AUTOREGULATION OF C-FES BY INTRAMOLECULAR INTERACTION OF ITS F-BAR AND SH2 DOMAINS
A novel role for the unique N-terminal region

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The c-Fes protein is a structurally unique non-receptor protein-tyrosine kinase encoded by the *fes/fps* proto-oncogene and is expressed in myeloid hematopoietic, vascular endothelial, and some epithelial and neuronal cell types. Downstream of a number of cytokines and growth factors, c-Fes plays a role in regulation of cell growth, differentiation, and chemotaxis. Dysregulation of c-Fes activity is implicated in a number of cancers, including acute myelogenous leukemia (AML) and some renal carcinomas. Structurally, c-Fes includes an amino-terminal F-BAR region—consisting of a Fes/CIP4 homology (FCH) domain and the first of two coiled-coil motifs (CC1)—followed by CC2 and the carboxy-terminal Src homology 2 (SH2) domain closely linked to a typical bilobate kinase domain (KD).

Unlike its oncogenic retroviral orthologs (v-Fes/Fps), c-Fes kinase activity is tightly regulated *in vivo,* yet lacks known negative regulatory features common to other cytoplasmic tyrosine kinases, such as a SH3 domain or regulatory tail tyrosine. In fact, a c-Fes truncation containing only the SH2 and KD is constitutively active which suggests a possible auto-inhibitory role for the unique N-terminal region. Deletion and mutation experiments reveal that loss of the predicted CC1 structure leads to upregulation similar to the c-Fes SH2-KD truncation. This would be the first case of autoinhibition by the coiled-coil; however, it is still unclear how the F-BAR acts *in cis* to accomplish autoinhibition. Substitution of the native c-Fes SH2 with that of v-Src (but not homologous v-Fps) displays an uninhibited catalytic activity level

analogous to the CC1 mutant, suggesting interdomain interaction between the F-BAR and SH2/KD may contribute to an inactive conformation. I collected evidence for this mechanism and began to develop tools to define the putative F-BAR:SH2 interface utilizing the bimolecular fluorescence complementation (BiFC) technique. Demonstrated by BiFC in 293T cells, the F-BAR interacts with the SH2 domain in a CC1-dependent manner. It may be that CC1 forms a critical interface with αB of the SH2 as preliminary data suggest that v-Src-mimetic mutations in αB weaken CC1-SH2 recognition. Further characterization of the specific intramolecular interactions involved in c-Fes regulation would advance new paradigms in modular domain relationships and could expand cancer drug discovery efforts.

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1.0 C-FES TYROSINE KINASE

In a distinct and small subfamily of the non-receptor protein-tyrosine kinome, the human *c-fes/fps* proto-oncogene encodes the unique c-Fes tyrosine kinase (1, 2). Though identified originally as the human homolog of transforming retroviral oncoproteins (3), c-Fes catalytic activity is finely tuned to certain cell-surface signaling pathways *in vivo* (discussed in section 1.3). The N-terminal region, making up over 55% of the protein, is a distinctive feature of c-Fes. At this end is a Fes/CIP4 Homology (FCH) region followed by two coiled-coil homology domains which have essential roles in the regulation of kinase activity and mediate c-Fes oligomerization *in vivo* (discussed in sections 1.2 and 1.3). Together the FCH and CC1 domains define an FCH-Bin/Amphiphysin/Rvsp (F-BAR) domain (see section 1.2.1). At the C-terminal end is a Src homology 2 (SH2) domain associated with the kinase domain. Examination of the crystal structure of the SH2 closely associated with the kinase domain has elucidated the mechanism of positive regulation of the kinase domain (discussed in section 1.3.1). Over the following five chapters, this dissertation reviews the characteristics of the Fes gene, protein structure, catalytic regulation, biological function, and role in disease.

1.1 CHARACTERIZATION OF THE C-FES GENE

1.1.1 Discovery and cloning of retrovirus and human genes.

On region 2, band 6, sub-band 1 of the long arm of the human chromosome 15 is *c-fes*, a 13.2 kb stretch that encodes a discrete member of the Fes/Fer subfamily (4). Initially, *fes* DNA was isolated as a transforming oncogene cloned from avian (*v-fps*, Fujinami) and feline (*v-fes*, Gardner-Arnstein [GA], Snyder-Theilen [ST], and Hardy-Zuckerman) sarcoma retroviruses (5-8). Several research groups in the first half of the 1980s independently identified the human gene (*c-fes*) by its high degree of sequence homology to the viral (*v-fes/fps)* oncogenes (3, 9-11). The sequence of the human *c-fes* gene was first reported by Roebroek et al. in 1985 and consists of 19 exons. The sequence revealed a translation start site in exon 2 and the translation stop codon (TGA) within exon 19, 200 bp upstream of a poly(A) site (12).

1.1.2 Sequence and structural homology with viral oncogene.

Despite significant sequence homology, human c-Fes is non-transforming and displays restricted catalytic activity, whereas the transforming v-Fes/Fps viral orthologs exhibit constitutive tyrosine kinase activity (2, 13-15). The overall structural organization of mammalian (c-Fes) and avian (c-Fps) orthologs are the same: an N-terminal unique region (F-BAR), including at least two coiled-coil domains, central SH2 domain, and C-terminal kinase domain. Shown in Figure 1 (below c-Fes) are the structures of three Fes-homologous oncoproteins: (top down) the Fujinami avian sarcoma virus v-Fps followed by the Gardner-Arnstein and Snyder-Theilen feline variants. In all three, the predominant structural modification is an N-terminal-

fused viral Gag sequence (3, 16-18). The *fes* gene in the feline viral strains lacks various exons that encode for N-terminal features like CC1 or CC2 in ST and GA, respectively. Any one or a combination of these structural variations could account for the activation of the kinase, not to mention more subtle changes and mutations. Transformation studies with mutant and chimeric constructs (discussed later) address these possibilities (18-22).

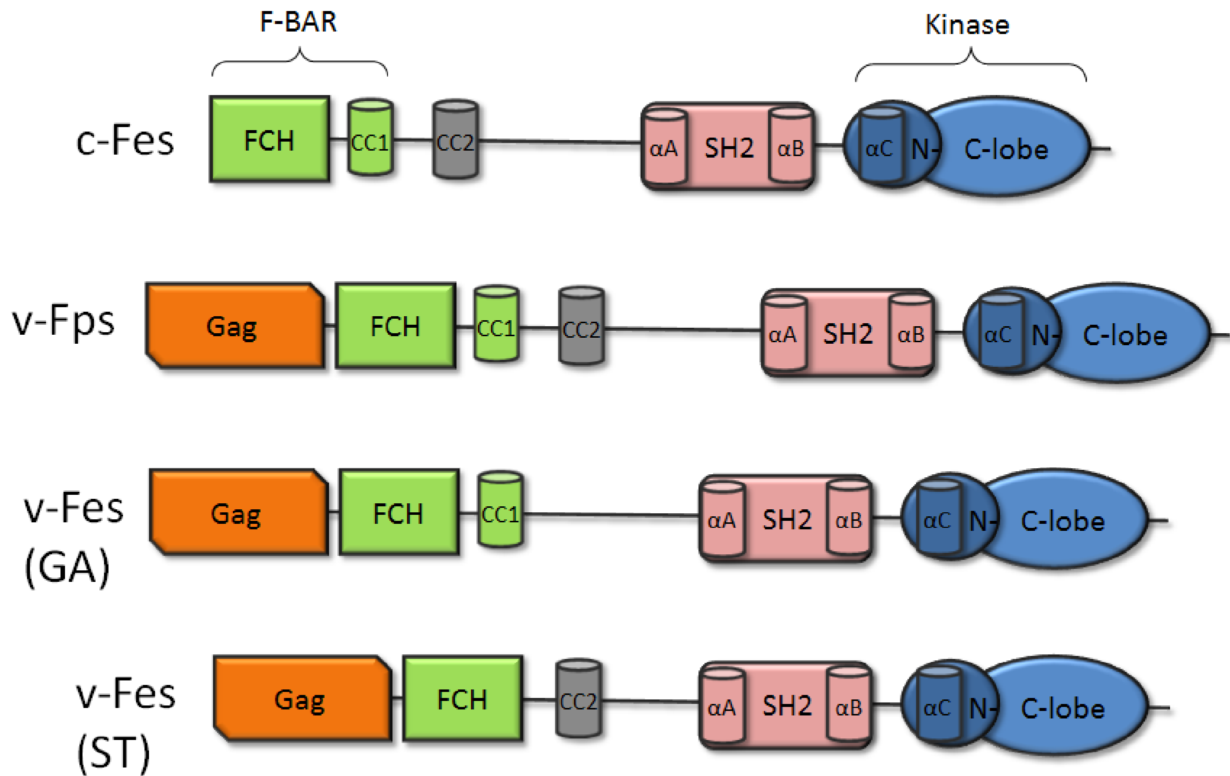


Figure 1. Structural aspects of c-Fes and viral oncogene homologs. From amino- to carboxyl-terminus: c-Fes is made up of an N-terminal F-BAR domain, which is composed of a FesCIP4 homology (FCH) domain and a coiled-coil (CC1), a second coiled-coil (CC2), a Src homology 2 (SH2) domain, and a bilobed tyrosine kinase domain. Homologous viral oncogenes *v-fps* and *v-fes* have viral gag sequences fused upstream of sequences derived from cellular genes. Below mammalian c-Fes, descending: v-Fps from Fujinami avian sarcoma virus, v-Fes from Gardner-Arnstein feline sarcoma virus, and v-Fes from Snyder-Theilen feline sarcoma virus. (Not drawn to scale or with true orientation of all features.)

1.1.3 *c-fes* gene region features.

The whole 13.2 kb human *c-fes* gene (15.q26.1) encompasses 19 exons (Figure 2). Exon 1 is noncoding, the site of transcription initiation (discussed above). The translational start site is found within exon 2, which encodes the N-terminal FCH domain. The first coiled-coil is encoded by exons 4 and 5, while exon 8 codes for the second. The central SH2 domain is coded for by exons 11 and 12 followed by exons 13 through 19 encoding the catalytic domain. Exon 19 harbors the translation stop codon (TGA) and a downstream polyadenylation signal. (12)

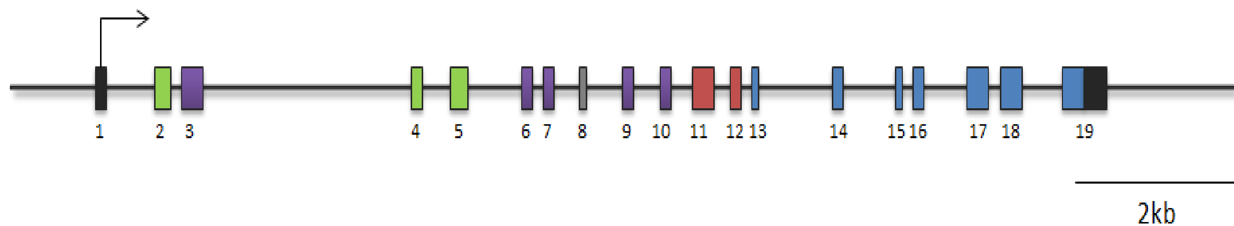


Figure 2. Major features of c-fes gene. The *c-fes* gene is arranged as 19 exons. The noncoding (black) 1st exon holds the transcription initiation site (arrow). Exon 2 (green) encodes the FCH domain. Exons 4-5 (green) encode CC1. Exon 8 (gray) encodes CC2. Exons 11-12 (red) encode the SH2 domain. Exons 13-19 (blue) encode the tyrosine kinase domain. The remainder of exon 19 is noncoding since it harbors the translation termination codon and poly(A) addition site.

The *c-fes* locus is a self-contained genetic element, containing all the necessary transcription regulatory elements in a space of 446 bp and tissue-specific promoters in less than 2 kb of 5'- and 3'-flanking sequences (23-25). In the *c-fes* promoter, 72-83 bp upstream of the 3' end of exon 1, there are two major transcription initiation sequences (26, 27). Having more than one initiation site can occur in cases where TATA box or Inr (initiator) sequences are lacking (28-30), like with c-Fes. The entire promoter is defined as -425/+91 from the first initiation site (+1), where the core promoter for the transcription initiation complex is located at -131/+91 (25, 31). Within this critical core promoter there is one *fes* expression factor (FEF; 5'-GAATCA-3'),

two hematopoietic-specific PU.1/Spi-1 (5'-AGGAA-3'), and three Sp1 (5'-CCGCCC-3') recognition sites (25, 32). The Sp1 transcription factor is ubiquitously expressed transcription factor Sp1 (25). While FEF and PU.1/Spi-1 factor expression is tissue-specific, mutation of their recognition sequences drastically reduces *c-fes* promoter activity in reporter gene assays (25, 32). Such studies suggest these transcription factors are critical to *c-fes* expression.

In terms of negative regulation of *c-fes* transcription, a region within intron 1 has been identified by deletion analyses (26). Reporter gene expression was halved when suppressed by this regulatory element (26). Southwestern and DNA footprinting analyses of K-562 myeloid cell nuclear extracts reveal the negative regulatory region localized to a 14 bp span starting at position +441 (5'-TGCGTGTGAGCGAG-3'). Interestingly, this sequence is homologous to Interleukin-3 gene negative regulatory elements (26, 33).

1.2 STRUCTURE OF THE C-FES PROTEIN

Human c-Fes (UniProtKB P07332) is an 822 amino acid (93 kDa) cytoplasmic protein that is made up of an N-terminal F-BAR domain (FCH and CC1), a second coiled-coil, and a C-terminal SH2 domain closely linked to a kinase domain (see Figure 1). It lacks an SH3 domain, a pleckstrin homology (PH) domain, or a negative regulatory tail tyrosine, which are common modular domains in other non-receptor tyrosine kinases (e.g. Abl, Src, Tec/Btk, and Zap70). The unique region of Fes/Fer is on the N-terminal end, upstream of the SH2. (1, 2, 15, 34)

1.2.1 F-BAR region.

The FCH domain component of the F-BAR is not found in other non-receptor protein tyrosine kinases, although originally named for Fes and Cdc42-interacting protein (CIP4) homology (35). Many FCH-containing proteins are adaptors associated with endocytosis, vesicular transport, and cytoskeletal remodeling (36). Due to these trends, it has been proposed that the c-Fes FCH domain has some role in microtubule dynamics. Indeed, FCH domain deletion results in loss of murine Fes co-localization with microtubule nucleation sites and abolished microtubule nucleation (37). Furthermore, reintroduction of Fes into Fes-deficient MEFs rescued the aberrant microtubule and centrosome phenotype. Later, it was shown that the FCH domain mediated interaction with tubulin monomers, which might suggest c-Fes facilitates tubulin polymerization *in vivo* (38).

Adjacent to c-Fes FCH domain is the first of two predicted coiled-coil domains (39, 40). A coiled-coil is a super-helical bundle of several parallel or antiparallel amphipathic α-helices coiled together (41). The α-helices consist of heptad repeats of amino acids arrayed in a pattern where every first and fourth residues form a hydrophobic interface, while the rest are hydrophilic, forming a solvent-exposed surface. In the superhelix, a hydrophobic sidechain packs into space shielded by the four surrounding hydrophilic residues like “knobs into holes” (41). There is evidence that coiled-coils are involved in numerous biological activities including protein oligomerization and scaffolding (42).

Previous work from our lab strongly implicates the c-Fes coiled-coil domains in the regulation of Fes oligomerization and kinase activity. When wild-type c-Fes is expressed in COS-7 cells, it is catalytically repressed and for the most part displays a diffuse cytoplasmic distribution. In striking contrast, a c-Fes CC1 point mutant (L145P) exhibits greatly elevated

catalytic activity and microtubular localization. This finding suggests that CC1 (and the F-BAR) may play a role in regulation of kinase domain activity.

Together, the FCH and CC1 domains comprise the F-BAR domain. Proteins in the F-BAR domain family are often at the cell membrane acting as multi-functional adapters involved in processes like endocytosis and cell motility (36).

There is no published literature on the X-ray crystal structure of the c-Fes F-BAR domain, (see Appendix A) however it is likely to be very similar to F-BAR domain subfamily members like Fer and $FCHSD_{1/2}$ (43). Homology modeling of the Fes F-BAR domain to FBP17 (44) identified conserved basic residues (R113/K114) implicated in binding phosphoinositides. *In vitro,* the F-BAR domain indeed binds phosphoinositides unless the conserved basic residues are mutated to uncharged residues, such as glutamine (45). In mast cells expressing the RK-QQ Fes mutant, FcepsilonRI-induced Fes tyrosine phosphorylation and Lyn-containing membrane fraction localization was defective (45).

1.2.2 CC2 and linker region.

Coiled-coil 2 follows CC1, but is separate from the F-BAR domain. This second coiled-coil may be important for oligomerization, but identical mutations in CC1 and CC2 have vastly different effects on kinase activity (22, 46). Like in oligomer transactivation, CC2 may help recruit other activators or substrates of Fes. For example, the transcriptional co-repressor KRAB-associated protein (KAP)-1 was pulled out of Fes CC2-baited yeast two-hybrid screens (47). Follow-up experiments in yeast demonstrated that KAP-1 interacts with c-Fes, and in Sf-9 cells the KAP-1 coiled-coil domain is sufficient for binding with the Fes N-terminal unique region. HL-60 myeloid leukemia cells express Fes and KAP-1 endogenously and the two were found in

association in that context. Finally, in human embryonic kidney cells, full length Fes and KAP-1 co-expression resulted in both Fes autophosphorylation and phosphorylation of KAP-1 tyrosine residues, suggesting KAP-1 may be both an activator and downstream effector of Fes. (47)

The CC2-SH2 linker is over 92 amino acids in length (48). Largely disordered, apart from a few predicted helices and a turn, this linker is probably flexible to accommodate *trans* interactors (substrate, adapters, etc.) and perhaps permit *cis* interactions between the features of the unique N-terminal region and the downstream SH2-kinase core.

1.2.3 SH2 domain.

Found in hundreds of proteins, SH2 domains are conserved signaling modules that recognize specific phosphotyrosine peptide sequences in order to mediate signal transduction (49-51). Phosphopeptide library screens, biophysical, biochemical, and genetic studies on the selectivity of SH2 domains for their phosphotyrosine substrates revealed that binding affinity can be attributed to the phosphate moiety of the target, while residues within two to six positions from that phosphotyrosine usually determine specificity (52-58). Recruitment of effectors by the SH2 domains of cytokine and growth factor receptors has been extensively characterized, but these modular domains were actually discovered in non-receptor tyrosine kinases like Fes (50). The v-Fps SH2 was one of the first described and shown to be necessary for full kinase activity (59) and substrate recognition (60). Like in viral Fps, the c-Fes SH2 domain plays a dual role in modulation of kinase activity and biological impact. *In vitro* Fes substrate phosphorylation and autophosphorylation are significantly impaired by deletion of the SH2 domain (61, 62). *In vivo*, substitution of the whole Fes SH2 with that of v-Src results in greatly upregulated Fes kinase activity (21). Together, these studies implicate the c-Fes SH2 domain function in both positive

and negative regulation of kinase activity. Unlike Src family kinases, the c-Fes SH2 domain is disengaged from the kinase domain in inactive Fes. This way the SH2 domain may be more accessible to other Fes domains, adapters, or substrates.

The close proximity of the SH2 and kinase domains prompted the idea that the two may interact *in cis* to bring about a more stable active kinase domain. Such speculation is consistent with the fact that mild proteolysis of v-Fps generates a stable SH2-kinase domain core fragment that highly active. Confirming the positive regulatory role of the SH2, mutations that affect kinase function likewise affect the SH2-kinase domain interaction (62, 63). X-ray crystallography provides direct evidence for the regulatory interaction of the c-Fes SH2 and kinase domains, revealing a helical interface between the SH2 domain and the N-terminal lobe of the kinase domain (64). More detail on this follows in section 1.3.1; refer to Figure 3.

In addition to c-Fes kinase domain regulation, an intact SH2 domain is also required for biological function. The c-Fes/v-Src SH2 chimera localizes to focal adhesions in transformed rodent fibroblasts (21), which suggests that a shift in subcellular localization may influence its catalytic activity (or vice versa) and the SH2 may influence both localization and catalysis. Additionally, wild-type c-Fes exhibits association and phosphorylation at focal adhesions, acting on p130 Cas and other macrophage focal adhesion proteins (65). The same is true in epithelial cells where the interacting partner and substrate is Ezrin, an adapter protein involved in membrane-cytoskeleton attachment (66), where the Fes SH2 domain recognizes Ezrin pTyr477. This relationship is necessary for HGF-induced cell scattering. In COS cells transfected with active mutants of c-Fes, the SH2 domain mediates association of Fes with the tubulin cytoskeleton (38). *In vitro* without any accessory proteins, purified recombinant c-Fes phosphorylates purified tubulin and catalyzes tubulin polymerization (38). Altogether, these data

support the idea that c-Fes regulates microtubule dynamics by phosphorylation and interactions dependent on its SH2 domain.

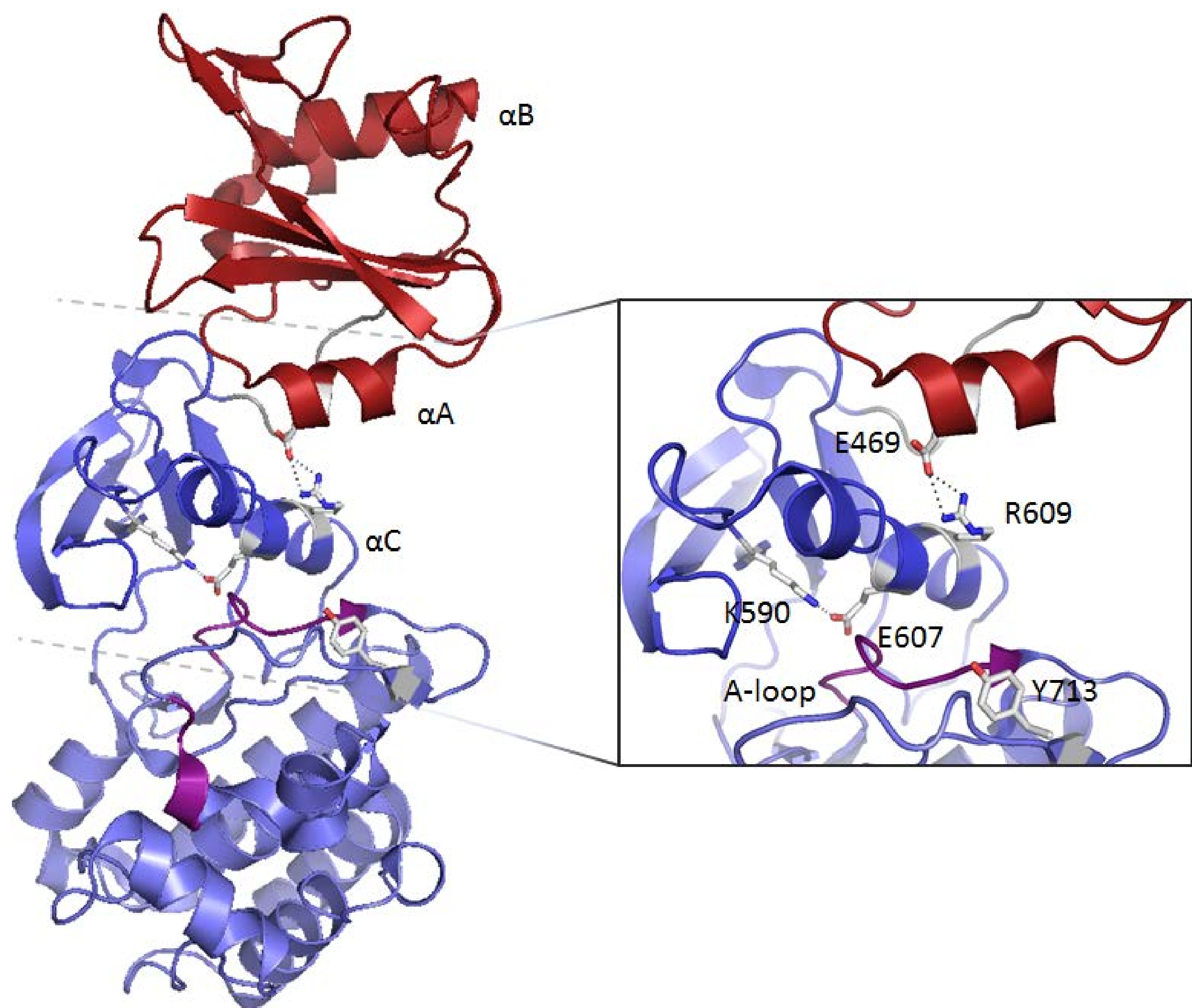


Figure 3. Crystal Structure of c-Fes SH2-kinase domain core. The full c-Fes SH2-kinase unit (amino acids 448-822) is shown on the left (PDB: 3BKB). The helical interface of the SH2 (red) and kinase (blue) domains is enlarged on the right. Interaction of the αA helix of the SH2 domain and the αC helix of the kinase domain N-lobe is stabilized by the SH2 E469 contacting the αC R609, maneuvering the αC helix into a pro-catalytic conformation. Salt bridging between K590 and E607 further stabilizes the primed state. The side chain of the activation segment (purple) autophosphorylation site, Y713, is also shown. Mutational analysis verified that both tight packing and electrostatic interactions shown are crucial for this stable, pro-catalytic form of the SH2-kinase core. (64)

1.2.4 Kinase domain.

Localized to the C-terminal region of c-Fes is a typical bilobed tyrosine kinase domain. (Refer to Figure 3, above.) This domain is comprised of 261 amino acids (561-822), including a highly conserved lysine (K590) within the ATP-binding pocket and the autophosphorylation site (Y713). (1, 67) The pTyr713 is located on the activation loop and is required for full activation of the kinase domain (61, 68), much like c-Src Y416 (69). This autophosphorylation aids in stabilization of the activation segment in its catalysis-competent conformation. Unlike some other non-receptor tyrosine kinase domains, Fes lacks a membrane-targeting signal (e.g. Abl myristate binding site) or regulatory tail (e.g. Src pY530).

Helix αC is located on the N-lobe of the kinase domain and forms a helical interface with the SH2 (discussed later). Cooperation of key residues, phosphotyrosines, a-loop, substrate, and SH2 domain achieves the most efficient kinase. Mutation or loss of any of these critical features can affect the enzymatic function of the kinase domain. Catalytically, the kinase domain coordinates phosphate transfer from ATP onto the target tyrosine sidechain acceptor: ATP + [protein]-L-tyrosine = ADP + [protein]-L-tyrosine phosphate. Reported substrates include BCR (70), HCLS1/HS1 (45), PECAM1 (71), STAT3 (72, 73), TRIM28 (47), tubulin (38), as well as Fes molecules (autophosphorylation). Fes kinase domain activity is implicated in several cellular functions discussed in section 1.4.3.

1.3 REGULATION OF C-FES ACTIVITY

Wild-type c-Fes catalytic activity is precisely regulated *in vivo* unlike the retroviral oncoproteins. Phosphoamino acid analyses of c-Fes immunoprecipitated from cells following metabolic labeling with $^{32}PO_4$ provided the first evidence of this phenomenon (74). Consistent with a complete lack of c-Fes autophosphorylation, no phosphotyrosine was detectable; only phosphoserine was found. The same conclusions were drawn with Rat-2 fibroblast transformation assays. Even when expressed to high levels, c-Fes was not sufficient to transform rodent fibroblasts (13). Transforming activity of c- Fes was not reported until exceedingly over-expressed in NIH 3T3 cells (14). The exact conformation adopted by full-length c-Fes when inactive or active, monomeric or oligomeric, wild-type or mutant is not known. What is known about c-Fes regulation provides the basis for hypothetical models.

1.3.1 Positive regulation by the SH2:Kinase domain interaction.

There are multiple modular domains comprising c-Fes, however the most proximal to the kinase domain is the SH2 domain. Several studies suggest that the central SH2 domain regulates c-Fes kinase activity. *En bloc* substitution of the Fes SH2 domain with variants results in altered Fes kinase activity: upregulation in the case of v-Src SH2 or downregulation with Ras-GAP SH2 (21, 46). Partial proteolysis of homologous v-Fps releases a stable, highly active 45 kDa core protein containing only the SH2 and kinase domains, which supports SH2-kinase domain interaction is necessary and sufficient for full kinase activity (61-63). The X-ray crystal structure of the stable, active c-Fes SH2-kinase unit shows that the SH2 domain sits atop the kinase domain, structurally organizing and stabilizing the kinase domain for activity. For example, the

critical α-helix C is packed close with αA of the SH2 domain (64). Accordingly, mutation of key residues solved in the SH2:kinase interface abrogates kinase activity. All the above findings support the notion that the SH2 domain is a positive regulator of c-Fes activity.

There is no crystal structure of full-length c-Fes, but the high-resolution structure of the SH2 domain and adjacent kinase domain from the Knapp group reveals many details of the active SH2-kinsae domain complex (64). Confirming previous reports that the SH2 domain positively regulates kinase activity, the crystal structure displays extensive, tight packing of the SH2 against the N-lobe of the kinase domain. Unlike the c-Abl or c-Src kinases, c-Fes has a short SH2-kinase linker and a glycine residue (G463) that allow for the close association of SH2 and kinase domains. This is stabilized by interdigitation and electrostatic interactions. SH2 αA helix glutamate residues (E469/472) interact with an arginine (R609) in the αC of kinase domain (see Figure 3), stabilizing the αC helix in a favorable orientation for communication (E607-K590) with a lysine in the active site. Thus the kinase domain is set for enzymatic activity. As predicted, mutation at these key sites can result in loss of both SH2-kinase domain interaction and catalytic activity. Predicted to hamper packing of the SH2 against kinase domain N-lobe, G463V abrogated kinase activity of the full-length Fes. Likewise, E to K mutation at 469 and 472 diminished kinase activity as there was not the SH2 salt bridge network with the N-lobe. Co-crystallization of the Fes SH2-kinase core with a substrate peptide demonstrated how substrate binding further stabilizes the activation-loop by inducing a β-sheet between the substrate peptide and activation-loop, cooperatively strengthening SH2:kinase N-lobe interaction and the active conformation. (64)

Supporting the role of the SH2 substrate recruitment in promoting catalytic activity, loss of SH2-ligand interaction resulted in loss of the SH2:kinase domain interaction, rendering the αC

helix highly mobile. Accordingly, abolishing phosphotyrosine binding by a point mutation in the SH2 domain greatly diminished Fes kinase activity in a cell-based assay. Therefore, it is reasonable to envision that a series of coordinated events is needed for induction of c-Fes kinase activity (see Figure 4). In the inactive state, the activation loop is disordered, helix αC is not engaged with the SH2 helix A, no substrate ligand is bound to the SH2 domain and Y713 is unphosphorylated. Substrate recruitment by the SH2 domain induces stable interaction with the kinase domain and positions αC for enzymatic activity. Then the primed kinase domain autophosphorylates, further stabilizing the activation loop, thus the kinase domain assumes the fully active state.

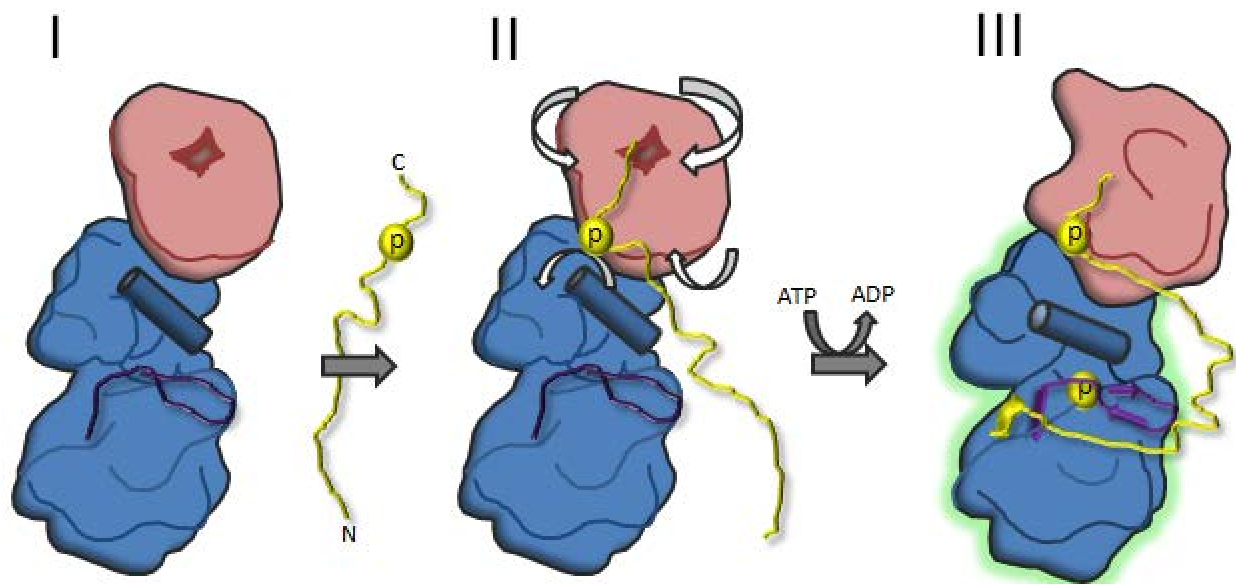


Figure 4. Model of c-Fes SH2, kinase domain, and substrate in concert during activation. I) In its least stable form, the SH2 is unligated and Y713 is unphosphorylated. The Fes SH2 domain (red), αC (dark blue), and activation loop (purple) of the kinase domain (light blue) remain disordered and inactive. II) Recognition of phosphorylated substrate (yellow) by the SH2 domain induces structural ordering (white arrows) of the SH2, stabilization of

SH2:KD interface, and rotation of αC into an orientation primed for activity (white arrow). Autophosphorylation of Y713 stabilizes the a-loop and is required for the fully active form. (64)

1.3.2 Oligomerization.

As discussed previously, the coiled-coil oligomerization motifs were suspected to mediate Fes oligomerization. Experimentally, deletion of the unique N-terminal region eliminated Fes oligomerization, while deletion of the SH2 and kinase domains did not alter the Fes oligomer profile (40). Gel filtration analyses of lysates derived from cells expressing full-length Fes revealed that active c-Fes was exclusively oligomeric and could form homopentameric complexes. Based on *in vitro* data (22, 46), the coiled-coil oligomerization domains mediate conversion of c-Fes between inactive monomers and active oligomers in one possible regulatory model (Figure 5). In this model, CC1 interacts with CC2 and holds c-Fes in an inactive monomeric state. When the negative regulatory CC1 domain no longer restrains CC2, c-Fes forms oligomers and undergoes autophosphorylation, achieving full activation. Transactivation in homo-oligomers would ramp up the response. A major difficulty with this model is since it is largely based on *in vitro* studies excluding the SH2 and kinase domains. Later studies showed that it was possible that wild-type c-Fes also forms constitutive oligomers (75, 76), which does not support a model in which c-Fes is a monomer in its inactive state. As it turns out, c-Fes forms oligomers in live cells independently of its activation status as demonstrated by BiFC (76). These data suggest that some other regulatory mechanism, rather than oligomerization, controls c-Fes activity *in vivo*.

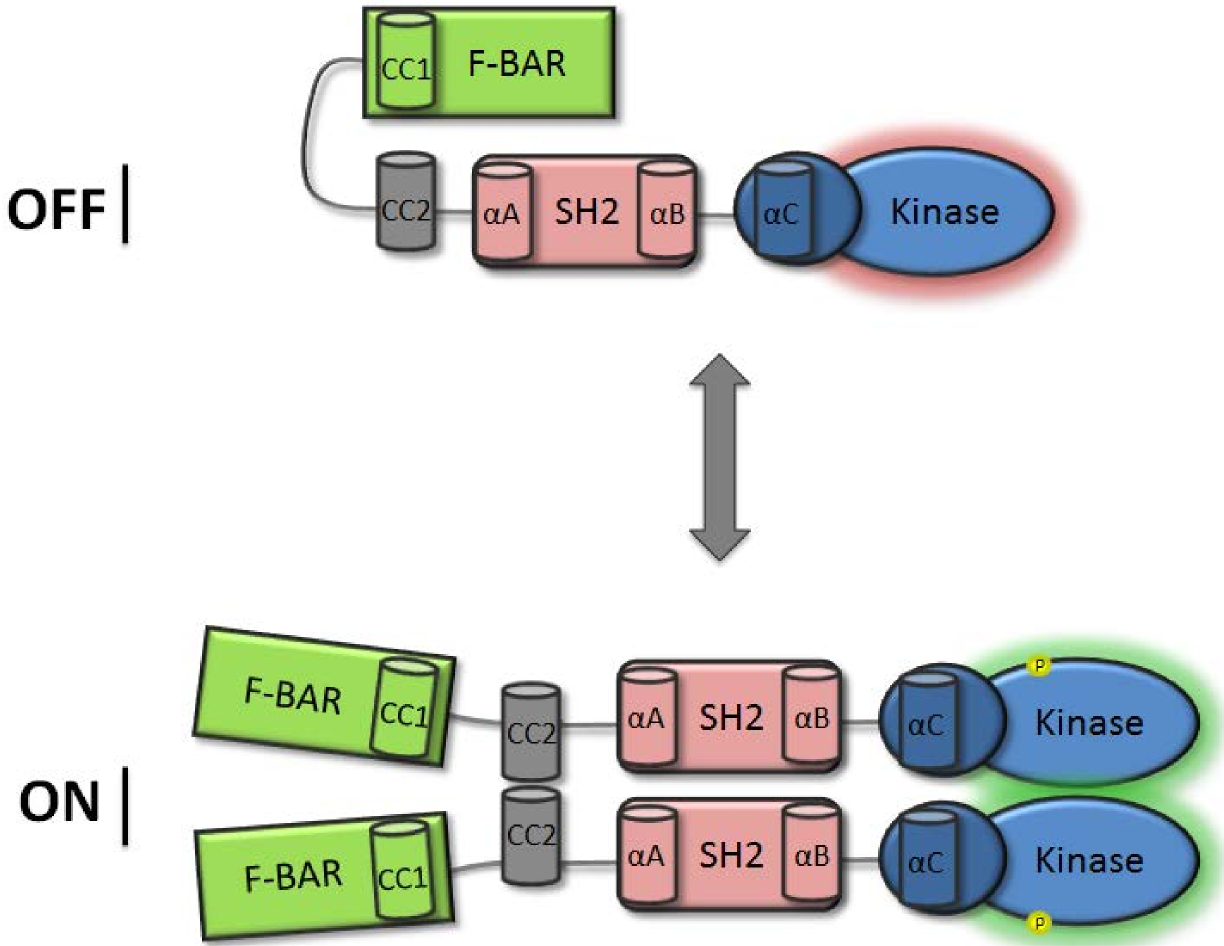


Figure 5. Cartoon of activation by oligomerization of c-Fes. A model for the regulation of c-Fes activity where CC1 interacts intramolecularly with CC2 in order to prevent oligomerization and activation. When CC1 no longer binds CC2 (perhaps by competitive binding with an upstream effector), c-Fes is free to form oligomers by CC2 dimerization. This drives autophosphorylation and transactivation to produce fully active, oligomeric Fes. (Dimer shown for simplicity. Cartoon not drawn to scale or with true orientation of all features.) While this model provides an attractive explanation for the role of coiled-coils in kinase regulation, subsequent studies have shown that c-Fes forms constitutive oligomers in cells regardless of its activation state. Thus, an alternative mechanistic explanation is required. This older model also ignores the positive impact of the SH2 domain on the kinase domain.

1.3.3 Autophosphorylation.

The major site of autophosphorylation in human c-Fes maps to Y713 (61, 68, 77). A phenylalanine substitution at this position, which lacks a hydroxyl group on the aromatic ring, abolishes autophosphorylation there. Even with an ideal substrate *in vitro*, Fes-Y713F substrate phosphorylation is less than 10% that of wild-type (61). Since Fes pY713 levels correlate so strongly with downstream Fes target phosphorylation and biological function, it is a reliable readout of Fes kinase activation. Later sections discuss the regulation of autophosphorylation and the role it plays in promoting full kinase activation.

1.3.4 Models of c-Fes downregulation.

Older biochemical and mutagenesis studies suggested a model of Fes regulation in which activity was controlled by interconversion of monomeric and oligomeric states. Gel filtration and cross-linking studies showed that Fes oligomerization depends on both CCs, while only loss of CC1 activates Fes (22, 76). Based on these, it was proposed that inactive, monomeric c-Fes was restrained by intramolecular CC1-CC2 binding. It was only a CC2 unhindered by CC1 that could mediation formation of active oligomers. However, subsequent BiFC studies showed that c-Fes forms oligomers irrespective of activation state *in vivo* and even oligomerization-defective Fes mutants exhibit transphosphorylation (76). While it is clear that CC1 plays an important role in downregulation of Fes kinase activity, the mechanism by which this occurs is not clear.

As described in previous sections, X-ray crystallography supports a mechanism for upregulation of Fes kinase activity via the cooperation of SH2 and kinase domains in assuming a stable, enzymatically active form. The difficulty in crystallizing full-length c-Fes and the

deficiency of structural data outside of the C-terminal SH2-kinase unit leaves the suggestions of other data as the foundation for models of c-Fes downregulation. The most salient observations are these: i) deletion or disorder of the CC1 domain results in strong activation of the kinase domain (22, 75), which supports an allosteric or intramolecular-interaction mechanism connecting the N-terminal F-BAR region and C-terminal SH2-kinase region of c-Fes; ii) *en bloc* substitution of the c-Fes SH2 domain with that of v-Src, but not v-Fps, also results in strong activation of the kinase domain (21). A model that explains these findings is that the F-BAR and SH2 domains interact to restrain SH2-dependent kinase activation. It could be proposed that the F-BAR domain competitively interacts with the SH2 or kinase domain at or near the SH2-kinase domain interface. It is also possible that the F-BAR domain binds elsewhere on the SH2 or kinase domain and by binding either alters the conformation of one partner's SH2-kinase domain interface or keeps the two physically separated by reorienting one partner to prevent the interaction. Kinase activation may then be permitted in this model by interaction of the N-terminal segment with cellular binding partners (47, 78), relieving its influence on SH2 or kinase domain and permitting the formation of the active SH2-kinase structure. In order to support this model with evidence, one must identify specific features of c-Fes that are required for the F-BAR:SH2/kinase domain interaction. (Discussion continued in sections 3 and 4.)

1.4 BIOLOGICAL FUNCTIONS OF C-FES

1.4.1 Expression pattern.

Where a protein is expressed plays a part in determining the functional role of that protein. There are many different tissues and cell types, each with their own unique complement of expressed gene products available for interaction. In the case of a kinase, what substrates are available and what happens to them after being phosphorylated is important. Over murine development, the expression pattern of c-Fes becomes more specific. In an adult, the c-Fes kinase is expressed in a discrete subset of tissues including myeloid hematopoietic, vascular endothelial, neuronal, and epithelial cells where it responds to cytokine and growth factor stimuli [reviewed in (2, 15)]. In mice, endogenous murine *fes* and transgenic human c-Fes are localized to the bone marrow, lymph system, spleen and lungs (23). In mouse embryos, *fes* is expressed in the vascular endothelium, skin epidermis, and ventricular linings in the brain (79). *In situ* hybridization and Northern blot analysis detected *c-fes* transcripts in liver, lung, skin, spinal cord, heart, gut and kidney (80). Diseased tissues may still express c-Fes, at normal levels or higher, while others may lose Fes expression. For example, in many cases of acute myeloid leukemia (AML) *c-fes* is highly expressed (81, 82). However, c-Fes expression is robust in normal colonic epithelial tissue and lost or diminished in tumor tissue from the same individual (83). The role of c-Fes in cancer is complex, and is covered in more detail below.

1.4.2 Subcellular distribution.

Like the tissues that make up an organism, the various cellular compartments, organelles, and cytostructural networks that a protein can be localized to are important to biological function. This subcellular localization may be tissue- or cell-specific or change as the greater organism advances through stages of development. Like human development, individual proteins are “born” from a ribosomal complex and are trafficked through a life cycle that may stop at the membrane, nucleus, or even outside the cell. In the case of c-Fes, it is predominantly localized to the cytoplasm in the downregulated state (21, 38). In a few cell types, c-Fes displays a diffuse cytoplasmic distribution with a perinuclear bias and some punctate projections that spread towards the cell membrane (79). A minority of publications report a c-Fes subpopulation that is nuclear in myeloid hematopoietic tissue, despite lacking any known nuclear localization sequence (84, 85).

The subcellular localization of c-Fes also appears to be activation-dependent. In Rat-2 cells, ectopic c-Fes displays a diffuse cytoplasmic distribution that shifts to focal adhesions when active (21). Activation of c-Fes coincides with relocalization from a diffuse cytoplasmic distribution to microtubules in COS-7 and neuronal cells (37, 86). In macrophages, c-Fes associates with the actin cytoskeleton (65). A unique activation-dependent relocalization event occurs in LLC-PK1 porcine kidney cells where activated c-Fes move between focal adhesions and cell-to-cell contact points depending on confluency (66).

1.4.3 Proliferation and differentiation.

Being the cellular homolog of viral oncoproteins, the implication is that c-Fes plays a role in growth and differentiation (2). Normal expression in myeloid hematopoietic tissue and involvement in certain leukemias suggests that c-Fes functions in hematopoiesis (87-89). Fes is implicated in signal transduction downstream of some hematopoietic cytokines and growth factors like granulocyte-macrophage colony-stimulating factor (GM-CSF), interleukin-3 (IL-3) and erythropoietin (85). However, it is not a perfectly clear case. When kinase-dead Fes was overexpressed in a couple growth factor-dependent cell-lines, TF-1 and 32D, proliferation in response to GM-CSF and IL-3, and the differentiation program induced by G-CSF were not altered (90). It may be a cell-line-specific result, but Fes catalytic activity is not critical for growth and differentiation in response to GM-CSF and IL-3 in these cells. Yet, the closely related kinase Fer is pervasively expressed (91) and may play redundant role (92). If the overexpressed phosphorylation-defective Fes does not overwhelm it, Fer may be sufficient in this case. That said, c-Fes null and knockout mice exhibited some cases of defect, but it is clear that c-Fes is not essential in innate immunity (2, 93). Naturally, differentiation varies in how it is manifested in different tissue lineages. However, c-Fes is involved in a few general biological activities as part of its cell- or signal-specific proliferation or differentiation function.

As discussed previously, c-Fes undergoes a subcellular redistribution to the cytoskeleton upon kinase activation in various cell types [Rat-2 to focal adhesions (21), COS-7 (37) & neuronal cells (86) to microtubules]. Rat-2 and COS cells do not normally express Fes, but ectopically expressed Fes exhibits concomitant activation and relocalization. In neuronal cells, endogenous c-Fes co-precipitates with soluble tubulin in whole cell lysates of neonatal rat brain and immunofluorescence microscopy shows endogenous c-Fes co-localizes with microtubule

fibers in cultured rat neurons (77). Based on these facts, c-Fes may function in regulating cellular architecture and motility. Along those lines, TF-1 cells expressing active c-Fes mutants undergo GM-CSF-independent cell attachment and spreading (75). It may be that wild-type c-Fes regulates microtubule dynamics in response to these cellular signals (37). When activated, c-Fes may catalyze both tubulin phosphorylation and polymerization, and then by the SH2 domain come into association with tyrosine-phosphorylated microtubules for recruitment to other substrates. It's possible that pTyr-tubulin Fes-SH2 recruitment is accomplished by other tyrosine kinases like c-Src. In fact, co-expression of c-Fes with an active mutant of the Hck stimulates Fes relocalization to microtubules in COS cells (38). Furthermore, endogenous c-Fes has been observed associating with the tubulin cytoskeleton without need for introduction of an active mutant SFK (37). Lastly, c-Fes was found aggregating at the microtubule organizing center and colocalized with γ-tubulin, which is essential for microtubule nucleation and orientation, when microtubular networks are damaged by demecolcine-treatment in COS cells. A naturally occurring event that disrupts microtubular networks is mitotic metaphase. Interestingly, active mutant FesL145P co-localizes with the mitotic spindle (38). Taken all together, the above evidence implicates c-Fes in cytoskeletal dynamics, especially microtubules.

Several lines of evidence support a role for c-Fes in angiogenesis, the complex process that leads to the development of new blood vessels. First, expression levels of endogenous c-Fes in primary human vascular endothelial cells are comparable to myeloid cell levels (24). Second, the angiogenic growth factors FGF-2 and angiopoietin-2 stimulate c-Fes activity in capillary endothelial cells. In the same cell type, simply over-expressing c-Fes is sufficient for chemotaxis and tubulation (94). In fact, such over-expression induces tube formation independent of FGF-2. Lastly, transgenic mice expressing a membrane-targeted, activated mutant of Fes were

hypervascular and developed hemangiomas (24). Consistent with these observations, c-Fes is implicated in angiogenesis.

1.5 PATHOLOGICAL ROLES OF C-FES

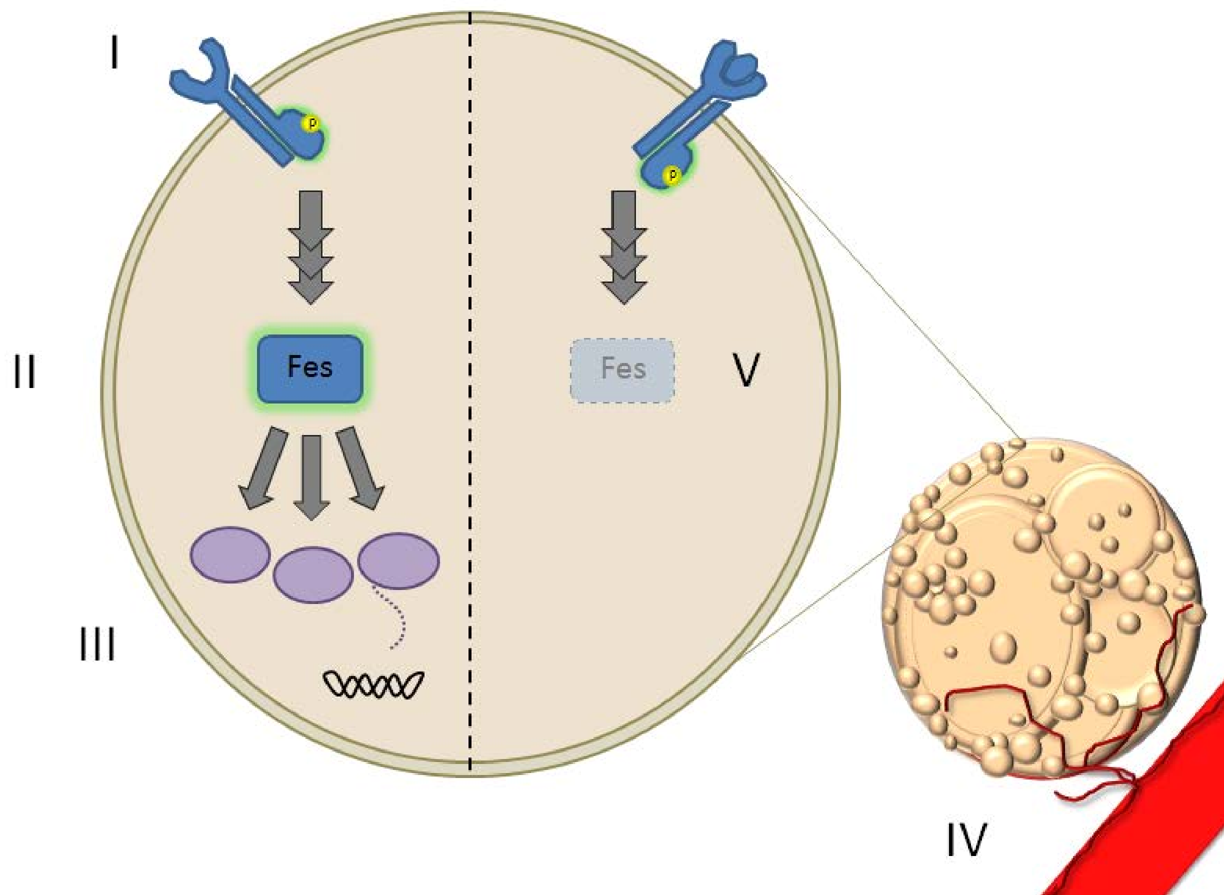


Figure 6. Possible modes of c-Fes involvement in oncogenesis. Depending on the type of cell and the type of cancer, Fes may be related to tumorigenesis in several ways: I) activated mutant upstream, like a ligand-independent receptor; II) Fes overexpression (there are no known activated mutants of Fes in human cancer); III) overexpression or over-activity of downstream targets that required Fes signaling; IV) support of tumor angiogenesis; V) loss of Fes expression or tumor suppression activity. Gain-of-function/expression (left): AML, renal carcinoma, breast cancer (IV). Loss-of-function/expression (right): CML, colorectal cancer.

1.5.1 Oncogenesis.

Human c-Fes is homologous to several retroviral oncoproteins named for their sarcoma-forming ability. The only major difference between the well-behaved c-Fes and the oncogenic viral products is an N-terminal gag derived from the virus. Given that c-Fes is strictly regulated, it stands to reason that uncontrolled Fes activity can be pathological. When those problems involve proliferation and differentiation, then the stage is set for oncogenesis. Indeed, extreme overexpression (14), viral gag fusion (19), or activated mutants of Fes (21, 75) alone are all sufficient for robust tyrosine kinase activity and fibroblast transformation (20, 95). Knockdown by siRNA targeting *c-fes* significantly slows renal carcinoma cell proliferation (96). Stable expression of activated mutant Fes in the TF-1 myeloid leukemia cell line, which normally requires media supplemented with GM-CSF, drives cytokine-independent cell growth and survival (75). However, wild-type c-Fes at endogenous levels can also contribute to oncogenesis. In acute myelogenous leukemia (AML) blasts and cell lines, c-Fes is an essential effector downstream from an activated mutant of the FLT3 receptor (97). Similarly, the ligand-independent activated mutant KIT(D816V) required c-Fes for cell survival and proliferation (98). These data are consistent with other clinical findings that implicate Fes (99), even in an ancillary role in the tumor niche (100).

1.5.2 Tumor suppression.

In addition to an oncogenic role for Fes, evidence also suggests that c-Fes may be involved in tumor suppression in some cell types (101). In chronic myelogenous leukemia (CML) cell lines, ectopic expression of c-Fes resulted in differentiation and suppressed cell

growth (102, 103). Bcr-Abl, the fusion protein primarily responsible for initiation of chronic phase CML, is then a potential target for c-Fes. Interestingly, Bcr-Abl protein levels drop in cells stably expressing c-Fes (77). Nucleotide sequence analysis of the tyrosine kinome in 182 colorectal cancer cell lines and xenografts identified *c-fes* along with only five others for consistently exhibiting kinase domain mutations (104). These kinase domain mutations (M704V, R706Q, V743M, and S759F) reduce or eliminate c-Fes enzymatic activity (83). In another epithelial tissue, Fes delays tumor onset in a mouse model of breast cancer, as onset is more rapid in mice with either null or kinase-dead *fes* mutations, while expression of a *fes* transgene rescues the slower tumor onset kinetics observed in *c-fes* null mice (101). Likewise, HT-29 and HCT 116 colorectal cancer cell line growth in soft agar is suppressed by Fes (83). While c-Fes is abundantly expressed in matched normal colonic epithelial cells from CRC patient tissue samples, expression is reduced or absent in the majority of colon tumor sections from the same group of individuals (83). The observations that Fes mutations associated with colorectal cancer decrease kinase activity, and that Fes gene expression is often silenced in colorectal tumors, suggest that Fes may play a tumor suppressor role in cancers of epithelial origin.

2.0 BIMOLECULAR FLUORESCENCE COMPLEMENTATION

Biochemical complementation has been in use since the mid-20th century. It was not until the turn of the millennium that a fluorescent protein was engineered for complementation assays (105). Shortly after, Hu et al. were the first to publish a fluorescence complementation study in live cells, with YFP, and named the technique Bimolecular Fluorescence Complementation (106). In this technique, cells are transfected with expression constructs encoding the two interacting molecules of interest, one which is fused to the N-terminal fragment of the Venus variant of YFP (VN) and the other to a complementary C-terminal fragment (VC). Importantly, these fragments do not fluoresce alone, nor do they interact and refold on their own (106, 107). Once expressed, interacting molecules bring the two non-fluorescent fragments into close proximity where they refold into the fluorescent protein (complementation). This stable reconstituted fluorescent protein emits a readily measured fluorescent signal indicative of the interaction (108), as pictured below in Figure 7.

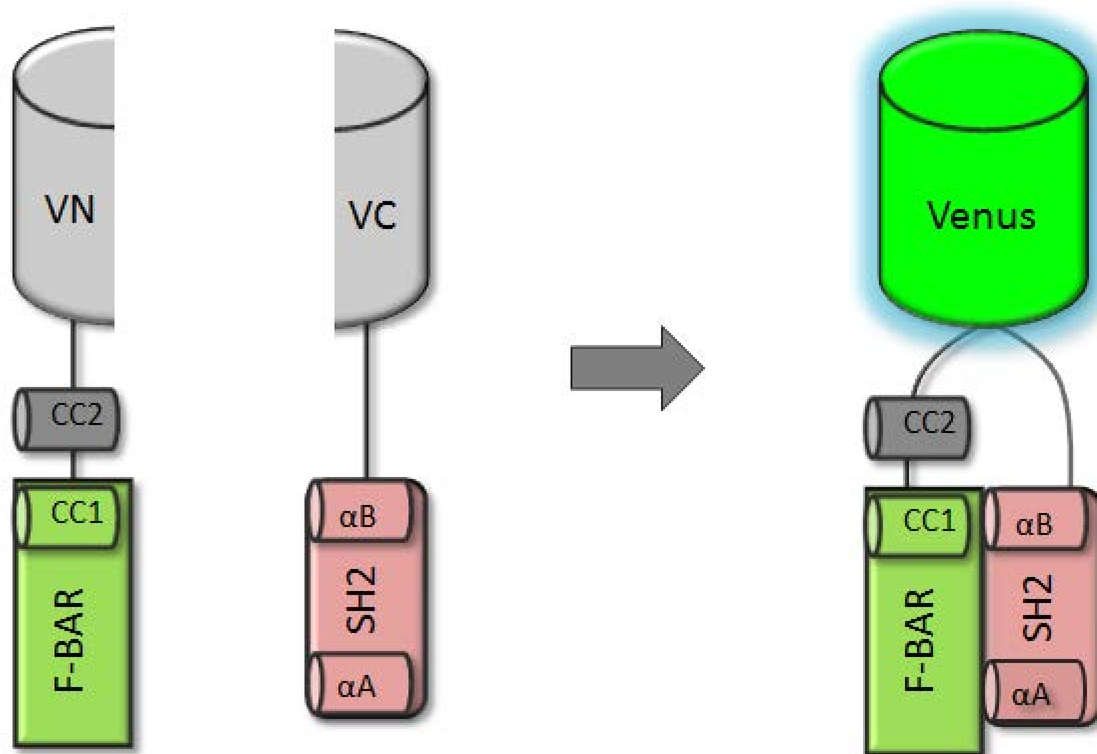


Figure 7. Bimolecular fluorescence complementation. Potential interacting partners (Fes domains in this example) are fused to non-fluorescent N-terminal (1-173) and C-terminal (155-238) fragments of the Venus YFP variant coding sequence (termed "VN" and "VC" respectively) and co-expressed in the same cell. If the domains interact, VN and VC are brought into close proximity, resulting in structural complementation and YFP fluorescence. (Not drawn to scale or with true orientation of all features.)

2.1 METHODOLOGY

In the BiFC approach, interacting partners (c-Fes domains in this case) are fused to non-fluorescent amino- and carboxyl-terminal segments of the Venus YFP coding sequence and co-expressed in the same cell. Interaction draws YFP fragments into close proximity, and then the two must refold into the native β-barrel structure in order to reconstitute a fluorophore capable of reporting the interaction by emission. The tertiary structure of the coupled fragments is highly

stable, so the interactions captured will be visible if there are enough formed. Images captured of the cells expressing the BiFC pair can be analyzed and quantified. Common to all protein–protein interaction assays, it is critical to identify suitable controls and parameters which identify and limit non-specific interactions and false positives. Ideally, controls include specific mutations that eliminate or severely reduce the interactions of interest. (109) It is also possible add untagged competitors to the experiment as a control.

2.2 ADVANTAGES

BiFC offers a useful, adaptable technique to directly examine protein-protein interaction in a more physiologically relevant environment. Conventional complementation, where the protein reporter divided into two non-functional fragments is usually an enzyme of some kind, usually involves a more complicated reporting method. BiFC combines the simplicity of a complementation assay with the power to visualize and quantify the fluorescent intensity and subcellular distribution in cells. Since the fragment folding is virtually irreversible, given enough expression time BiFC is sensitive enough to detect even transient interactions between proteins expressed at physiological levels (106, 110). BiFC is also versatile as there are many fluorescent proteins to work with. BiFC can be obtained between fragments of *Aequorea* variants (CFP, GFP, and YFP) and *Anthozoa* or *Discosoma* red-shifted proteins (mCherry). (109) Thus the spectrum of fluorescence excitation and emission wavelengths is broad. In the case of YFP, engineers are attempting to optimize refolding through mutation, e.g. Venus (111). The enhanced complementation efficiency allows for experiments to be performed at 37°C, compatible with culture conditions for most mammalian cells (112, 113) Reducing the need to treat the cells in

unusual or harsh ways can increase efficiency and validity. For example, protein complexes can be identified by biochemical approaches like co-immunoprecipitation, but these invariably involve cell lysis and often lack temporal and subcellular resolution. With BiFC, the investigator can directly view interaction in live cells. Additionally, untagged competitors that may disrupt the protein–protein interaction can only prevent the earliest onset of BiFC after association begins, suggesting that this first phase is relatively short (half-time of seconds to minutes) and again largely irreversible thus trapping even transiently formed complexes (106). Thus it is possible for BiFC to detect more binding events like SH3 domain and ligands. Fluorescence resonance energy transfer (FRET) techniques are also applicable in PPI studies. In contrast, BiFC involves relatively simple measurements with a single excitation/emission (unless you opt for multicolor BiFC with non-overlapping excitation/emission spectra). In contrast to FRET, BiFC is more tolerant of the relative orientation of the distinct tags while retaining comparable distance constraints (114). BiFC signals are appropriate for analysis by advanced imaging techniques and high-content analysis. One can even expand investigation to more than two protein partners using multicolor BiFC or BiFC combined with FRET.

2.3 APPLICATION

Generally, BiFC is utilized in protein-protein interaction studies. Basically, complexes are visualized in cells, but combined with complementary techniques and controlled variables BiFC can be information rich. For example, BiFC followed by comparative mutagenesis of the suspected dimerization interface may be used to delineate features of the site of interaction (115, 116). BiFC has been applied to a wide variety of complexes in numerous cell types (115). Many

interactions assessed by BiFC have been relatively stable ones, such as transcription factor dimerization (117), ubiquitin modifications (118), or association between Akt and Smad3 (119). Some have undertaken high-content BiFC, like in quantifying 7TM receptor signaling recruitment of β-arrestin2 (109). In this case, BiFC provided data consistent with the dual sensor model of β-arrestin binding to 7TM receptors (120). In the Smithgall lab, Fes oligomerization (76) and Nef dimerization (121) have been studied in a BiFC context. The possibilities for this tool grow with each new designer fluorescent reporter, new techniques to enhance or couple with BiFC, and as new questions are asked and answered.

3.0 A NOVEL ROLE FOR THE UNIQUE N-TERMINAL REGION IN THE AUTO-REGULATION OF C-FES

3.1 BACKGROUND AND SIGNIFICANCE

High levels of c-Fes are found in myeloid leukemias, presumably representing expansion of a c-Fes-positive cell population (26, 74, 99). Although active c-Fes can be transforming, ectopic expression of wild-type c-Fes in fibroblasts does not result in transformation. The activity of full-length c-Fes is tightly regulated *in vivo*, with the catalytic domain assuming an inactive state (13) Crystal structure analysis revealed that specific association of the SH2 and kinase domain is critical to the maintenance of the active state (64). In cells, the 45 kDa SH2-kinase domain unit is highly active, suggesting the N-terminal unique region restrains this coupling. The crystal structure of full-length c-Fes has not been reported and it is not known what (if any) interactions may exist between the N-terminal F-BAR domain and the SH2-kinase domain. That said, consideration of the activities of Fes SH2 and F-BAR domain mutants support a model in which F-BAR interaction with the SH2 domain may act to repress kinase activity (Figure 8).

Regulated properly	Dysregulated activated	Dysregulated inactive
wt	Gag-Fes	E469K
YFP-Fes	v-Fps	K590E
v-Fps SH2	v-Src SH2	Y713F
	ΔF-BAR	Ras-GAP SH2
	ΔCC1	
	L145P	

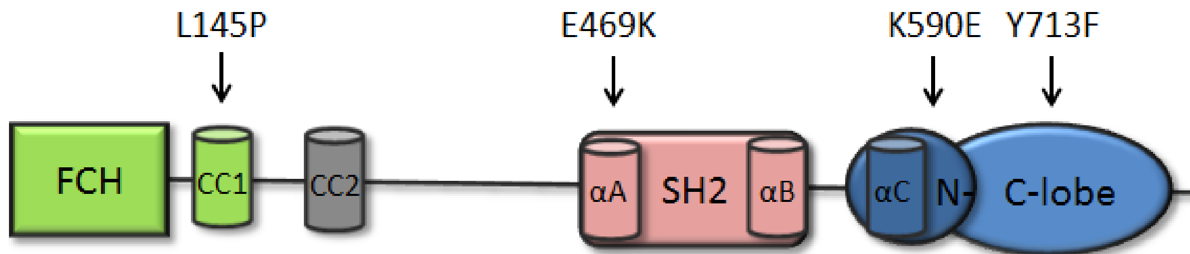


Figure 8. Activating and inactive mutants of Fes. The tight regulation of c-Fes kinase activity can be overcome in multiple ways as summarized here. N-terminal fusion of viral Gag sequences generates a chimeric protein with robust tyrosine kinase and transforming activities in fibroblasts (19, 20, 95). Possibly, viral Gag fusion impedes negative autoregulation and/or relocalizes Fes to a subcellular compartment (e.g., plasma membrane, cytoskeleton) essential for Fes signaling. However, myristoylation and plasma membrane targeting alone are insufficient for fully active Fes kinase in cells (21, 22), Targeting of c-Fes to focal adhesions by substitution of the native SH2 domain with that of v-Src results in stimulation of the kinase (21). Similarly, mutagenesis or deletion of the first coiled-coil domain in the F-BAR causes drastic upregulation of Fes kinase activity, and the mechanism by which this N-terminal modification impacts the C-terminal kinase domain is not clear yet. A possible explanation is laid out in this dissertation.

3.2 HYPOTHESIS

3.2.1 The F-BAR domain plays a key role in autoinhibition of c-Fes kinase activity through specific interactions with the SH2 domain.

Since structural and mutational analyses of c-Fes reveals that the specific association of the SH2 and kinase domain is critical to the maintenance of the active state (2, 51, 64) and the SH2-kinase unit is active in cells while full-length wild-type c-Fes is not, this positive regulation of c-Fes kinase domain activity by SH2 must be suppressed in the full length protein somehow. Intramolecular interactions involving the F-BAR and SH2 domains could explain this phenomenon. Unlike SH2-mediated activation, the structural basis of c-Fes inhibition is poorly understood. Figure 9 and the section below go into detail about this hypothetical model of c-Fes autoregulation.

3.2.2 Proposed model of c-Fes autoregulation.

The auto-stimulatory SH2-kinase domain interaction is required for upregulated activity; therefore a mechanism of c-Fes autoregulation is such that the F-BAR binds the SH2 region in a manner that is auto-inhibitory, preventing the positive regulation of the kinase by the SH2. It may be that the first coiled-coil of the F-BAR is essential to this. The hypothetical model explains the following prior observations: a) full-length, wild-type c-Fes is tightly regulated *in vivo*, assuming the inactive state (13), yet b) truncated c-Fes containing only the SH2 and kinase domains is constitutively active (64); c) mutation that upsets CC1 of the F-BAR results in uninhibited activity (46, 75), and d) SH2 replacements that lack critical, conserved residues in

the αB helix disinhibits c-Fes (21, 46), while e) substitution of the SH2 domain with a species (e.g. Ras-Gap) that bears critical dissimilarities from c-Fes in the αA helix is not active (21, 46) and f) SH2-deletion results in diminished activity, but not inhibition as seen in the wild-type c-Fes (21).

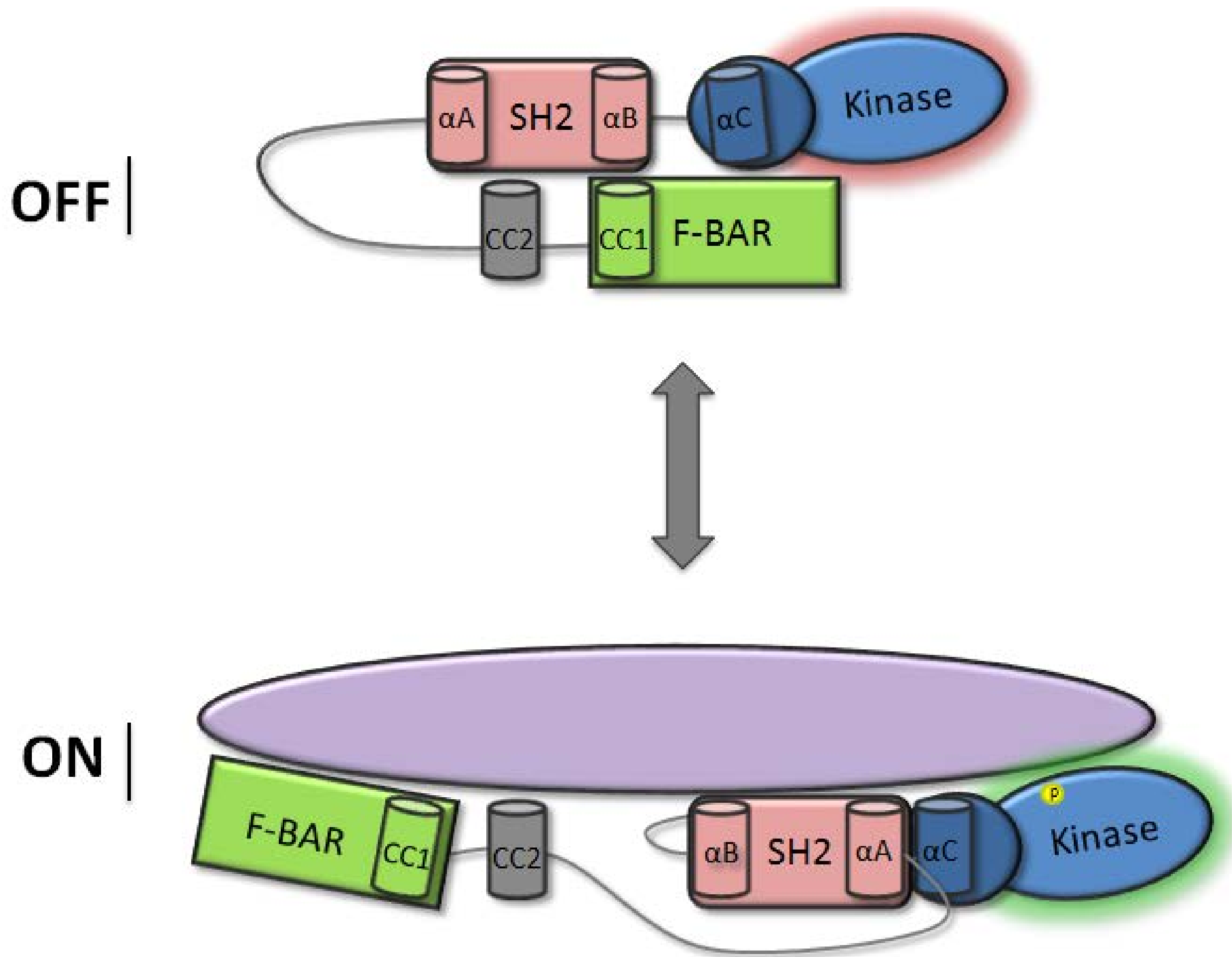


Figure 9. Hypothetical model of c-Fes autoregulation. The inactive (OFF) conformation of c-Fes may be such that the F-BAR interacts with the SH2, keeping the SH2-kinase stimulation from occurring. One way in which c-Fes could be turned ON would be some adapter or substrate (perhaps in complex) disrupts the F-BAR:SH2 interface, freeing the SH2 to cooperate with the N-lobe to promote catalytic activity. In this case, the F-BAR and CC2 could then participate in oligomerization that accompanies Fes activity. (Not drawn to scale or with true orientation of all features.)

3.3 SPECIFIC AIMS

3.3.1 Test for Fes F-BAR:SH2 interaction.

Mutagenesis studies and X-ray crystallography show that the auto-stimulatory SH2:kinase domain interaction is required for c-Fes kinase activity (64); therefore, the mechanism of c-Fes autoregulation may be that the F-BAR (in particular the CC1 domain) binds to the SH2 region in a manner that prevents positive regulation. This model explains why loss of the F-BAR or CC1 results in uninhibited activity (46, 75) and why substitution of the Fes SH2 with that of v-Src releases kinase activity (21, 46). Based on the model, some component including CC1 of the N-terminal region of c-Fes interacts with the SH2-kinase region. A BiFC approach can test this mechanism (Figure 7). Interaction can be visualized directly and mutants known to deregulate Fes activity (L145P) can be compared to wild-type pairings.

3.3.2 Develop tools to test the effect of F-BAR:SH2 interaction on Fes activity.

The SH2 domain is required for full kinase activity and x-ray crystallography demonstrates that the SH2 domain interacts with the kinase domain, stabilizing a critical helix in the N-lobe, and promotes the active conformation of the kinase domain. Introducing mutations at key residues involved in the interaction of the SH2 and kinase domains revealed by the crystal structure results in reduced catalytic activity (64). Given that the F-BAR region interacts with the SH2 domain, mutations of amino acids contained within these two that increase kinase activity may be at the interaction interface of the F-BAR and SH2. Conversely, mutations found to diminish this interdomain interaction may cause upregulated catalytic activity. Substitution of the

Fes SH2 with that of v-Src releases Fes kinase activity in cells when wild-type c-Fes would be repressed. Substitution with the v-Fps SH2, however, does not affect Fes regulation. Therefore, differences between the c-Fes and v-Src SH2 domains not found in v-Fps may be responsible for the activity of the Fes/v-Src SH2 chimera. Sequence alignments and structural overlays assisted this comparison and reveal a few amino acid candidates for mutagenesis (see Figures 12 and 13). These Src-like mutations introduced into the Fes BiFC constructs allows for testing their effect on interdomain interaction. Mutations that diminish the F-BAR:SH2 interaction could then be introduced into full-length Fes for kinase activity studies.

4.0 EXPERIMENTATION

4.1 F-BAR:SH2 INTERACTION DEMONSTRATED BY VENUS BIFC IN CELLS

4.1.1 Construction of Plasmids.

To create the vectors required for BiFC analysis, sequences encoding regions of c-Fes from the N-terminal region ("F-BAR", bp 1-1053, encoding aa 1-351; "F-BAR-L", bp 1-1341, encoding aa 1-447), SH2 domain (bp 1342-1653, encoding aa 448-551), kinase ("KD", bp 1654-2466, encoding aa 552-822), SH2-kinase core, ("SH2-KD", bp 1342-2466, encoding aa 448-822) and full length c-Fes (2466 bp; 822 aa) were amplified by PCR from a pcDNA3.1(+)Fes-FLAG parent plasmid and subcloned into both pcDNA3.1(-)VN173 and VC155 vectors. This way each of the Fes cDNAs were fused to the N-terminus of both VN173 and VC155 Venus YFP fragments in order to perform BiFC experiments with many combinations. PCR site-directed mutagenesis (Stratagene QuikChange II XL) with point mutation primers (sense 5'-GC CAG TAC CGA GCT C***C***G GCA CGG GAC AGT GC-3'; antisense 5'-GC ACT GTC CCG TGC C***G***G AGC TCG GTA CTG GC-3') generated L145P mutants of the F-BAR and F-BAR-L Venus constructs. Plasmids were confirmed by sequencing (Genewiz) and amplified for experiments by Maxiprep (Invitrogen).

4.1.2 Cell Culture and Transfection.

HEK-293T cells were maintained at 37 °C in humidified incubators (5% CO_2) and cultured in 5% FBS-supplemented DMEM (Invitrogen). Transient transfection was performed as follows: 293T cells ($3x10^5$) were plated in 12-well plates for BiFC (or 100 mm dishes for cell lysates) and transfected the next day (at 50-70% confluency) with 1 µg of total plasmid DNA—0.5 ug of each of the two, VC and VN—delivered in 3 µl X-tremeGENE transfection reagent (Roche). All transfections were performed using serum-free media as diluent. Following incubation at 37 °C for 24 hours, transfected cells were viewed, then fixed with 4% paraformaldehyde and immunostained to detect expression levels (section 4.1.3, below). Transiently transfected 293T cells in 100 mm dishes were washed with PBS, suspended 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 Na_3VO_4, 25 mM NaF) and sonicated for 10 sec on ice. The cell lysates were cleared of debris by centrifugation, mixed with 2X SDS-PAGE buffer (125 mM Tris-HCl pH 6.8, 20% glycerol, 4% SDS, 172 mM βME, 0.05% bromophenol blue), boiled at 95 °C for 2 min, then separated by SDS-PAGE and transferred to PVDF membrane. Western blots were treated with antibodies directed against GFP or actin (1:1000, Santa Cruz), then washed and incubated with AP-conjugated secondary antibodies (1:10,000), in order to check for expression and even loading. NBT/BCIP treatment visualized these immunostained protein bands.

4.1.3 Immunofluorescent staining.

The transfected cells in multi-well plates were fixed with 4% PFA-PBS solution for 10 minutes followed by washes with cold PBS and permeabilization by 0.2% Triton X-100 (in PBS)

treatment for 15 min. Following 2% BSA-PBS blocking for 30 minutes, cells were incubated at 4 °C overnight with anti-GFP (1:500 dilution in 2% BSA-PBS), which again recognizes both VC and VN fragments. Immunostained cells were visualized with Texas Red-conjugated secondary antibodies (1:1000) so that IF counterstain was in a distinct channel from the green BiFC.

4.1.4 Fluorescence Microscopy and Imaging.

Images were captured of cells using an inverted epifluorescence microscope (Nikon TE300) accompanied by a SPOT Advanced charge-coupled digital camera and software (Diagnostic Instruments). Grayscale images of both IF red- and BiFC green-channels at multiple spots in each well, which represent a single experimental BiFC pair, were acquired and saved as TIFF files for later analysis.

4.1.5 BiFC Quantification.

In order to collect quantifiable data for analysis and graphical representation, both IF (protein expression) and BiFC (interaction) images of a single spot for a given co-expression were opened in ImageJ software. The 100.0 rolling-ball operation automatically removes image background for both IF and BiFC images. Then, the threshold intensity is set on the IF image so that only fields (cells and cell clusters) exhibiting adequate IF (expression) are measured. The program can then record the mean gray value (and several other measurements) of all IF-positive cells/regions on the image, effectively measuring fluorescence intensity of many cells. In the next step, the exact same image fields and measurement parameters were applied to the corresponding BiFC image. This way only cells expressing the BiFC constructs were quantified,

eliminating false negatives. In Excel (Microsoft), all the matched IF-BiFC mean values were analyzed. The ratio of BiFC intensity to IF intensity indicates interaction efficiency—the extent to which the co-expressed VN- and VC-fused Fes domains interact and YFP complementation occurs. The entire data set of BiFC:IF ratios for each tested pair were averaged and the correlation coefficient found. BiFC as a function of IF was plotted to display correlation as the slope of a line fit to the scattered points of individual cells.

4.1.6 Results.

Previous studies suggest that deletion or mutation of the F-BAR results in a highly active SH2-kinase domain coupling (64, 75), so direct evidence of the F-BAR interacting with either the SH2 or kinase domain would be consistent with a mechanism of autoregulation. To determine if these domains interact, I applied a BiFC-based strategy in which complementary fragments of Venus YFP were fused to truncations of c-Fes containing its various domains and expressed in HEK-293T cells (which do not express endogenous c-Fes). Approximately 24 hours post-transfection, a BiFC signal was visible from c-Fes oligomerization (and GST dimerization) BiFC, which was expected (76). Transfected cells in the same 12-well plates were fixed and immunostained for GFP, which recognizes both fragments of Venus. (The X-tremeGENE co-transfection protocol is highly efficient and immunoblots of cell lysates confirm expression.) ImageJ data analysis of corresponding immunofluorescence (IF) and BiFC images generated sets of mean intensity values for IF-positive cells. In Figure 10 on the top left are representative images (pseudo-colored red and green for IF and BiFC, respectively) of equivalent exposure times for F-BAR-VC co-expressed with SH2-VN. (Note: a longer linker on the F-BAR may allow for more efficient BiFC, as the F-BAR-L construct was preferred in these experiments.) In

this BiFC experiment, it is clear that the F-BAR interacts with the SH2 as most cells expressing the two display a strong signal. The mean BiFC:IF ratios of individual cells were plotted and show a positive correlation between the level of expression and complementation. Compared to wild-type, the VC-fused L145P mutant does not produce a robust BiFC signal when co-expressed with the SH2 domain-VN fusion. Unlike with wild-type F-BAR-VC, there is not a strong positive correlation (slope of the line) between the expression of this pair and BiFC. This greatly abated BiFC to IF ratio indicates that a mutation which kinks CC1, releasing Fes kinase activity, also disrupts an F-BAR:SH2 interaction.

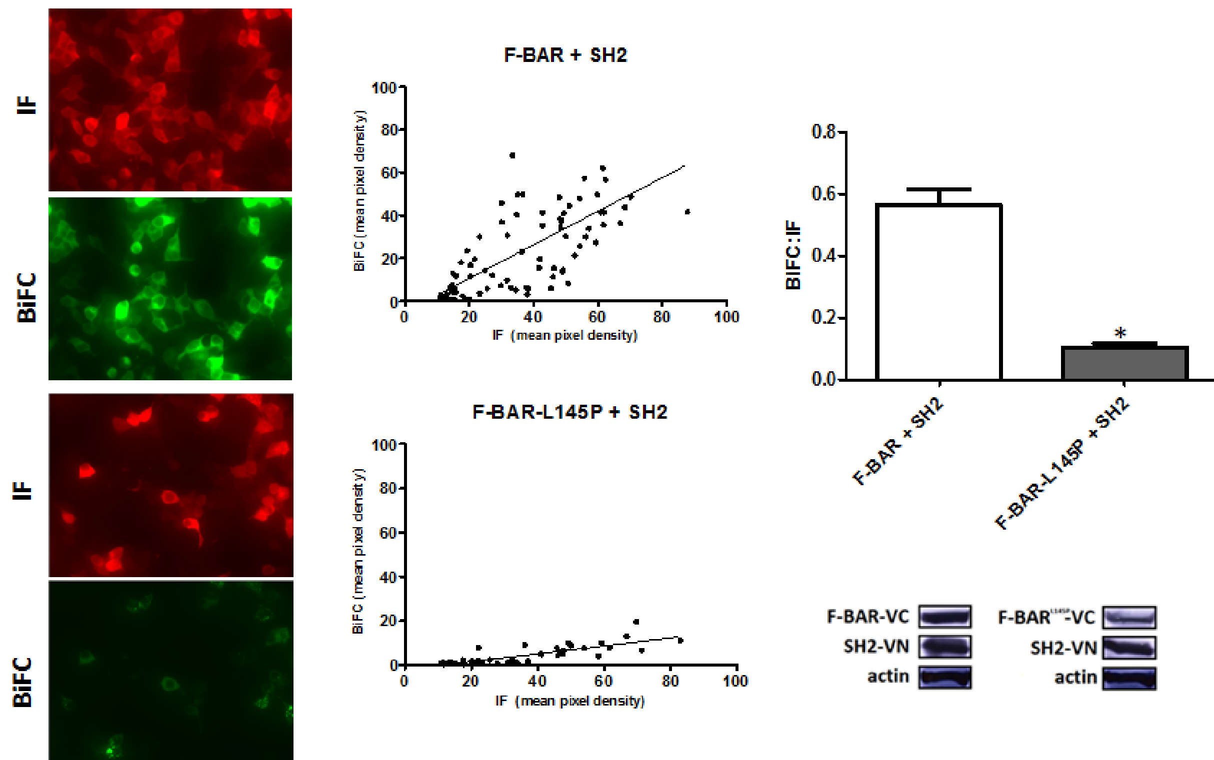


Figure 10. F-BAR interacts with SH2 domain in CC1-dependent manner. Venus BiFC expression constructs were co-expressed in 293T cells, which were fixed and immunostained. Immunofluorescence (IF) and BiFC were visualized by fluorescence microscopy. Shown (left) are representative IF and BiFC images. Grayscale mean pixel densities of individual IF-positive cells were measured using ImageJ. Plots (middle) and statistics were generated in GraphPad PRISM 5. There is a linear correlation (top right) between expression and F-BAR:SH2

interaction in wild-type that is abrogated in the L145P mutant. (*$p < 0.0001$) Expression of the individual BiFC members was additionally confirmed by western blot (bottom right).

Since the interaction of the SH2 and kinase domain of c-Fes are well characterized (51, 64), the result of co-expression of the SH2-KD Venus pair was expected (Figure 11, top). Two domains of Fes known to associate with one another *in cis* also form a complex in cells. This result validates our approach taken to investigate Fes interdomain interaction.

It was not known whether the N-terminal region of Fes interacted with either the SH2 or kinase domains, although hypothetically one or both (individually or as a contiguous unit, SH2-KD) could have. I tested many combinations of the various regions of c-Fes (with both orientations of VN and VC), such as F-BAR-L-VN -VC dimers, and the kinase domain did not exhibit any appreciable complementation with the F-BAR region (Figure 11, bottom). It is likely that the SH2 is the central hub for c-Fes intramolecular interactions.

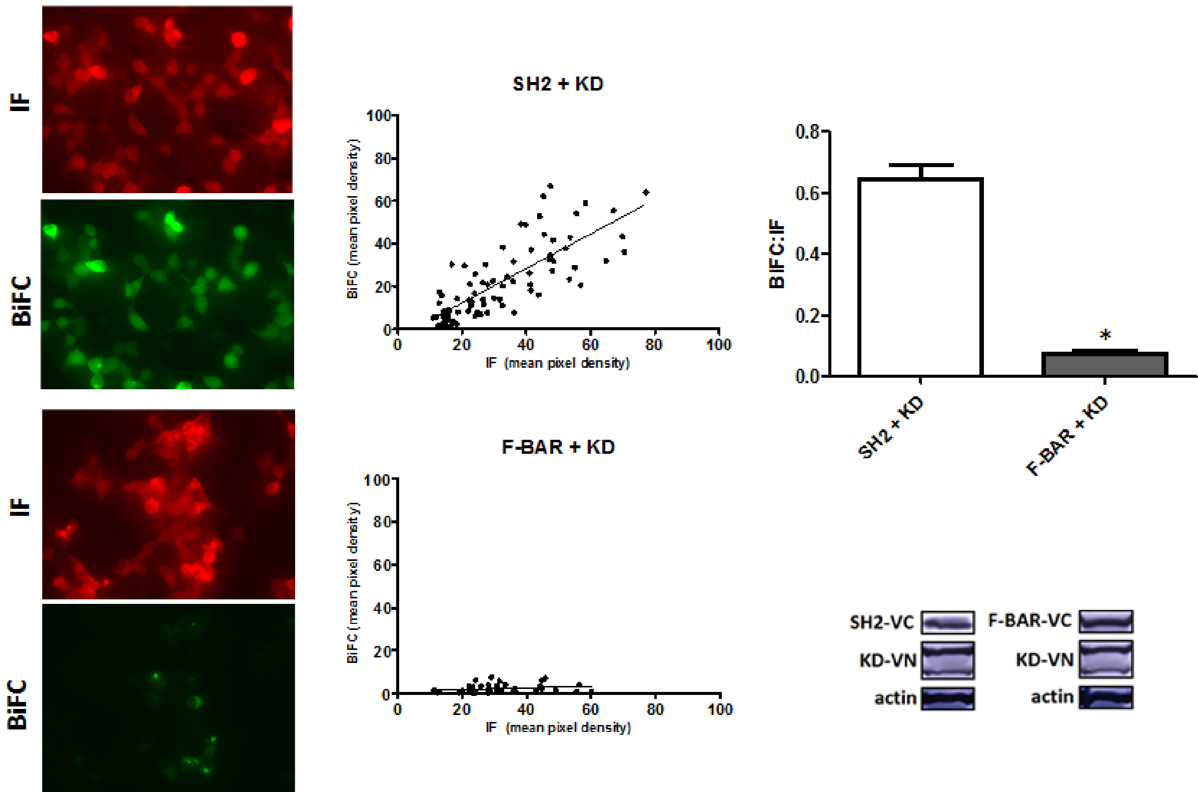


Figure 11. The Fes kinase domain (KD) interacts with the SH2 domain but not the F-BAR domain. As in the previous figure, shown are representative IF and BiFC images pseudo-colored green and red, respectively, for Venus-fragment-fused Fes domain co-expressions of the SH2 + KD and F-BAR-L + KD. Mean pixel densities were measured using ImageJ and analyzed in GraphPad as before. As expected (see crystal structure) there is a positive relationship between expression and SH2:KD interaction whereas co-expressed F-BAR-L and KD BiFC vectors show no appreciable complementation.*$p < 0.001$

4.1.7 Discussion.

The fact that the c-Fes F-BAR region interacts with its SH2 domain in cells is an important discovery in Fes biology. Many other non-receptor tyrosine kinases have an SH2 and SH3, or more, with well-known regulatory interactions. This report is the first with direct

evidence for the Fes SH2 interacting with any other non-catalytic domain within c-Fes. As I have proposed, it may be that these two domains coordinate the regulation of c-Fes by a novel intramolecular mechanism, supported by the results of CC1 mutation in BiFC and full-length Fes kinase assays. Consistent with these findings, the F-BAR region (or a portion of it) may sequester the SH2 domain, thus preventing its role in kinase domain stimulation. When the F-BAR region is deleted or mutated in a way that such that it can no longer hold the SH2 domain, the SH2 is free to conform to the kinase core and aid in stabilizing a pro-catalytic state. If L145P mutant and 45 kDa SH2-kinase core proteins are constitutively active due to this loss of auto-inhibition, then it may be that binding of adapter proteins, oligomerization, substrate recruitment, or another event is the trigger of c-Fes activation in physiological conditions. Oligomerization and subcellular redistribution indeed coincide with c-Fes autophosphorylation, perhaps preceding it.

One possible alternative interpretation is that some other protein somehow regulates c-Fes kinase activity in an F-BAR-dependent manner; however, studies in yeast indicate that c-Fes regulation is intrinsic, not requiring any host cell factor (46). There remain many things unknown about the Fes F-BAR, such as its conformation within the full-length protein, but now there is a more solid basis for our working model and future studies.

4.2 DEVELOPMENT OF TOOLS TO TEST THE EFFECT OF F-BAR:SH2 INTERACTION ON FES KINASE ACTIVITY

The loss of F-BAR features or replacement of SH2 have profound effects on Fes activity (21, 64). In the case of CC1, structural predictors suggest the Fes L145P mutant loses its

structure (75). The results of the previous section suggest that this coiled-coil is essential to interaction with the SH2, which has two α-helices of its own. Having a reciprocal mutation in the SH2 would strengthen the case for this interaction and its role in autoregulation. Mutants of the SH2 are tools needed for BiFC and full-length kinase assays to better understand c-Fes autoregulation.

4.2.1 SH2 sequence and structural alignments.

In the case of the Fes/Src SH2 substitution, a conserved glutamic acid in helix A critical to association with the kinase domain helix C (64) might explain why this chimera was capable of being so highly active. The other helix (αB) of the SH2 faces away and could be involved in F-BAR binding. In order to determine candidate SH2 mutants for use in future experiments, I performed comparative amino acid sequence analysis by ClustalW (122) alignment of c-Fes, v-Fps, v-Src, and Ras-Gap SH2 domains (in Figure 12). Furthermore, 3D structural overlays of the Fes versus Src SH2 domains in PyMol (Figure 13) revealed differences in the crystal structures.

SH2	αA		αB
c-Fes	WYHGAIPRAEVAELL	...	SIPLLIDHLLSTQ
v-Fps	WYHGAIPRSEVQELL	...	TIPLLIDHLLQSQ
v-Src	WYFGKITRRESERLL	...	SLQQLVAYYSKHA
Ras-GAP	WYHGKLDRTIAEERL	...	SLSDLIGYYSHVS

Figure 12. Sequence variance between c-Fes, v-Fps, v-Src, and Ras-Gap SH2 domains. Yellow highlight added for emphasis of critical positions on c-Fes which are identical (or similar) in v-Fps while uniquely variant in v-Src αB or Ras-Gap in αA. Light blue highlight emphasizes conservation in αA between c-Fes, v-Fps, and v-Src lacking in Ras-Gap, which may be critical to the auto-stimulatory SH2:KD interaction; whereas red

highlights positions in v-Src where conservation between c-Fes and v-Fps is not shared there, which may be important in the auto-inhibitory F-BAR:SH2 interaction.

Based on the X-ray crystallography (64) and these alignments, there are number of SH2 mutations that can be made to probe the F-BAR:SH2 interface. Since only the v-Src SH2 substitution leads to the active kinase, significant differences in the amino acid sequence of the αB helix in v-Src versus c-Fes may be responsible for the loss of inhibition. Instead of completely replacing the entire helix with the v-Src sequence, I designed primers to generate four mutants: S532A, T533A, Q534A, and triple STQ/AAA. These three residues protrude outward from the αB helix, and are therefore most likely to form the proposed negative regulatory interface with CC1. The Fes SH2 packs against the kinase N-lobe, forming a critical interface coordinated by polar interactions between the helix αA of the SH2 and αC of the kinase domain stabilized by acidic residues in the SH2 αA (E469 and E472) and αC of the kinase domain (R609). Therefore, these E/K mutations were part of the mutagenesis performed in this work in order to have well-characterized control mutations.

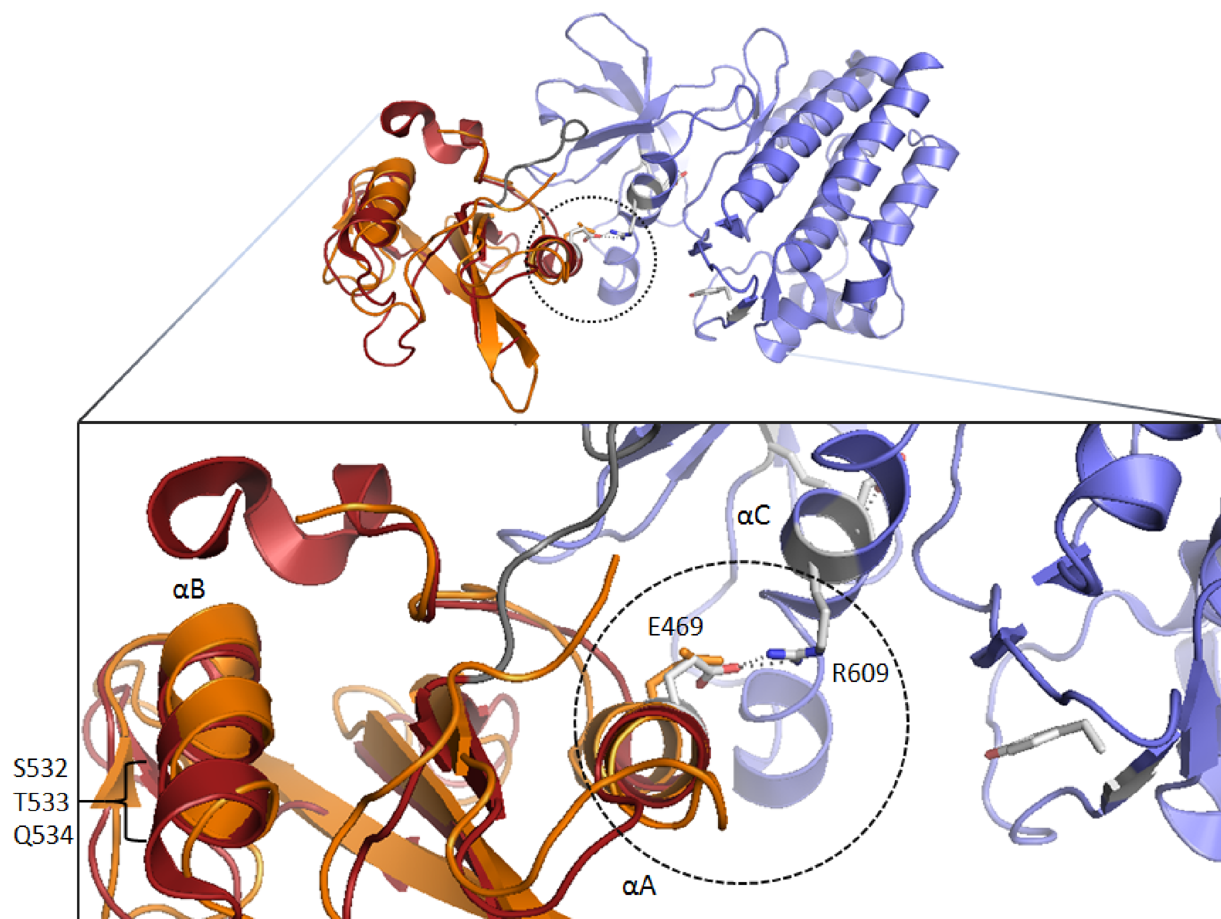


Figure 13. Alignment of c-Fes SH2-kinase domain crystal structure with v-Src domain. Top) Full-view of the X-ray crystal structure of the Fes SH2-kinase unit (PDB.3BKB). The Src SH2 domain (PDB:1SHB, orange) was aligned to the Fes SH2. The Fes SH2 (red) forms an interface with the kinase N-lobe (blue). Polar interactions between the helices are stabilized by E469 and R609. Bottom) Close-up view of the overlaid SH2 domains, with the v-Src shown in orange. A stretch of three residues of helix B (STQ) are not the same in v-Src. Docking of the Src SH2 domain onto that of c-Fes positions a glutamate residue in the Src SH2 αB helix in close proximity to R609 in the Fes N-lobe helix αC. This observation suggests that the v-Src SH2 domain can still form a productive SH2:N-lobe interface required for kinase activity.

4.2.2 Site-directed mutagenesis.

PCR site-directed mutagenesis was performed on the pcDNA3.1 Fes SH2-BiFC and a c-Fes-FLAG plasmids with 16 primers (8 mutations) to generate 19 new constructs. Primers were designed to be oligomers between 20-30 bp in length, with a high GC content, melting around 80 °C, with G/C ends, and central mismatches. The primers (sense 5'-3'): for helix B were S532A (C GAC CAC CTA CTG ***GC***C ACC CAG CAG CCC C), T533A (C CAC CTA CTG AGC ***G***CC CAG CAG CCC C), Q534A (C CTA CTG AGC ACC ***GC***G CAG CCC CTC ACC), and STQ/AAA (C GAC CAC CTA CTG ***GC***C ***G***CC ***GC***G CAG CCC CTC ACC); for helix A E469K (C CCG AGG GCA ***A***AG GTG GCT GAG C), E472K (GCA GAG GTG GCT ***A***AG CTG CTG GTG C), and EE/KK (CCG AGG GCA ***A***AG GTG GCT ***A***AG CTG CTG G); for kinase dead Fes, K590E (C CTG GTG GCG GTG ***G***AG TCT TGT CGA GAG ACG). The E-K and K59E mutations were also introduced into L145P Fes-FLAG plasmids to test these changes in a Fes background that is already activated. Small-scale bacterial preparation preceded sequence confirmation, followed by large-scale preparations.

4.2.3 Results.

The mutations were successfully introduced into the BiFC and full-length Fes plasmids generating VN-SH2 S/T/Q→A, VN-SH2 E/E→K, as well as FLAG-Fes and L145P Fes counterparts with these mutations. Preliminary data gathered with a few of these shows that they can be used as tools to learn more about Fes regulation. In a BiFC experiment with the F-BAR, the Q534A and AAA mutants of SH2 helix B displayed extremely weak BiFC compared to the

wild-type SH2. However, mutation on the other side of the SH2 (αA EE/KK) did not significantly affect the F-BAR interaction.

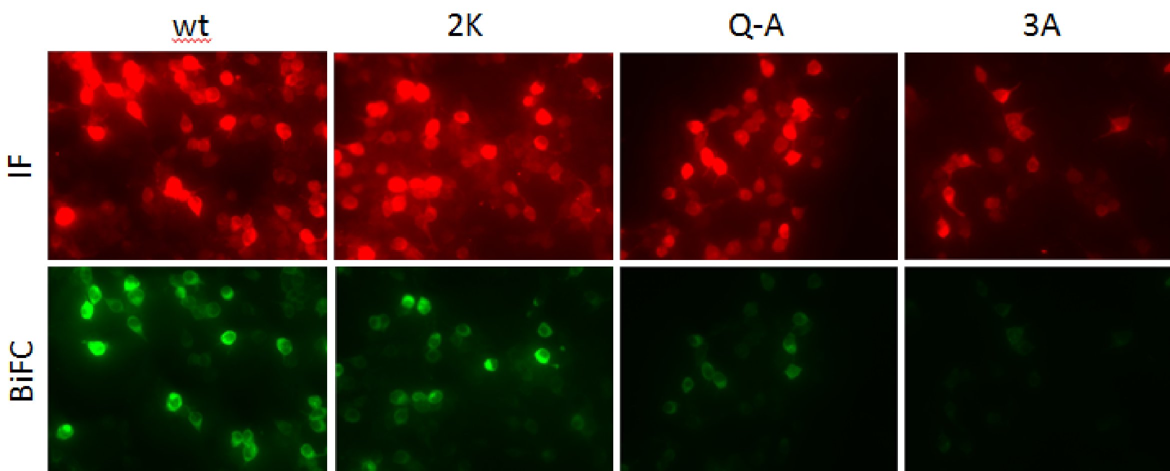


Figure 14. Src-like mutations in αB diminish F-BAR:SH2 BiFC. 293T cells were transfected with F-BAR-VC and SH2-VN as before, along with wells for the SH2-VN mutants. After 24 hours of expression, fixing, and immunostaining 6 sec exposures were captured. Consistent with an αB:CC1 helical interaction, mutations in helix B seem to reduce BiFC.

4.2.4 Discussion.

The successful BiFC experiment verifies the utility of the SH2-VN mutants. The same cannot be said for the Fes-FLAG mutants as there may be stability (or expression) issues. Attempts at western blots, particularly for pTyr713, were highly variable. It may be more prudent to use YFP-Fes, as it may be more stable and more easily detected in COS-7 experiments to observe subcellular localization and pTyr immunofluorescence. The full-length Fes SH2 mutants would be critical to phosphorylation activity studies of Fes with respect to the

hypothesis that the F-BAR inhibits the kinase by SH2 association. An experiment showing that the results seen in L145P mutation are similar in SH2 helix B mutants would fit in nicely.

The preliminary result (shown in Figure 14) is consistent with the idea that the F-BAR interacts with the SH2 by recognizing helix B. This would explain why the v-Src SH2 chimera was activated while the v-Fps SH2 chimera was not. The αA mutant performed like wild-type when it came to F-BAR interaction, so not just any mutation of the SH2 will destabilize it. Future experiments might show that this EE/KK does not interact with the kinase domain as demonstrated by BiFC, which would be consistent with previous studies.

4.3 FUTURE DIRECTIONS

4.3.1 Translation of BiFC results with interdomain interaction mutants into full-length c-Fes activity studies.

In order to connect Fes interdomain interactions shown by BiFC to regulation of Fes kinase activity, the mutations that affect BiFC should be tested in full-length Fes. Autophosphorylation in the activation loop of Fes at tyrosine residue 713 is required for activity and indicates the upregulated kinase, so will serve as the primary readout of functionality. It can be detected by phospho-specific immunostaining of western blots or fixed cells (21, 46, 75). Comparison between immunoblots of lysates from cells expressing wild-type c-Fes versus Fes bearing mutations that have resulted in reduced BiFC between regulatory domains will clarify whether the intramolecular interactions are linked to catalytic activity. L145P will exemplify positive activity, while the K590E kinase dead mutant will serve as a negative control.

Constitutively active or dysregulated c-Fes has been implicated in some cancers, like AML, and has been shown to drive transformation in a number of cell types. As a model for transformation, wild-type and mutant forms of full-length Fes could be subcloned into retroviral vectors for transduction of rodent fibroblasts, followed by focus-forming and soft agar colony formation assays (21, 75). To evaluate the mutants in a context relevant to human cancer, TF-1 myeloid leukemia cells may be infected with retroviruses expressing mutant Fes (75). TF-1 cell-attachment, spreading, and growth in the absence of GM-CSF would indicate downstream activation of survival pathways of these leukemia cells as a model for AML. RNAi knockdown of the mutant resulting in reversal of the effect the mutant had would serve as confirmation. This approach may also be applied to transformed cells stably expressing KITD816V or Flt3 (97, 98). These in vitro and in vivo kinase activity experiments are necessary to couple BiFC findings with c-Fes autoregulation.

4.3.2 Complementary F-BAR *en bloc* substitution experiments.

F-BAR domain proteins are typically thought of as regulators of membrane curvature and dynamics, implicated in endocytosis, exocytosis, vesicular transport, cellular morphogenesis, and motility (36, 43, 123, 124). However, here I present findings to implicate the Fes F-BAR in regulation of its catalytic function through interaction with the SH2 domain. Structural data on the F-BAR is pending and no crystal structure exists for full-length c-Fes, where it may be revealed how exactly the F-BAR interacts with the SH2 domain and what happens to the SH2-kinase domain interaction as a consequence in the downregulated state. (There is, however, a recently uploaded PDB:4DYL that has resolved much of the N-terminal region of c-Fes, see Appendix A.) In the meantime, experiments with homologous F-BAR domains swapped in for

the Fes N-terminal region could be very informative, as SH2 substitution has been in the past (21). Closely related F-BARs include FCHSD1 and TOCA2, while more taxonomically distant are Pacsin1 and FCHO (43). It would be ideal to find a few homologous F-BAR domains that show diverse effects on the Fes kinase activity. In that way, comparison of the sequences and structures of these F-BAR domains might identify specific features or residues that are involved in Fes regulation.

4.3.3 Development of a BiFC-based platform for c-Fes allosteric small molecule modulators.

Since tyrosine kinase domains are highly conserved, inhibitors directed against the kinase domain often lack specificity (125). Even in cases where a relatively high level of target selectivity has been achieved, resistance mutations arise in patients following long-term drug exposure (e.g. imatinib, which inhibits Bcr-Abl in CML). In that case, combination therapy with allosteric inhibitors may be effective (126). In the case of Fes, novel allosteric inhibitors and activators that target its unique regulatory regions—SH2 interfaces with the kinase domain and F-BAR, respectively—represent powerful new tools in the study of c-Fes signaling pathways and potentially new treatments for Fes-related cancers. Due to the differing tissue-specific roles of c-Fes in cancer, small molecule modulators of this kinase present utility in both investigation of its function and anti-cancer therapy. The BiFC strategy developed for investigating interdomain interactions of c-Fes could be adapted to identify allosteric small molecule modulators of c-Fes activity (Figure 15). An advantage of a BiFC-based screen is that it occurs in a mammalian cellular context. The ArrayScan II (Thermo Scientific) can be adapted to BiFC in an 384-well format. The BiFC pair to be screened against, either F-BAR:SH2 or SH2:kinase domain, would

be expressed from a single 2A plasmid. Self-cleaving viral 2A sequences (127) separate the two BiFC-fragment-fused domains and a marker RFP gene so that the BiFC pair will be expressed in equimolar quantities. Thus, ArrayScanII can identify expression-positive cells, which will be gated on the RFP signal. This 2A-BiFC expression platform has been successfully developed by our laboratory as a screening assay for small molecules interfering with HIV Nef homodimerization with a Z'-factor of ~0.7, which is indicative of a robust screening assay (J Poe & T Smithgall, manuscript submitted). In the case of the Fes screen, the wild-type F-BAR-VC:SH2-VN BiFC pair, expressed in the absence of library compounds (DMSO present), and BiFC-negative F-BARL145P-VC:SH2-VN transfections will serve as plate controls to define the screening window on each plate. For the SH2-kinase pairing, the E469K mutant may be an acceptable negative control. To find small molecules that act on the non-catalytic regulatory domains of c-Fes, one might screen the National Cancer Institute Diversity Set III library, ChemDiv Targeted Diversity Peptidomimetic collection, or some other set of compounds with highly diverse scaffolds (not designed for kinases). Chemical compounds may be auto-fluorescent, so initial hits will be added to wells containing cells transfected with YFP only to identify such compounds. The goal of the screen is to identify small molecules that specifically target the domains of Fes, and not the BiFC reporter directly. Therefore, tentative hit compounds will also be counter-screened against an irrelevant GST-BiFC dimer. The GST-BiFC counterscreen will eliminate any compounds that artificially quench BiFC without actually binding Fes. Once false-positive and nonspecific compounds are sorted out, one might perform 10-point concentration-response experiments with the true hits in the same BiFC assay used in the primary screen to determine the most effective concentration for use in secondary activity-based screens. Lead candidates will be small molecules that most effectively alter BiFC at the

lowest concentration. Hits will be prioritized further based on cytotoxicity, where the drugs least toxic to mammalian cells will be preferred and tested for effects on activity of the full-length Fes. Secondary screens would involve full-length c-Fes and appropriate control mutants—L145P activated and K590E kinase dead mutants—to be assayed for kinase activity by Rat-2 transformation in the presence of candidate small molecule modulators. The readout will be colony-forming activity, correlated with Fes kinase activity by pY713 immunoblotting. Hits that reduce F-BAR:SH2 BiFC would be c-Fes activators. In contrast, small molecules reduce SH2:kinase domain BiFC would be inhibitors.

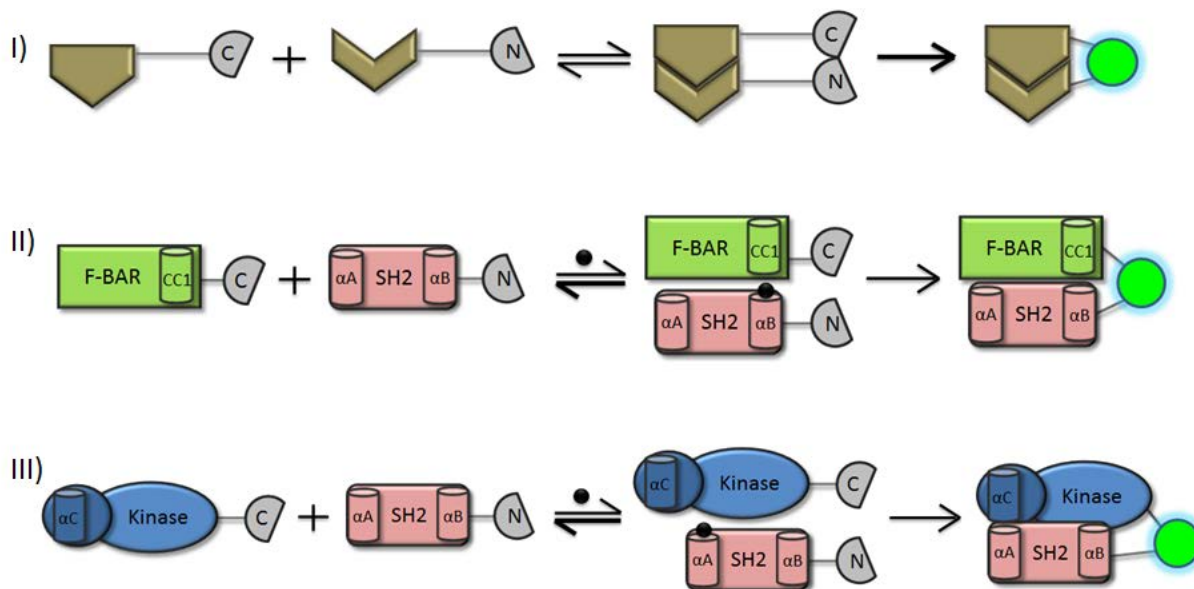
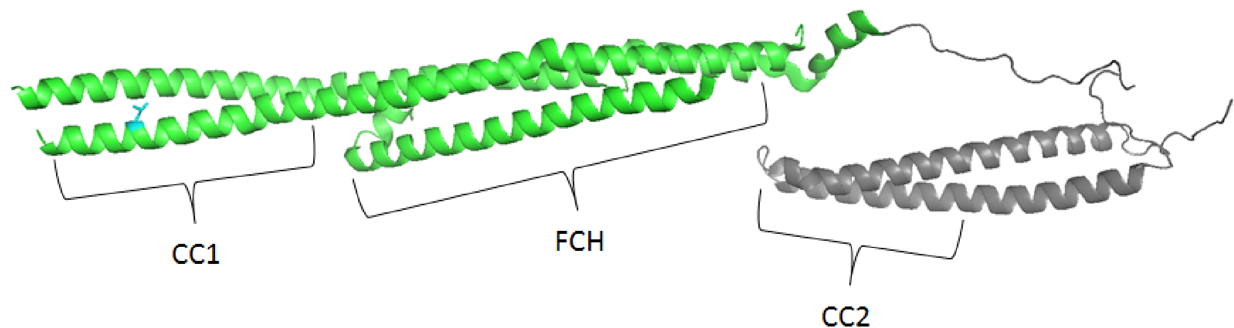


Figure 15. BiFC-based screen concept for allosteric modulators of Fes activity. I) In DMSO-only wells, the interacting partners generate a normal BiFC signal for that pairing. II) In the presence of a small molecule activator of Fes, the BiFC between F-BAR and SH2 would be reduced. III) In the case of an inhibitor, the SH2-kinase domain BiFC would be impaired. (Not drawn to scale or with true orientation of all features.)

APPENDIX A

SUPPLEMENTAL FIGURES



Supplemental Figure 1. First X-ray diffraction crystal structure of human FES F-BAR. Shown are residues 1-405 of c-Fes. FCH and CC1 (green) make up the F-BAR, while CC2 and linker segments (gray) were also resolved to within 2.18Å. The leucine at position 145 (cyan) is highlighted for reference as proline substitution is predicted to kink CC1. (PDB:4DYL; Ugochukwu, E., Salah, E., Elkins, J., Barr, A., Krojer, T., P., Filippakopoulos, Weigelt, J., Arrowsmith, C.H., Edwards, A., Bountra, C., von Delft, F., Knapp, S., Structural Genomics Consortium, publication pending)

Since crystal structures of both the F-BAR region and the SH2-kinase core have been solved, and BiFC captures the F-BAR:SH2 interaction, it may be plausible to try co-crystallization of F-BAR and SH2 or SH2-kinase complexes. *In silico* binding and folding analysis may predict the conformation of the Fes F-BAR in complex with the SH2 and kinase domains.

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