characterization of the c-*fes* Gene

More than twenty-six years ago, various groups independently identified a single 13.2 kb human gene (named c-*fes*) with significant sequence homology to the ST and GA v-*fes* transforming retroviral oncogenes (24-26). Upon sequencing the entire c-*fes* gene in 1985, Roebroek and colleagues organized c-*fes* into 19 exons (the first of which is non-coding), identified a poly(A) addition signal 200 bp downstream of the TGA termination codon in exon 19, and located a translation initiation sequence in exon 2 (Figure 2) (4).

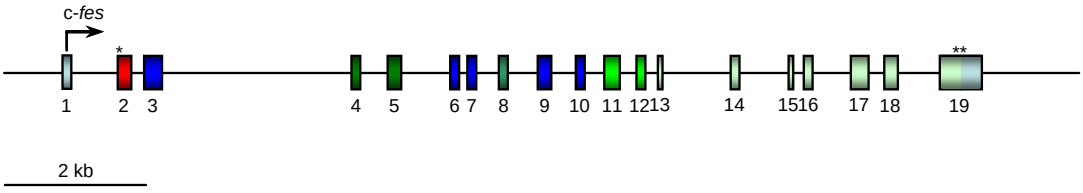

Figure 2: Organization of c-*fes* Gene

The 13.2 kb human c-*fes* gene (chromosome 15.q26.1) is organized into 19 exons. Exon 1 (gray), the site of c-*fes* transcription initiation (indicated by the arrow) is noncoding. Exon 2 (red) harbors the translational start site (*) of c-Fes and encodes the FCH domain. Exons 4 and 5 (green) code for the first coiled-coil (CC1) domain. Exon 8 (sea green) encodes the second coiled-coil (CC2) domain. Exons 11 and 12 (bright green) encode the SH2 domain. Exons 13-19 (light green) encode the kinase domain. The translation termination codon and poly(A) addition signals (**) are found in exon 19.

Strikingly compact, the c-*fes* locus is a self-contained genetic element that harbors all transcription regulatory and tissue-expression specific controls in only 446 bp and 1.54 kb of 5'- and 3'-flanking sequences, respectively (27-29). The sequence of the c-*fes* promoter is described in Figure 3. Two major transcription initiation sites are found within the c-*fes* promoter (bold/italicized), both of which are 72-83 bp upstream of the 3' boundary of exon 1 (uppercase) defined by Roebroek and colleagues (30,31). The presence of multiple initiation sites is not uncommon as the c-*fes* promoter does not contain either TATA box or initiator (Inr) sequences (32,33). Defining the first initiation site as +1, the entire c-*fes* promoter is -425/+91 and the critical core of the c-*fes* promoter is -131/+91 (29,30).

Regulation of c-*fes* expression in myeloid cells has been well characterized, and various transcription factors recognize sequences within the robustly active c-*fes* core promoter including the ubiquitously expressed transcription factor Sp1, the hematopoietic cell-specific factor PU.1/Spi-1, and a c-*fes* expression factor (FEF) that is not present in human epithelial cells (29,34,35). In total, three Sp1 (5'-CCGCCC-3'), two PU.1/Spi-1 (5'-AGGAA-3'), and one FEF (5'-GAATCA-3') recognition sequences are found within the core promoter (Figure 3: boxed). Reporter gene expression assays have revealed the critical nature of the PU.1/Spi-1 and FEF sites, as mutation of their respective recognition sequences greatly decreases the activity of the c-*fes* promoter (29,34). The chicken lysozyme locus is the only other known gene whose expression is regulated by FEF.

Figure 3: Features of the c-*fes* Promoter and Intron 1

The c-*fes* promoter and intron 1 extend -426 to +577 relative to the first transcription initiation site and contain all transcription regulatory units for c-*fes* expression. Binding sites for the Sp1 (5'-CCGCCC-3'), Spi-1/Pu.1 (5'-AGGAA-3'), and Fes Expression Factor (FEF: 5'-GAATCA- 3') transcription factors (boxed) are located within the critical core of the c-*fes* promoter (-131 to +91). Exon 1 (+1 to +85: uppercase) harbors two transcription initiation sequences (+1 and +11: bold/italicized). Intron 1 (+86 to +577: lower case) contains an orientation specific negative regulatory region (underlined). Exon 2 (uppercase) begins at base +578 and contains the c-Fes translational start site (+587: uppercase/bold/italicized).

In addition to the positive regulatory elements found in the core c-*fes* promoter, a region associated with negative regulation of c-*fes* transcription is found in intron 1 (Figure 3: underlined) (30). He and colleagues used DNA footprinting analyses of K-562 myeloid cell nuclear extracts to determine that the sequence +441/+454 (5'-TGCGTGTGAGCGAG-3') inhibits the activity of the c-*fes* promoter nearly 50% in an orientation specific manner (30). This region shares sequence homology with negative regulatory regions found in the LD87α and IL-3 cytokine genes (30). The c-Fes translation start site (+587) (Figure 3: uppercase/bold/italicized) is located 3' to the negative regulatory region in exon 2.

1.1.2 c-Fes Structure

Structurally, the 822 amino acid c-Fes protein consists of amino-terminal Fes/Fer/CIP4 homology (FCH) domain followed by two coiled-coiled (CC) motifs, a central Src-homology 2 (SH2) domain, and a carboxyl-terminal tyrosine kinase domain (Figure 1). Together, the FCH and the first CC motif define an F-BAR domain, a structural region found in at least 21 human genes. c-Fes, along with Fer, constitute one of six F-BAR protein classes (36). Proteins harboring an F-BAR domain are considered to be multi-functional adapters at the membrane-cytosol interface that are involved in cellular processes including endocytosis, exocytosis, and motility (36,37). In contrast to other non-receptor tyrosine kinases family members such as Src, Abl, Tec/Btk, and Syk/Zap70, Fes does not possess an SH3 domain, a pleckstrin homology (PH) domain, or a negative regulatory tail tyrosine residue (12,13).

1.1.2.1 FCH Domain

FCH domain containing proteins have been implicated in a variety of cellular responses including cytoskeletal rearrangement, vesicular transport, and endocytosis [reviewed in (12)]. Originally described as a region of homology between Fes, Fer, and the Cdc42-interacting protein (CIP4) (38), two related roles have been proposed for the c-Fes FCH domain in the modulation of microtubule dynamics. Takahashi and colleagues found that murine Fes co-localized with microtubule nucleation sites in an FCH domain dependent manner as deletion of the FCH domain abrogated normal microtubule nucleation and centrosome formation events in COS-7 cells (39). This group also determined that re-expression of Fes in Fes-deficient mouse embryonic fibroblasts restored normal microtubule centrosome formation. A subsequent study by Laurent and colleagues suggested that the FCH domain was dispensable for the interaction of human c-Fes with the microtubule network *in vivo* and instead mediated the associated of c-Fes with soluble unpolymerized tubulin (40). Similar to c-Fes, CIP4 interacts with microtubules through its FCH domain, and this interaction potentially facilitates the association of the Wiskott-Aldrich Syndrome Protein (WASP) with microtubules and sites of substrate adhesion in hematopoietic cells (41).

1.1.2.2 CC Domains

Located immediately downstream of the c-Fes FCH domain are two coiled-coil oligomerization motifs. Coiled-coils consist of two to five parallel or antiparallel amphipathic α-helices that are wound into super helical bundles (42,43). These α-helices exhibit a heptad repeat pattern in the chemical nature of their sidechains as the first and fourth residues of the heptad repeat form the hydrophobic interface of the superhelix, whereas the remaining residues form the

hydrophilic or solvent-exposed region (43). This arrangement is referred to as "knobs-into-holes" since one helical sidechain packs into a space surrounded by four sidechains of the facing helix (43).

Coiled-coils are implicated in a broad array of biological functions including protein oligomerization, structural formations, scaffolding, and binding to non-native proteins (44). Read and colleagues were the first group to suggest the presence of coiled-coils domains within the c-Fes protein (45). Using the COILS algorithm, which measures the probability of an amino acid and its surrounding sequencing of forming a coiled-coil amphipathic α-helix (46), two regions were identified within the unique amino-terminus of c-Fes that exhibited strong homology to coiled-coil domains (45). This study went on to implicate the amino-terminus, and in extension the coiled-coil motifs, as the mediator of c-Fes oligomerization. While deletion of the unique amino-terminus abolished Fes oligomerization, deletion of the SH2 and kinase domains failed to alter the oligomeric profile of c-Fes (45). Gel filtration analyses of clarified cellular lysates expressing full-length recombinant c-Fes demonstrated that active c-Fes was exclusively oligomeric and could form high molecular weight structures up to a pentamer.

In addition to mediating c-Fes oligomerization, the coiled-coil domains also contribute to the regulation of c-Fes catalytic activity (14,47,48). Through deletion, insertion, and point mutation analyses, the first coiled-coil domain (CC1) has been defined as a negative regulator of c-Fes kinase activity *in vivo*, whereas the CC2 domain has been implicated in substrate recruitment. An in depth analysis into how the coiled-coil domains contribute to c-Fes activation is found in Section 1.1.3.

1.1.2.3 SH2 Domain

Located in the central region of c-Fes is an SH2 domain. SH2 domains are compact protein modules that mediate protein-protein interactions within tyrosine kinase signaling pathways (49-51). Conserved among most non-receptor protein-tyrosine kinases, SH2 domains contact target proteins through two binding surfaces, conceptually similar to the engagement of a two-pronged plug with an electrical socket (52). The first surface involves a deep pocket lined with basic residues to accommodate the phosphotyrosine residue. This pocket contains an invariant arginine residue (Arg-483 in c-Fes) that forms hydrogen bonds with the phosphate oxygens of the phosphotyrosine residue. The second binding surface contacts the three to six amino acids immediately following the phosphotyrosine residue to confer optimal substrate binding. As recognition sequences overlap, substrate selection also depends on localized protein concentration as well as other domains within the interacting proteins (49). For example, the SH2 domain of c-Fes exhibits high affinity for the sequence Y^{P}EXV/I (53). Among SH2 domain containing proteins, the v-Fps SH2 domain was the first to be characterized and contributes to both kinase regulation and substrate selectivity (54,55).

1.1.2.4 Tyrosine Kinase Domain

Immediately carboxyl-terminal to the SH2 domain is the tyrosine kinase domain of c-Fes. The bilobate tyrosine kinase domain catalyzes the transfer of a phosphate to a target tyrosine residue (56). The small lobe of the kinase domain binds ATP molecules, the large lobe binds substrate molecules by interacting with residues flanking the tyrosine target, and catalysis of the phosphate transfer occurs in the cleft between the two lobes. For c-Fes, the optimal substrate sequence correlates well with the optimal SH2 domain binding sequence (57).

c-Fes autophosphorylation occurs via an intermolecular mechanism (58). Two consensus autophosphorylation sites are located at Tyr-713 and Tyr-811 (17,58,59). Tyr-713, the major site of autophosphorylation, is critical for kinase activity as a Y713F point mutation reduces the kinase activity of c-Fes by more than 90% (59,60). While Tyr-713 primarily regulates kinase activity, Tyr-811 may function as a docking site for SH2 domain containing proteins as the sequence immediately carboxyl-terminal to Tyr-811 partially matches ten predicted SH2 binding motifs (58). Together, each c-Fes structural domain contributes to substrate selectivity and regulation of kinase activity.

1.1.3 Catalytic Regulation of c-Fes

c-Fes naturally adopts an inactive conformation, and various studies support the conclusion that c-Fes activity is strictly regulated in living cells. Foster et al. determined an avian variant of c-Fes (c-Fps) failed to induce the transformation of chicken embryo fibroblasts, as measured by focus forming and growth assays (61). Only when c-Fps was activated via mutation or fusion to a viral gag sequence did transformation occur. Also, when ectopically expressed in rodent fibroblasts, c-Fes exhibits restrained protein tyrosine kinase activity and fails to induce transformation. However, as with the avian system described above, ectopic over-expression of activated Fes induces tissue hyperplasia and morphological tumorigenic transformation in rodent fibroblasts (14-17). The question remains as to what governs the catalytic activation of c-Fes in cells.

In vitro analyses have suggested a regulatory model in which the coiled-coil oligomerization motifs control the interconversion of c-Fes between inactive monomeric and active oligomeric configurations (Figure 4) (14,45,47). In this model, CC1 interacts

intramolecularly with CC2 to hold c-Fes in an inactive monomeric conformation. When the negative regulation imparted by the CC1 domain is released, c-Fes oligomerizes through its CC2 domain, inducing autophosphorylation and activation. However, this model is largely based on *in vitro* studies including gel filtration analyses of the inclusive coiled-coil domains that exclude potential regulatory mechanisms imparted by the SH2 and kinase domains. Further, the inclusive CC1 + CC2 region eluted as a mixture of monomeric and oligomeric forms suggesting the possibility that wild-type c-Fes may exist as a constitutive oligomer (14).

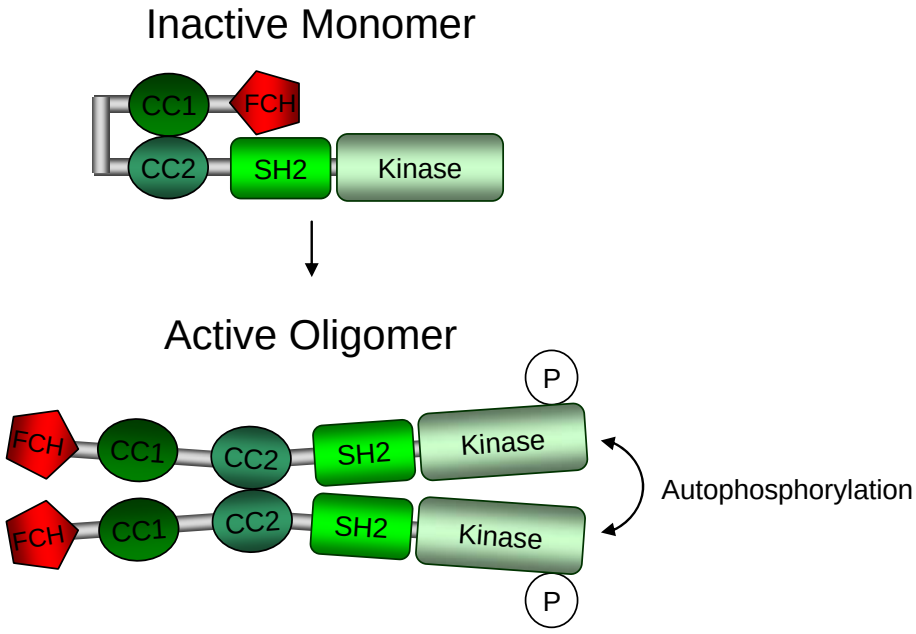

Figure 4: Model for the Regulation of c-Fes Catalytic Activity

A model for the regulation of c-Fes kinase activity, based largely on *in vitro* evidence, is as follows. When c-Fes is inactive, CC1 interacts intramolecularly with CC2, holding c-Fes as a monomer. Upon release of the negative regulation imparted by CC1, c-Fes oligomerizes through

its CC2 domain, inducing autophosphorylation and activation. A dimer is shown for simplicity; however, c-Fes can form higher order structures up to a pentamer.

In addition to the coiled-coil domains, the central SH2 domain also modulates c-Fes kinase activity. Substitution of the SH2 domain results in either upregulation (v-Src SH2 domain substitution) or downregulation (Gap SH2 domain substitution) of c-Fes kinase activity (17,48). Also, c-Fes has been shown to bind directly to its own SH2 domain (59), and this interaction is mediated at least in part by the kinase domain (T. Smithgall, unpublished results). In addition, early studies involving a viral counterpart of c-Fes (v-Fps) suggest that the SH2 domain interacts in *cis* with the kinase domain to form the active kinase conformation (13). Mild proteolysis of v-Fps released a stable globular fragment containing the SH2 and kinase domains, consistent with the idea that SH2-kinase domain interaction is essential for full kinase activity (62,63).

Most recently, the inclusive c-Fes SH2-kinase region X-ray crystal structure was solved revealing that the SH2 domain contacts the amino-terminal lobe of the kinase domain at multiple locations, including the critical αC helix involved in conformational regulation of the active site (64) (Figure 5A). Upon mutation of key residues involved in at the SH2:kinase interface, c-Fes kinase activity was dramatically reduced. Of interest is that the SH2:kinase interaction interface of active c-Fes is similar to that of active c-Abl (Figure 5B) suggesting that this conformation may be a common regulatory mechanism of certain non-receptor tyrosine kinases (64). Collectively, these data suggest the SH2 domain is a positive regulator of c-Fes kinase activity. Experiments in Aim 1 will identify the contribution of each c-Fes domain to the regulation of its oligomerization and kinase activation.

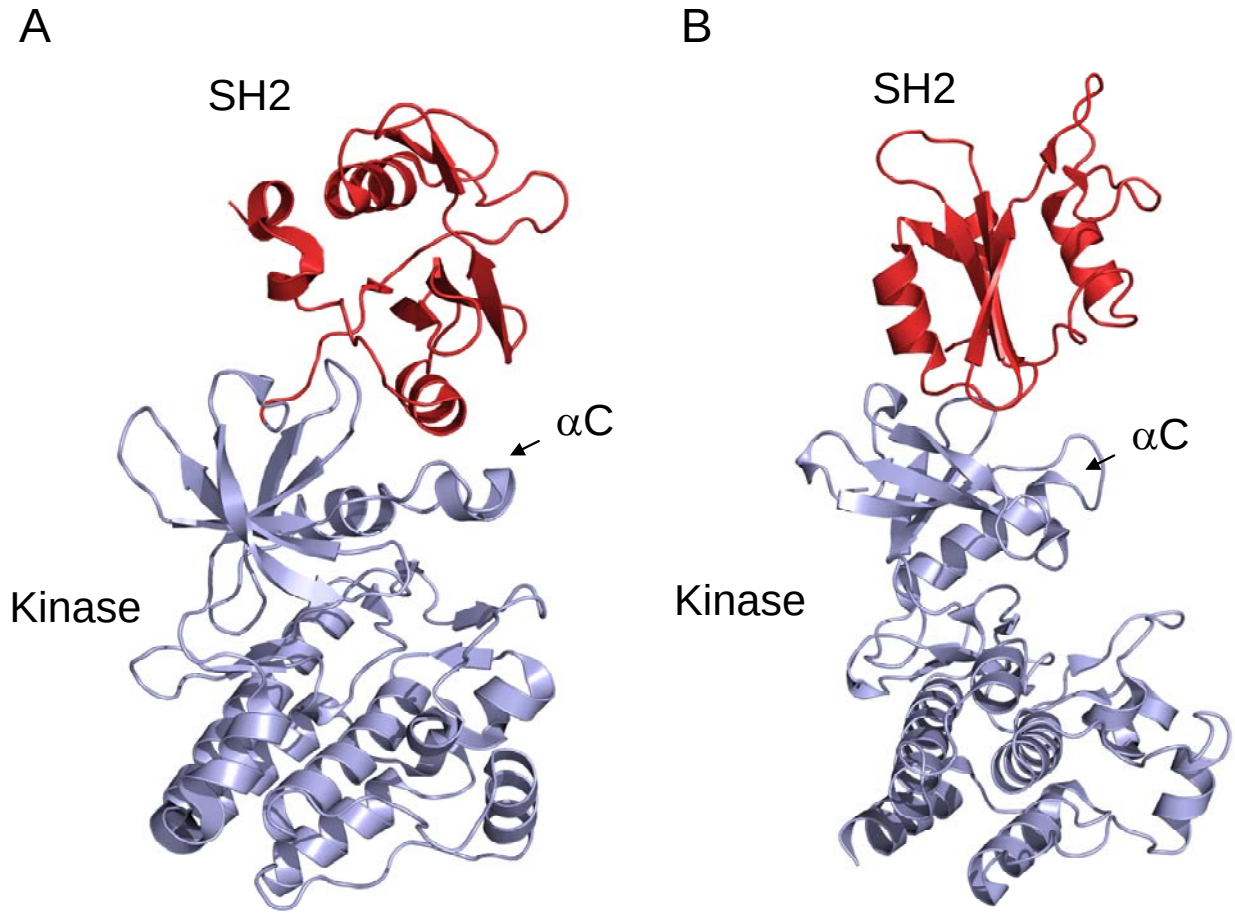

Figure 5: Structures of c-Fes and c-Abl SH2-Kinase Domains in the Active Conformation. Structural models for the SH2-kinase domains of active c-Fes (A) and c-Abl (B) are shown. For both proteins, the SH2 domain (red) contacts the kinase domain (blue) at multiple locations. A prominent point of contact involves the critical αC helix of the kinase domain.

1.1.4 c-Fes Biological Functions

The c-Fes kinase is expressed in a variety of cells including myeloid hematopoietic, vascular endothelial, neuronal, and epithelial cells (Table 1) where it undergoes activation in response to signaling imparted by a variety of cytokines and growth factors [reviewed in (12,13)]. In these cell types, c-Fes has been linked to signaling pathways that control differentiation, oncogenesis, and tumor suppression. Patterns of Fes expression have also been established for both human and mouse tissue. Greer and colleagues localized endogenous murine *fes* and transgenic human c-Fes to the bone marrow, spleen, lymph node, and lung tissue (27). Haigh et al. additionally determined that *fes* was expressed in the developing vascular system, epidermis of the skin, and the lining of ventricles of the developing brain in mouse embryos (65). Further, Caré and colleagues initiated northern blot and in situ hybridization analyses to determined the spatial and temporal distribution of c-*fes* RNA during human and murine development and found c-*fes* transcripts in lung, liver, spinal cord, skin, gut heart and kidney (66). Most recently, Delfino et al. analyzed the expression of c-Fes in colonic epithelial tissue and observed robust c-Fes expression in normal tissue and reduced or absence of c-Fes expression in tumor tissue from the same individual (67).

Cell Line	Species	mRNA	Protein	Reference
Myeloid				
AML	Human	Yes	Yes	(28)
HEL	Human	ND	Yes	(68)
HL-60	Human	Yes	Yes	(29,68)
K-562	Human	No	No	(29,68)
KG-1	Human	ND	Yes	(68)
TF-1	Human	ND	Yes	(69,70)
THP-1	Human	Yes	Yes	(29,68)
U-937	Human	Yes	Yes	(29,68)
Vascular Endothelial				
Bac.2F5	Murine	Yes	Yes	(28)
EOMA	Murine	Yes	Trace	(28)
HUVEC	Human	Yes	Yes	(28)
IBE	Murine	ND	Yes	(71)
Neuronal				
Hippocampal	Rat	ND	Yes	(39)
PC-12	Rat	ND	Yes	(72)
Epithelial				
CACO-2	Human	ND	Trace	(67)
COLO 320	Human	ND	No	(67)
DLD-1	Human	ND	No	(67)
HCT 116	Human	ND	No	(67)
HT-29	Human	ND	No	(67)
SNU 1040	Human	ND	No	(67)
HeLa	Human	No	ND	(29)
LLC-PK1	Pig	ND	ND	(73)

Table 1: c-Fes Cellular Expression Summary

Expression of c-Fes mRNA and protein in myeloid, vascular endothelial, neuronal, and epithelial cells. ND: no data.

1.1.4.1 Subcellular Distribution

Within the cell, c-Fes predominantly exists as a cytoplasmic non-receptor tyrosine kinase. Multiple studies confirm this distribution (17,39,40) and one study in particular (65) found that c-Fes exhibited a diffuse cytoplasmic distribution with strong localization to the perinuclear region as well as punctuate projections that extend towards the plasma membrane. However, a few isolated experiments also suggest a subset of c-Fes molecules localizes to the nucleus in myeloid hematopoietic cells even though a nuclear localization sequences has not been identified (74,75).

c-Fes subcellular localization also appears to be modified as a result of kinase activation (17,39,40,73). In Rat-2 cells, ectopically expressed c-Fes exhibits a diffuse cytoplasmic distribution that changes to focal adhesion localization upon activation (17). In COS-7 cells, wild-type c-Fes naturally exhibits an inactive, diffuse cytoplasmic distribution. However, upon catalytic activation, c-Fes relocalizes to the prominent COS-7 microtubule network. Of note is that c-Fes has also been demonstrated to localize to the microtubule network in neuronal cells as well (39,76). In line with this subcellular redistribution, a recent analysis of c-Fes expression in porcine kidney LLC-PK1 cells found that active c-Fes localization shifted between focal adhesions and cell-cell contacts, depending on cellular confluency (73). Further, Jucker and colleagues found that wild-type c-Fes associated with cytoskeletal components (77). Taken together, all of these observations suggest a role for c-Fes in regulating cellular cytoplasmic architecture and possibly cell movement.

1.1.4.2 Differentiation

The role of c-Fes in cellular differentiation responses is well characterized. A foremost example involves the highly undifferentiated chronic myelogenous leukemia (CML) cell line (K-

562) (78,79) that does not express c-Fes (Table 1). Upon re-introduction of c-Fes, these cells undergo terminal differentiation as measured by the appearance of phagocytic activity, Fc receptors, nitro blue tetrazolium (NBT) reduction, MAC-1 immunofluorescence, and lysozyme production as well as the increases in the expression of the CD13 and C33 myelomonocytic surface antigens (47,60,80). Similarly, stable expression of active c-Fes mutants in the cytokine-dependent myeloid leukemia cell line TF-1 promotes GM-CSF independent cell growth and survival, cell attachment, and cell spreading as well as increases in the CD13 and CD33 antigens (14). Also, expression of an active c-Fes mutant in the myeloid progenitor cell line U-937 induces cell adherence, terminal macrophage differentiation, and differentiation marker (CD11b, CD11c, CD18, and CD14) expression (81). Further, antisense oligonucleotide inhibition of c-*fes* in the promyelocytic leukemia cell line HL-60 or FDC-P1/MAC-11 murine myeloid precursor cell line blocks PMA-induced differentiation (82,83). In a neuronal cell context, over-expression of wild-type c-Fes accelerates NGF-induced neurite extension in PC-12 cells, while active c-Fes mutants induce spontaneous neurite formation in this cell line, suggestive of a role in neuronal differentiation (40,72). This process has been linked to the PI3K-dependent activation of the small G-proteins Rac and Cdc2. Last, over-expression of wild-type c-Fes induces FGF-2-independent tube formation by cultured brain capillary endothelial cells, implicating c-Fes in angiogenesis (71).

1.1.4.3 Oncogenesis

As suggested by Sangrar and colleagues (84), c-Fes may suffer from an "identity crisis" as both oncogenic and tumor suppressor roles (discussed in the next section) have been proposed for c-Fes. In addition to its viral counterparts that are sarcoma-inducing oncoproteins, over-expression of wild-type or activated c-Fes mutants causes oncogenic transformation in rodent

fibroblasts as well as tissue hyperplasia in transgenic mice (14-17). Further, fusion of viral gag sequences to the amino-terminal regions of either c-Fes or c-Fps results in fibroblast transformation and strong tyrosine kinase activity (61,85,86). Last, a recent study implicated c-Fes as a promoter of renal carcinoma cell proliferation as siRNA knockdown of c-*fes* significantly slows the renal carcinoma cell growth (Dr. Shigeru Kanda, University of Nagasaki, Japan, personal communication).

1.1.4.4 Tumor Suppression

Studies also suggest a tumor suppressor role for c-Fes in both myeloid hematopoietic and epithelial cells. Over-expression of wild-type Fes in K-562 myeloid leukemia cells suppresses cell growth and supports differentiation (discussed above), implicating Fes as a potential suppressor of chronic myelogenous leukemia (17,80,87). A potential target for c-Fes has been suggested to be the Bcr-Abl protein (the 9;22 chromosomal translocation gene product responsible for chronic myelogenous leukemia initiation) as Bcr-Abl protein levels decrease upon stable expression of c-Fes (60).

With respect to epithelial cells, Bardelli and colleagues identified c-*fes* as one of only seven genes exhibiting consistent colorectal cancer-associated kinase domain mutations following nucleotide sequence analysis of the tyrosine kinome in 182 colorectal cancer cell lines or xenografts (88). Initially, these kinase domain mutations (M704V, R706Q, V743M, and S759F) were predicted to be activating and promote colorectal cancer tumorigenesis, as the c-Fes protein is the tightly regulated normal cellular homolog of transformation retroviruses. However, subsequent studies established that these mutations reduced or eliminated c-Fes kinase activity (67,84). Sangrar and colleagues went on to characterize the role of Fes in a mouse model of breast epithelial cancer and found that tumor onset occurs more rapidly in mice targeted with

either null or kinase-inactivating *fes* mutations (84). In addition, this group determined a *fes* transgene restores the kinetics of tumor onset in the c-*fes* null mice (84). In a parallel study, our laboratory established c-Fes as a suppressor of Fes-negative HT-29 and HCT 116 colorectal cancer (CRC) cell line growth in soft agar (67). Our study also showed that while c-Fes is strongly expressed in normal colonic epithelial cells from CRC patient tissue samples, expression is reduced or absent in 67% of colon tumor sections from the same group of individuals (67). In line with this, c-Fes protein expression is significantly reduced or absent in five of six CRC cell lines examined, with CACO-2 cells being the notable exception (67). Collectively, these data support a role for c-Fes as a tumor suppressor in colonic epithelial cells.

1.2 COLORECTAL CANCER

Cancer of the colorectum, the final portion of the digestive system extending from the small intestine illeocecal valve to the anus, is the fourth most common cancer in the United States. In 2008, an estimated 148,810 men and women will develop colorectal cancer (CRC) and 49,960 will die from the disease (89). From a different perspective, someone will be diagnosed with CRC every 3.5 minutes, and someone will die from CRC every nine minutes (90). Fortunately, CRC incidence rates have been decreasing over the past two decades and have fallen nearly 2.1% per year from 1998-2003 due in part to improved staging, surgical therapies, and adjuvant therapies (89). However, the full complement of improperly activated oncogenes and inactivated tumor suppressor genes associated with the multi-step process of CRC carcinogenesis remains to be defined. Molecular analyses, such as the Bardelli study described

above that associated c-Fes with colorectal cancer, continue to identify new players in CRC progression (88).

1.2.1 CRC Screening and Risk Factors

Colorectal cancer is a multi-stage process of carcinogenesis that involves the gradual accumulation of both genetic and epigenetic alterations that transform the normal colonic epithelium to metastatic cancer (Figure 6). As a result, most cases of colorectal cancer are treatable as long as the disease is detected early in the process of tumorigenesis. The American Cancer Society recommends that average risk individuals should be screened at age 50 (more than 92% of CRC cases are diagnosed after this age) and high risk persons should be screened earlier (91,92). Unfortunately, most average risk persons do not heed this warning despite the clear benefit of early CRC detection. Beginning at age 50, the American Cancer Society (ACS) recommends that average-risk adults have a fecal occult blood test (FOBT) or fecal immunochemical test (FIT) every year, a flexible sigmoidoscopy (FSIG) every 5 years, a double-contrast barium enema every 5 years, and a colonoscopy every 10 years (89,91,92). Emerging screening technologies include the molecular detection of genetically mutated or epigenetically altered genes associated with CRC and virtual colonoscopies (computed tomography colonography) (93). However, it remains to be seen whether these technologies will be cost effective when compared to the current screening recommendations.

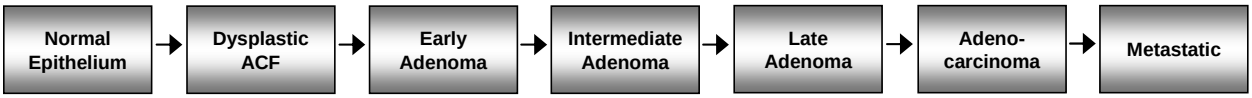

Figure 6: Colorectal Carcinogenesis.

Adaptation of the "Vogelgram" originally proposed by Fearon and Vogelstein in 1990 (94) that describes the progression of normal colonic epithelium transforming to metastatic cancer. ACF – Aberrant crypt foci. Early adenomas – adenomas smaller than 1.0 cm in size. Intermediate adenomas – adenomas greater than 1.0 cm in size that do not contain foci of carcinoma. Late adenomas – adenomas greater than 1.0 cm in size that contain foci of carcinoma.

Various risk factors exist for developing colorectal cancer. Individuals who are more than 50 years old, have previously had CRC, have colonic polyps, have a family history of either adenomas or CRC, or have an inflammatory bowel disease such as ulcerative colitis or Crohn's disease are at an increased risk for CRC (90). Further, diets high in fat or diets low in fruits and vegetables, a sedentary lifestyle, obesity, smoking, or alcohol consumption increase the risk of developing CRC. According to the American Cancer Society, CRC risk may be reduced through the regular use of nonsteroidal anti-inflammatory drugs or hormones such as estrogen and progestin (89).

1.2.2 CRC Prognostic Indicators and Treatment

The prognosis for a CRC patient depends on the degree of tumor penetration, whether the cancer has spread to lymph nodes, and whether the cancer has metastasized. As a result, the most

comprehensive staging tool for CRC is the American Joint Committee on Cancer (AJCC) TNM system that describes a cancer based on the primary tumor growth (T), spreading to regional lymph nodes (N), and metastasis to other body organs (M). Table 2 summarizes the staging, treatment recommendations, and five-year survival rates associated with each CRC stage (0-IV) (90,93). As mentioned above, the early detection of premalignant colonic adenomas is of great importance, as colon and rectal cancers detected in their early stages are often curable (95).

Stage	TNM	Definition	Treatment	Five-Year Survival (%)
0	TisN0M0	Intraepithelial or intramucosal carcinoma; no lymph node involvement; no metastasis	Surgery	70
I	T1N0M0	Tumor invasion no deeper than submucosa; no lymph node involvement; no metastasis	Surgery	69
	T2N0M0	Infiltration of muscularis; no penetration of colonic wall; no lymph node involvement; no metastasis		
IIA	T3N0M0	Subserosa invasion or into the nonperitonealized pericolic or perirectal tissue; no lymph node involvement; no metastasis	Surgery ± Chemotherapy	59
IIB	T4N0M0	Tumor directly invades other organs or structures or perforates the visceral peritoneum; no lymph node involvment; no metastasis		
IIIA	T1-2N1M0	Submucosa invasion or muscularis infiltration; metastasis in one to three lymph nodes; no distant metastasis	Surgery + Chemotherapy	41
IIIB	T3-4N1M0	Extension through colonic wall; metassis in one to three lymph nodes; no distant metastasis		
IIIC	TXN2M0	Any tumor; metastasis in four more lymph nodes; no distant metastasis		
IV	TXNXM1	Any tumor; any node; distant metastasis	Chemotherapy ± Surgery	5

Table 2: American Joint Committee on Cancer (AJCC) TNM Staging System

Colorectal cancer is staged in accordance with the TNM classification system laid forth by the AJCC. The staging and associated TNM classification along with the definition, treatment, and five-year survival are included. T: tumor, N: node, M: metastasis.

Various treatment options exist for CRC patients. Surgical resection offers the greatest potential cure for patients with invasive CRC and radiation therapy is useful for patients with rectal cancer. Chemotherapeutic options include: fluorouracil (5-FU), which in combination with leucovorin has been a mainstay chemotherapeutic agent for decades in the treatment of advanced CRC; capecitabine, a 5-FU prodrug that mimics the continuous infusion of 5-FU; irinotecan, a topoisomerase inhibitor with activity in metastatic CRC; oxaliplatin, which in combination with 5-FU and leucovorin inhibits DNA synthesis; cetuximab, a chimeric monoclonal antibody that dysregulates cell signaling of many epithelial malignancies by binding to the external growth factor receptor (EGFR); and bevacizumab, a human chimeric antibody that hinders angiogenesis (93). Epigenetic therapies aimed at disrupting CRC carcinogenesis are discussed in the following section.

1.3 CARCINOGENESIS

Knudson's two-hit hypothesis suggests that at least two genetic "hits", such as deletions or mutations that either activate normally silenced oncogenes or inactivate normally expressed tumor suppressors, combine to promote carcinogenesis (96,97). Additionally, epigenetic modifications which alter gene expression through mechanisms that do not change the actual DNA sequence are recognized as a "third pathway" in Knudson's model of tumor suppressor inactivation in cancer (98). As mentioned above, colorectal cancer tumorigenesis is a multi-step process where genetic and epigenetic events accumulate to transform the normal colonic epithelium into metastatic cancer.

1.3.1 Genetic Hits

Genetic alteration of tumor suppressors and oncogenes accumulate to promote CRC. A prominently mutated gene in both familial (inherited) and sporadic (non-inherited) CRC is the adenomatous polyposis coli (*APC*) tumor suppressor gene. The "gatekeeper" of cell proliferation in the colorectum, *APC* mutations are present in 60% to 80% of sporadic CRC cases, and inherited *APC* mutations result in familial adenomatous polyposis (FAP), which is a syndrome that accounts for 1% to 2% of all CRC cancer patients (90). A patient afflicted with FAP develops hundreds to thousands of polyps prior to turning age 30, and is likely to develop CRC by age 39 if their colon is not surgically removed. Inherited DNA mismatch repair (MMR) gene mutations can cause Lynch syndrome/hereditary nonpolyposis colon cancer (HNPCC). HNPCC accounts for 3-5% of all CRC cases, and HNPCC patients have a 70% to 80% chance of developing CRC and usually develop CRC by age 44 (90). Additional genes that are altered include the tumor suppressors *p53*, *DCC*, *SMAD2*, and *DPC4*/*SMAD4* and as well as the *K-ras* proto-oncogene (99). Further, as mentioned earlier, c-*fes* was one of the seven genes that exhibited consistent kinase domain mutations in a screen of colorectal cell lines that were found to suppress the catalytic activity of c-Fes, implicating c-Fes as a CRC tumor suppressor (67,84,88).

1.3.2 Epigenetic Hits

In addition to genetic events that accumulate to promote CRC, epigenetic events including histone modifications and DNA methylation alter gene function without changing the DNA sequence. In addition to various cancers (including CRC), epigenetic diseases include

ATR-X-syndrome, Fragile X syndrome, ICF syndrome, Angelman's syndrome, Prader-Will syndrome, BWS, Rett syndrome, α-Thalassaemia, Leukemias, Rubinstein-Taybi syndrome, Coffin-Lowry syndrome (100).

1.3.2.1 Histone Modifications

Histones, the protein component of chromatin around which chromosomal DNA is wound, undergo epigenetic modifications including acetylation and methylation of conserved lysine residues on the amino-terminal tail domains. Histone acetylation, regulated by histone acetyltransferases (HATs) and histone deacetyltransferases (HDACs), controls the structure of chromatin. In the absence of HATs, DNA (146 bp) is wrapped tightly around a core of histone octamers, preventing gene transcription. However, upon acetylation of conserved amino-terminal tail lysine residues of the core H2A, H2B, H3, and H4 histones, the affinity of DNA for the histone core is relaxed, permitting gene transcription (101). Histone methylation can also modulate gene transcription. Methylation of the amino-terminal lysine 9 of histone H3 (H3-L9) is indicative of silenced DNA transcription that is often found in heterochromatic DNA regions and the inactive X chromosome, while methylation at the Histone H3 lysine 4 indicates active DNA transcription and is often found in the promoters of transcribed genes (100). Histone modifications are often linked with DNA methylation, an additional epigenetic modification, and DNA methylation can trigger methylation of histone H3-L9 (102-104).

1.3.2.2 DNA Methylation

DNA methylation typically occurs at carbon 5 of cytosine in the context of CpG (5'-CG-3') dinucleotide sequences (105). The methyl group addition reaction is summarized in Figure 7. The cysteine residue within the active site of the DNA methyltransferase (DNMT) forms a

covalent bound with the sixth carbon (C6) of the target cytosine. Electron flow then increases to C5, resulting in an attack on the methyl group of AdoMet. From there, a proton is abstracted from C5 and β-elimination allows C5-C6 double bond to reform, releasing the DNMT enzyme from the methylated DNA.

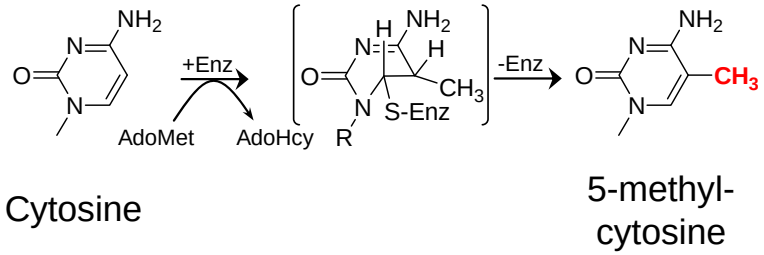

Figure 7: Cytosine Methylation Reaction.

Within the active site of the DNMT, a cysteine residue covalently binds to the cytosine carbon 6 (C6). Following this, the flow of electrons to carbon 5 (C5) increases, resulting in an attack on the methyl group of AdoMet. A proton is then abstracted from C5 and β-elimination to allow the C5-C6 double bond to reform. The DNMT enzyme is then released from the methylated DNA.

In organisms with low complexity genomes, such as *Drosophila*, *Caenorhabditis elegans*, and yeast, DNA methylation has not been detected (106). However, as vertebrate genomes increase in complexity, DNA methylation and DNA-histone interactions organize genomes into transcriptionally active (non-methylated) and inactive (methylated) zones.

In normal human cells, methylation at CpG dinucleotides regulates embryonic gene expression and silences incorporated viral genomes such as EBV and HIV (107-111).

Throughout time, the CpG nucleotide has been depleted via a deamination process in which 5-methylcytosines are converted to thymines, leaving the human genome with only 10% of its predicted number of CpG dinucleotides. Of these remaining CpG dinucleotides, 70 to 80% are methylated (112). However, CpG islands (CpG-rich stretches of DNA approximately 1 kb in length that do not have the typical underrepresentation of CpG dinucleotides) within 40% of mammalian gene promoters are rarely methylated in normal cells and are associated with transcriptionally active human genome zones (113). However, this is not the case in the context of cancer, as both genome-wide hypomethylation and regional hypermethylation events occur. Genome-wide hypomethylation leads to genomic instability and CpG island hypermethylation represses the transcription of downstream tumor suppressor genes, promoting carcinogenesis (113-116). In fact, methylation of a CpG island in a tumor suppressor gene promoter often leads to irreversible inhibition of expression (105,117-119). Colonic cancers have been suggested to have the one of the highest frequencies of CpG island promoter hypermethylation and exhibit CpG Island Methylator Phenotype (CIMP), which described the simultaneous methylation of several gene promoters at once (120). Genes that are methylated in CRC include the *hMLH1* and *MGMT* DNA repair genes as well as the tumor suppressors *APC*, $p16^{INK4}$, $p14^{ARF}$, *HPP1*/*TPEF*, *RIZ1*, and *HLTF* (121-128).

DNA Methyltransferases

In mammals, three DNA methyltransferase (DNMT) enzymes (DNMT1, DNMT3a, and DNMT3b) modulate the methylation patterns of CpG dinucleotides (98). In cancer, research has yet to conclusively establish how global hypomethylation and CpG hypermethylation events occur (129). One study suggests that hypomethylation occurs when catalytically inactive DNMT3b variants shield DNA from active DNMTs (130). Other studies suggest that increases in

the mRNA and protein levels of both DNMT1 and DNMT3b correlate with hypermethylation (131,132). Regardless, both DNMT1 and DNMT3b have been shown to maintain abnormal gene hypermethylation in cancer cells (133,134).

DNMT1 is primarily involved in the maintenance of DNA methylation patterns as it maintains CpG dinucleotide patterns during DNA damage repair and DNA replication as part of the DNA replication complex (135,136) and exhibits a 5- to 40-fold preference for hemi-methylated DNA (137,138). However, DNMT1 is also involved in the establishment of de novo methylation patterns involving embryonic CpG dinucleotides (138), non-CpG dinucleotide cytosines (139), and CpG dinucleotides found within CpG islands (140,141). Disruption of the *DNMT1* gene in embryonic stem cells results in a three-fold decrease in cytosine C5 methylation levels, but does not alter cellular viability, growth, or morphology (142).

DNMT3a and DNMT3b of the DNMT3 family additionally maintain CpG dinucleotide methylation patterns, but are essential for the establishment of de novo methylation patterns observed for CpG dinucleotides (143,144). Inactivation of both DNMT3a and DNMT3b in ES cells eliminates de novo methylation activity (144). Further, both DNMT3a and DNMT3b are required for embryonic development as mice truncated at DNMT3a are runted and die after four weeks and mice truncated at DNMT3b exhibited growth and neural tube defects (144). Combining the DNMT3a and DNMT3b truncations resulted in smaller embryos with altered morphology that died before day 8.5 (144). An additional DNMT3 homologue, DNMT3L (DNA methyltransferase 3-like), is expressed specifically in germ cells (145). Although it does not possess methyltransferase activity, DNMT3L is essential for the establishment of male and female germ cell methylation patterns (146). Further research has suggested that DNMT3L enhances the de novo methylation activity of both DNMT3a and DNMT3b (147-151).

Mechanisms of Methylation-mediated Transcriptional Repression

CpG island promoter hypermethylation represses gene expression by either denying access of transcription factors or permitting the binding of nucleoproteins that recruit transcriptional repression complexes to otherwise actively transcribed genes. Various experiments suggest that certain transcription factors are unable to bind their target sequence when it is methylated (152,153). These factors include AP-2, CREB, E2F, NF-kB, and c-Myc (98). A second possible mechanism by which promoter methylation downregulates gene expression may involve methylation-dependent recruitment of nucleoprotein factors such as the methylated CpG binding proteins MeCP1 (154) and MeCP2 (155), which in turn deny access to transcription factors either directly or by inducing inaccessible DNA conformations. MeCP1, first identified by Meehan and colleagues in 1989, is a 120 kDa nucleoprotein that selectively binds to DNA sequences containing methylated CpG dinucleotides (154). MeCP1 does not display affinity for non-methylated CpG dinucleotides and exhibits only weak affinity for hemi-methylated CpGs, instead preferring DNA sequences with clusters of methylated CpG dinucleotides (at least 12 symmetrically methylated CpGs being required) (154,156,157). Similar to MeCP1, MeCP2 (an 84 kDa nucleoprotein characterized in 1992 by Lewis et al.) exhibits no affinity for non-methylated CpG dinucleotides or non CpG 5-methylcytosine residues (155). Unlike MeCP1, however, MeCP2 can bind to one symmetrically methylated CpG dinucleotide per probe molecule (155). MeCP2 contains a transcriptional-repression domain (TRD) (158) and this activity relies in part on histone deacetylation prompting Nan and colleagues to describe MeCP2 as a “mechanistic bridge” between DNA methylation and histone deacetylation (158).

Neither CpG binding protein is influenced by the sequences surrounding their target methyl-CpG residues.

1.3.2.3 Methylation Biomarkers and Therapies

Promoter methylation marks a distinct clinical and epidemiological pathway and has potential use as a clinical diagnostic biomarker for the early stages of carcinogenesis. Aberrant gene promoter methylation associated with the early stages of carcinogenesis can be detected in older patients, livers of patients with chronic viral hepatitis, the lungs of heavy smokers, and in Barrett's esophagus, which are all associated with an increased risk for the development of CRC cancer (98). Further, promoter hypermethylation can be detected in specimens taken from patients (sputum, serum, urine, and breast ductal fluid). Demethylation therapies are described below.

The cytidine analog methylation inhibitors (Figure 8A), 5-azacytidine (5-aza-C: azacytidine) and 5-aza-2'-deoxycytidine (5-aza-2'-dC: decitabine), irreversibly inhibit the DNMT driven methylation reactions by incorporating into DNA and covalently binding to the active site of the DNMT, preventing resolution of the 5,6-dihydropyrmidine intermediate (Figure 8B). Generally, 5-aza-2'-dC is considered to be a more specific inhibitor than 5-aza-C, since 5-aza-C also incorporates into RNA to interfere with protein synthesis, whereas 5-aza-2'dC is solely incorporated into DNA (159). In a mouse model of colorectal cancer, reduction of DNA methylation through genetic or pharmacologic intervention has been shown to have tumor-preventative effects (160).

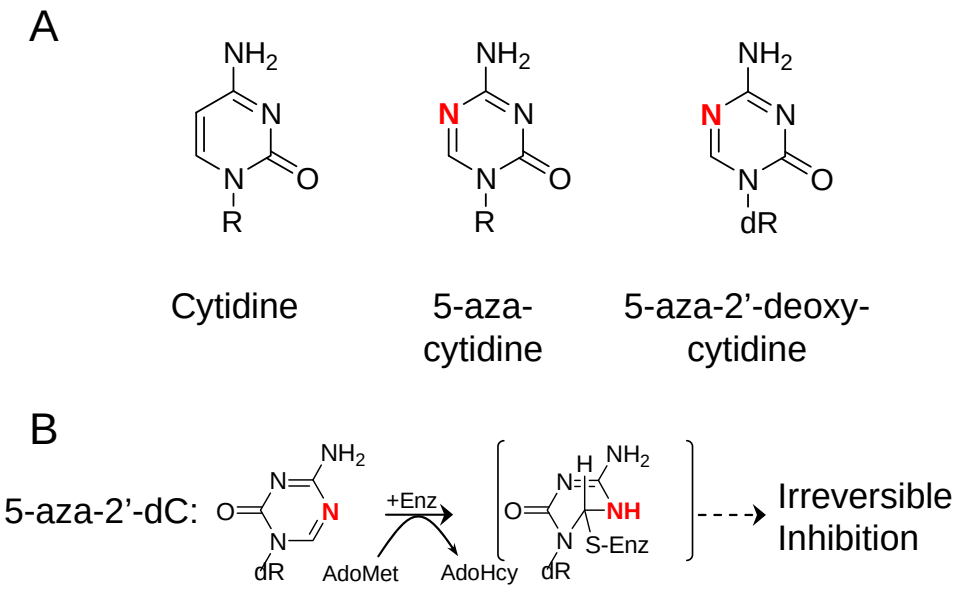

Figure 8: Mechanism of 5-aza-2'-deoxycytidine Activity

A) Structural comparison of cytidine with synthetic analogs. R – Ribose. dR – Deoxyribose. B) 5-aza-2'-deoxycytidine methylation inhibition reaction. 5-aza-2'-dC incorporates into DNA and irreversibly binds to the DNMT. This prohibits the 5,6-dihydropyrimidine intermediate reaction from resolving, resulting in irreversible inhibition of the DNMT activity.

Clinically, azacytidine and decitabine are highly effective hypomethylating agents with proven anticancer effects. Since the 1960s, azacytidine had been used in clinical trials for the treatment of β-thalassaemia, sickle cell anemia, leukemias (CML, AML, myelodysplasia), metastatic lung cancer, EBV-associated malignancies, androgen insensitive prostate cancer, metastatic lung cancer, cervical cancer, testicular cancer, colorectal cancer, head and neck cancer, renal, malignant melanoma, ovarian cancer, and AIDs (105). Azacytidine and decitabine

have both been granted FDA approval for the treatment of myelodysplastic syndromes (MDSs), a condition where hypermethylation events promotes leukemic transformation (161,162). Original azanucleoside clinical trials were administered at maximum tolerated doses, which were effective in myelosuppression but caused severe side effects (105). On the other hand, current studies instead rely on low-dose treatment regimes that favor hypomethylation over cytotoxicity with primarily hematological side effects (159,163). 5-aza-2'-dC is also useful in a laboratory setting for identifying novel tumor suppressors and was used in Chapter 3 to determine the mechanism by which c-Fes expression is lost in colorectal cancers.

1.4 HYPOTHESES AND SPECIFIC AIMS

1.4.1 Hypotheses

The c-*fes* proto-oncogene encodes the 93 kDa non-receptor protein-tyrosine kinase c-Fes. While *in vitro* research has suggested a model where the coiled-coil domains mediate the conversion of c-Fes from an inactive monomeric to active oligomeric kinase, little is known about the strict *in vivo* regulation of c-Fes catalytic activity. Further, gel filtration analyses of the inclusive wild-type coiled-coil domains suggest the possibility of c-Fes adopting a constitutively oligomeric conformation. **Due to this, I propose to test the hypothesis that the coiled-coil domains modulate the oligomerization status of c-Fes in living cells.** Regarding c-Fes biological activity, historical data largely supports the role of c-Fes in oncogenesis based in part on its relationship to transforming retroviral oncogenes. As a result, the colorectal cancer-associated tumor suppressor role for c-Fes is in its nascent stages, and mechanisms responsible

for the downregulation of c-Fes expression in colorectal cancers have not been explored. **Thus, I also propose to test the hypothesis that promoter methylation downregulates c-Fes expression in colorectal cancer.** To address these hypotheses, I have set forth the following aims: (1) to test the hypothesis that the coiled-coil domains mediate the *in vivo* oligomerization of c-Fes, a constitutive oligomer; and (2) to define the mechanism by which c-Fes expression is lost in colorectal cancer.

1.4.2 Specific Aims

Aim 1: to test the hypothesis that the coiled-coil domains mediate the *in vivo* oligomerization of c-Fes, a constitutive oligomer

c-Fes, a 93 kDa non-receptor protein-tyrosine kinase, consists of an amino-terminal FCH domain and a coiled-coil domain (collective referred to as an F-BAR domain), a second coiled-coil domain, a central SH2 domain, and a carboxyl-terminal kinase domain. In living cells, c-Fes kinase activity is tightly regulated by an unknown mechanism. Adapting a YFP bimolecular fluorescence complementation (BiFC) assay for c-Fes analysis, (Figure 9), I defined the *in vivo* oligomerization interface of c-Fes through a series of point mutants. Further, I was able to hypothesize a relationship between oligomerization and catalytic activation.

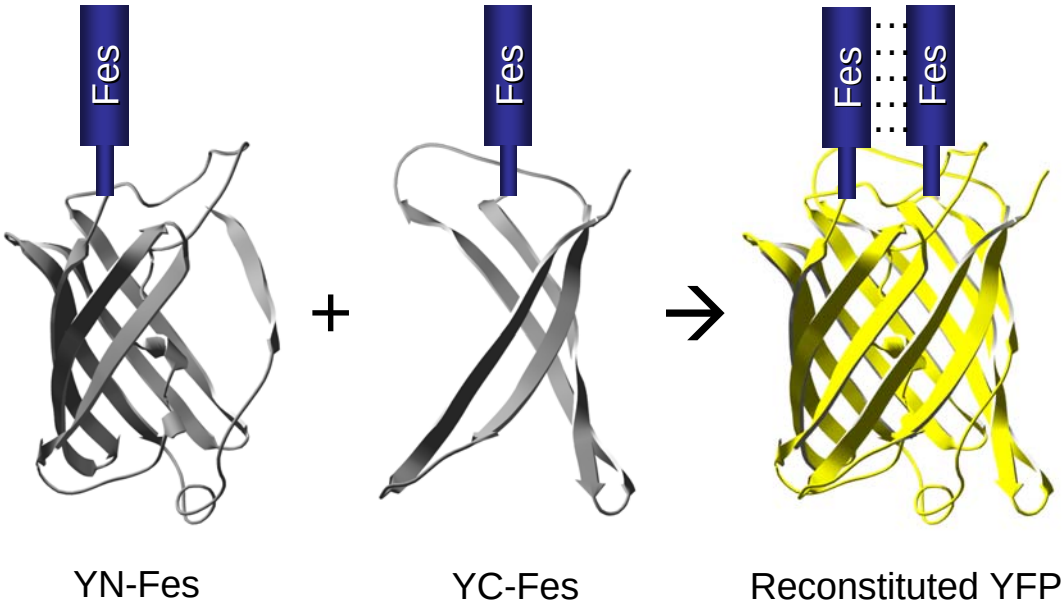

Figure 9: c-Fes Bimolecular Fluorescence Complementation (BiFC) Assay

Potential interacting partners (Fes) are fused to non-fluorescent amino-terminal (1-154) and carboxyl-terminal (155-238) portions of the YFP coding sequence (termed "YN" and YC" respectively) and co-expressed in the same cell. If oligomerization of the fused proteins occurs, YN and YC are brought into close proximity, resulting in structural complementation and YFP fluorescence.

Aim 2: to define the mechanism by which c-Fes expression is lost in colorectal cancer

Previous research has demonstrated that c-Fes expression is lost in cancerous tissue and colorectal cancer cells, a finding common among tumor suppressors. Further, past data suggests a CpG island exists within the c-*fes* promoter. As a result, promoter methylation may downregulate c-*fes* expression. Using demethylation treatments, bisulfite sequencing, and *in*

vitro methylation assays, I was able to conclusively establish that c-*fes* promoter methylation downregulates c-Fes expression in colorectal cancer.

2.0 BIMOLECULAR FLUORESCENCE COMPLEMENTATION DEMONSTRATES THAT THE c-FES PROTEIN-TYROSINE KINASE FORMS CONSTITUTIVE OLIGOMERS IN LIVING CELLS[*]

2.1 ABSTRACT

The c-*fes* proto-oncogene encodes a non-receptor protein-tyrosine kinase (c-Fes) that contributes to the differentiation of myeloid hematopoietic, vascular endothelial, and some neuronal cell types. Although originally identified as the normal cellular homolog of the oncoproteins encoded by avian and feline transforming retroviruses, c-Fes has recently been implicated as a tumor suppressor in breast and colonic epithelial cells. Structurally, c-Fes consists of a unique aminio-terminal region harboring two coiled-coil motifs, a central SH2 domain, and a carboxyl-terminal kinase domain. In living cells, c-Fes kinase activity is tightly regulated by a mechanism that remains unclear. Previous studies have established that c-Fes forms high molecular weight oligomers *in vitro*, suggesting that the dual coiled-coil motifs may regulate the interconversion of inactive monomeric and active oligomeric states. Here we show for the first time that c-Fes forms oligomers in live cells independently of its activation status

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using a YFP bimolecular fluorescence complementation assay. We also demonstrate that both amino-terminal coiled-coil regions are essential for c-Fes oligomerization in transfected COS-7 cells as well as HCT 116 colorectal cancer and K-562 myeloid leukemia cells. Together, these data provide the first evidence that c-Fes, unlike c-Src, c-Abl and other non-receptor tyrosine kinases, is constitutively oligomeric in both its repressed and active states. This finding suggests that conformational changes, rather than oligomerization, govern its catalytic activity *in vivo*.

2.2 INTRODUCTION

The human c-*fes* proto-oncogene encodes a structurally unique, 93 kDa non-receptor protein-tyrosine kinase (c-Fes) expressed in myeloid hematopoietic, vascular endothelial, and some neuronal cells where it has been linked to signaling pathways controlling differentiation (12,13). Early work showed that restoring wild-type c-Fes expression in the chronic myelogenous leukemia cell line K-562 suppresses cell proliferation and primes the cells for differentiation to macrophages by phorbol esters (47,80). Similarly, active c-Fes mutants induced GM-CSF-independent proliferation in addition to cell attachment and spreading in the cytokine-dependent myeloid leukemia cell line TF-1, consistent with differentiation along the monocyte-macrophage pathway (14). In the monocytic precursor cell line U-937, an active c-Fes mutant also induced cell adherence, macrophage morphology, and differentiation marker expression (81). Over-expression of wild-type c-Fes accelerated NGF-induced neurite extension in PC12 cells, while active c-Fes mutants induced spontaneous neurite formation in this cell line, suggestive of a role in neuronal differentiation (40,72). Finally, over-expression of wild-type c-

Fes induced FGF-2-independent tube formation by cultured brain capillary endothelial cells, implicating c-Fes in angiogenesis (71).

More recently, c-Fes expression has been detected in epithelial cells, where it may serve a tumor suppressor function. Greer and colleagues determined that tumor onset in a breast cancer model occurred more rapidly in mice targeted with either null or kinase-inactivating c-*fes* mutations, and that a c-*fes* transgene restored the latency of tumor formation (84). Our group found that c-Fes protein is strongly expressed in normal human colonic epithelial cells, while expression was reduced or absent in 67% of colon tumor sections from the same individuals (67). Furthermore, re-expression of wild-type or active c-Fes suppressed anchorage-independent growth of two colorectal cancer cell lines, HCT 116 and HT-29, both of which are negative for c-Fes protein expression (67). Kinase-inactivating mutations have also been reported for c-Fes in colorectal cancer cell lines (67,84,88), providing further support for a tumor suppressor function for c-Fes in some tissue types.

Structurally, c-Fes consists of a long unique amino-terminal region, with a tubulin-binding FCH domain and two coiled-coil homology motifs, followed by a central Src-homology 2 (SH2) domain and a carboxyl-terminal kinase domain (12,13) (Figure 1). The c-Fes FCH and first coiled-coil domain together have recently been recognized as an F-BAR domain (37). Unlike other non-receptor protein-tyrosine kinases, c-Fes lacks negative regulatory features such as an SH3 domain or the negative regulatory tail associated with Src-family kinases (164). Despite this, c-Fes kinase activity remains strictly regulated in mammalian cells. When wild-type c-Fes is ectopically expressed in rodent fibroblasts, little or no transforming activity is apparent (14-16). However, mutation or deletion of the first coiled-coil domain results in strong upregulation of kinase activity and release of transforming potential in fibroblasts (14,47).

Similarly, *en bloc* substitution of the c-Fes SH2 domain with that of v-Src also causes loss of negative regulation *in vitro* (17), implicating the SH2 domain as well as the amino-terminal coiled-coil motif in the regulation of kinase activity.

Gel-filtration and cross-linking studies have established that c-Fes forms higher order oligomers *in vitro* (up to a pentamer), and that the two amino-terminal coiled-coil domains are responsible for oligomerization (14,45). In particular, gel-filtration analyses involving a small fragment of the amino-terminal region showed that point mutations disrupting both coiled-coil motifs prevent oligomerization (14). This result led to a hypothetical model for c-Fes activation in which the coiled-coil domains mediate interconversion between inactive monomeric and active oligomeric states (14). In this model, the more amino-terminal coiled-coil was proposed to interact intramolecularly with the second to hold c-Fes in an inactive monomeric conformation. Supporting this model is the observation that negative regulation imparted by the first coiled-coil domain can be relieved by mutation (45,58). However, no evidence for an inactive monomer has been generated either *in vitro* or *in vivo*, and it is unclear how oligomerization modulates c-Fes activity in living cells.

In this study, we investigated c-Fes oligomerization in live cells using a bimolecular fluorescence complementation (BiFC) assay based on yellow fluorescent protein (YFP) as originally developed by Kerppola and colleagues for the study of transcription factors (165,166). BiFC provides a useful technique to examine protein-protein interaction in a normal cellular environment, as it is sensitive enough to detect interactions between proteins expressed at physiological levels (165,166). In this approach, oligomerization partners (c-Fes in this case) are fused to non-fluorescent amino- and carboxyl-terminal portions of the YFP coding sequence and co-expressed in the same cell. Oligomerization brings the two YFP fragments into close

proximity, resulting in structural complementation and fluorescence. Using the BiFC technique, we made the surprising discovery that c-Fes exists as a constitutive oligomer in cells, regardless of its autophosphorylation state. We further demonstrated that both of the c-Fes coiled-coil regions are essential for oligomerization in transfected COS-7 cells as well as cell lines where c-Fes is known to exert biological effects (HCT 116 colorectal cancer cells and K-562 CML cells). Together, these data demonstrate that c-Fes oligomerization is independent of activation and suggest that conformational changes, rather than oligomerization, govern c-Fes kinase activation and downstream signaling *in vivo*. The discovery that c-Fes exists as a pre-formed oligomer *in vivo* makes it unique among non-receptor protein-tyrosine kinases, and suggests new strategies for the design of small molecules that may enhance its activity *in vivo*. Such compounds may be of utility for the differentiation therapy of certain types of tumors.

2.3 EXPERIMENTAL PROCEDURES

2.3.1 Construction of Plasmid Vectors.

Fes point mutants L145P, L334P, L145P-L334P (2LP), R483L, and K590E have been described elsewhere (14,40,58) (Figure 10A). To create the vectors required for BiFC analysis, sequences encoding the non-fluorescent amino- and carboxy-terminal portions of YFP (YN: amino acids 1-154, YC: amino acids 155-238) were amplified by PCR from pEYFP-C1 (Clontech) and subcloned into separate pcDNA3.1(+) vectors (Invitrogen). Full-length YFP was cloned in a similar manner for creation of YFP-Fes fusion plasmids. Each of the c-Fes cDNAs was fused to the carboxyl-terminal end of either YN or YC, creating YN-Fes and YC-Fes BiFC

fusion pairs. Each BiFC fusion pair was subcloned from pcDNA3.1+ into the mammalian expression vector pIRES (Clontech). In this construct, YC-Fes was subcloned directly downstream of the CMV promoter with YN-Fes downstream of the IRES sequence (Figure 10B). This cloning strategy was also applied to the pIRES-Fes BiFC control plasmids, which either express YC-Fes or YN-Fes alone. All c-Fes constructs used in this study encode a carboxyl-terminal FLAG epitope tag.

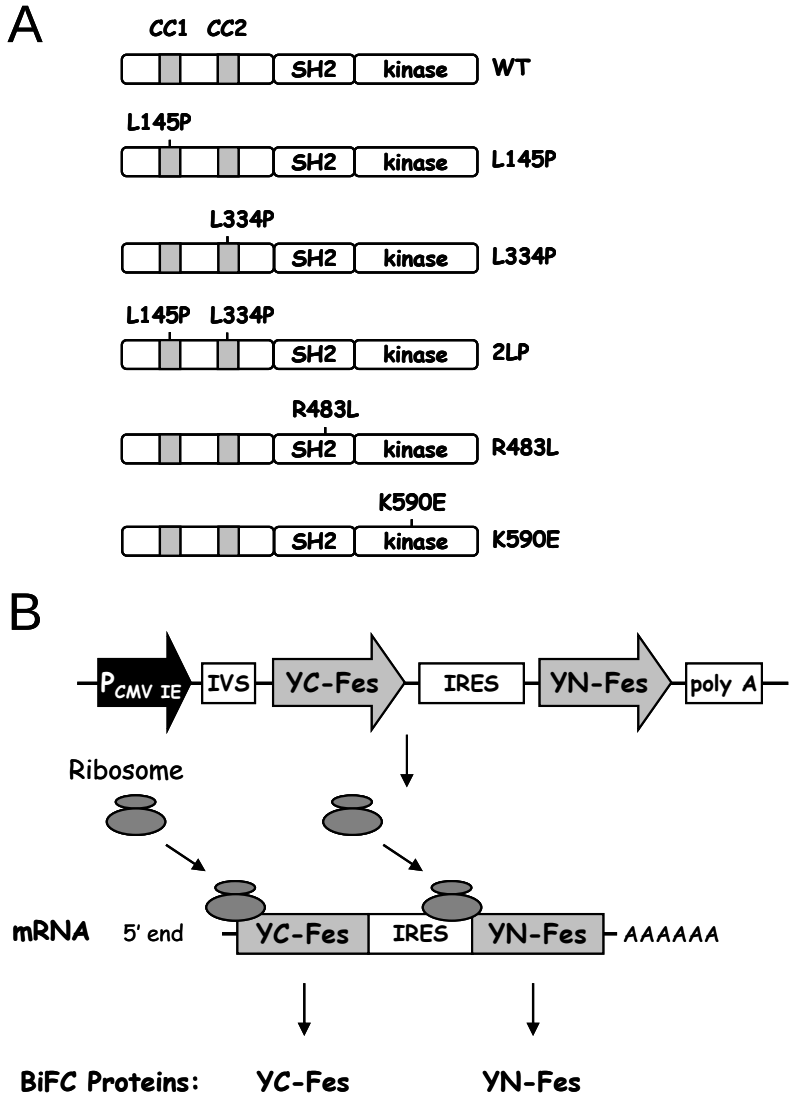

Figure 10: c-Fes Constructs and BiFC Experimental Design

A) The structure of the wild-type (WT) c-Fes protein is shown at the top, which includes a unique amino-terminal region with two coiled-coil motifs (CC1 and CC2), a central SH2 domain, and a carboxyl-terminal kinase domain. Coiled-coil domain mutants include leucine to proline substitutions in CC1 (L145P) and CC2 (L334P), as well as the corresponding double mutant (2LP). c-Fes proteins with inactivating mutations in the SH2 domain (R483L) as well as the kinase domain (K590E) are also shown. B) pIRES YFP BiFC system. The coding sequences for c-Fes were fused in frame with the nucleotide sequences of the amino-terminal portion of YFP (encoding amino acids 1-154) to create the YN-Fes fusion protein and to the sequences of the carboxyl-terminal part of YFP (encoding amino acids 155-238) to create YC-Fes. The resulting c-Fes fusion proteins were subcloned into the pIRES vector as shown. Use of the IRES construct ensures simultaneous expression of both YN-Fes and YC-Fes BiFC partners from the same transcript as shown.

2.3.2 Cell Culture and Transfection.

Cell lines were maintained at 37 °C in a 5% CO_2 humidified incubator. COS-7, HCT 116, and K-562 cells were obtained through the ATCC and cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen), McCoy's Modified 5A medium (Invitrogen), or RPMI 1640 (Invitrogen), respectively. All culture media were supplemented with 10% fetal bovine serum (FBS; Atlanta Biological) and Antibiotic/Antimycotic (Invitrogen). Transient transfection was

performed as follows: COS-7 cells ($2.25x10^5$) were plated in 60 mm dishes and transfected one day later with 2 μg of total plasmid DNA using Fugene 6 (Roche). HCT 116 cells ($6x10^5$) were seeded in 6-well plates and transfected one day later with 2 μg of total plasmid DNA using Lipofectamine 2000. K-562 cells ($6x10^5$) were seeded in 12-well plates and transfected immediately with 2 μg of total plasmid DNA using Lipofectamine 2000 (Invitrogen). All transfections were performed using serum-free Opti-MEM (Invitrogen) as diluent, and transfected cells were grown in Antibiotic/Antimycotic-free medium. Following incubation at 37 °C for 20 h, transfected cells were switched to room temperature for 2 h to promote fluorophore maturation prior to fluorescence microscopy.

2.3.3 Immunofluorescence Microscopy and Fluorescence Imaging.

Transfected COS-7 or HCT 116 cells were fixed with 4% paraformaldehyde in PBS for 10 min followed by 2 washes with PBS and permeabilized with 0.2% Triton X-100 in PBS for 15 min. Cells were then blocked in PBS containing 2% BSA for 30 min and incubated for 60 min with either anti-Fes (1:250 dilution; Fes C19, Santa Cruz Biotechnology) or anti-Fes phosphospecific primary antibodies [pFes; 1:1,000 dilution; recognizes phosphotyrosine 713 in the activation loop (48)]. Immunostained cells were visualized with secondary antibodies conjugated to Alexa Fluor 594 (Invitrogen) or Texas red (Southern Biotech). Fluorescent images were recorded using a Nikon TE300 inverted microscope with epifluorescence capability and a SPOT CCD high-resolution digital camera and software (Diagnostic Instruments). Following acquisition of black & white images, appropriate color palettes were applied (green for YFP or BiFC, red for c-Fes protein, pTyr-713, or RFP), and minimal histogram, brightness, and contrast

adjustments were performed to improve image clarity. Identical manipulations were applied to all images in a given experiment.

2.3.4 Immunoblotting and Antibodies.

Transiently transfected COS-7, K-562, and HCT 116 cells were washed with PBS, resuspended in Fes lysis buffer [50 mM Tris/HCl (pH 7.4), 1 mM EDTA, 50 mM NaCl, 1 mM $MgCl_2$, 0.1% Triton X-100, 2 mM PMSF, 2 mM sodium orthovanadate, 25 mM sodium fluoride and Protease Inhibitor Cocktail Set III (Calbiochem)], and sonicated for 10 s at 4 °C. The cell lysates were clarified by centrifugation, diluted with 2X SDS-PAGE sample buffer (125 mM Tris-HCl, pH 6.8, 20% glycerol, 4% SDS, 172 mM 2-mercaptoethanol, 0.05% bromophenol blue), and heated to 95 °C for 10 min. Lysates were subjected to immunoblot analysis with antibodies to c-Fes (C-19; 1 μg/ml), pFes (1:1000 dilution), phosphor-tyrosine (PY99; 1 μg/ml, Santa Cruz Biotechnology), and Hck (N30; 1 μg/ml, Santa Cruz Biotechnology) as required. Lysates were also blotted with anti-actin antibodies (Chemicon MAB1501; 1:10000 dilution) as a loading control. Immunoreactive bands were detected using an alkaline phosphatase-conjugated secondary antibody followed by colorimetric detection with NBT/BCIP.

2.4 RESULTS

2.4.1 Visualization of c-Fes Oligomers in Live Cells.

Previous data from our laboratory suggested that c-Fes kinase activity may be regulated by interconversion of inactive monomeric and active oligomeric configurations (see Introduction) (14,45,47). These studies implied that wild-type c-Fes, whose kinase activity is strongly repressed in cells, naturally adopts a monomeric conformation, as is the case for the downregulated forms of several other non-receptor tyrosine kinases such as c-Abl (167,168), Hck (169,170), and c-Src (171,172). To determine the oligomerization status of wild-type c-Fes in live cells, we applied a BiFC approach in which complementary fragments of YFP (YN: amino acids 1-154, YC: amino acids 155-238) were fused to the amino-terminus of full-length wild-type c-Fes and subcloned into a single pIRES expression vector (Figure 10B). We also created a plasmid for expression of full-length YFP fused to c-Fes for use as a positive control. COS-7 cells were transfected with plasmids encoding YFP-Fes, YN-Fes alone, YC-Fes alone, as well as both YN-Fes plus YC-Fes and monitored by fluorescence microscopy (Figure 11A). Transfected cultures were also immunostained to identify c-Fes-positive cells. The number of cells exhibiting YFP fluorescence was then normalized to the total number of Fes-positive cells present in each culture (Figure 11B). Cells expressing YN-Fes or YC-Fes alone failed to exhibit detectable fluorescent signals. In contrast, approximately 50% of the cells expressing both YN-Fes and YC-Fes fusion proteins exhibited a strong, cytoplasmic fluorescent signal relative to cells transfected with the YFP-Fes fusion protein, consistent with wild-type c-Fes oligomerization *in vivo*. In addition, the diffuse cytoplasmic distribution of the c-Fes BiFC signal is very similar to that of YFP-Fes, consistent with previous data for cells transfected with wild-

type, inactive c-Fes (17,39,40,65). This observation suggests that the YN and YC fusions do not alter c-Fes subcellular localization. Immunoblots shown in Figure 11C confirm expression of full-length YFP-Fes, YN-Fes, and YC-Fes. Note that YN-Fes is expressed at much lower levels than YC-Fes, most likely because its translation is controlled by the IRES in the bicistronic transcript (Figure 10B). This observation shows that c-Fes oligomerization *in vivo* is not an artifact of over-expression, as the c-Fes BiFC signal is readily observed despite the limiting amount of YN-Fes protein present. These results demonstrate for the first time that wild-type c-Fes forms oligomers in living cells.

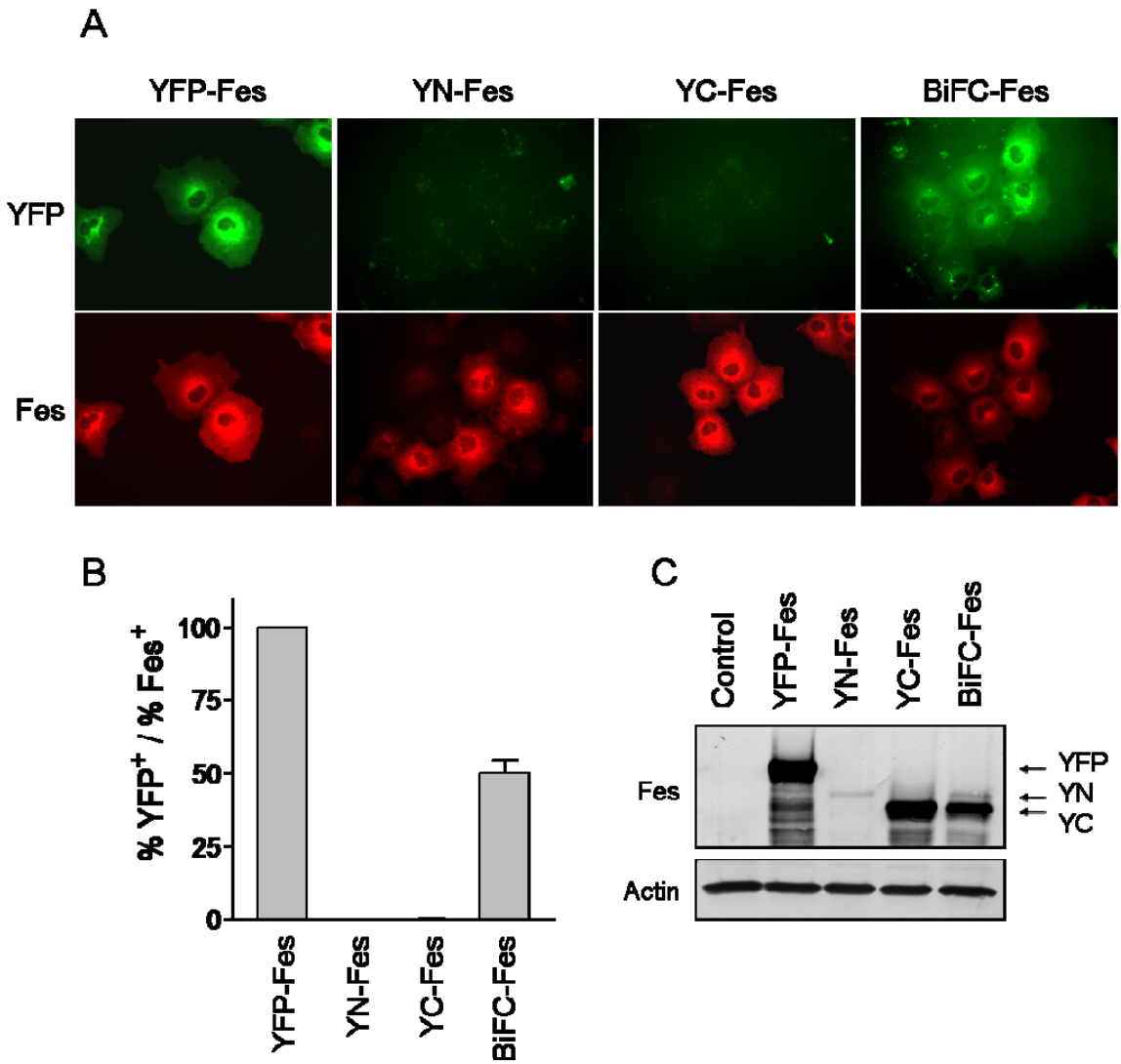

Figure 11: Wild-type c-Fes Forms Oligomers in Living Cells.

COS-7 cells were transfected with plasmids encoding YFP-Fes, YN-Fes alone, YC-Fes alone, and YN-Fes + YC-Fes (BiFC-Fes). Twenty hours after transfection, cells were incubated at room-temperature for 2 hours and either fixed and immunostained with a c-Fes-specific antibody or lysed for immunoblot analysis. A) Fluorescent images of representative cells expressing YFP-Fes, YN-Fes, YC-Fes, and BiFC-Fes. YFP-Fes, YN-Fes, YC-Fes, and BiFC-Fes proteins were examined for YFP fluorescence (top row). Secondary antibodies conjugated to Alexa Fluor 594

were used to visualize transfected cells immunoreactive for the c-Fes antibody (bottom row). YFP/BiFC and Alexa Fluor 594 are represented as green and red, respectively. B) The number of cells exhibiting a YFP fluorescent signal in each transfection condition was normalized to the total number of c-Fes-positive cells present. At least 250 cells were counted for each condition. Each experiment was repeated in triplicate and the results are presented as the mean ratios ± S.D. C) Lysates from the transfected cell populations shown in part A were analyzed by immunoblotting with antibodies to c-Fes (top) and to actin as a loading control (bottom). The arrows indicate the positions of the YFP-Fes (YFP), YN-Fes (YN), and YC-Fes (YC) fusion proteins.

2.4.2 Activation does not influence wild-type c-Fes oligomerization.

Results presented in Figure 11 strongly suggest that c-Fes naturally adopts an oligomeric conformation *in vivo*. To evaluate the effect of kinase activation on c-Fes oligomerization in living cells, the YFP-Fes and BiFC-Fes plasmids described in Figure 11 were co-transfected with a plasmid encoding an active form of Hck (Hck-YF) (173). Hck is a member of the Src kinase family expressed in myeloid cells that has been previously demonstrated to activate wild-type c-Fes *in vivo* (40,71). To identify cells expressing active c-Fes, the transfected cultures were also immunostained with a phosphospecific antibody (pFes) that recognizes autophosphorylation of Tyr-713 in the c-Fes activation loop (48). As shown in Figure 12A, YFP-Fes alone exhibited a diffuse cytoplasmic localization and only a trace amount of staining with the phosphospecific antibody. On the other hand, co-expression with Hck-YF induced strong YFP-Fes activation as judged by pFes antibody staining. In addition, active YFP-Fes relocalized to the prominent

microtubule network present in COS cells, consistent with our previous observations (40). Paralleling results for YFP-Fes, Figure 12B shows that cells co-expressing YN-Fes plus YC-Fes exhibited a strong BiFC signal but only a trace of autophosphorylation indicating that wild-type c-Fes oligomers are catalytically inactive. Co-expression with Hck-YF induced strong phosphorylation of the c-Fes oligomers at Tyr-713, as judged by phosphospecific antibody staining. In addition, activation of c-Fes by Hck-YF induced localization to microtubules, demonstrating that active c-Fes remains oligomeric in living cells and moves to microtubules as an oligomer. Immunoblot analysis confirms that wild-type YFP-Fes, YN-Fes and YC-Fes are weakly autophosphorylated when expressed alone, but are robustly phosphorylated in the presence of Hck-YF (Figure 12C). These results illustrate that activation does not alter wild-type c-Fes oligomerization in living cells, as c-Fes remains oligomeric irrespective of kinase domain autophosphorylation.

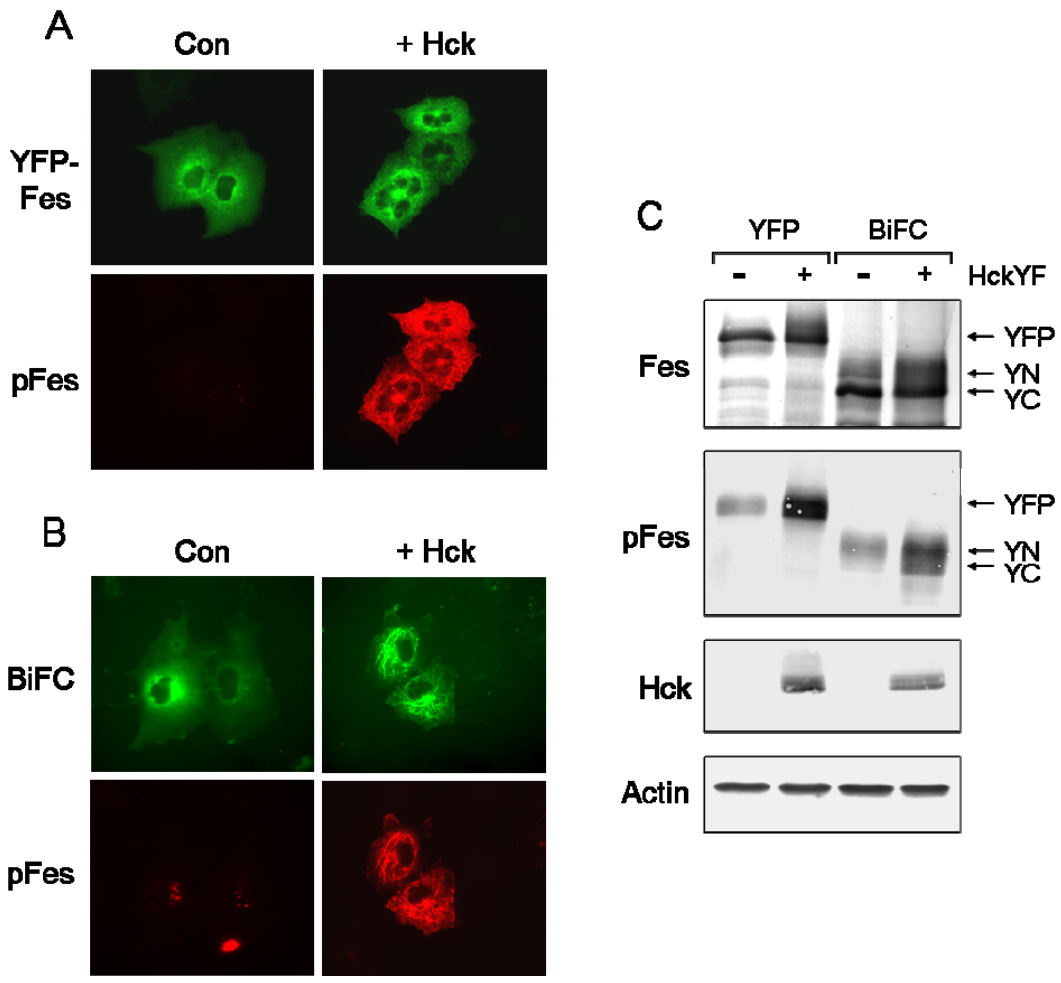

Figure 12: Active Wild-type c-Fes Remains Oligomeric *in vivo*.

Wild-type YFP-Fes and c-Fes BiFC fusion vectors were transiently expressed in COS-7 cells. Cells were also co-transfected with an active form of the Src family kinase Hck as indicated. Twenty hours later, cells were incubated at room-temperature for 2 hours and either fixed and stained with c-Fes phosphospecific antibodies or lysed for immunoblot analysis. A) Representative fluorescent images of COS-7 cells expressing YFP-Fes plus and minus (Con) active Hck. Secondary antibodies conjugated to Texas Red were used to visualize pFes staining. YFP-Fes and Texas red (pFes) fluorescence are represented as green and red, respectively. B) Representative fluorescent images of COS-7 cells expressing BiFC-Fes (YN-Fes & YC-Fes) plus

and minus (Con) Hck. The BiFC-Fes fluorescent signal (BiFC) and Texas red (pFes) fluorescence are represented as green and red, respectively. C) Immunoblot analyses of cell lysates from part A for c-Fes protein levels (Fes) and c-Fes autophosphorylation with the phosphospecific antibody (pFes); arrows indicate the position of YFP-Fes (YFP), YN-Fes (YN), and YC-Fes (YC). Blots were also performed for Hck protein expression and actin as a loading control.

2.4.3 The coiled-coil homology domains are essential for c-Fes oligomerization in living cells.

Located within the unique amino-terminal region of c-Fes are two coiled-coil motifs that mediate c-Fes oligomerization *in vitro* and play a key role in the regulation of c-Fes kinase activity *in vivo* (14,45,47). To assess the role of the coiled-coil motifs in c-Fes oligomerization in cells, BiFC assays were performed using coiled-coil leucine to proline point mutants previously shown to disrupt the function of these domains (14) (Figure 10A). Expression vectors that co-express both YN-Fes and YC-Fes coiled-coil mutants from the same transcript were generated as described above, and tested for oligomerization via BiFC in COS-7 cells. As shown in Figure 13, less than 5% of cells co-expressing YN- and YC-fused Fes-L145P, Fes-L334P, or Fes-2LP were fluorescent, indicating a requirement for both coiled-coil domains in maintaining c-Fes oligomerization *in vivo*. These results also provide an essential control for the wild-type c-Fes BiFC result, as they show that mutations in the coiled-coil domains of c-Fes previously established to contribute to oligomerization *in vitro* are required for fluorescence complementation in cells. Thus homotypic c-Fes interactions, and not the YFP fragments

themselves, are the driving force behind the BiFC signal. A final interesting feature of the coiled-coil mutants is that while they fail to form oligomers *in vivo* (negative BiFC signal), they all show a staining pattern consistent with localization to the microtubule network. This result suggests that association with microtubules does not require oligomerization, and is consistent with our previous data that c-Fes movement to microtubules is dependent upon both Fes-mediated tubulin phosphorylation and subsequent binding via the c-Fes SH2 domain (40). Indeed, immunoblots show that the YN- and YC-Fes coiled-coil domain mutants are strongly autophosphorylated, consistent with this idea (Figure 13C).

In addition to the coiled-coil domains, we also assessed the contribution of the c-Fes SH2 and kinase domains to oligomerization using mutant proteins. Unlike the coiled-coil mutants, inactivating mutations in the SH2 domain (R483L) or kinase domain (K590E) did not alter the capacity of c-Fes to form oligomers, as both mutants produced positive BiFC signals in a similar percentage of cells as wild-type c-Fes. These mutants exhibited a diffuse cytoplasmic distribution and failed to track to microtubules, consistent with lack of kinase activity *in vivo*. Immunoblots shown in Figure 13C are consistent with this conclusion, as neither of these mutants reacted with the c-Fes phosphospecific antibody. Taken together, these results strongly suggest that both of the c-Fes coiled-coil domains, but neither the SH2 nor the kinase domain, are required for c-Fes oligomerization in living cells.

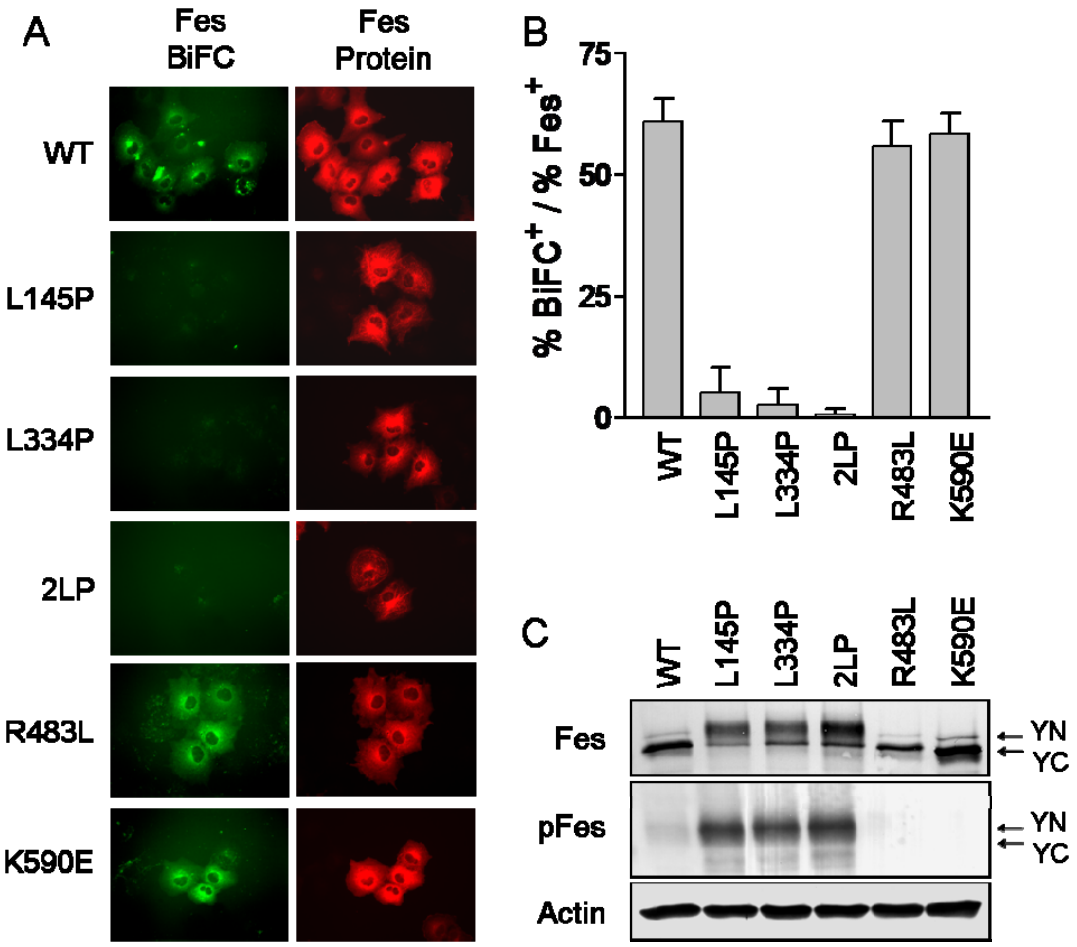

Figure 13: Coiled-coil Homology Domains Mediate c-Fes Oligomerization *in vivo*.

Plasmids encoding wild-type, L145P, L334P, 2LP, R483L, and K590E c-Fes BiFC partners (YN and YC fusions; see Figure 10) were transiently expressed in COS-7 cells. Twenty-hours after transfection, cells were incubated at room-temperature for 2 hours and either fixed and stained with a c-Fes-specific antibody or lysed for immunoblot analysis. A) Representative fluorescent images of cells expressing the c-Fes BiFC partners for each of the point mutants shown as well as the wild-type (WT) control (top). Secondary antibodies conjugated to Alexa Fluor 594 were used to visualize c-Fes-positive immunostained cells. The BiFC signal and Alexa Fluor 594 fluorescence are represented as green and red, respectively. B) The number of cells exhibiting a

positive BiFC signal in each transfection condition was normalized to the total number of c-Fes-positive cells present. At least 250 cells were counted for each condition. Each experiment was repeated three times, and the results are presented as the mean ratios ± S.D. C) Lysates from the transfected cell populations shown in part A were analyzed by immunoblotting for c-Fes protein (top), c-Fes autophosphorylation (pFes; middle) and actin as a loading control (bottom). The arrows indicate the positions of the YN-Fes (YN), and YC-Fes (YC) fusion proteins.

2.4.4 c-Fes forms coiled-coil-mediated oligomers in human colorectal cancer and chronic myelogenous leukemia cell lines.

Previous work has demonstrated that c-Fes is expressed in normal colonic epithelium as well as myeloid hematopoietic cells, and that loss of c-Fes expression correlates with tumor progression in both colorectal cancer and CML (67,68,80). Restoring c-Fes expression to HCT 116 colorectal cancer and K-562 CML cells results in a growth-suppressive effect (47,67,80). To determine whether c-Fes forms oligomeric complexes in these biologically relevant cellular contexts, BiFC analysis was performed using wild-type c-Fes and the double coiled-coil c-Fes mutant (2LP). Transient expression of the YN- and YC-Fes fusion proteins as well as the YFP-Fes control was achieved in HCT 116 cells using the pIRES vector system. Figure 14A shows that wild-type c-Fes exhibited strong BiFC-dependent fluorescence similar to the YFP-Fes control in HCT 116 cells, with a positive BiFC signal present in over 50% of cells expressing the wild-type c-Fes BiFC partners (Figure 14B). On the other hand, cells co-expressing the YN- and YC-Fes-2LP proteins failed to exhibit detectable YFP complementation in HCT 116 cells.

Together, these results indicate that c-Fes forms oligomers in colorectal carcinoma cells in a coiled-coil-dependent fashion.

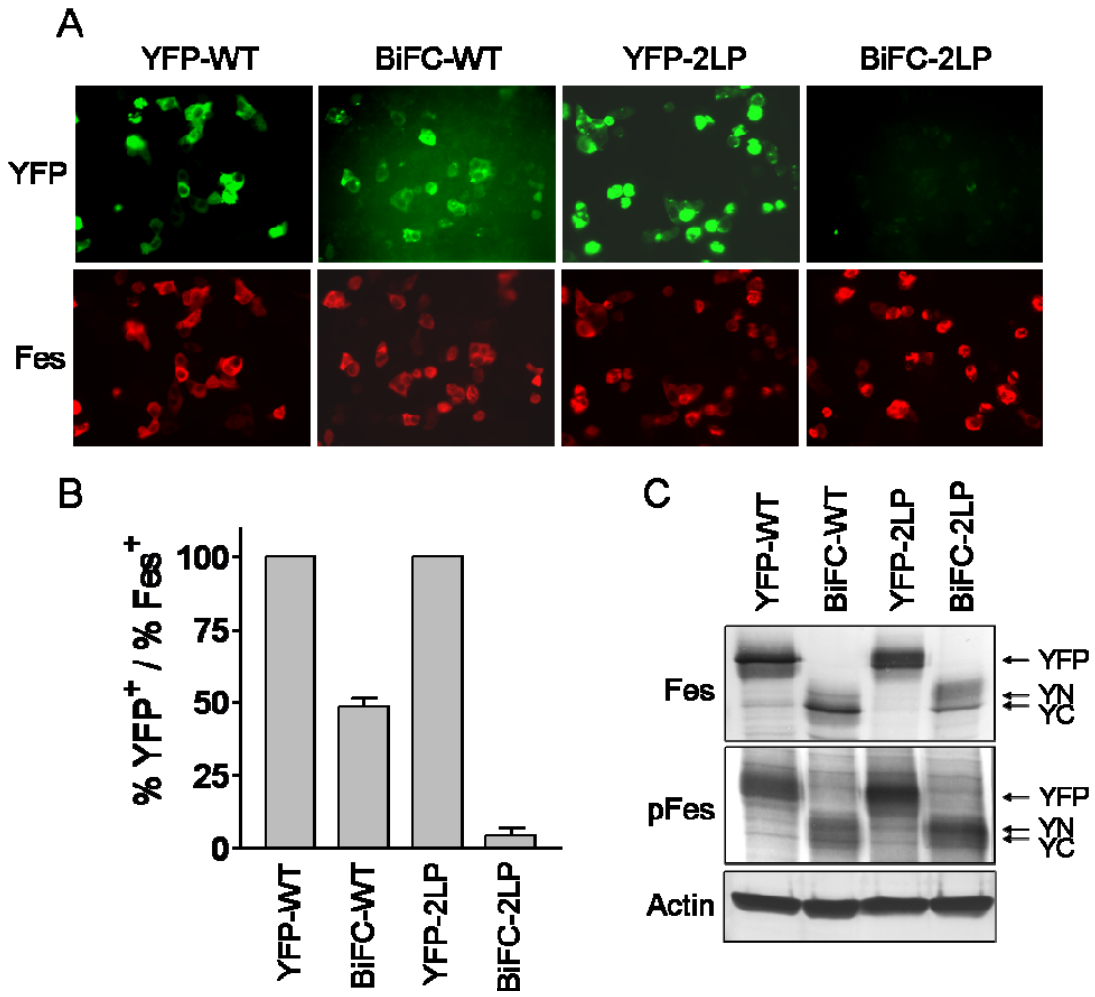

Figure 14: Coiled-coil-dependent Oligomerization of c-Fes in HCT 116 Colorectal Carcinoma Cells.

HCT 116 cells were transfected with plasmids encoding wild-type c-Fes or the double coiled-coil domain mutant (2LP) as YFP fusions or as BiFC partners using the pIRES vector shown in Figure 10. Twenty hours after transfection, cells were incubated at room temperature for 2 hours and either fixed and stained with a c-Fes-specific antibody or lysed for immunoblot analysis. A) Representative fluorescent images of cells expressing wild-type YFP-Fes (YFP-WT) or the corresponding BiFC partners (BiFC-WT) as well as the YFP fusion of the coiled-coil domain double mutant (YFP-2LP) or the BiFC partners of this mutant (BiFC-2LP). Secondary antibodies conjugated to Alexa Fluor 594 were used to visualize Fes-positive immunostained cells. YFP/ BiFC and Alexa Fluor 594 fluorescence are represented as green and red, respectively. B) The number of cells exhibiting a YFP fluorescent signal in each transfection condition was normalized to the total number of Fes-positive cells present. At least 250 cells were counted for each condition. Each experiment was repeated three times, and the results are presented as the mean ratios ± S.D. C) Lysates from the transfected cell populations shown in part A were analyzed by immunoblotting for c-Fes protein (top), c-Fes autophosphorylation (pFes; middle) and actin as a loading control (bottom). The arrows indicate the positions of the YFP-Fes (YFP), YN-Fes (YN), and YC-Fes (YC) fusion proteins.

Immunoblots were performed on HCT 116 cell lysates to verify the expression and activation status of the c-Fes fusion proteins. As shown in Figure 14C, the YFP, YN, and YC fusion proteins of both the wild-type and 2LP c-Fes proteins are clearly present. Interestingly,

immunoblots of the same cell extracts with the c-Fes phosphospecific antibody (pFes) show that wild-type c-Fes is strongly phosphorylated on activation loop tyrosine residue Tyr-713. This is in contrast to transfected COS cells, where wild-type c-Fes autophosphorylation is downregulated in comparison to the coiled-coil domains mutants [Figures 12 and 13; see also Ref. (40)]. Activation of wild-type c-Fes in transfected colon carcinoma cells may be due to direct phosphorylation by Src-family kinases, which are often constitutively active in colorectal cancer cells (174).

In K-562 CML cells, we were unable to detect translation of the YN-Fes protein from the pIRES plasmid (data not shown). To analyze c-Fes oligomerization in this cell line, therefore, separate expression vectors encoding the YN-Fes and YC-Fes fusion proteins were co-transfected together with an RFP expression plasmid to monitor transfection efficiency. As in previous experiments, wild-type and 2LP mutant YFP-Fes fusion proteins were included as positive controls. As shown in Figure 15A, K562 cells co-expressing the wild-type c-Fes BiFC partners exhibited strong fluorescence, similar to that observed with the YFP-Fes control. In contrast, co-expression of YN- and YC-2LP did not result in fluorescence complementation, indicating that c-Fes oligomerization is coiled-coil-dependent in cells of myeloid lineage as well. Note that transfected cells are clearly present in the BiFC-2LP cell population, as indicated by strong fluorescence from the RFP control. Immunoblot analysis of cell lysates verified expression of the YN-, YC- and YFP-Fes fusion proteins in each of the transfected cell populations (Figure 15B). Immunoblots with the pFes phosphospecific antibody showed that wild-type YFP-Fes autophosphorylation was repressed in K-562 cells, but strongly activated by the double coiled-coil (2LP) mutation. In K-562 cells expressing the c-Fes BiFC partners, a low

level of autophosphorylation was observed with wild-type c-Fes which was enhanced with the YN- and YC-Fes 2LP mutants, consistent with the results in COS cells.

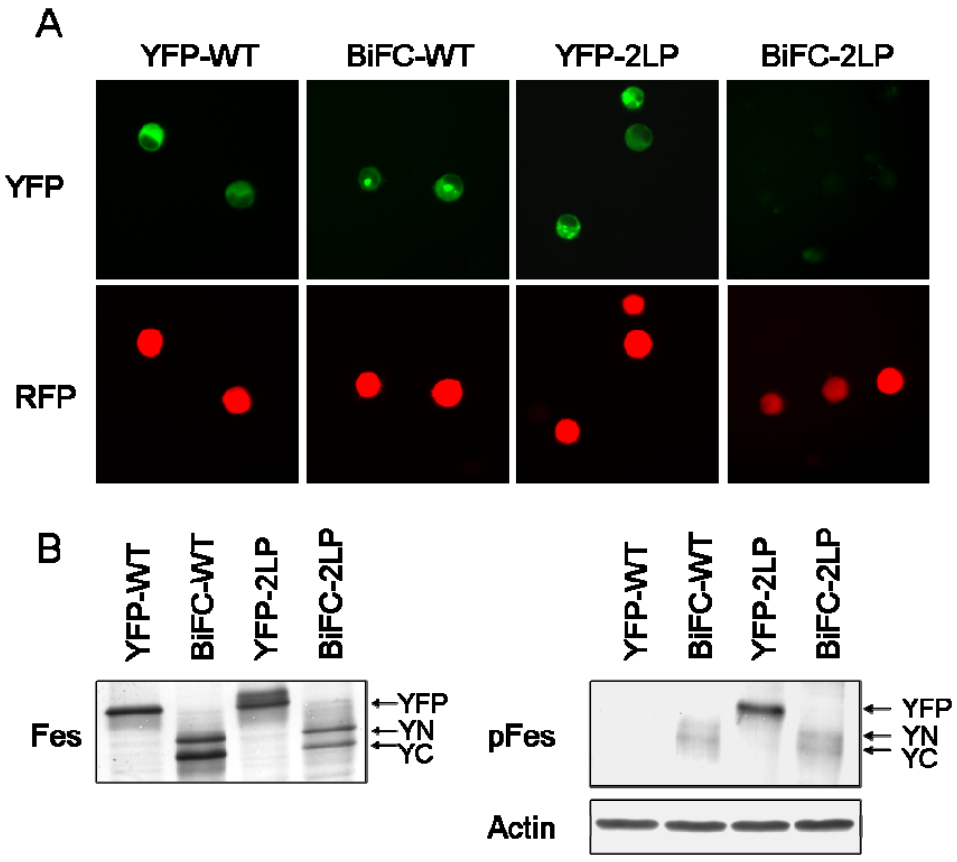

Figure 15: Coiled-coil-dependent Oligomerization of c-Fes in K562 Chronic Myelogenous Leukemia Cells.

K-562 cells were transfected with individual plasmids encoding wild-type c-Fes or the double coiled-coil domain mutant (2LP) as YFP fusions or as BiFC partners (YN-Fes + YC-Fes fusions). Cells were co-transfected with RFP as a marker for transfection efficiency. A) Representative fluorescent images of cells expressing wild-type YFP-Fes (YFP-WT) or the corresponding BiFC partners (BiFC-WT) as well as the YFP fusion of the coiled-coil domain

double mutant (YFP-2LP) or the BiFC partners of this mutant (BiFC-2LP). YFP/BiFC and RFP fluorescence are represented as green and red, respectively. B) Lysates from the transfected cell populations shown in part A were analyzed by immunoblotting for c-Fes protein (left), c-Fes autophosphorylation (pFes; right top) and actin as a loading control (right bottom). The arrows indicate the positions of the YFP-Fes (YFP), YN-Fes (YN), and YC-Fes (YC) fusion proteins.

2.5 DISCUSSION

The non-receptor protein-tyrosine kinases encoded by the three classic tyrosine kinase proto-oncogenes, c-Src, c-Abl, and c-Fes, are strictly regulated with respect to kinase activity *in vivo*. In the case of the c-Src and c-Abl, extensive X-ray crystallographic studies have provided tremendous insight as to the structural mechanisms responsible for downregulation of kinase activity *in vivo* (164,175). In both cases, intramolecular interactions cause these kinases to adopt a monomeric, downregulated conformation. In contrast, no structural information is available for the full-length c-Fes kinase, and the mechanism responsible for suppression of its kinase activity *in vivo* remains unclear. Previous work from our laboratory has implicated the coiled-coil domains as key regulators of c-Fes kinase activity *in vivo*, and initially led us to a model in which c-Fes self-regulates its kinase activity through coiled-coil-mediated monomer (inactive) to oligomer (active) transition (14,47,48). Prior to our study, however, the oligomeric nature of c-Fes had not been directly examined in living cells, and no evidence for the putative inactive monomer existed.

In this study, we explored c-Fes oligomerization in live cells by developing a YFP bimolecular fluorescence complementation (BiFC) assay, based on previous work of Kerppola

and colleagues for the analysis of transcription factors that also contain coiled-coil oligomerization domains (165,166). Here we establish for the first time that c-Fes, unlike c-Src and c-Abl, is a constitutive oligomer in living cells. Using COS-7 cells as a model system, we demonstrate that wild-type c-Fes adopts an oligomeric conformation irrespective of kinase domain autophosphorylation. Using previously described point mutations that disrupt the function of each major c-Fes domain (14,40,58), we determined that both coiled-coil domains are required for oligomerization in living cells. While mutation of either coiled-coil domain alone substantially reduced the percentage of oligomeric c-Fes molecules, the double coiled-coil mutant (2LP) virtually eliminated c-Fes oligomerization as reflected in the nearly complete loss of the BiFC signal (Figure 13). This result agrees with previous gel-filtration experiments using the same mutations in the context of a shorter c-Fes amino-terminal protein construct encompassing only the coiled-coils and the intervening protein sequence (14). In these prior studies, mutation of both coiled-coils was required for elution of the recombinant protein as a monomer. In contrast to the coiled-coil domains, disruption of either SH2 function (R483L) or kinase activity (K590E) was without effect on oligomerization, as both of these mutants formed oligomers with BiFC efficiencies similar to wild-type c-Fes *in vivo* (Figure 13). Previous chemical cross-linking studies have also suggested that the SH2 and kinase domains are not involved in the oligomerization of c-Fes (45).

Upon establishing the coiled-coil domains as mediators of c-Fes oligomerization in living cells, we next assessed whether c-Fes also form oligomers in cell lines where it has been shown to produce a biological effect. Previous studies have demonstrated that re-expression of c-Fes suppresses anchorage-independent growth of HCT 116 colorectal cancer cells and causes growth arrest and terminal differentiation in K-562 CML cells (47,67,80). In these cell lines, we

determined that wild-type c-Fes forms coiled-coil mediated oligomers (Figures 14 and 15), paralleling our results in COS-7 cells and strongly suggesting that oligomerization is essential to c-Fes function. Unexpectedly, wild-type c-Fes was also found to be constitutively autophosphorylated upon expression in HCT 116 cells (Figure 14C). This observation may be due to the presence of active Src-family kinases in colorectal cancer cell lines (174), which can directly activate c-Fes by phosphorylating its activation loop tyrosine residue (Figure 12) (40).

With the surprising revelation that c-Fes intrinsically adopts an oligomeric conformation regardless of the activation status of its kinase domain, the question remains as to what mechanism governs the tight regulation of c-Fes catalytic activity *in vivo*. c-Fes may self-regulate its catalytic activity through conformational changes involving interplay of the unique amino-terminal region and SH2-kinase unit, an idea originally suggested by Greer (12). When c-Fes is inactive, the coiled-coil domains may lock c-Fes in a conformation that hinders the association of the SH2 domain with the kinase domain. In response to upstream stimuli, such as proteins that bind to the coiled-coils in *trans* (176,177), the conformational restraints imparted by the coiled-coil domains may be disrupted, enabling the SH2 domain to interact with and prime the c-Fes kinase domain for *trans*-autophosphorylation and cellular signaling. This revised model suggests that small molecules designed to specifically bind to the coiled-coil domains may be potent c-Fes agonists. Such molecules may have utility in the differentiation therapy of tumor cells where the c-Fes protein is expressed but its catalytic activity is repressed. The proposed role of the SH2 domain in this model of c-Fes regulation strikes a contrast to that of c-Src and c-Abl, where it contributes to negative regulation of catalytic activity (164,175).

Support for the activation mechanism described above comes from several previous studies as well as new data presented here. First, deletion of the c-Fes SH2 domain has been

shown to diminish autophosphorylation and substrate phosphorylation both *in vitro* and in cell-based assays (48,59). Furthermore, an intact SH2 domain is required for c-Fes biological activity (fibroblast transformation), as an activated c-Fes variant (myristoyl-Fes) can be rendered biologically inert by SH2 domain deletion or substitution with a heterologous SH2 domain (17). Second, c-Fes has been shown to bind directly to its own SH2 domain (59), and this interaction is mediated at least in part by the kinase domain (T. Smithgall, unpublished results). In addition, early studies involving a viral counterpart of c-Fes (v-Fps) suggest that the SH2 domain interacts in *cis* with the kinase domain to form the active kinase conformation (13). Mild proteolysis of v-Fps released a stable globular fragment containing the SH2 and kinase domains, consistent with the idea that SH2-kinase domain interaction is essential for full kinase activity (62,63).

Very recently, the X-ray crystal structure of the c-Fes SH2-kinase region has been solved (64). This structure reveals that the SH2 domain indeed makes multiple contacts with the amino-terminal lobe of the kinase domain, including the critical αC helix involved in conformational regulation of the active site. Mutagenesis of key residues at the SH2:kinase interface dramatically reduced kinase activity, firmly establishing that c-Fes kinase domain function is dependent upon interaction with the SH2 domain. When considered in the context of the full-length structure, it is reasonable to postulate that amino-terminal sequences may interfere with the formation of this positive regulatory interface, thus repressing kinase activity. Along these lines, data presented in Figure 13 show that mutational disruption of the amino-terminal coiled-coil domains causes a complete loss of oligomerization (no BiFC signal), yet result in very strong kinase domain autophosphorylation as well as c-Fes movement to the microtubule network. [Note that our previous work has established that microtubule association of c-Fes correlates strongly with kinase activation in COS cells (40)]. Thus the coiled-coil domains

contribute not only to oligomerization, but also to repression of kinase activity, possibly by affecting the SH2:kinase domain interaction described above.

Alternatively, catalytic activation of c-Fes may involve an equilibrium shift from lower-order to higher-order oligomers. Cross-linking studies suggest that c-Fes forms trimers, while gel-filtration experiments support the existence of oligomers as large as pentamers (45). Note that the BiFC analysis used here cannot distinguish between individual oligomerization states. Rather, BiFC only reports in a binary fashion as to whether or not c-Fes has interacted with itself *in vivo*. While further clarification of the contribution of oligomerization to c-Fes catalytic activity and biological function is required, it is tempting to speculate that c-Fes is primed for autophosphorylation and signaling through the constitutive oligomeric nature imparted upon its overall structure by its unique amino-terminal coiled-coil oligomerization domains.

2.6 FOOTNOTE

†This work was supported by National Institutes of Health Grant CA123756.

3.0 PROMOTER METHYLATION BLOCKS C-*FES* PROTEIN-TYROSINE KINASE GENE EXPRESSION IN COLORECTAL CANCER*

3.1 ABSTRACT

The c-*fes* locus encodes a unique non-receptor protein-tyrosine kinase (c-Fes) traditionally viewed as a proto-oncogene but more recently implicated as a tumor suppressor in colorectal cancer (CRC). Recent studies have demonstrated that while c-Fes is expressed in normal colonic epithelium, expression is lost in tumor tissue and colorectal cancer cell lines, a finding common among tumor suppressors. Here we provide compelling evidence that promoter methylation is an important mechanism responsible for down-regulation of c-*fes* gene expression in colorectal cancer cells. Treatment with the DNA methyltransferase inhibitor 5-aza-2'-deoxycytidine resulted in the expression of functional c-*fes* transcripts in all CRC cell lines examined, including Caco-2, COLO 320, DLD-1, HCT 116, SNU-1040, SW-480, and HT-29. Bisulfite sequencing of genomic DNA isolated from 5-aza-2'-deoxycytidine-treated HT-29 cells identified methylated CpG dinucleotides immediately upstream from the c-*fes* transcription initiation sites. In contrast, this region of the c-*fes* promoter was hypomethylated in genomic DNA from normal colonic epithelium. In addition, methylation completely blocked the activity

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of the c-*fes* promoter in reporter gene assays. Promoter methylation is a previously unrecognized mechanism by which c-*fes* expression is suppressed in CRC cell lines, and is consistent with a tumor suppressor role for c-*fes* in this tumor site despite its tyrosine kinase activity.

3.2 INTRODUCTION

The human c-*fes* locus encodes a 93 kDa protein-tyrosine kinase (c-Fes) expressed in myeloid, vascular endothelial, neuronal, and epithelial cells (12,13,65,67). The c-*fes* gene was first identified as the normal cellular homolog of transforming oncogenes found in avian and feline retroviruses (12,13). Unlike its transforming viral counterparts, which exhibit constitutive protein-tyrosine kinase activity, c-Fes kinase activity is strictly regulated in mammalian cells (15,16). However, ectopic over-expression of wild-type Fes or of activated Fes mutants causes oncogenic transformation of rodent fibroblasts as well as tissue hyperplasia and hemangioma formation in transgenic mice (14,15,28). These earlier findings led to the view that c-*fes* functions as a proto-oncogene. However, over-expression of wild-type Fes in K-562 myeloid leukemia cells suppresses cell growth and restores differentiation, implicating Fes as a potential suppressor of chronic myelogenous leukemia (17,80,87).

Recent studies have proposed a novel role for c-Fes as a tumor suppressor in epithelial cells as well. Bardelli and colleagues discovered that c-*fes* was one of only seven genes exhibiting consistent colorectal cancer-associated kinase domain mutations following nucleotide sequence analysis of the tyrosine kinome of 182 colorectal cancers (88). While these mutations were initially predicted to be activating and contribute to tumorigenesis, subsequent studies have established that these mutations rendered c-Fes either catalytically inactive or had no effect on

kinase activity (67,84). Using a mouse breast epithelial cancer model system, Greer and colleagues determined that tumor onset occurred more rapidly in mice targeted with either null or kinase-inactivating c-*fes* mutations and that a c-*fes* transgene restored the kinetics of tumor onset in the c-*fes* null mice (84). Our group determined that re-expression of wild-type or activated Fes suppressed the growth of the Fes-negative HT-29 and HCT 116 colorectal cancer (CRC) cell lines in soft agar (67). Our study also showed that while c-Fes was strongly expressed in normal colonic epithelial cells from CRC patient samples, expression was reduced or absent in 67% of colon tumor sections from the same group of individuals (67). Similarly, Fes protein expression was significantly reduced or absent in five of six CRC cell lines examined (67). Together, these results suggest that loss of c-Fes expression is a common finding in colorectal cancer, an observation that fits with a tumor suppressor function for c-Fes in this tumor site. However, the mechanisms responsible for c-Fes protein loss in colonic epithelial cells are currently unknown.

Epigenetic silencing of tumor suppressor gene transcription, through DNA methylation and histone modifications, is well-recognized as a 'third pathway' in Knudson's model of tumor suppressor inactivation in cancer (98). DNA methylation events typically occur at carbon 5 of cytosine in CpG (5'-CG-3') dinucleotide sequences, a reaction that is catalyzed by DNA (C5) methyltransferases. In normal cells, CpG islands (CpG-rich stretches of DNA approximately 1 kb in length) within a gene promoter are rarely methylated (113). However, when promoter CpG islands become hypermethylated, transcription of downstream genes is often compromised (113,116). In fact, methylation of a CpG island in a tumor suppressor gene promoter often leads to irreversible inhibition of expression (105,117-119).

In this study, we investigated promoter methylation as a possible mechanism responsible for the loss of c-*fes* gene expression associated with colorectal cancer. We first established that

the absence of c-Fes protein in CRC cell lines correlates with the loss of full-length c-*fes* transcripts. Computational analysis of the c-*fes* promoter region revealed the presence of a putative CpG island surrounding the transcription initiation sites. Subsequent 5-aza-2'-deoxycytidine demethylation experiments restored Fes gene and protein expression in all of CRC cell lines analyzed, and bisulfite sequencing experiments identified key methylated CpG dinucleotides within the c-*fes* promoter region that may be responsible for gene silencing. Finally, *in vitro* methylation completely blocked the activity of the c-*fes* promoter in reporter-gene assays, directly implicating methylation as a major mechanism suppressing c-*fes* expression in colorectal cancer.

3.3 MATERIALS AND METHODS

3.3.1 Cell Culture

Cell lines were maintained at 37 °C in a 5% CO_2 humidified incubator. Caco-2 and 293T cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen). COLO 320, DLD-1, SNU-1040, and SW-480 cells were grown in RPMI 1640 medium (Invitrogen). HCT 116 and HT-29 cells were grown in McCoy's Modified 5A medium (Invitrogen). TF-1 and K-562 cell culture has been previously detailed (14,47). All cell culture media were supplemented with 10% fetal bovine serum (FBS) (Atlanta Biological) and Antibiotic/Antimycotic (Invitrogen). All cell lines were obtained from the American Type Culture Collection.

3.3.2 RT-PCR

Total RNA was isolated using the RNeasy Plus Mini Kit (Qiagen) and treated with TURBO DNase (Ambion) to remove contaminating traces of DNA. Random decamer-primed cDNA was synthesized from 1 μg of total RNA using the RETROscript Kit (Ambion). For semiquantitative analysis, one-tenth of each RT reaction was used in a 50 μl PCR reaction. Amplification of Fes and GAPDH were performed as follows: 94 °C for 2 min; 35 or 40 cycles at 94 °C for 1 min, 59 °C or 65 °C for 30 sec, and 72 °C for 1 min; and a final extension at 72 °C for 10 min. The primer pair used to amplify a 3' portion of the c-*fes* transcript (280 bp PCR product from the kinase domain coding region) was 5'-GGACCTGGCTGCTCGGAACTG-3' (sense) and 5'-CCTTCTCCACAAACTCCCGTGTC-3' (antisense). The primer pair used to amplify a 5' portion of the c-*fes* transcript (266 bp PCR product encompassing non-coding exon 1 through the FCH domain coding region in exon 2) was 5'-GAGGAGGAAGCGCGGAATCAG-3' (sense) and 5'-CTCGAATTCTCACCCACTG-TCCTGCAGGG-3' (antisense). The GAPDH control was amplified using the primer pair 5'-CCCTTCATTGACCTCAACTACATGGT-3' (sense) and 5'-GAGGGGCCATCCACAGTCTTCTG-3' (antisense) to generate a 470 bp product. Aliquots (10 μl) of each reaction were run on 2% agarose gels and stained with ethidium bromide.

3.3.3 Southern Blot Analysis

Genomic DNA was isolated using the PureLink Genomic DNA Purification Kit (Invitrogen). Southern blot analysis was performed as previously described (178). Briefly,

aliquots of genomic DNA (10 μg) were digested overnight with 300 U each of *Eco*RI and *Bam*HI at 37 °C. DNA fragments were resolved on an 0.8% agarose gel, denatured, and transferred to a nylon membrane by the capillary method in 20X SSC overnight. The DNA was subsequently crosslinked to the membrane by ultraviolet irradiation (Spectrolinker XL-1000; Stratgene). The membrane was then prehybridized for 2 hours at 42 °C in prehybridization buffer (50% formamide, 3X SSC, 10X Denhardt's solution, 2% SDS, and 40 μg/ml of heat-denatured herring sperm DNA). Hybridization was conducted overnight at 42 °C in hybridization buffer (50% formamide, 3X SSC, 1X Denhardt's solution, 5% dextran sulfate, 2% SDS, and 40 μg/ml of heat-denatured herring sperm DNA) containing 10^6 cpm/ml of each of two ^{32}P-labeled c-*fes*-specific probes described previously (178). After hybridization, the membrane was washed twice in 2X SSC/0.1% SDS at 42 °C for 15 min, followed by two 15 min washes in 0.2X SSC/0.1% SDS at 55 °C. Detection of c-*fes* sequences required autoradiography for five days using intensifying screens.

3.3.4 5-aza-2'-deoxycytidine (5-aza-2'-dC) Treatment

Freshly plated cells were treated with 5-aza-2'-dC (Sigma) at a final concentration of 10-15 μM (121). The 5-aza-2'-dC treatments were renewed every 24 hours. Total RNA, protein, or genomic DNA was isolated from the treated cells for analysis of c-*fes* mRNA levels (4 days), c-Fes protein levels (4 days), and c-*fes* promoter methylation status (8 days).

3.3.5 Immunoprecipitation, Immunoblotting, and Antibodies

Cultured cells were washed with PBS, harvested by centrifugation, and sonicated on ice in radioimmune precipitation assay (RIPA) buffer [50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% SDS, 1% sodium deoxycholate, 2 mM sodium orthovanadate, 25 mM sodium fluoride and Protease Inhibitor Cocktail Set III (EMD Biosciences)]. Fes immunoprecipitation assays were performed as described elsewhere (177). In brief, clarified protein lysates were incubated with a rabbit polyclonal antiserum raised against the Fes amino-terminal and SH2 domains (65) for 1 h at 4 °C and subsequently incubated for an addition hour at 4 °C with 30 μl of protein G-Sepharose beads (AP Biotech; 1:1 w/v slurry). Following three washes with RIPA buffer, the protein complexes were eluted from the beads by heating in SDS sample buffer. Immunoprecipitates were subjected to immunoblot analysis with antibodies to c-Fes (Santa Cruz Biotechnology, C-19; 1 μg/ml). Lysates were blotted with actin antibodies (Chemicon MAB1501; 1:1000 dilution) to control for equivalent amounts of input protein in each immunoprecipitation reaction.

3.3.6 Tissue Staining

Formalin-fixed, paraffin-embedded normal human colon surgical specimens (obtained from the University of Pittsburgh Health Sciences Tissue Bank) were deparaffinized in xylenes and rehydrated through a graded alcohol series. Tissue sections were either stained with Harris hematoxylin solution and eosin Y solution (Sigma-Aldrich), or immunostained for c-Fes expression. For immunofluorescent staining, antigen retrieval was performed in sodium citrate

buffer using a microwave oven. Cells were then blocked in PBS containing 1% normal serum for 1 h and incubated overnight at 4 °C with an anti-Fes primary antibody (Santa Cruz Biotechnology, C-19; 1:250 dilution). Immunostained tissue was visualized with secondary antibodies conjugated to Alexa Fluor 594 (Invitrogen). Fluorescent images were recorded using a Nikon TE300 inverted microscope with epifluorescence capability and a SPOT CCD high-resolution digital camera and software (Diagnostic Instruments).

3.3.7 Bisulfite Genomic DNA Sequencing

Genomic DNA was extracted directly from paraffin-embedded thin sections of normal colonic epithelial tissue using the Pinpoint Slide DNA Isolation System (Zymo Research). Genomic DNA from 5-aza-2'-dC treated and untreated HT-29 cells was isolated using the PureLink Genomic DNA Purification Kit (Invitrogen). Genomic DNA aliquots were then treated with sodium bisulfite using the EZ DNA Methylation-Gold Kit (Zymo Research). The targeted region of the c-*fes* promoter [-104 to +126 relative to the first transcription initiation site (30)] was then amplified using a fully-nested Hot-Start Taq (Qiagen) PCR protocol. For the first PCR round, 2 μl of bisulfite-treated genomic DNA was amplified using the following PCR conditions: 95 °C for 15 min, followed by 35 cycles at 94 °C for 1 min, 50 °C for 30 sec, 72 °C for 1 min plus a final extension at 72 °C for 10 min. Primers designed to recognize the bisulfite-modified 362 bp (-183 to +180) region of the c-*fes* promoter were 5'-GTTGGGTTATTTTTTTCGGTT-3' (sense) and 5'-TAAATAAATCTCTAACCCTC-3' (antisense). For the nested PCR, the following PCR conditions were used: 95 °C for 15 min, followed by 35 cycles at 94 °C for 1 min, 56 °C for 30 sec, and 72 °C for 1 min with a final extension at 72 °C for 10 min. Nested

primers designed to amplify within the first round PCR product were 5'-CCCAAG-CTTGGAGTAGGGGGGTTGGTAGG-3' (sense) and 5'-CCCGAATTCCCTACTCTACCCCTACC-TACC-3' (antisense), which contain *Hind*III and *Eco*RI restriction sites, respectively. The PCR products (257 bp: -104 to +126) were then subcloned into pcDNA3.1(+) (Invitrogen) and used to transform DH5α *E. coli*. Plasmid DNA was isolated from individual colonies and sequenced to determine the methylation status of the CpG dinucleotides within this proximal c-*fes* promoter region (30).

3.3.8 Plasmid Construction and *in vitro* Methylation

A previously characterized minimal c-*fes* promoter region (-425 to + 91) (30) was amplified using the following primers, which contain *Nhe*I and *Hind*III restriction enzyme sites, respectively: 5'-CCCGCTAGCAATTCCGTGAGGTGGGGAGGG-3' (sense) and 5'-CCCAAGCTT-GTACCCGCACGGGCAGCTGCT-3' (antisense). The resulting PCR product was subcloned into pcDNA3.1(+), and the nucleotide sequence was verified. This c-*fes* minimal promoter was then digested from pcDNA3.1(+), purified, and aliquots incubated in the presence or absence of *Sss*I methylase (New England BioLabs) (179). The efficiency of the methylation reaction was verified by resistance to cleavage by the methylation-sensitive restriction enzyme *Bstu*I. The methylated and unmethylated c-*fes* promoter regions were then purified and ligated into the pGL4.14 Luciferase Assay Vector (Promega). The pGL4.14 vector contains the firefly luciferase reporter gene, but lacks promoter and enhancer sequences. The pGL4.14 parent vector lacking a promoter insert served as the negative control. This approach was based on previous work by Pogribny and colleagues (179).

3.3.9 Transient Transfection and Luciferase Assay

The ligation reactions from the methylated and unmethylated c-*fes* promoter fragments and pGL4.14 were combined with 3 μl of Fugene 6 in 50 μl of Opti-MEM (Invitrogen). To normalize for transfection efficiency, the ligation products were cotransfected with 0.12 μg of pGL4.74 *Renilla* luciferase vector. Following a 30 min room temperature incubation, the Fugene 6/DNA transfection complex was added to 2 x 10^5 293T cells. Forty-eight h later, the cells were washed with PBS, harvested by scraping into a passive lysis buffer (Promega), and lysed according to the manufacturer's protocol. Cell extracts were assayed in a Victor2 1420 multilabel counter (Perkin Elmer) for firefly and *Renilla* luciferase activity using the Dual-Luciferase Reporter Assay System (Promega).

3.4 RESULTS

3.4.1 c-*fes* Transcripts are Reduced or Absent in CRC Cells

Recent work from our laboratory established that c-Fes protein expression is reduced or absent in CRC cell lines as well as primary tumor samples (67). To determine whether loss of c-Fes protein correlates with loss of c-*fes* mRNA, RT-PCR experiments were conducted on RNA isolated from seven CRC cell lines. RNA isolated from the human myeloid leukemia cell lines TF-1 and K-562 served as positive and negative controls, respectively (13,29). As shown in Figure 16A, c-*fes* expression was significantly reduced or absent in the CRC cell lines Caco-2,

DLD-1, HT-29, SNU-1040, and SW-480, as well as the control K-562 cell line as measured by RT-PCR amplification of the 3' end of the c-*fes* transcript. Surprisingly, 3' c-*fes* RT-PCR products were observed in COLO 320 and HCT 116 at levels similar to TF-1 cell positive control, possibly suggesting that post-transcriptional events are responsible for the lack of Fes protein previously reported for these two CRC cell lines (67). However, amplification of a 5' portion of the c-*fes* transcript (exon 1 through FCH domain in exon 2) established c-*fes* expression was significantly reduced or absent in all of the CRC cell lines (Figure 16B), including COLO 320 and HCT 116 (Figure 16B). These observations imply that the 3' PCR products observed in Figure 16A with COLO 320 and HCT 116 cells are derived from incomplete transcripts and are non-functional. To verify the integrity of the c-*fes* gene, Southern blot analysis was performed as previously described (178). Figure 16C shows that probes specific for the 5' and 3' ends of the c-*fes* gene detected restriction fragments of the expected lengths (5', 5.05 kb; 3', 4.16 kb) for all of the samples tested, confirming that the c-*fes* gene is present and not grossly rearranged in the CRC or myeloid cell lines used in this experiment.

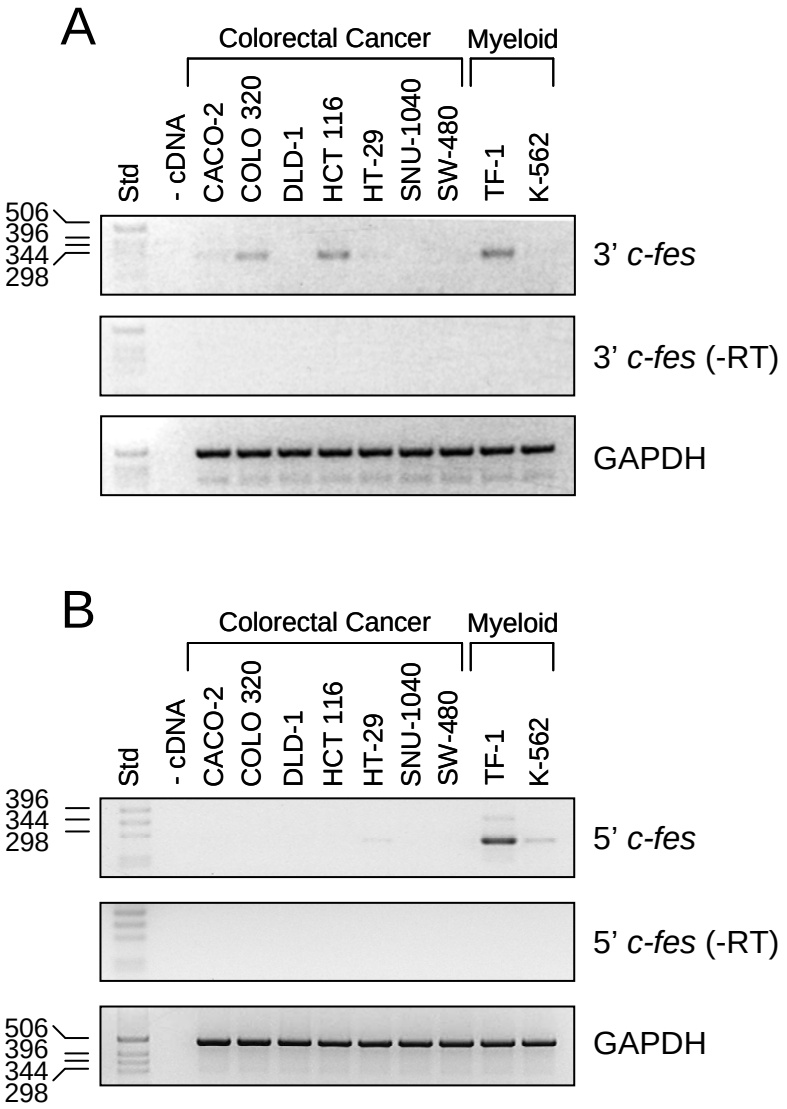

A
Colorectal Cancer
Myeloid
Std
- cDNA
CACO-2
COLO 320
DLD-1
HCT 116
HT-29
SNU-1040
SW-480
TF-1
K-562
506
396
344
298
3' c-fes
3' c-fes (-RT)
GAPDH
B
Colorectal Cancer
Myeloid
Std
- cDNA
CACO-2
COLO 320
DLD-1
HCT 116
HT-29
SNU-1040
SW-480
TF-1
K-562
396
344
298
5' c-fes
5' c-fes (-RT)
506
396
344
298
GAPDH
C
Colorectal Cancer
Myeloid
CACO-2
COLO 320
DLD-1
HCT 116
HT-29
SNU-1040
SW-480
TF-1
K-562
5'- 5.05 kb
3'- 4.16 kb

Figure 16: Transcription of c-*fes* is Low or Absent in Colorectal Cancer Cell Lines.

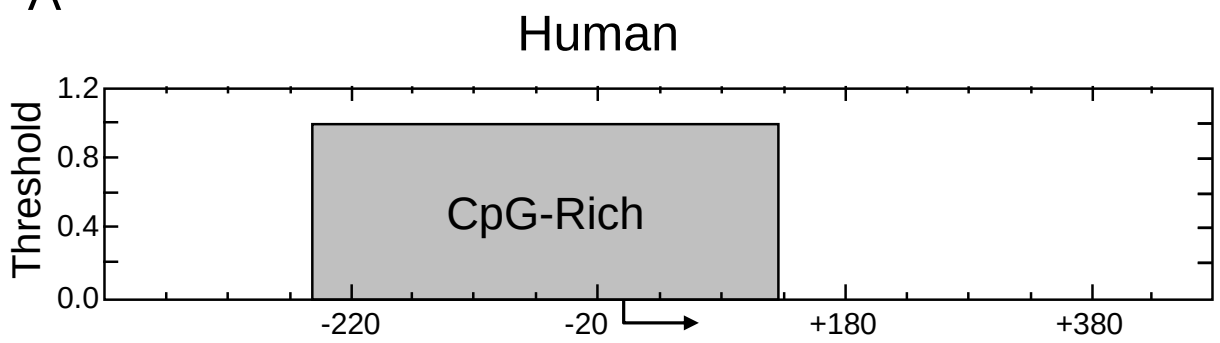
Total RNA and genomic DNA were isolated from colorectal cancer (Caco-2, COLO 320, DLD-1, HCT 116, HT-29, SNU-1040, and SW-480) and myeloid leukemia (TF-1 and K-562) cell lines for RT-PCR and Southern blot analyses, respectively. A) RT-PCR analysis of c-*fes* (3' region: 280 bp) and GAPDH (470 bp) mRNA levels. Control reactions lacking reverse transcriptase (-RT) are also shown. PCR reactions were analyzed after 35 cycles. Representative images of ethidium bromide-stained agarose gels are shown; images inverted for clarity. The experiment was repeated three times with comparable results. B) RT-PCR analysis of c-*fes* (5' region: 266 bp) and GAPDH (470 bp) mRNA levels. Control reactions lacking reverse transcriptase (-RT) are also shown. PCR reactions were analyzed after 40 cycles for c-Fes and 35 cycles for GAPDH. Representative images of ethidium bromide-stained agarose gels (inverted for clarity) are shown. The experiment was repeated two times with comparable results. C) Southern blot analysis of the c-*fes* gene in colorectal cancer and myeloid leukemia cell lines. Genomic DNA (10 μg) from each cell line were digested with *Eco*RI and *Bam*HI, resolved on an agarose gel, and transferred to a nylon membrane. The membrane was probed with DNA fragments specific for the 5' and 3' ends of the c-*fes* gene (178). Comparable results were obtained from three independent experiments.

3.4.2 The c-*fes* Promoter Harbors a Potential CpG Island

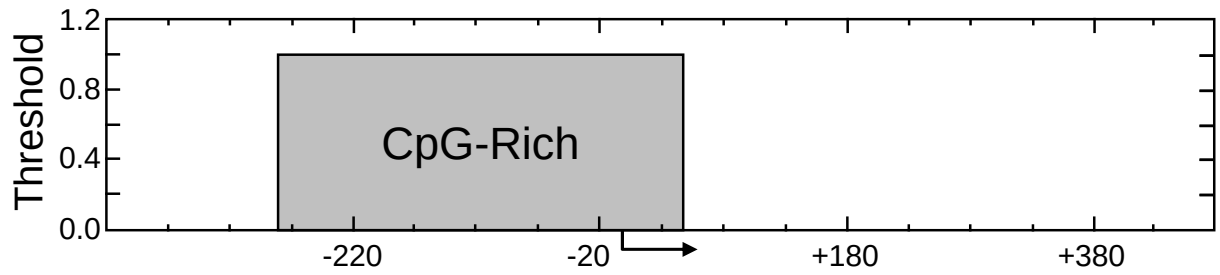
Normally unmethylated CpG islands may become hypermethylated in tumors, leading to irreversible inhibition of gene expression (105,118). Previous findings have suggested that a CpG island may exist at the 5' end of the human c-*fes* locus (180). To determine whether a CpG

island exists within the c-*fes* promoter, the DNA sequence was analyzed using the EMBOSS CpGPlot program, which detects regions of genomic DNA sequences that are rich in the CpG dinucleotide pattern (181). As shown in Figure 17A, a 375 bp CpG island was identified in the human c-*fes* promoter at nucleotide positions -249 to +126 relative to the first transcription initiation site (30). Feline and mouse c-*fes* promoter sequences were also analyzed for the presence of CpG islands. Figure 17B shows that a putative 323 bp CpG island is also present within feline c-*fes* promoter, while sequence analysis failed to detect a CpG island within the mouse c-*fes* promoter (data not shown). Whether or not promoter methylation is a unique regulatory feature of the human and cat promoters and does not operate in mice will require further investigation. For the human c-*fes* promoter, note the high density of CpG dinucleotides (red) located near the transcription initiation sites (underlined) (Figure 17C). Methylation of even a small core region near a transcription start site is often sufficient for gene silencing (118).

A



B



C

Human

```
-250 gcgccccttccggtgttccggggcgctgccgggccctggggcctgcgg
-200 ggcgcgggcggctcttggctgggccattctttccggccccctcctccct
-150 tccgtttccgtggccgtgcggccggctagaggctgcggcccagcgcggag
-100 caggggggctggcaggcgtcgggacggtcgggccggtcccgcccgccccct
 -50 tcccctccacaggcccgcccggggcctgggccaactgaaaccgcgggag
  +1 GAGGAAGCGCGGAATCAGGAACTGGCCGGGGTCCGCACCGGGCCTGAGTC
 +51 GGTCCGAGGCCGTCCCAGGAGCAGCTGCCCGTGCGGgtacctctagcccc
+101 ggggcctggaggagcggtgggagctggggggcgcg
```

Figure 17: Computational Analysis Reveals Potential CpG Islands within the Human and Feline c-*fes* Promoters.

A) A 375 bp CpG-rich region (-249 to +126 relative to the first transcription initiation site) was identified in the 1 kb human c-*fes* promoter region using the EMBOSS CpGPlot program (181). The position of the first transcriptional start site is indicated by the arrow. B) A potential 323 bp CpG island also exists with the feline c-*fes* promoter. C) Potentially methylated cytosines (red) are indicated within the human CpG island (bold). Exon 1 is indicated by upper case letters. The transcription initiation sites in exon 1 are underlined (30).

3.4.3 Demethylation Restores Expression of Functional c-*fes* Transcripts

To establish a role for DNA methylation in the repression of c-*fes* gene expression observed in Figure 16, the same panel of CRC and myeloid leukemia cell lines was treated with the demethylation reagent 5-aza-2'-deoxycytidine (5-aza-2'-dC) followed by RT-PCR analysis of the 3' and 5' regions of the c-*fes* transcript as in Figure 16. 5-aza-2'-dC treatment leads to rapid loss of DNA cytosine-C5 methyltransferase activity, because the enzyme becomes irreversibly bound to 5-aza-2'-dC upon incorporation into DNA [reviewed in (105)]. As shown in Figure 18A, RT-PCR analysis revealed that the 3' region of the c-*fes* transcript was restored in Caco-2, DLD-1, HT-29, SNU-1040, SW-480, and K-562 cells following four-day treatment with 5-aza-2'-dC. In addition, RT-PCR products corresponding to the 5'end of the c-*fes* transcript were restored in all seven CRC cell lines as well as K562 cells upon 5-aza-2'-dC treatment, suggesting that functional transcripts are now present in each of these cell lines (Figure 18B). The nucleotide sequences of all c-*fes* RT-PCR products were confirmed (data not shown).

To determine whether the c-*fes* RT-PCR products were derived from functional mRNA transcripts, lysates from 5-aza-2'-dC-treated cells were examined for c-Fes protein by immunoprecipitation followed immunoblotting. As shown in Figure 18C, 5-aza-2'-dC treatment restored c-Fes protein in Caco-2, DLD-1, HT-29, SNU-1040, SW-480, COLO 320, HCT 116, and K-562 cells, demonstrating the functionality of the c-*fes* transcripts. Regarding the two cells lines that exhibited 3' but not 5' transcripts in Figure 16, no truncated c-Fes protein products were observed in COLO 320 suggesting the 3' transcripts were not functional. On the other hand, two c-Fes truncation variants at ~90 and 92 kDa were observed in HCT 116 cells, suggesting that the observed 3' RT-PCR products are amplified from partial c-*fes* transcripts. TF-1 cells were used as a positive control for c-Fes protein expression in this experiment. These results demonstrate that expression of functional c-*fes* transcripts in colorectal cancer cell lines, as well as K-562 CML cells, is restored in response to treatment with a DNA methyltransferase inhibitor.

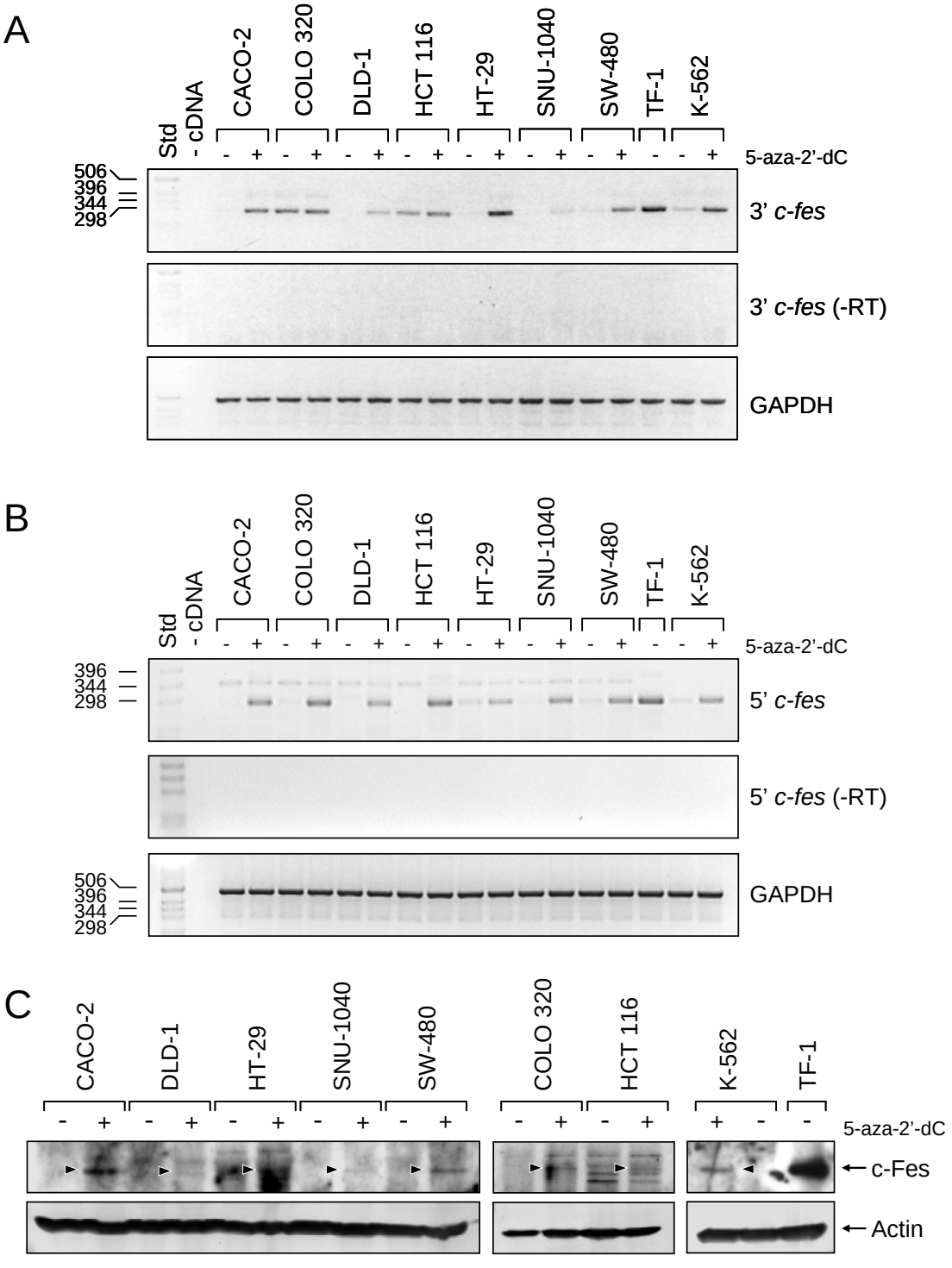

A
Std
- cDNA
CACO-2
COLO 320
DLD-1
HCT 116
HT-29
SNU-1040
SW-480
TF-1
K-562
5-aza-2'-dC
506
396
344
298
3' c-fes
3' c-fes (-RT)
GAPDH
B
Std
- cDNA
CACO-2
COLO 320
DLD-1
HCT 116
HT-29
SNU-1040
SW-480
TF-1
K-562
5-aza-2'-dC
396
344
298
5' c-fes
5' c-fes (-RT)
506
396
344
298
GAPDH
C
CACO-2
DLD-1
HT-29
SNU-1040
SW-480
COLO 320
HCT 116
K-562
TF-1
5-aza-2'-dC
c-Fes
Actin

Figure 18: 5-aza-2'-deoxycytidine Treatment Restores Functional c-*fes* Transcripts in Colorectal Cancer Cell Lines.

Colorectal cancer (Caco-2, COLO 320, DLD-1, HCT 116, HT-29, SNU-1040, and SW-480) and myeloid leukemia (TF-1 and K-562) cell lines were incubated with (+) or without (-) 10 μM 5-aza-2'-dC for four days. A) RT-PCR analysis of c-*fes* (3' region: 280 bp) and GAPDH (470 bp) transcript levels from treated vs. untreated cells. Controls reactions lacking reverse transcriptase (-RT) are also shown. PCR reactions were analyzed after 35 cycles. Representative images of ethidium bromide-stained agarose gels are inverted for clarity. B) RT-PCR analysis of c-*fes* (5' region: 266 bp) and GAPDH (470 bp) transcript levels from treated vs. untreated cells. Controls reactions lacking reverse transcriptase (-RT) are also shown. PCR reactions were analyzed after 40 cycles for c-Fes or 35 cycles for GAPDH. Representative images of ethidium bromide-stained agarose gels are inverted for clarity. C) c-Fes kinase protein was immunoprecipitated from control and 5-aza-2'-dC treated cells using an antibody generated against its amino-terminal and SH2 regions. Immunoprecipitates were then immunoblotted with an antibody raised against the carboxyl-terminus of c-Fes (upper panel). Fes was immunoprecipitated from TF-1 myeloid leukemia cells as a positive control (far right lane). The position of the immunoreactive 93 kDa c-Fes bands from the 5-aza-2'-dC-treated cultures are indicated with the arrowheads. Cell lysates were blotted with an anti-actin antibody to ensure equivalent levels of input protein for each immunoprecipitation reaction (lower panel). All experiments were repeated two or three times with comparable results.

3.4.4 The c-*fes* Promoter is Extensively Methylated in CRC Cells

We next investigated whether the putative CpG dinucleotides predicted to lie within the c-*fes* promoter were hypermethylated in CRC cell lines. First, we established the baseline methylation pattern of the c-*fes* promoter under physiological conditions by performing sodium bisulfite sequencing on genomic DNA isolated from normal human colonic epithelium. For these experiments, thin sections of formalin-fixed paraffin-embedded normal human colon tissue were immunostained to confirm c-Fes expression. A representative section is shown in Figure 19A, and displays both normal colonic microanatomy as well as strong epithelial staining for c-Fes protein. Genomic DNA was isolated directly from a serial section of this sample, subjected to bisulfite treatment, and the c-*fes* promoter sequence surrounding the transcription start site was amplified by PCR. The PCR product was then subcloned into a plasmid vector and 26 individual clones were sequenced. The methylation status of the eleven CpG dinucleotides immediately upstream of the first c-*fes* transcription start site are presented in Figure 19B. These c-*fes* promoter CpG dinucleotides are largely demethylated, consistent with the strong Fes staining observed in colonic epithelium. Note that 13 of the 26 clones were completely unmethylated, with an additional eight sequences exhibiting only a single methylated CpG dinucleotide at a distance of 76 nucleotides or greater from the transcriptional start site.

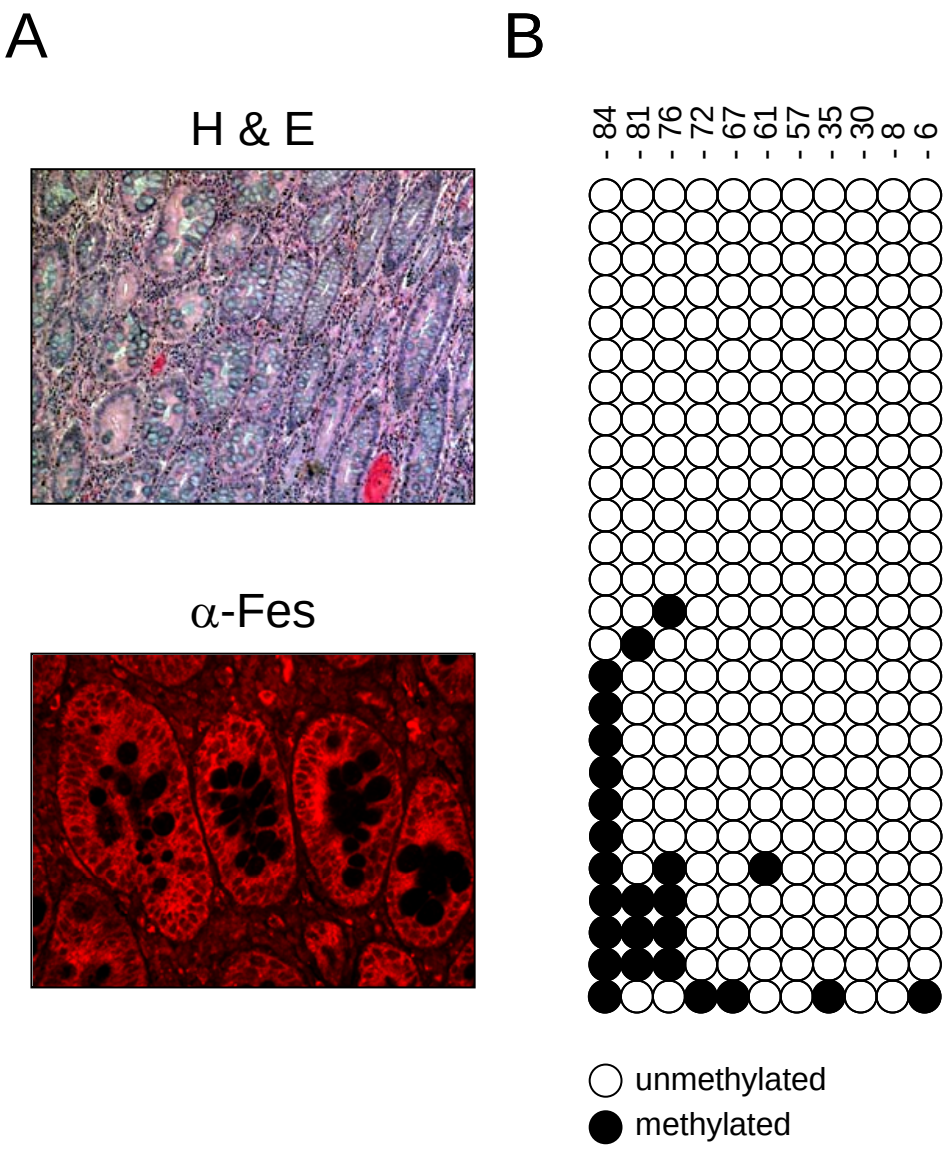


Figure 19: c-Fes Protein Expression in Normal Colonic Epithelium Correlates with Hypomethylation of the c-*fes* Promoter.

A) Thin sections of formalin-fixed paraffin-embedded normal colonic epithelial tissue were stained with hematoxylin and eosin (H&E; 100X; top) or immunostained with c-Fes antibodies and visualized with secondary antibodies conjugated to Alexa Fluor 594 (α-Fes; 400X; bottom). No immunofluorescence was observed without the primary Fes antibody (data not shown). B) Methylation status of the eleven CpG dinucleotides immediately preceding the first c-*fes*

transcription initiation site. Genomic DNA was isolated directly from the normal colonic epithelial tissue sections and treated with sodium bisulfite. The c-*fes* promoter region was PCR-amplified and subcloned into the plasmid vector pcDNA3.1. The rows of circles summarize the bisulfite sequencing results from each of 26 independent clones. Open circles represent unmethylated CpG dinucleotides, while filled circles represent methylated CpG sites. The position of each CpG nucleotide relative to the first c-*fes* transcription initiation site is indicated at the top.

To determine whether CpG dinucleotides near the start site of c-*fes* transcription are hypermethylated in colorectal cancer cells, DNA sequence analysis was performed on individual clones of bisulfite-treated genomic DNA isolated from both untreated and 5-aza-2'-dC-treated HT-29 cells as described above for normal colonic epithelium. As shown in Figure 20A, the proximal c-*fes* promoter from untreated HT29 cells was heavily methylated in comparison to normal colonic epithelium (Figure 20B), with only 3 of 30 clones unmethylated (10%) and many of the remaining clones showing multiple sites of methylation. In contrast, treatment with 5-aza-2'-dC induced a dramatic decrease in methylation at nine of eleven CpG sites, with the extent of methylation at seven of the sites reduced by more than 50% compared to the untreated control (Figure 20). Note that complete demethylation of all eleven CpG sites was observed in 13 of 34 clones (38%) from HT-29 cells treated with 5-aza-2'-dC. This reduction in promoter methylation in response to 5-aza-2'-dC treatment correlates with the re-expression of the c-*fes* gene (Figure 18), strongly suggesting that methylation directly controls c-*fes* gene expression.

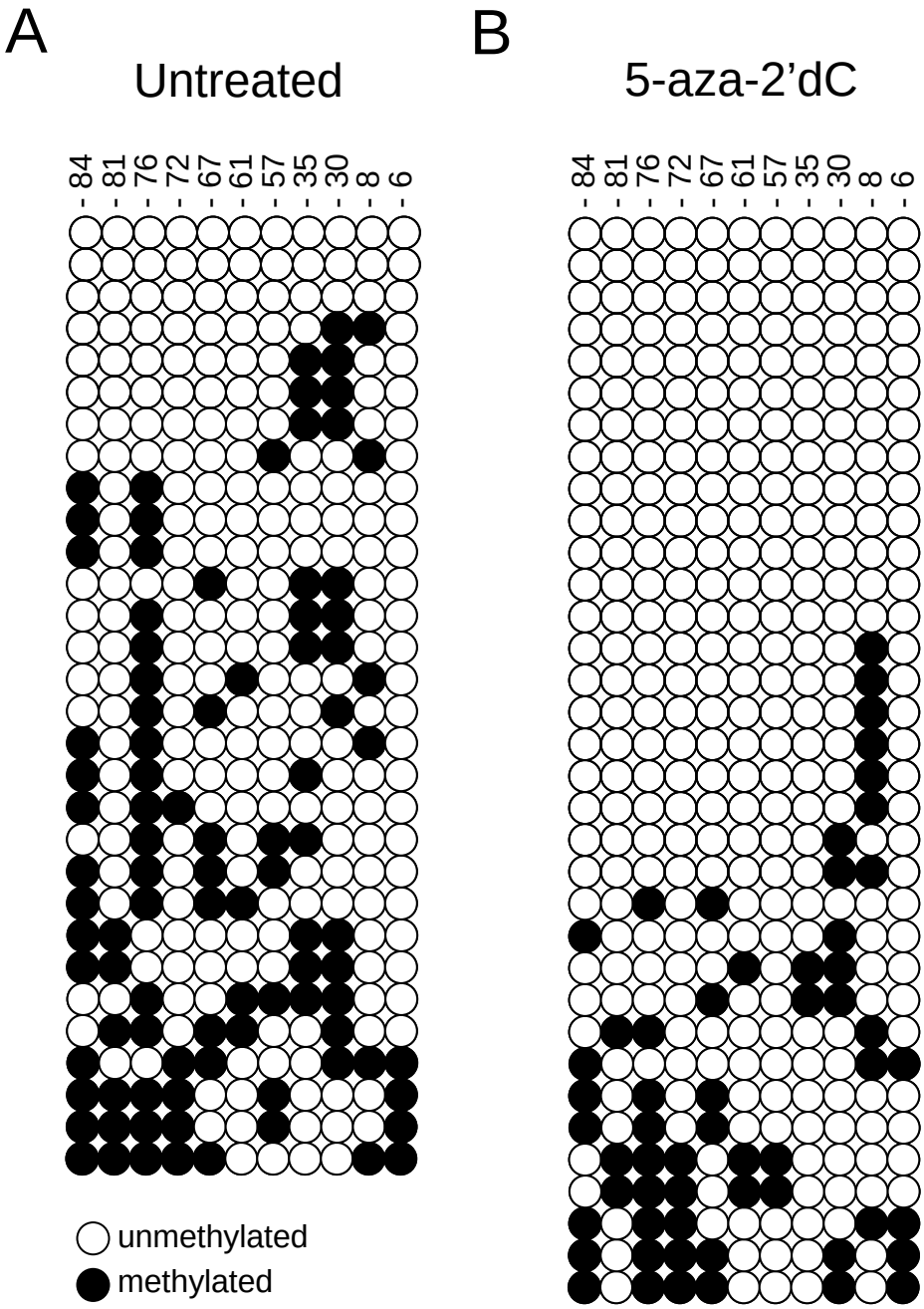

A
Untreated
-84
-81
-76
-72
-67
-61
-57
-35
-30
-8
-6
unmethylated
methylated
B
5-aza-2'dC
-84
-81
-76
-72
-67
-61
-57
-35
-30
-8
-6

Figure 20: Bisulfite Sequencing Reveals Extensive c-*fes* Promoter Methylation in HT-29 Cells Which is Reversed by 5-aza-2'-dC Treatment.

HT-29 cells were grown in the presence or absence of 15 μM 5-aza-2'-dC for eight days. Genomic DNA was isolated, treated with sodium bisulfite, and the c-*fes* promoter region was PCR-amplified and subcloned into the plasmid vector pcDNA3.1. A) Methylation status of the eleven CpG dinucleotides immediately preceding the first c-*fes* transcription initiation site in untreated HT-29 cells. The rows of circles summarize the bisulfite sequencing results from each of 30 independent clones. Open circles represent unmethylated CpG nucleotides, while filled circles represent methylated CpG sites. The position of each CpG nucleotide relative to the first c-*fes* transcription initiation site is indicated at the top. B) Methylation status of the proximal c-*fes* promoter in 5-aza-2'-dC treated HT-29 cells. Bisulfite sequence analysis of the c-*fes* promoter was performed as in part A, except 34 individual clones were sequenced. This entire experiment was repeated twice with comparable results.

3.4.5 *In vitro* Methylation Blocks c-*fes* Promoter Activity

To determine whether methylation directly impacts c-*fes* promoter activity, an *in vitro* methylation assay was performed using the dual-luciferase reporter assay. A previously defined minimal c-*fes* promoter (-425 to +91) with robust activity (30) was methylated *in vitro* using the *Sss*I methylase, and ligated upstream of the firefly luciferase coding sequence in the pGL4.14 vector. The efficiency of the methylation reaction was verified by resistance to *Bstu*I restriction enzyme cleavage (data not shown). Human 293T epithelial cells were then transfected with the ligation products and cells extracts were assayed for firefly luciferase activity. Luciferase activity

from the methylated c-*fes* promoter and vector control are expressed relative to activity of the unmethylated c-*fes* promoter. As shown in Figure 21, methylation completely blocked c-*fes* promoter activity, to the same level as the promoterless vector control. This result strongly implies that promoter methylation is one important mechanism that directly governs expression of the c-*fes* gene in colorectal cancer and possibly other tumor sites where c-*fes* is normally expressed (e.g., breast epithelium, myeloid cells) (12,13,65,67).

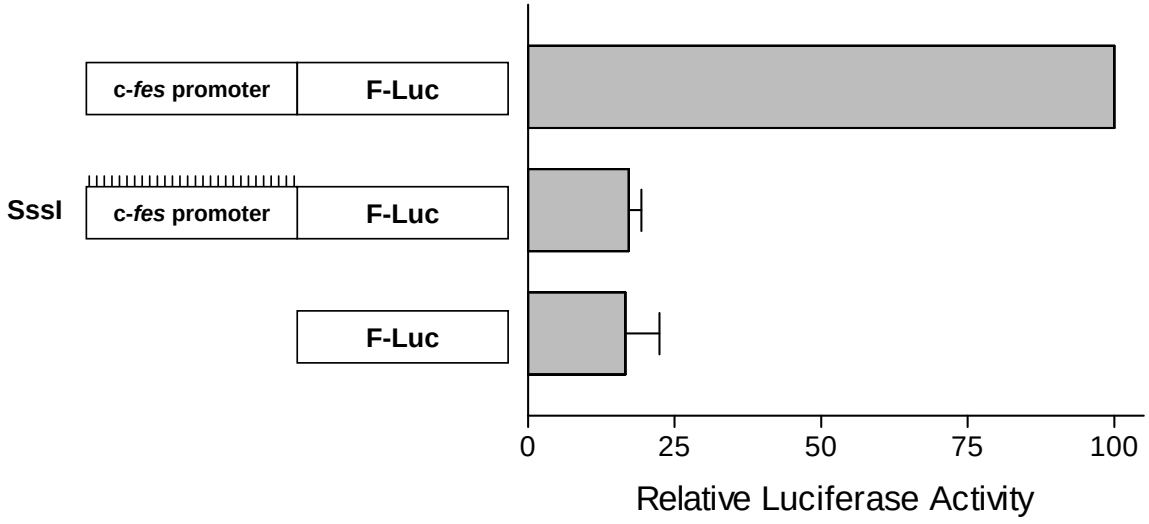


Figure 21: Methylation Regulates c-*fes* Promoter Activity *in vitro*.

Relative firefly luciferase activity after *in vitro* methylation of c-*fes* promoter fragments using the Dual Luciferase Reporter Assay System (Promega). A minimal c-*fes* promoter region (-425 to + 91) was methylated *in vitro* using *Sss*I methylase. Control and methylated c-*fes* promoter regions were then purified, ligated into the pGL4.14 Luciferase Assay Vector, and transfected into 293T cells along with the *Renilla* luciferase control vector. Forty-eight h later, the cells were washed and assayed for luciferase activity. Firefly luciferase activity was normalized to the

Renilla luciferase control, and activity from the methylated c-*fes* promoter and vector control are expressed relative to the unmethylated c-*fes* promoter (set to 100%). Tick marks represent methylation of c-*fes* promoter CpG dinucleotides by the *Sss*I methylase. Data presented represent the mean $\pm$ S.D. of three independent experiments.

3.5 DISCUSSION

Although c-*fes* has been historically viewed as a proto-oncogene because of its protein-tyrosine kinase activity, several recent reports have established a tumor suppressor function for c-*fes* in epithelial cancers (67,84). Greer and colleagues determined that null or kinase-inactivating c-*fes* mutations accelerated tumor onset in a mouse breast epithelial cancer model system (84). Importantly, the kinetics of tumor onset in targeted c-*fes* null mice was restored with a c-*fes* transgene in this study, allowing direct attribution of the effect on tumor latency to c-*fes* gene loss. Recent work from our group has demonstrated that loss of Fes protein expression is a common feature of both CRC cell lines as well as primary colon tumor specimens (67). We also determined that re-expression of wild-type or activated Fes in the CRC cell lines HT-29 and HCT 116 suppressed transformed colony growth in soft agar (67). Furthermore, re-expression of wild-type or activated Fes in HCT 116 cells almost completely suppresses invasion through a matrigel matrix, without affecting cell proliferation or viability (J. Shaffer and T. Smithgall, unpublished observation). While these previous studies support a tumor suppressor role for c-Fes in colorectal and other epithelial cancers, the mechanism responsible for the loss of c-Fes expression in tumor cells has not been investigated.

Data presented here are the first to define a mechanism by which c-*fes* gene expression is repressed in colorectal cancer. First, we established that full-length c-*fes* transcripts are absent in seven independent colorectal cancer cell lines, suggesting that the loss of c-Fes protein previously observed in these cell lines (67) results directly from down-regulation of c-*fes* gene expression (Figure 16). Based on EMBOSS CpGPlot identification of a CpG island in the human c-*fes* promoter (Figure 17), we hypothesized that methylation of CpG dinucleotides within the c-*fes* promoter downregulates c-Fes expression in CRC cell lines. Using the potent demethylation agent 5-aza-2'-dC, we re-established c-*fes* gene expression in each CRC cell line (Figures 18A and 18B). These data directly implicate promoter methylation as a key mechanism governing c-*fes* transcription in colorectal cancer cell lines. Treatment with 5-aza-2'-dC also restored expression of full-length (93 kDa) c-Fes protein in each CRC cell line and in K562 CML cells, demonstrating that the RT-PCR products were derived from functional c-*fes* transcripts (Figure 18C). Of interest is the observation that truncated variants of c-Fes were observed in untreated HCT 116 cells. However, full-length c-Fes was only expressed in HCT 116 cells upon 5'-aza'-2-dC treatment, suggesting that expression of the full-length protein is controlled by promoter methylation in this cell line.

In addition to the CRC cell lines, we also observed that 5-aza-2'-dC treatment restored functional c-*fes* transcripts in the cell line K-562, which was derived from the blast crisis phase of chronic myelogenous leukemia (78). Previous work has established that c-*fes* expression is undetectable in K-562 cells, despite being of myeloid origin (30,68,80) and having an intact c-*fes* locus (178). Re-introduction of c-*fes* has been shown to cause growth suppression and differentiation in K562 cells, suggesting a tumor suppressor function for c-*fes* in CML as well. Consistent with our observations, Alcalay et al. reported that the c-*fes* promoter was

hypomethylated in the myeloid leukemia cell lines HL-60, KG-1, and U937 (180), all of which strongly express c-Fes (68).

In order to attribute c-*fes* gene downregulation to methylation of specific CpG dinucleotides within the c-*fes* promoter CpG island, we performed sodium bisulfite sequencing on the c-*fes* promoter from 5-aza-2'-dC-treated HT-29 cells. Using the methylation pattern of CpG dinucleotides from the c-*fes* promoter in normal colonic epithelial cells for comparison (Figure 19B), we found that several CpG sites within the c-*fes* promoter were heavily methylated in HT-29 cells (Figure 20A). These sites consistently exhibited reduced methylation following 5-aza-2'-dC treatment (Figure 20B). The actual degree of demethylation is most likely an underestimate, as 5-aza-2'-dC inhibits DNA (cytosine-C5) methyltransferase activity but does not remove pre-existing methylated cytosine residues (105). These methylated CpG dinucleotides lie in regions that can inhibit c-*fes* gene transcription through one of two mechanisms. First, transcription factor binding may be inhibited by methylated CpG dinucleotides. While transcription factors controlling c-*fes* gene expression in colonic epithelial cells are not known, factors that regulate c-*fes* in myeloid cells have been extensively characterized. These include the ubiquitous transcription factor Sp1, the hematopoietic cell-specific factor PU.1/Spi-1, and a c-*fes* expression factor (FEF) that is not present in human epithelial cells (29,34,35). Note that the DNA binding and transcriptional activities of Sp1, whose consensus binding site contains a central CpG site, are not influenced by methylation (182,183). However, methylation may influence the DNA binding and transcriptional activities of tissue-specific transcription factors that drive c-*fes* expression both in myeloid and epithelial cells. A second possible mechanism by which promoter methylation down-regulates c-*fes* expression may involve methylation-dependent recruitment of nucleoprotein factors such as the

methyl-CpG-binding proteins MeCP1 and MeCP2, which in turn deny access to transcription factors (154-158,184). Future studies will define the precise mechanism by which methylation inhibits c-*fes* expression.

Data presented here also provide direct evidence that methylation governs c-*fes* promoter activity. *In vitro* methylation of a robustly active c-*fes* promoter (30) that mimics methylation patterns observed by bisulfite sequencing of HT-29 cell genomic DNA completely blocked the activity of the c-*fes* promoter in a reporter gene assay (Figure 21). This result suggests that methylation directly governs c-*fes* gene expression in CRC cell lines.

DNA methylation is a well-documented epigenetic mechanism altering gene expression in a variety of tumor types. Hypermethylation of tumor suppressor genes effectively abolishes their transcription, while hypomethylation of proto-oncogenes increases their transcription (117,119). In colorectal cancers, hypermethylation is a frequent event resulting in the silencing of well known tumor suppressors such as *P16*/*CDKN2A*/*INK4A* and *P14*/*ARF* (98,185-188). Here, we identify for the first time a candidate protein-tyrosine kinase tumor-suppressor gene that is hypermethylated in colorectal cancers. Through demethylation treatment, sodium bisulfite sequencing, and *in vitro* methylation assays, we have established that loss of c-*fes* expression in colorectal cancers may be due in part to methylation of CpG sites within the c-*fes* promoter. Selective re-expression of the c-*fes* tyrosine kinase gene with demethylation agents or other small molecules may be of value in CRC therapy.

3.6 FOOTNOTE

This work was supported by NIH grant CA123756. The authors wish to thank Drs. Nathan Bahary and Scott Lowe of the University of Pittsburgh School of Medicine for providing formalin-fixed, paraffin-embedded human colon surgical specimens and Dr. Will Walker of the Department of Cell Biology and Physiology at the University of Pittsburgh School of Medicine for reagents and advice regarding the luciferase assays.

3.7 FES PROMOTER METHYLATION AND PROTEIN EXPRESSION ANALYSIS IN HUMAN COLON SURGICAL SPECIMENS

As described in the first part of Chapter 3, promoter methylation regulates Fes gene and protein expression in colorectal cancer cell lines. To summarize our key findings, treatment of seven CRC cell lines with the potent demethylation agent 5-aza-2'-deoxycytidine resulted in expression of both the c-*fes* gene and full-length c-Fes protein products (Figure 18). This correlates strongly with a decrease in c-*fes* promoter methylation as measured by sodium bisulfite sequencing (HT-29 CRC cells: Figure 20). In this section of Chapter 3, we extended our analysis to human surgical specimens. Using colorectal cancer patient surgical specimens, our laboratory previously determined that c-Fes expression was reduced or absent in 67% of tumor tissues when compared to matched normal tissue (67). To define a mechanism responsible for this reduced expression, we analyzed the status of c-*fes* promoter methylation in colorectal cancer patient-derived tissue samples. In conjunction with this, we performed

immunofluorescence staining analysis of c-Fes protein expression on the same patient samples to establish a mechanism by which c-Fes expression is lost in human colorectal cancers.

3.8 MATERIALS AND METHODS

3.8.1 Cell Culture and 5-aza-2'-deoxycytidine Treatments

Cell lines were maintained at 37 °C/5% CO_2 in a humidified incubator and grown in either McCoy's Modified 5A (HT-29) or RPMI (COLO 320) medium that was supplemented with 10% FBS (Gemini Biosciences) and Antibiotic/Antimycotic (Invitrogen). To induce demethylation, freshly plated cells were treated at a final concentration of 10 μM 5-aza-2'-dC for 4 days. The treatments were renewed every 24 hours.

3.8.2 Immunofluorescence Staining

Formalin-fixed, paraffin-embedded normal and matched tumor human colon surgical specimens (obtained from the University of Pittsburgh Health Sciences Tissue Bank) were deparaffinized in xylenes and rehydrated through a graded alcohol series. Tissue sections were either stained with Harris hematoxylin solution and eosin Y solution (Sigma-Aldrich), or immunostained for c-Fes expression. For immunofluorescence staining of tissue, antigen retrieval was performed in sodium citrate buffer using a microwave oven. For immunofluorescence staining of colorectal cancer cell lines, HT-29 and COLO 320 cells were fixed with 4 °C paraformaldehyde in PBS and permeabilized with 0.2% Triton X-100. Cells

were then blocked in PBS containing 1% normal serum for 1 h and incubated overnight at 4 °C with an anti-Fes primary antibody (Santa Cruz Biotechnology, C-19; 1:250 dilution). Immunostained cells and tissue were visualized with secondary antibodies conjugated to Alexa Fluor 594 (Invitrogen). Fluorescent images were recorded using a Nikon TE300 inverted microscope with epifluorescence capability and a SPOT CCD high-resolution digital camera and software (Diagnostic Instruments).

3.8.3 Bisulfite Genomic DNA Sequencing

Genomic DNA from normal and tumor colonic epithelial tissue was extracted directly from paraffin-embedded thin sections of from the same patient using the Pinpoint Slide DNA Isolation System (Zymo Research). In this system, a solution is applied to deparaffinized tissue of interest. Once the solution is dried, underlying cells are embedded within. Following Proteinase K digestion and DNA purification, the DNA is ready for use. Genomic DNA aliquots were then treated with sodium bisulfite using the EZ DNA Methylation-Gold Kit (Zymo Research). The targeted region of the c-*fes* promoter [-104 to +126 relative to the first transcription initiation site (30)] was then amplified using a fully-nested Hot-Start Taq (Qiagen) PCR protocol. For the first PCR round, 2 μl of bisulfite-treated genomic DNA was amplified using the following PCR conditions: 95 °C for 15 min, followed by 35 cycles at 94 °C for 1 min, 50 °C for 30 sec, 72 °C for 1 min plus a final extension at 72 °C for 10 min. Primers designed to recognize the bisulfite-modified 362 bp (-183 to +180) region of the c-*fes* promoter were 5'-GTTGGGTTATTTTTTTTCGGTT-3' (sense) and 5'-TAAATAAATCTCTAACCCTC-3' (antisense). For the nested PCR, the following PCR conditions were used: 95 °C for 15 min,

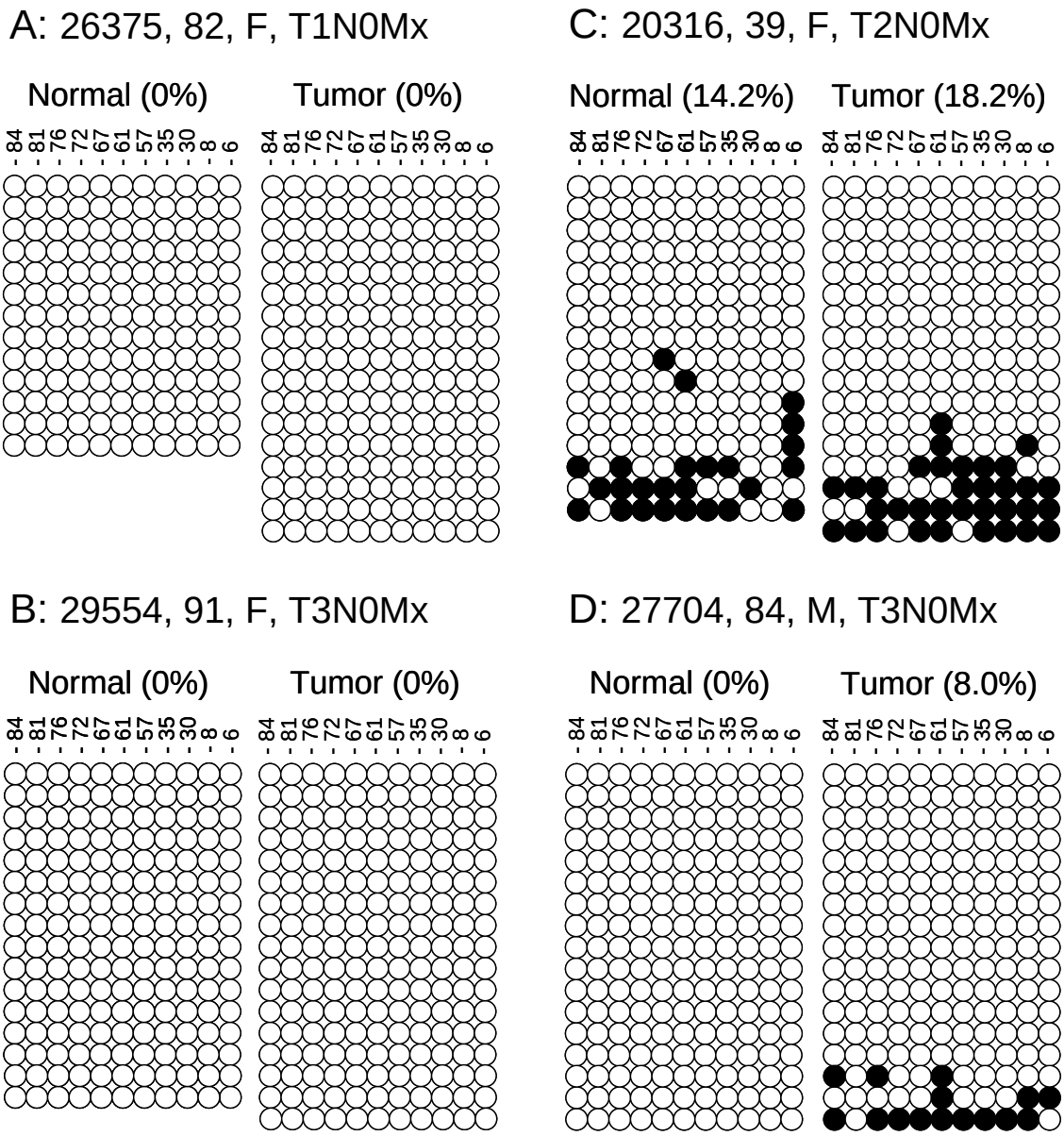
followed by 35 cycles at 94 °C for 1 min, 56 °C for 30 sec, and 72 °C for 1 min with a final extension at 72 °C for 10 min. Nested primers designed to amplify within the first round PCR product were 5'-CCCAAG-CTTGGAGTAGGGGGGTTGGTAGG-3' (sense) and 5'-CCCGAATTCCCTACTCTACCCCTACC-TACC-3' (antisense), which contain *Hind*III and *Eco*RI restriction sites, respectively. The PCR products (257 bp: -104 to +126) were then subcloned into pcDNA3.1(+) (Invitrogen) and used to transform DH5α *E. coli*. Plasmid DNA was isolated from individual colonies and sequenced to determine the methylation status of the CpG dinucleotides within this proximal c-*fes* promoter region (30).

3.9 RESULTS

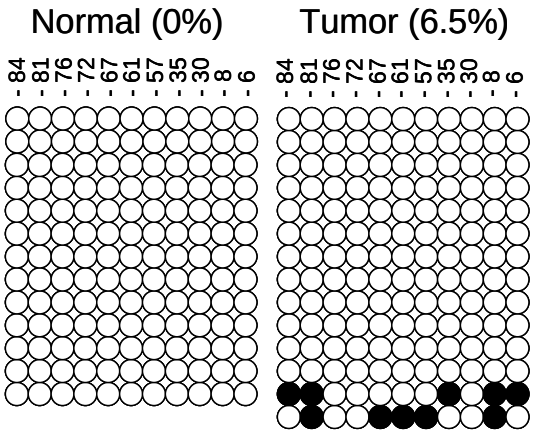
3.9.1 c-*fes* Promoter is Hypermethylated in Colon Tumor Tissue

Colorectal cancer cell line analyses suggest a role for c-*fes* promoter methylation in the downregulation c-Fes expression. To determined whether the c-*fes* promoter is hypermethylated in tumors from human colorectal cancer patients, sodium bisulfite DNA sequencing was performed on genomic DNA isolated from matched normal and tumor colonic epithelial tissue (eight surgical specimens total) (Figure 22). Two patient cases (A: 26475 and B: 29554) exhibited no methylation of the c-*fes* promoter in either normal- or tumor-derived genomic DNA. In one patient case (C: 20316), methylation of the c-*fes* promoter was detected in both normal- and tumor-derived genomic DNA, with a slight increase observed for the tumor-derived gDNA. For five patient cases, an increase in methylation was observed for tumor-derived genomic DNA in comparison to genomic DNA isolated from normal tissue. Of note is that while two cases (D:

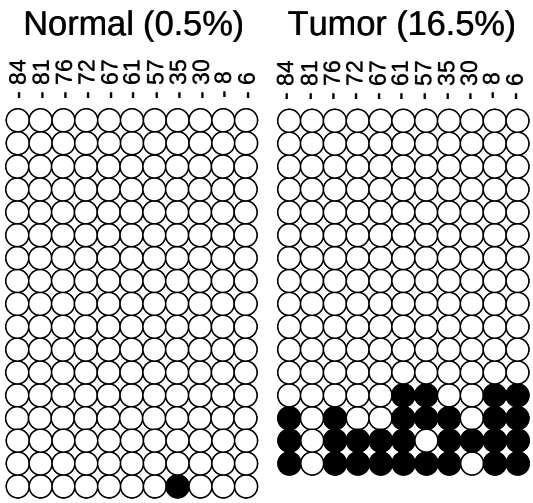
27704 and E: 1066) had a mild (1-10%) increase in c-*fes* promoter methylation in tumor tissue-derived genomic DNA, three patient cases (F: 27941, G: 27208, and H: 25465) had moderate (11-20%) increases in methylation of the c-*fes* promoter in tumor-derived genomic DNA relative to normal tissue. In summary, the c-*fes* promoter was hypermethylated in genomic DNA isolated from tumors, relative to normal tissue, in five of eight colorectal cancer patients.



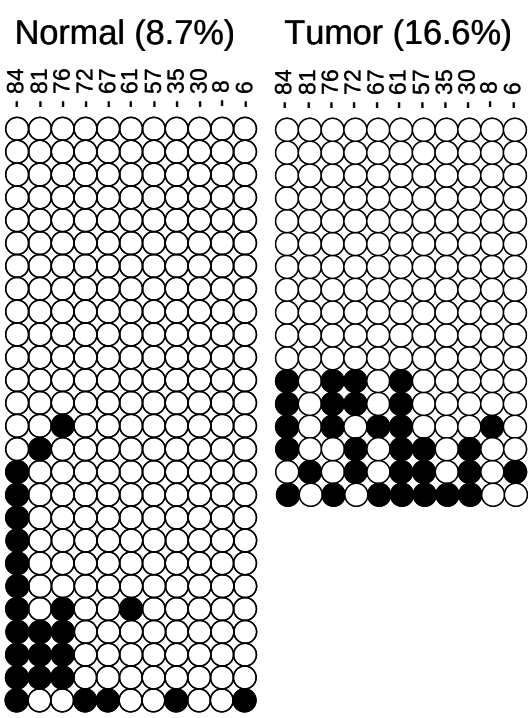
E: 1066, 69, M, T2N0Mx



G: 27208, 67, F, T2N0Mx



F: 27941, 70, M, T2N0Mx



H: 25465, 68, M, T3N2Mx

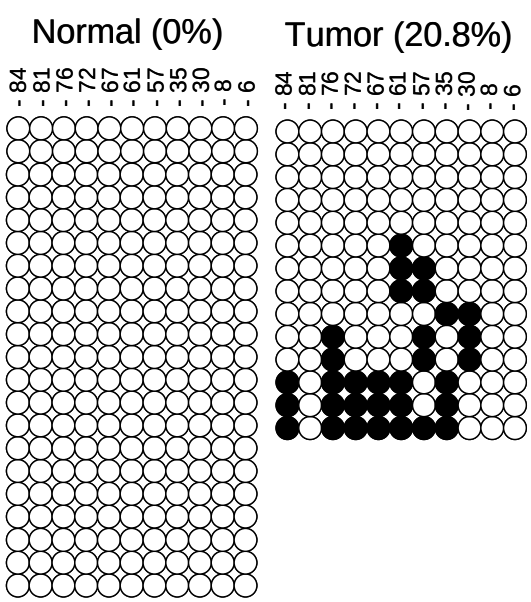


Figure 22: c-*fes* Promoter is Hypermethylated in Colonic Epithelium Tumors

Methylation status of the eleven CpG dinucleotides immediately preceding the first c-*fes* transcription initiation site was analyzed in genomic DNA samples isolated directly from both normal and tumor-tissue sections in eight human surgical samples (A-H). The samples were treated with sodium bisulfite, PCR-amplified, and then subcloned into the pcDNA3.1(+) plasmid vector. The rows of circles represent the bisulfite sequence results of each individual clone. Open circles are representative of unmethylated CpG dinucleotides, and filled circles represent methylated CpG sites. Clinical details (case #: age, gender, stage) are provided for each patient case as well as the percentage of methylated CpG dinucleotides relative to the total CpG dinucleotides analyzed per sample.

3.9.2 c-Fes Protein Expression is Lost in Colon Tumor Tissue

To correlate c-Fes protein expression in human colorectal cancer patient samples to the methylation status of the c-*fes* promoter, the aforementioned colorectal cancer patient samples were immunostained with an antibody directed at the carboxyl-terminus of c-Fes (Fes C19; Santa Cruz Biotechnology). As controls, two colorectal cancer cell lines that express c-Fes protein only upon treatment with 5-aza-2'-dC [HT-29 and COLO 320: (67) and Figure 17C] were immunostained. Similar to the immunoprecipitation experiments, c-Fes expression was not observed in untreated cells (Figure 23: Untreated), but was observed in 5-aza-2'-dC treated cells (Figure 23: Treated). In line with past c-Fes expression data, the pattern was largely a diffuse cytoplasmic distribution pattern in both cell lines (17,39,40,65). In addition, cell periphery

staining was observed for c-Fes in a subset of COLO 320 cells (demarked by arrows), an observation previously made for CACO-2 cells over-expressing c-Fes (Frank Delfino, unpublished observation). This localization potentially implicates c-Fes in the modulation of cell-cell adhesion, a process intimately associated with cancer cell invasion that c-Fes has been demonstrated to suppress (J. Shaffer, unpublished observation).

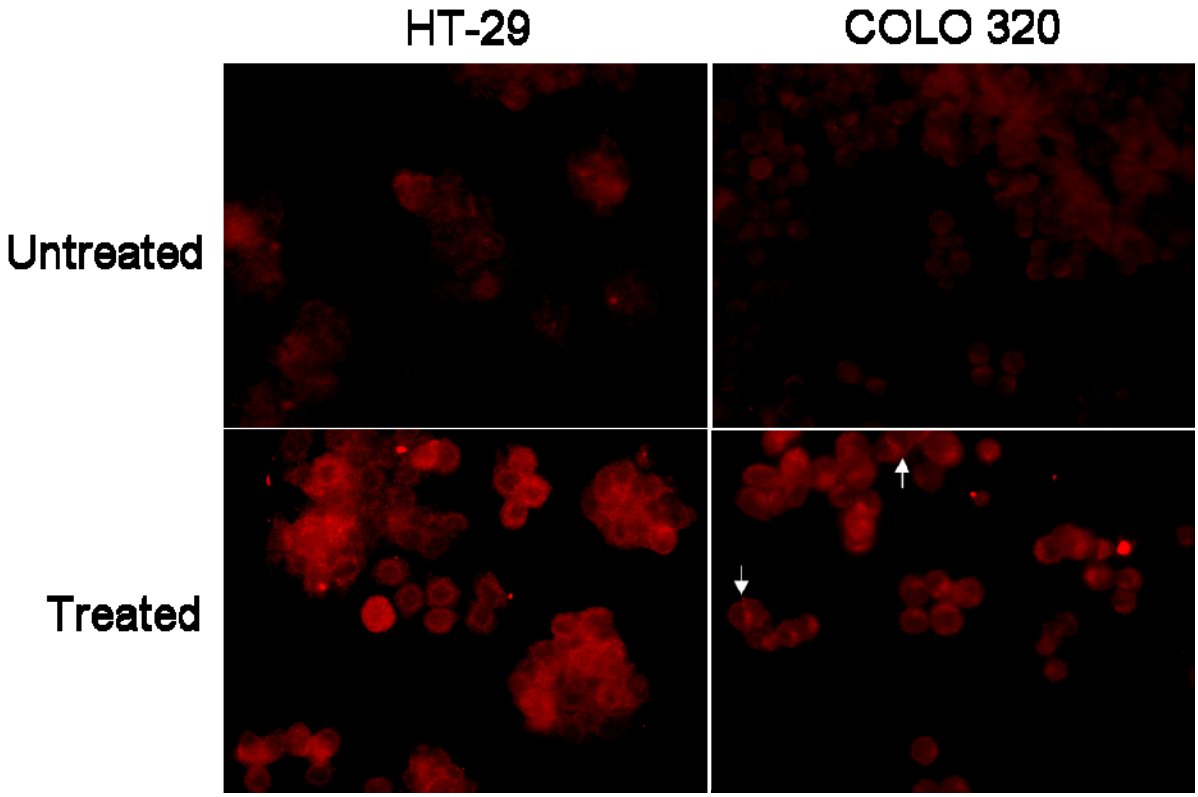


Figure 23: c-Fes Protein is Expressed in Colorectal Cancer Cells Treated with 5-aza-2'-dC

Colorectal cancer (HT-29 and COLO 320) cell lines were incubated with (Treated) or without (Untreated) 10 μM 5-aza-2'-dC for four days and then fixed and stained with a c-Fes-specific antibody. Representative fluorescent images of immunostained cells are shown. Secondary

antibodies conjugated to Alexa Fluor 594 were used to visualize c-Fes-positive immunostained cells (red).

For the human surgical specimens, three expression profiles were observed (Figure 24). For two patient samples (A: 26375 and B: 29554), undetectable/very weak c-Fes expression was observed in both normal and tumor colon tissue. For three samples (C: 20316, D: 27704, and E: 1066), weak expression of c-Fes was detected in both normal and tumor colon tissue. Last, for three samples (F: 27941, G: 27209, and H: 25465), moderate to strong expression of c-Fes was observed in normal colon tissue, while weak to no expression was observed for c-Fes in matched tumor tissue. In Summary, loss of c-Fes expression is associated with colon tumors in patients where c-Fes is moderately to strongly expressed in the normal colonic epithelium. In these three cases, loss of c-Fes protein expression in the tumor tissue correlates with increases in methylation of the c-*fes* promoter (discussed below).

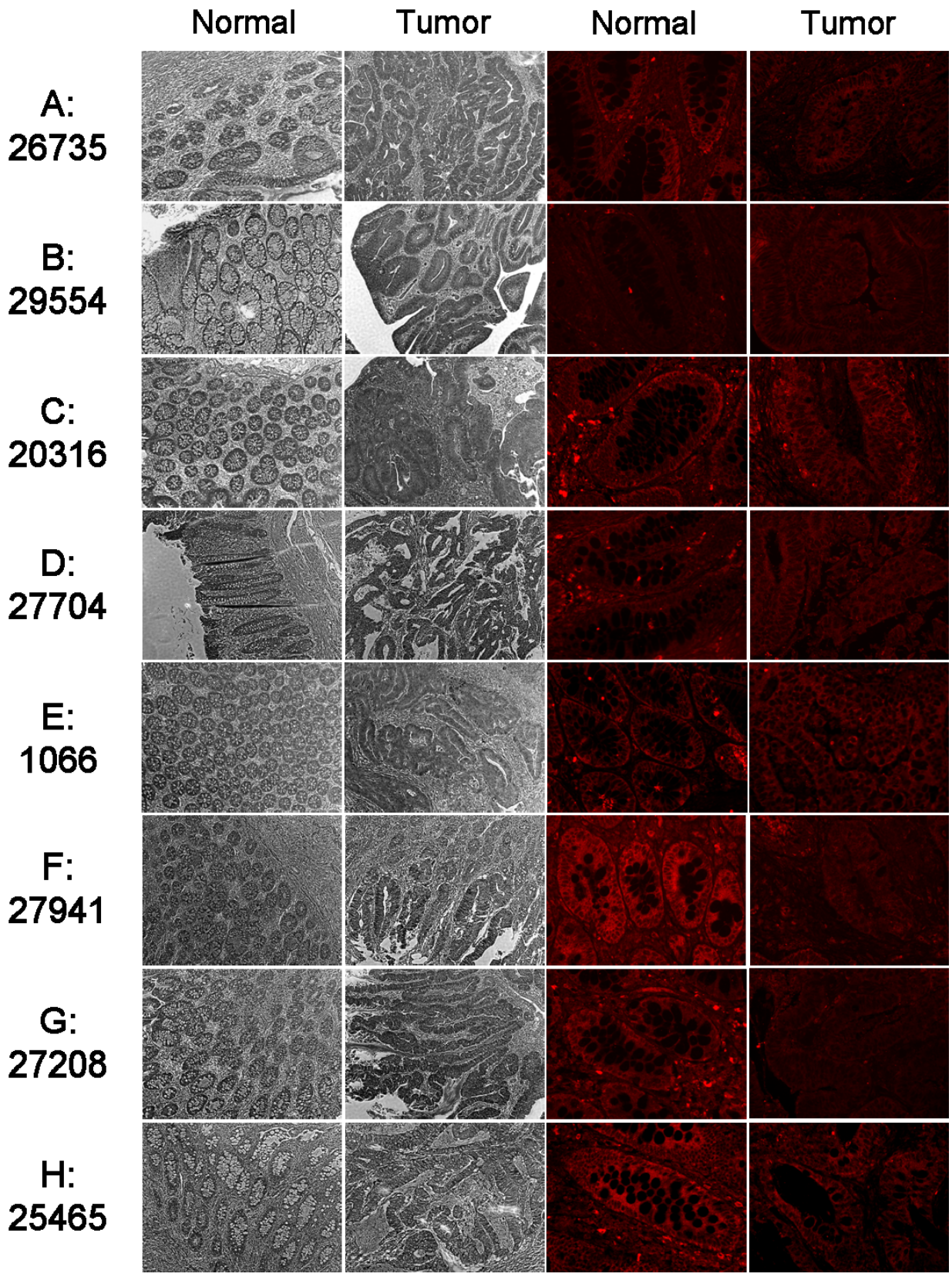

Normal
Tumor
Normal
Tumor
A:
26735
B:
29554
C:
20316
D:
27704
E:
1066
F:
27941
G:
27208
H:
25465

Figure 24: c-Fes Protein Expression is Reduced in Colorectal Cancer Tissue.

Adult human colon serial sections were either stained with hematoxylin & eosin (Columns 1 & 2; 100X) to identify normal and tumor histology or an antibody raised against the carboxyl-terminus of c-Fes to detect c-Fes expression (Columns 3 & 4; 400X). Cells positive for c-Fes were visualized with a secondary antibody conjugated to Alexa Fluor 594. Clinical details (case #: age, gender, stage): A) 26375: 82, F, T1N0Mx. B) 29554: 91, F, T3N0Mx. C) 20316: 39, F, T2N0Mx. D) 27704: 84, M, T3N0Mx. E) 1066, 69, M, T2N0Mx. F) 27941: 70, M, T2N0Mx. G) 27208: 67, F, T2N0Mx. H) 25465: 68, M, T3N2Mx.

3.10 DISCUSSION

A role for c-Fes as a tumor suppressor in colorectal cancer continues to emerge as c-Fes has been demonstrated to suppress both the growth in soft agar (67) and invasion through matrigel (J. Shaffer, unpublished observation) of colorectal cancer cell lines. In addition, Greer and colleagues have implicated c-Fes as a tumor suppressor using a mouse model of breast epithelial cancer (84). Also of interest is that loss of c-Fes expression is associated with colorectal cancer cell lines and patient derived tumors, a finding common among tumor suppressors (67). In the first part of Chapter 3, we determined that Fes gene and protein expression is suppressed in colorectal cancer cell lines through c-*fes* promoter methylation. However, the mechanism associated with repression of c-Fes in human surgical specimens has not been analyzed.

In this portion of Chapter 3, the role for c-*fes* promoter methylation in suppressing c-Fes expression was analyzed. Three patterns of c-*fes* promoter methylation were observed for the

human surgical specimens (Figure 22). First, c-*fes* promoter methylation was undetectable in either normal or tumor tissue (two cases). Second, methylation of the c-*fes* promoter was observed in both normal and tumor tissue (one case). Third, the c-*fes* promoter is mild to moderately methylated in the colon tumor tissue while weakly or not methylated in the matched normal tissue (five cases). The prevalence of the third observation suggests that methylation of the c-*fes* promoter increases as normal colonic epithelium transforms into tumors, implicating c-Fes in the process of colorectal tumorigenesis.

With respect to c-Fes protein expression, three observations were also made (Figure 24). First, c-Fes expression was very weak to undetectable in both normal and tumor colon tissue (two cases). These cases also had no detectable c-*fes* promoter methylation suggesting gross gene rearrangement at the c-*fes* locus or a post-transcriptional processing event is responsible for the silencing of c-Fes expression. Second, c-Fes was weakly expressed in both normal and tumor colon tissue (three cases). Third, c-Fes was moderately to strongly expressed in normal colon tissue, while weakly or not expressed in matched tumor tissue (three cases). In line with previous findings of our laboratory (67), this final observation suggests that c-Fes expression is reduced or absent in colorectal tumor tissue when compared to normal tissue.

Of interest is the strong correlation of c-*fes* promoter methylation to the status of c-Fes protein expression (Table 3). When the c-*fes* promoter exhibited mild methylation (1-10% of total CpG dinucleotides analyzed), c-Fes protein expression persisted in tumors (Figures 22 and 24: D & E). For these cases, an additional level of c-Fes regulation (i.e. coding domain mutations) may be present. This possibility is addressed in Section 4.2.2. However, when the c-*fes* promoter was moderately (11-20%) methylated in tumor tissue (Figures 22 and 24: F-H), c-Fes expression was reduced or absent suggesting that c-*fes* promoter methylation downregulates

c-Fes protein expression. This being said, we must exercise caution in interpreting our data as our sample size (eight patient samples, paired normal and tumor tissue) is quite small. To satisfy power (90%) and significance ($\alpha = 0.05$) requirements, at least 30 patient samples (each consisting of paired normal and tumor tissue) should be analyzed as determined by a one-sided Spearman rank correlation (Dr. James Schlesselman and The Minh Luong, University of Pittsburgh Cancer Institute Biostatistics Facility, personal communication). Nonetheless, this exciting finding further substantiates c-Fes as a tumor suppressor in colorectal cancer and warrants addition studies of c-Fes in the colon (see Future Directions).

				c-*fes* methylation		c-Fes staining	
Case	**Age**	**Sex**	**Stage**	**Normal**	**Tumor**	**Normal**	**Tumor**
26375	82	F	T1N0Mx	--	--	--	--
29554	91	F	T3N0Mx	--	--	--	--
20316	39	F	T2N0Mx	++	++	+	+
27704	84	M	T3N0Mx	--	+	+	+
1066	69	M	T2N0Mx	--	+	+	+
27941	70	M	T2N0Mx	+	++	+++	--
27208	67	F	T2N0Mx	--	++	++	--
25465	68	M	T3N2Mx	--	++	++	(+)

Table 3: Summary of c-*fes* Methylation and c-Fes Expression in Human Colon Surgical Specimens

Colon tissue sections from eight colorectal cancer patients were either bisulfite sequenced to determine c-*fes* promoter methylation or immunostained with an antibody raised against a c-Fes carboxyl-terminal peptide. Relative c-*fes* promoter methylation was scored using the following

scale (Methylation Percentage: Methylated CpG dinucleotides relative to the total number of CpG dinucleotides analyzed) : --, no methylation (0%); +, mild (1% to 10%); ++, moderate (11% to 20%); +++ , strong (21% to 30%). Relative c-Fes staining was scored using the following scale: --, undetectable expression; +, weak expression; ++, moderate expression; +++, strong expression.

4.0 DISCUSSION

4.1 SUMMARY OF MAJOR FINDINGS

c-Fes is a unique non-receptor tyrosine kinase, both in terms of its structure and biological function. Unlike other non-receptor tyrosine kinases, c-Fes does not possess an SH3 domain, pleckstrin homology (PH) domain, or negative regulatory tail tyrosine residue (12,13). Instead, c-Fes harbors a unique amino-terminal region consisting of an FCH domain followed by two coiled-coil oligomerization motifs, a central SH2 domain, and a carboxyl-terminal kinase domain. Based on *in vitro* experiments conducted in our laboratory the coiled-coil domains were originally proposed to control the interconversion of c-Fes between inactive monomeric and active oligomeric forms [described in (12,13)]. Nonetheless, the mechanism regulating c-Fes kinase activity has not been directly examined in living cells. Regarding it biological function, c-Fes has both oncogenic and tumor suppressor tendencies. On one hand, c-Fes is the normal cellular homolog of transforming sarcoma inducing retroviruses (6-11). On the other hand, c-Fes has recently been implicated as a colorectal cancer-associated tumor suppressor (67,84). This tumor suppressor role for c-Fes, however, is in its nascent stages, and mechanisms governing the downregulation of its expression in colorectal cancers have yet to be defined. Due to these knowledge gaps, the goals I set forth for my thesis project were to establish mechanisms by which c-Fes tyrosine kinase activation and colorectal cancer gene expression were regulated.

4.1.1 c-Fes Catalytic Regulation

To directly define the role of oligomerization in regulating the *in vivo* activity of c-Fes, I adapted the YFP bimolecular fluorescence complementation assay original described by Kerppola and colleagues (165,166) for use with c-Fes. For this assay, non-fluorescent amino- and carboxyl-terminal portions of the YFP coding sequence were fused to potential oligomerization partners (c-Fes or c-Fes mutants where each functional domain was mutated) and co-expressed in the same cell. The experimental readout is straightforward: fluorescence indicates oligomerization of the fused partners, while a lack of fluorescence indicate a failure of the partners to oligomerize. In contrast to the accepted dogma that wild-type, inactive c-Fes adopts a monomeric conformation [described in (12,13)], I found that wild-type, inactive c-Fes naturally adopted an oligomeric conformation. Following this, I co-expressed Hck-YF, a potent activator of c-Fes (40,71), with wild-type c-Fes and determined that oligomerization was independent of the c-Fes activation status. From there, I used point mutations to disrupt the function of each characterized c-Fes domain (CC1: L145P, CC2: L334P, SH2: R483L, and Kinase: K590E) and found that both coiled-coil domains mediate c-Fes oligomerization *in vivo* whereas mutation of either the SH2 or kinase domain did not affect c-Fes oligomerization. Last, in an effort to characterize whether oligomerization was a general property of c-Fes in physiologically relevant cellular contexts, BiFC oligomerization assays were performed in both K-562 CML and HCT 116 CRC cell lines, suggesting that c-Fes naturally adopts coiled-coil dependent oligomeric complexes *in vivo*. In summary, these findings suggest a new "twist" in the thinking of how c-Fes catalytic activity in regulated in living cells (discussed below).

4.1.2 Regulation of c-Fes Expression in Colorectal Cancer

To define the mechanism by which c-Fes expression is lost in colorectal cancers, I performed a comprehensive examination of the c-*fes* gene expression status in seven common colorectal cancer cell lines (CACO-2, COLO 320, DLD-1, HCT 116, HT-29, SNU 1040, SW-480). First, I used RT-PCR targeted at a 3' region of the c-*fes* transcript and found that the c-*fes* gene was absent or significantly reduced in five of seven lines (the exceptions being COLO 320 and HCT 116). However, upon targeting the extreme 5' region of the c-*fes* transcript, I determined that the transcripts observed in COLO 320 and HCT 116 cells were not properly initiated, suggesting that a loss of functional c-*fes* transcripts is a common finding in all tested colorectal cancer cell lines. After using Southern blot analyses to rule out loss or gross rearrangement of the c-*fes* gene, I used the EMBOSS CpG plot algorithm (181) to determine a CpG island (CpG-rich stretches of DNA approximately 1 kb in length) existed within the c-*fes* promoter. As a result of this, I treated the colorectal cancer cells with the potent demethylation agent 5-aza-2'-deoxycytidine (5'-aza-2'-dC) which irreversibly binds and inhibits DNA methyltransferases (105) to determine whether methylation inhibits c-*fes* expression in CRC cell lines. Without fail, I was able to re-express c-*fes* transcripts each CRC cell lines (and an additional CML cell lines K-562) upon treatment with 5-aza-2'-deoxycytidine as measured by RT-PCR targeting the 5' and 3' regions of the c-*fes* gene. In addition, I was able to re-express full-length c-Fes protein products in each CRC cell line. From there, I performed bisulfite sequencing on 5-aza-2'-dC treated and untreated HT-29 cells to directly implicate specific CpG dinucleotides of the c-*fes* promoter in the methylation of the c-*fes* promoter. In this assay, I found that methylation of the c-*fes* promoter dramatically decreased upon treatment with the 5-aza-2'-dC demethylation reagent. To conclude the cell line portion of this study, I used an *in vitro*

system methylation analysis to determine that methylation blocks the activity of the c-*fes* promoter. As a finale to this study, I performed bisulfite sequencing analysis on a limited number of matched normal and tumor colorectal cancer patient derived tissue samples (eight total pairs) suggesting that methylation of the c-*fes* promoter increases as normal tissue transforms to invasive adenocarcinoma. Further, this increase in methylation correlated well with loss of c-Fes protein expression.

4.1.3 Implications for c-Fes Kinase Regulation

Prior to this thesis, the working model for the regulation of c-Fes catalytic activity involved the coiled-coil domains modulating the conversion of c-Fes from an inactive, monomeric form to an active, oligomeric kinase in response to stimuli (13). This model tied the regulation of c-Fes to mechanisms governing other non-receptor tyrosine kinases such as c-Src and c-Abl where intramolecular interactions lock the kinases in downregulated, monomeric forms (164,175). However, unlike c-Src and c-Abl, structural information of full-length c-Fes has not been resolved, and the c-Fes model has been inferred from *in vitro* experiments instead of live cellular data (14,45,47) and does not account for contributions of the SH2 and kinase domains. Here, I present a new model for the regulation of c-Fes catalytic activity. As c-Fes naturally adopts an oligomeric conformation in living cells, the coiled-coil domains may lock c-Fes in a conformation where the SH2 domain cannot interact with the kinase domain, which is required for full kinase activity of c-Fes (13,17,59,62,63). Upon activation through external stimuli, conformation restraints imparted by the coiled-coil domains may be relaxed, affording the SH2 the ability to interact with the kinase domain and fully activate the catalytic activity of c-Fes. This mechanism of c-Fes regulation may be distinct among non-receptor tyrosine kinases.

As a result, small molecules that activate c-Fes may be of use in colorectal cancer cases where c-Fes protein is present, as activation of c-Fes further enhances the growth suppression of colorectal cancer cell growth (67).

4.1.4 Implications for c-Fes Expression Regulation

Recently, c-Fes has been proposed to function as a novel tumor suppressor in colorectal cancers where its loss of expression is a common finding in both colorectal cancer cell lines and patient tumor samples (67,84). In this thesis, I have defined a mechanism by which c-Fes expression is lost in colorectal cancers. Promoter methylation, a common mechanism by which tumor suppressor expression is downregulated in cancer, downregulates Fes gene and protein expression in colorectal cancer cell lines. Further, in a small sample of patients, c-*fes* gene methylation appears to increase as normal colonic epithelium is transformed to adenocarcinoma, and this increase correlates with the loss of c-Fes protein expression. As mentioned above, the full complement of improperly activated oncogenes and inactivated tumor suppressor genes associated with the multistep process of CRC carcinogenesis remains to be defined. Here, we have defined how a novel tumor suppressor is inactivated in colorectal cancers. As research has definitely implicated c-Fes as a suppressor of colorectal cancer cell growth (67) and invasion (J. Shaffer, unpublished observations), selectively re-expressing c-Fes may play a key role in the inhibition of CRC tumorigenesis.

4.2 FUTURE DIRECTIONS

4.2.1 c-Fes Oligomerization

As my thesis works suggests, conformational changes rather than oligomerization appear to regulate c-Fes catalytic activation. From this, the role that oligomerization plays in the biological responses of c-Fes remains to be defined. Possibly, c-Fes oligomerization is intimately associated with the cellular differentiation and tumor suppressor roles observed for c-Fes. Past research suggests that expression of wild-type c-Fes induces cellular differentiation (K-562 myeloid leukemia cells) or growth suppression (HCT 116 and HT-29 colorectal cancer cells) irrespective of c-Fes kinase activity (17,60,67). As a result, the main regulatory factor may be the oligomeric nature of c-Fes. To test this, hematopoietic differentiation assays (17), soft agar assays (67), or matrigel invasion assays (189) will be performed using wild-type c-Fes or point mutant c-Fes variants (L145P, L334P, 2LP) to define the role of oligomerization in Fes-mediated biological responses.

Also proposed within my thesis is the hypothesis that the coiled-coil domains within the unique amino-terminal F-BAR domain may lock c-Fes in a conformation that hinders the SH2 domain from associating with the catalytic domain to induce an active kinase. As a result, an interaction interface may be present between the amino-terminus region and the SH2-kinase domain unit. To test this, a modified PCR reaction (Diversify® PCR Random Mutagenesis Kit - Clontech) will be used to randomly insert mutations (2-8 mutations per 1,000 bp depending on buffer conditions) within the unique amino-terminus of c-Fes (190,191). The PCR products will then be cloned into a mammalian expression vector already containing the SH2-kinase domain portion of c-Fes to create a random mutagenesis library of full-length c-Fes. Using a COS-7 cell

based system where c-Fes naturally adopts an inactive conformation (40), we will first screen for mutations that activate c-Fes without disrupting oligomerization. Amino acids whose mutations meet these guidelines may be involved in a "downregulation interface" of the unique amino-terminus with the SH2-kinase domain unit.

4.2.2 c-Fes and the Colon

My thesis suggests that promoter methylation is a mechanism by which c-*fes* expression is downregulated in colorectal cancer cells, and demethylation treatments enable expression of c-*fes*. While the transcription factors that regulate c-*fes* expression in hematopoietic cell lines have been well characterized [the ubiquitous transcription factor Sp1, the myeloid specific transcription factor PU.1/Spi-1, and the c-Fes expression factor (FEF) combine to regulate c-*fes* expression (29,34,35)], the transcription factor(s) associated with c-*fes* expression in colonic or other epithelial cell types have not been characterized. Based on a transcription factor database search (http://motif.genome.jp/MOTIF.html) for proteins that interact with the c-*fes* promoter, the gut-enriched Kruppel-like factor (GKLF) that is highly expressed in the colonic epithelium may be a candidate factor that regulates c-*fes* expression within the colon (192-195). This possibility will be evaluated using chromosome immunoprecipitation (ChIP). In addition, RNase protection assays will be performed to establish what transcription factor interact with the c-*fes* promoter to induce transcription within the colonic epithelium.

Further, with regards to promoter methylation, the specific CpG dinucleotides whose methylation drives the suppression of c-*fes* transcription in colorectal cancer have not been isolated. To determine potential candidates, specific *in vitro* methylase reactions (*FnuD*II, *Hha*I, and *Hpa*II), similar to our *Sss*I *in vitro* methylation reaction performed in Figure 21, will be

performed in conjunction with gene reporter assays (179). This will also lend insight into which transcription factors are prohibited from binding the c-*fes* promoter to induced transcription either directly or through the binding of methyl binding proteins. In addition, ChIP assays will be performed to determine whether MeCP1 and MeCP2 are involved in repressing c-*fes* transcription in colorectal cancer cell lines.

In addition to regulation of c-Fes expression at the level of the c-*fes* gene, c-Fes catalytic activity is regulated through coding region mutations. Initially, c-Fes was linked to colorectal cancer as being one of only seven genes that exhibited consistent kinase domain mutations in 182 colorectal cancer cell lines or xenografts that were analyzed (88). These mutations were subsequently determined to downregulate or abolish c-Fes kinase activity (67,84). Due to this, the coding region of each of our colorectal cancer cell lines was sequenced for mutation. In two cell lines, DLD-1 and SNU 1040, the A at bp 2068 was substituted with a C resulting in a Leu to Met change at amino acid 690 that also killed c-Fes kinase activity (data not shown). As a result, it is also pertinent to establish whether other coding sequence mutations of c-Fes exist that are associated with colorectal cancer. To determine this, total RNA from patient derived tissue samples will be isolated and reverse-transcribed. From there, we will PCR amplify and sequence the coding region of c-Fes for mutation. Any observed mutations will be screened for their effects on c-Fes oligomerization and kinase activity as well as the apparent colorectal cancer growth and invasion suppression phenotypes associated with c-Fes expression in colorectal cancer cell lines.

Finally, as the role of c-Fes in colorectal cancer continues to evolve, various research avenues are open including what function does c-Fes possess when expressed in the normal colonic epithelium and what are c-Fes tumor suppressor targets. Based on its history of

involvement with cellular differentiation responses of myeloid, vascular endothelial and neuronal cells (see overall introduction), it is tempting to speculate that c-Fes functions similarly in the colon. We will be able to test this hypothesis in two ways. First, wild-type or activated variants of c-Fes can be over-expressed in normal colonic epithelial cells (196,197) to test for changes in gene or protein expression of differentiation markers such as MSH2, BCL2, Ki67, p27, p21, Chromogranin A, and Lysozyme (198-201). Second, once the expression of c-Fes has been verified in the normal colonic epithelial cells, siRNA directed at c-Fes can be performed to determine whether inhibition of c-Fes leads to the suppression of differentiation markers. Regarding tumor suppressor targets, one possibility is APC. APC is expressed in the gut epithelium, and its microtubule regulation capacity plays a prominent role in the migration, division, and differentiation processes of the gut (202). Without the microtubule stabilization of APC, gut epithelial cells may accumulate in the initial transformation stages. In line with this, the APC microtubule binding domain is deleted in transforming mutants of APC that result in sporadic and familial colon cancer progression (203-205). Like c-Fes, APC possesses an amino-terminal coiled-coil oligomerization domain and a carboxyl-terminal microtubule binding domain (202). c-Fes, through its coiled-coil motifs and microtubule binding, could function as a tumor suppressor by complementing this microtubule regulation activity when APC is truncated. Preliminary data indicates that both c-Fes and APC localize to microtubules in transfected COS-7 and that c-Fes can strongly phosphorylate a truncated APC variant in Sf-9 insect cells (data not shown). The consequences of either these microtubule localizes are unknown; however, c-Fes could either act in place of truncated APC to regulate microtubules, or restore the localization of APC to microtubules by interacting with APC through their common coiled-coil motifs.

4.3 CLOSING REMARKS

More than twenty-six years ago, c-*fes* was first identified as the normal cellular homolog of the transforming oncogene associated with avian and feline retroviruses. Since that time, the strictly regulated c-Fes kinase has been implicated in hematopoietic, vascular endothelial, and neuronal differentiation responses. Most recently, a tumor suppressor role for c-Fes in colorectal cancers has been proposed. However, prior to this thesis, mechanisms regulating c-Fes catalytic activity in cells and colorectal cancer expression have remained largely undefined.

This thesis fills c-Fes knowledge gaps in two ways. First, the mechanism of *in vivo* c-Fes catalytic regulation has been further defined. Through direct *in vivo* fluorescence studies, I was able to propose a mechanism by which conformation rather than oligomeric changes govern c-Fes kinase activity. Second, as the tumor suppressor role for c-Fes in colorectal cancers is in its nascent stages, the mechanisms governing the downregulation of its expression in cancer were assessed. Through extensive promoter demethylation analyses, I was able to conclude that methylation governs c-Fes expression in colorectal cancer. This revelation further substantiates a tumor suppressor role for c-Fes, as promoter methylation is a common mechanism by which tumor suppressors are inactivated in cancers.

In conclusion, the unique non-receptor tyrosine kinase c-Fes has an increasingly broad biological role in the human body. It is with great hope that this thesis advances the knowledge of a novel tumor suppressor gene associated with colorectal cancers.

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