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FES/FER kinase signaling in hematopoietic cells and leukemias

Andrew W.B. Craig[1]

[1]*Department of Biochemistry, Queen's University, Division of Cancer Biology and Genetics, Queen's Cancer Research Institute, Kingston, Ontario K7L 3N6 Canada*

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1. ABSTRACT

FES and FES-related (FER) comprise a unique subfamily of protein-tyrosine kinases (PTKs) that signal downstream of several classes of receptors involved in regulating hematopoietic cell development, survival, migration, and inflammatory mediator release. Activated alleles of FES are potent inducers of myeloid differentiation, however FES-deficient mice have only subtle differences in hematopoiesis. This may reflect overlapping function of other kinases such as FER. Studies of FES- and FER-deficient mice have revealed more prominent roles in regulating the activation of mature innate immune cells, including macrophages and mast cells. Recently, new insights into regulation of FES/FER kinases has emerged with the characterization of their N-terminal phospholipid-binding and membrane targeting FER/CIP4 homology-Bin/Amphyphysin/Rvs (F-BAR) and F-BAR extension (FX) domains. The F-BAR/FX domains regulate subcellular localization and FES/FER kinase activation. FES kinase activity is also enhanced upon ligand binding to its SH2 domain, which may lead to further phosphorylation of the same ligand, or other ligand-associated proteins. In mast cells, SH2 ligands of FES/FER include KIT receptor PTK, and the high affinity IgE receptor (FcεRI) that trigger rapid activation of FES/FER and signaling to regulators of the actin cytoskeleton and membrane trafficking. Recently, FES/FER have also been implicated in growth and survival signaling in leukemias driven by oncogenic KIT and FLT3 receptors. With further definition of their roles in immune cells and their progenitors, FES/FER may emerge as relevant therapeutic targets in inflammatory diseases and leukemias.

2. INTRODUCTION

FES was originally cloned as a retroviral oncogene from feline (v-FES) and avian (v-FPS) sarcomas (1-5). This led to identification and cloning of the cellular FES genes (also referred to as FPS) in birds and mammals (1, 2, 6). Subseqently, cloning of novel PTKs resulted in identification of FER PTK that was highly related to FES (7, 8). In mammals, FES and FER represent the only members of a subfamily of PTKs (subgroup IV) defined by a unique N-terminal domain comprising an Fer/CIP4 Homology (FCH) motif (9) and predicted coiled-coil (CC) domains, followed by an SH2 domain, and a C-terminal kinase domain (10). However, recent studies support the reclassification of the N-terminal domains of FES/FER as a F-BAR (FCH + CC1) domain, and a FX domain (CC2/CC3). Together these domains promote oligomerization and membrane targeting (11, 12). The FES/FER subfamily is represented by a single ortholog in early metazoans, including the marine sponge Sycon raphanus (Fes/FER_SR) (13), *Schistosoma*-family helminths (smFES) (14, 15), and Drosophila melanogaster (16-18). In terms of orthologues with homology to the F-BAR domain of FES/FER, this Pombe Cdc15 homology (PCH) family predates the evolution of PTK-based signaling mechanisms (19).

3. FES/FER EXPRESSION AND ISOFORMS

Although similar in domain organization (Figure 1A), FES and FER have distinct expression patterns. While FES expression is highest in hematopoietic cells and their progenitors, it is also detectable in epithelial,

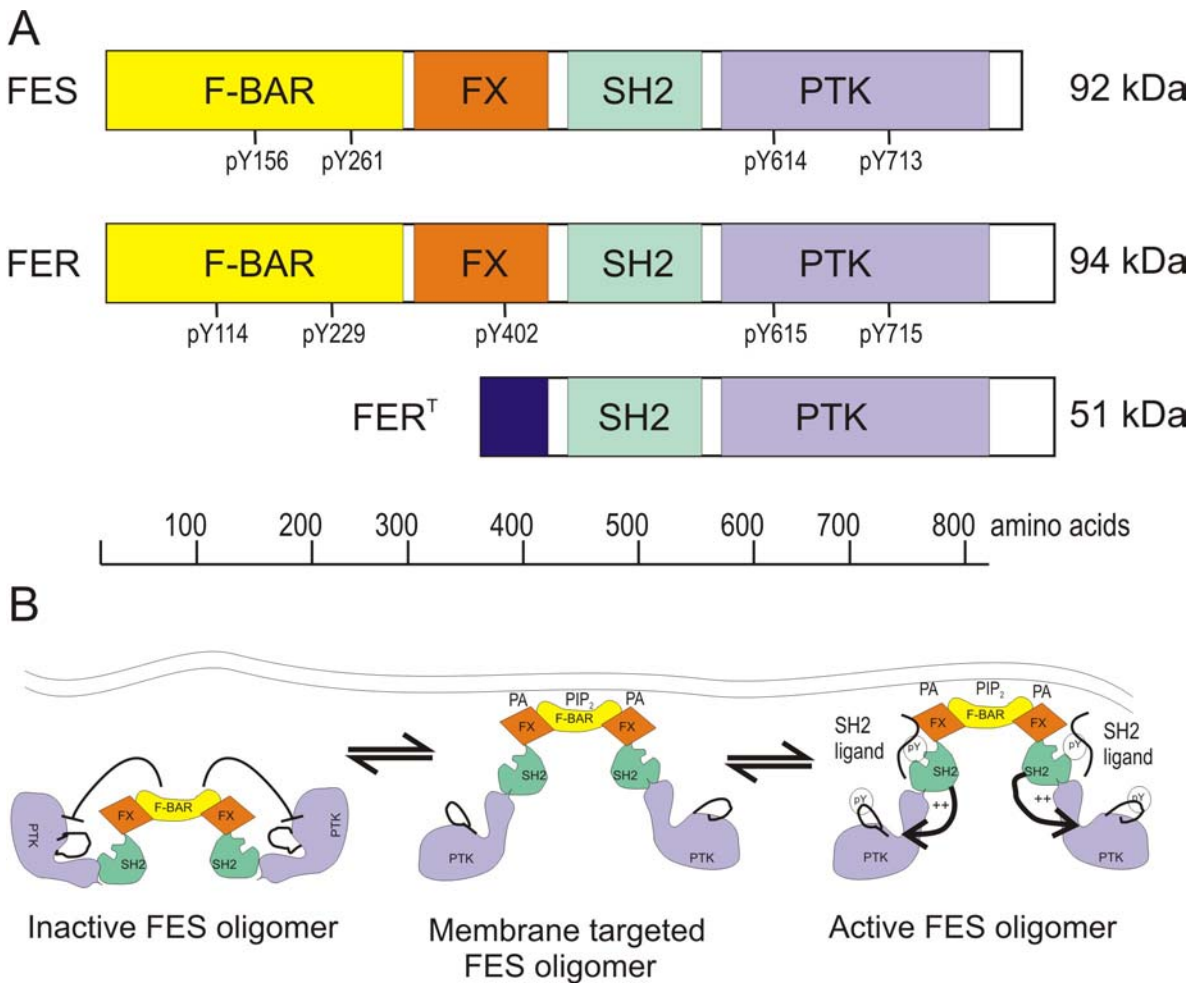

Figure 1. Domain organization and model for FES/FER activation. A. FES and FER share a similar domain organization : F-BAR, FX, SH2, PTK domains. FER^T is a testis-specific isoform of FER that encodes a unique 51 amino acid N-terminus, followed by SH2 and PTK domains. Sites of tyrosine phosphorylation identified in FES and FER are shown (see text for details), and their molecular masses are indicated on the right. B. Hypothetical model depicting potential steps leading to FES activation. FES is shown as a dimer mediated by its F-BAR domain, but may exist in higher order oligomers. FES N-terminal domain represses its kinase activity, which can be relieved by proline-insertion mutations that disrupt oligomerization. *In vivo*, the repressed FES oligomer may undergo conformational changes upon membrane targeting via F-BAR domain interactions with $PI(4,5)P_2$, or FX domain binding to PA (as reported for FER (11)). This open form of FES may more readily interact with SH2 ligands leading to kinase activation via the SH2-PTK interface. Although FER is not clearly repressed by its N-terminus, we predict that aspects of this model could be applied to FER activation as well.

endothelial and neuronal cells (20). FES is also expressed in hematopoietic stem cells since transgenic mice expressing Cre recombinase under the control of the human FES gene, was shown to delete floxed *phosphatidylinositol glycan class A* alleles in all blood cells (21). A recent study implicates promoter methylation as a mechanism for repression of FES expression in colorectal carcinomas compared to the colonic epithelium (22). This correlates with observed tumor suppressor roles of FES in colorectal carcinoma and mammary carcinoma (23, 24). Upregulation of FES expression was also observed during lactation in mouse mammary gland, and lactation defects were reported in *fes*-null mice (25). Although several *fes* mRNA species have been reported in granulocytes, myeloid leukemia cell lines, and lymphomas (26-28), there is limited evidence of alternative splice isoforms of Fes. Mammalian gene collection large scale sequencing efforts identified only 2 transcripts for *fes* in mice (29). In addition to a 2.45 kb transcript encoding full length *fes*, a 0.7 kb variant was also identified that potentially encodes a portion of the N-terminal F-BAR domain (amino acids 1-213; AAH38130.1 GenBank accession). Interestingly, expression of a truncated *fes* allele lacking SH2 and PTK domains in mouse embryonic stem (ES) cells, led to expansion of myeloid progenitors *in vitro*, and embryonic lethality in chimeric mice (30). It would be interesting to determine whether this truncated FES acts through disruption of membrane trafficking of cytokine receptors during differentiation of the ES cells. Currently, for the human *fes* gene, 3 alternate splice isoforms are described.

They are thought to arise from exon skipping, and encode for in-frame deletions within F-BAR, SH2, and PTK domains. These putative *fes* isoforms would be expected to be kinase inactive, and have potential dominant-negative effects on FES signaling.

In contrast with FES, FER is expressed in most tissues and cell types. In mammals, an internal testis-specific promoter in *fer* results in expression of a truncated FER isoform called FER^T (31). FER^T contains a unique N-terminus, but shares the SH2-kinase domain portion of FER (Figure 1A), and is highly expressed during spermatogenesis (32, 33). Although this suggests a possible role for FER or FER^T in male fertility, we were surprised to find that knock-in of a FER kinase-inactivating D743R mutation ($fer^{DR/DR}$) in mice results in no defects in fertility (34). Since these mice express only a kinase-dead form of FER and FER^T, this raises the possibility of a kinase-independent role of FER^T. Another possibility is that another PTK provides a redundant function within the testis, notably the Src family kinase Fyn shares a similar expression and localization in the acrosome of spermatids (35). It is interesting to consider that in *C. elegans*, the FER orthologue FRK-1 resembles FER^T (lacks an N-terminal F-BAR domain), and like the mammalian FER (36), it promotes β-catenin retention within adherens junctions to limit the Wnt signaling pathway within the endoderm (37).

4. FES/FER DOMAIN ORGANIZATION AND REGULATION

The last several years has seen great advances towards defining the structure and regulation of the FES/FER kinase family. The FCH and CC1 motifs have now been reclassified as an F-BAR domain, which is a conserved module found in all PCH proteins (38-40). With the exception of FES/FER, most PCH proteins are adaptor proteins that recruit actin regulatory proteins to sites of membrane curvature and regulate endocytosis (41). We and others have shown that the N-terminal F-BAR and FX domains of FES and FER bind phospholipids and target these PTKs to membranes (11, 12, 42). With crystal structures solved for F-BAR domains from several PCH proteins (43, 44), we generated a homology model for the FES F-BAR domain and showed that it functions in phospholipid binding, membrane targeting, and FES activation downstream of FcεRI in mast cells (12). In FER, the F-BAR/FX domains target FER to lamellipodia and is required for FER-induced cell motility (11). This is consistent with motility defects in FER-deficient embryonic fibroblasts (45) and mast cells (46). Membrane targeting of FER/FES is likely regulated in normal cells, since constitutive membrane targeting of FES (e.g. v-FES/v-FPS) results in a dramatic increase in PTK activity, and transforming ability compared to cellular FES (47, 48). Addition of a Src-like myristoylation sequence in FES (FES^{MF}) also confers increased transforming ability of FES *in vitro*, and leads to developement of cardiovascular defects, and vascular tumors (hemangiomas) in transgenic mice (fes^{MF}) (49). The Drosophila ortholog, Dfer also localizes to membranes, but did not require myristoylation to confer malignant transformation activity in fibroblasts (18).

Regulation of F-BAR domain targeting to membranes was recently reported (50). The authors solved a crystal structure for PCH adaptor protein Syndapin/PACSIN that contained the crescent shaped F-BAR domain dimer bound to its SH3 domain. In addition, mutations to charged residues within this interface led to a profound increase in membrane tubulating activity of Syndapin/PACSIN. Importantly, a similar effect was found upon addition of a proline-rich peptide from Dynamin (an SH3 domain ligand of several PCH proteins), which increased the liposome tubulating activity of the F-BAR domain. Intramolecular interactions of BAR domain adaptors Amphyphysin and Endophillin were also reported, suggesting that this mode of regulation may apply more broadly to the BAR domain superfamily. Since FES/FER lack SH3 domains, the mode of regulation of their F-BAR domains are likely distinct. However, intermolecular interactions with SH3 domains of BAR or F-BAR proteins that share similar subcellular localization as FES/FER are possible. In addition to homotypic interactions, the N-terminal domains of FES and FER mediate interactions with several proteins, including p120-catenin (36), BCR (51), Tubulin (52), and Plectin (53). In most of these examples, the binding partners are also potential substrates of FES or FER. In contrast, interaction of FER with the cytolinker protein Plectin was reported to suppress FER kinase activity, and this was dependent on the F-BAR domain region of FER (53). However, this could also be due to indirect effects since Plectin-deficient cells have alterations in focal adhesions and filamentous-actin (F-actin) (54). FER not only regulates focal adhesions (36, 55), but also is negatively regulated by F-actin in some cell models (56).

The N-termini of FER and FES are best characterized for mediating homotypic oligomerization (57-61). Unlike most RTKs, kinase activities of FES and FER do not correlate with their abilities to form oligomers (52, 58, 59, 61). In fact, proline insertions that disrupt oligomerization via the predicted CC motifs in FES (e.g. L145P), were shown to cause increased kinase activity, transforming ability, and cytokine-independent growth of myeloid leukemia cells (57, 58). The L145P mutation in FES may therefore disrupt intramolecular interactions that suppress FES activity (Figure 1B), or interactions with other binding partners that function in repression (10, 62). With the discovery of the FES F-BAR domain, it will be important to revisit the nature of the effects of the L145P mutation, and whether potential effects on membrane targeting may contribute to the activating phenotype observed. We hypothesize that membrane targeting via F-BAR/FX domains of FES/FER could lead to formation of a more open oligomer, that is poised for activation by engaging with SH2 domain ligands (Figure 1B). The L145P mutation might also promote this open conformation, increasing access of FES to ligands and substrates. Within the FES F-BAR homology model, L145 is located near the tips of the dimer in the α3 helix. The proline insertion may completely disrupt this helix, and the

F-BAR dimer. However, it is worth noting that F-BAR domains all share a conserved proline residue within α4 helix (P206 in human FES) that generates the characteristic crescent shaped F-BAR dimer (44). Since all F-BAR domains form dimers, it is worth noting that the L145P mutation alone did not disrupt oligomerization of a FES N-terminal fragment *in vitro* (58). However, in more recent bimolecular fluorescence complementation (BiFC) experiments, the L145P mutation did disrupt FES BiFC *in vivo*, suggesting that it can either block oligomerization entirely, or alter FES conformation enough to prevent the fluorescence complementation (61). Interestingly, the L145P mutation also mimics the effects of activated Src family kinase Hck on promoting FES activation and microtubule localization (52, 61). Since microtubule localization of FES was largely directed by SH2 domain interactions (52), this may suggest that activation of Src kinases *in vivo* (or L145P mutation *in vitro*), leads to FES SH2 interactions that disrupt an autoinhibited FES oligomeric state (Figure 1B). Src kinases may also act directly on FES by phosphorylating tyrosines within the activation loop region (Y713 in FES) (52), or the currently uncharacterized sites within the F-BAR and kinase domains (Y156, Y261, Y614) that were recently identified by mass spectrometry (Figure 1A) (63, 64). Another possiblility is that without active Src kinases, FES SH2 domain ligands are insufficient to trigger FES conformational changes and kinase activation.

The Knapp and Pawson groups recently solved structures for the SH2-kinase domain fragment of FES in both active and inactive conformations (65). They showed that residues within the SH2 domain form a key interface with the kinase domain to tightly control FES kinase activity. It was shown that formation of the FES catalytic site is positively regulated by SH2 interactions with pY-containing ligands. This cooperativity was lost with mutations disrupting the SH2-PTK interface (eg. G463V, E469K/E472K), or the pY-binding pocket within the SH2 domain (eg. R483M). These mutations dramatically reduce FES PTK activity in transfected cells, and the transformation phenotype induced by v-FES (65). Further, these results imply that FES activation will be tightly linked physically and temporally to signaling-induced tyrosine phosphorylation of SH2 domain ligands within cells (Figure 1B). The conservation of key SH2-PTK interface residues in FER, suggests that this allosteric mode of regulation of FES will also apply to FER. Nir and co-workers demonstrated that mutations in an Hsp90 recognition loop within the FER kinase domain abolished FER kinase activity (66). However, these mutations encompass the predicted SH2-PTK interface in FER, and disruption of this interface may also explain the loss of kinase activity. Thus, a more comprehensive analysis of FES/FER SH2 domain ligands in various cell models, will help identify potential nodes of FES/FER signaling.

Mechanisms controlling FER kinase activition are still being elucidated, and show some differences from those described for FES. For example, insertion of proline residues within predicted CC motifs in FER resulted in a block in oligomerization without affecting FER autophosphorylation (59). This may reflect that FER kinase activity is less restricted by N-terminal domain interactions than FES, at least in these conditions of ectopic expression (59). Recently, Itoh and co-workers reported that the F-BAR domain of FER is unable to bind phosphoinositides (e.g. $PI(4,5)P_2$), and that the FX domain mediates binding to phosphatidic acid (PA) (11). They showed that PA binding promotes FER kinase activity *in vitro*, suggesting positive cooperativity between lipid binding at the N-terminus and kinase activation. They also implicate Phospholipase D (PLD) as an upstream regulator of FER recruitment to lamellipodia via PA accumulation, and that PLD and FER act in a common pathway of chemotaxis (11). Given the activation of the PLD-PA axis downstream of many receptors, including G-protein coupled receptors (67), FER signaling roles may extend beyond pY-based receptor signaling pathways. One caveat to the Itoh study (11) involves the use of 1-butanol as an inhibitor of PLD. Treatment with 1-butantol is known to also deplete $PI(4,5)P_2$ levels (68), which is a well characterized F-BAR domain ligand. Thus, the potent effects on FER localization to lamellipodia or autophosphorylation may arise from depletion of both phosphoinositides and PA. Along with RNA interference approaches, the development of PLD-specific small molecule inhibitors (69, 70), should allow for this to be tested directly. It is worth noting that we demonstrated that the F-BAR and F-BAR/FX regions of FES are able to bind $PI(4,5)P_2$-containing liposomes. Likewise, a previous study reported effective $PI(4,5)P_2$ binding via the FER F-BAR domain (42). Thus, future studies will be needed to clarify the phospholipid ligands and membrane targeting mechanisms that regulate FER localization and activation in cells. It is also worth noting that proteomics studies of EGFR signaling and hepatocellular carcinomas identified two phosphorylation sites within the F-BAR domain (Y229 and Y114; Figure 1A) (71, 72). It will be important to determine the effects of phosphorylation of these residues on the FER F-BAR domain, and FER activity. FER phosphorylation within the FX domain (Y402; Figure 1A) has also been reported in a variety of cancers (11), and in mast cells activated via IgE/antigen (73). Given the effects of substituting adjacent basic residues within the FX domain of FER on PA binding (11), it is possible that phosphorylation of Y402 alters lipid binding activity or membrane targeting of FER. FER may also be regulated via proteolysis, as IL-10 signaling led to FER stabilization in macrophages (74). Nir and co-workers also showed that Hsp90 interacts with FER and protects FER from proteolysis (66). We observed decreased FER levels in SCF-treated BMMCs, which correlated with FER pY downstream of KIT (75). It will be important to determine whether this destabilization of FER involves loss of association with Hsp90 in SCF-treated mast cells. Interestingly, Hsp90 is recruited to oncogenic KIT (76), and is a viable therapeutic target in KIT-driven cancers, including acute myeloid leukemia, mast cell leukemia, and gastrointestinal stromal tumors (77-79).

5. FES/FER AS MODULATORS OF HEMATOPOIESIS AND IMMUNITY

FES is highly expressed in myeloid cells and their progenitors, and has been implicated in promoting

myeloid differentiation of leukemic cell lines (58, 80, 81). FES is activated downstream of receptors for key cytokines, including GM-CSF (82, 83). In the bipotent U937 cell system, that can differentiate into either neutrophils or macrophages, expression of an activated allele of FES induced macrophage differentiation via activation of the PU.1 pathway (84). Interestingly, PU.1 also promotes expression of FES in myeloid cells (85). Raising the possibility of a positive feedback loop involving FES and PU.1. Although the mechanism of PU.1 activation by FES has not been defined, the SH2 domain and kinase activity were required for this effect (84). One possible explanation is that activated FES can promote tyrosine phosphorylation and activation of STAT3 (86). Recent studies have implicated STAT3 in directly activating the PU.1 promoter (87, 88). This may explain in part how activated FES promotes myeloid differentiation via the PU.1 master regulatory pathway.

Studies with loss-of-function approaches suggest a more modest regulatory role for FES in myeloid and erythroid differentiation *in vivo*. Fes-null (*fes*$^{-/-}$) mice were developed by both Celeste Simon's and Peter Greer's laboratories. Simon's group targeted the 5' end of the *fes* gene (promoter and exons 1-3), and reported that altough *fes*$^{-/-}$ mice were viable, there was some skewing from Mendelian ratios, and some examples of runted *fes*$^{-/-}$ pups (89). They observed a reduction in B lymphoid, and increased GR-1^{+} granulocytes and CD11b^{+} monocytes in bone marrow, peripheral blood, and spleen in *fes*$^{-/-}$ mice compared to control mice. This correlated with inflammatory infiltrates in skin, and increased susceptibility to *Borrelia burgdorferi* infection (89). Greer's *fes*$^{-/-}$ mice (also referred to as *fps*$^{-/-}$) were generated by targeting the kinase domain-encoding exons, and showed no loss of viability or overt phenotypes on both an outbred CD-1 background or inbred 129/SvJ background (90). These dramatic differences in phenotypes reported for *fes*$^{-/-}$ mice may reflect differences in strain backgrounds, or possible generation of a hypomorphic *furin* allele by the Simon lab's targeting strategy. The *furin* gene is located immediately upstream of the *fes* promoter (91), and encodes an essential gene that regulates secretion of key regulators of embryonic heart development and inflammation (92, 93). Thus, impaired Furin function could explain the embryonic lethality and skin lesions. The kinase domain-targeted *fes*$^{-/-}$ mice were extensively characterized with cohorts of *fes*$^{-/-}$ mice and *fes*$^{-/-}$ crossed with a human *c-fes* transgene (rescue). There were small but significant reductions in peripheral blood monocytes, neutrophils and basophils in *fes*$^{-/-}$ compared to wild-type or rescue (90). Although there is no evidence that a truncated FES protein is expressed from kinase domain-targeted allele, it is possible that a truncated FES protein supports some kinase-independent functions in these mice. This was tested further in mice by knock-in of a kinase-inactivating K588R mutation (*fes*$^{KR/KR}$) resulting in normal levels of kinase-dead FES, which also revealed no major defects in hematopoiesis (94). We hypothesized that FER may compensate for loss of FES, and by crossing *fes*$^{KR/KR}$ mice with FER-deficient (*fer*$^{DR/DR}$) mice (34), we generated compound mutant mice lacking both FES and FER kinase activities. These compound FES/FER mutant mice are viable, but display more profound defects than the single mutants, including increased granulocyte progenitors, and circulating neutrophils (95). These mice also developed an abnormal B cell population in the thymus that increased with age (A. Craig & P. Greer, unpublished). Since *fer*$^{DR/DR}$ mice express low levels of a kinase-dead FER^{DR} protein (34), it will be important in future to generate *fer*-null mice to test more clearly the kinase-dependent and potential kinase-independent roles of FER.

Some of the most profound phenotypes associated with FES- and FER-deficient mice have involved immune challenges with lipopolysaccharide (LPS). Zirngibl *et al.* reported increased susceptibility of *fes*$^{-/-}$ mice in an endoxemia model (90). LPS-treated *fes*$^{-/-}$ mice exhibit increased levels of TNFα and reduced IL-10 in serum, compared to LPS-treated wild-type mice (96). In *fes*$^{-/-}$ peritoneal macrophages, LPS-induced internalization of TLR4 was delayed, allowing for extended NFκB activation and increased TNFα release (96). Another study showed enhanced transendothelial migration of leukocytes in LPS-treated *fes*$^{-/-}$ mice compared to control mice (97). Interestingly, a similar result was observed in LPS-treated *fer*$^{DR/DR}$ mice, with increased leukocyte adhesion and transendothelial migration (98). These studies illustrate a potential model by which FES or FER limit the innate immune response at least in part by promoting internalization of activated TLR4 or other key receptors. With the more recent discovery of F-BAR domains in adaptor proteins that coordinate membrane invagination and actin assembly (39, 40, 99), we have a better molecular understanding of how FES may regulate receptor internalization. F-BAR adaptor proteins induce or stabilize membrane invaginations, while recruiting actin assembly machinery (e.g. WASP/N-WASP). Branched actin supports the neck of the invagination and along with Dynamin promotes vesicle scission (41). FES/FER may cooperate with F-BAR adaptors via phosphorylation of Cortactin (or the hematopoietic cell-specific homolog HS1) that displaces WASP/N-WASP to stabilize actin branch points. Cortactin functions in many cellular processes driven by actin assembly, including cell migration and endocytosis (100). We hypothesize a model whereby F-BAR adaptors and F-BAR kinases cooperate to regulate actin-based processes including endocytosis, lamellipodia formation, and cell migration (Figure 2). Future studies will be required to test for cooperative effects FES/FER with F-BAR adaptors in regulating these cellular processes.

6. FES/FER SIGNALING DOWNSTREAM OF KIT RECEPTOR IN MAST CELLS

Mast cells are bone marrow-derived myeloid cells that have a characteristic granule-rich cytoplasm that reside near portals of entry for pathogens (eg. skin or mucosa). Mast cells serve as sentinels, and function in both innate and adaptive immunity (101). The KIT receptor PTK and its ligand stem cell factor (SCF) are required for mast cell differentiation from committed progenitors, and are also required for growth and survival signaling in mature mast cells (102). We and others have identified

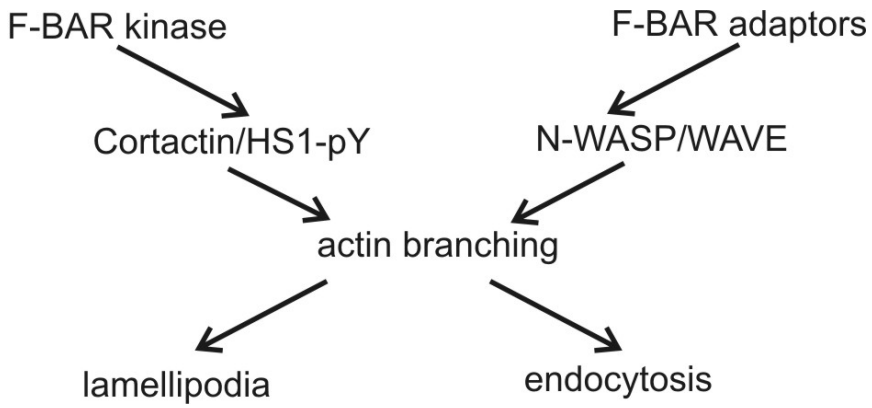

Figure 2. Potential cooperation of F-BAR adaptors and kinases in actin regulation. F-BAR adaptors interact with actin assembly machinery (eg. N-WASP, WAVE) to link membrane invaginations or vesicles to actin polymerization. F-BAR kinases (FES/FER) have been shown to phosphorylate Cortactin, and its hematopoietic cell-specific homolog HS1. Given the role of Cortactin in stabilizing actin branch points, we hypothesize that FES/FER function in part through regulating actin-based processes, including endocytosis, lamellipodia formation and cell migration.

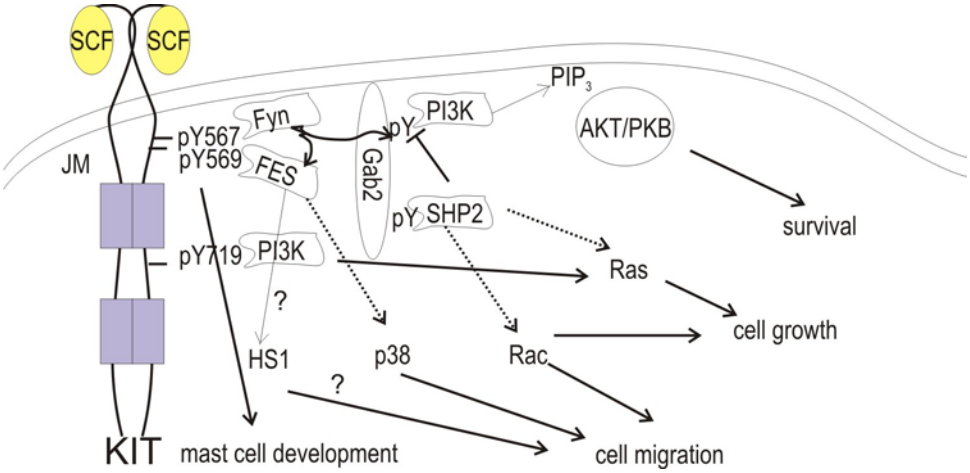

Figure 3. KIT juxtamembrane signaling pathways and FES activation. SCF binding to KIT induces dimerization and autophosphorylation at many tyrosines (for simplicity, only the juxtamembrane (JM) sites (Y567/Y569), and the direct PI3K recruitment site (Y719) are shown. KIT JM pathways are critical for mast cell development *in vivo*, and to cell migration *in vitro*. FES is recruited to KIT via SH2 domain interactions with JM sites, leading to FES activation via an upstream kinase (likely Fyn). FES promotes downstream signaling to p38 mitogen activated-protein kinase, and cell migration. We hypothesize that this involves signaling to SH2 domain-binding partners, such as HS1 or HEF1 adaptor protein (not shown).

FER and FES as downstream effectors of KIT receptor in mast cells, erythroid progenitors, or TF-1 myeloid leukemia cells (46, 103, 104). FER and FES also signal downstream of oncogenic KIT receptor in mast cell leukemia (HMC-1) cells (105, 106). Using a combination of approaches, we know that recruitment of FES (and likely FER) involves SH2 domain-mediated recruitment to juxtamembrane sites (Y567/Y569) in activated KIT (Figure 3) (75, 104). The juxtamembrane sites are known to also recruit Src family kinases (eg. Fyn and/or Lyn) (107, 108), adaptor proteins (eg. Shc, Grb2/Gab2) (109), and protein-tyrosine phosphatases (eg SHP1, SHP2) (110). Subsequent FES activation involves phosphorylation by an upstream kinase, which we identified as Fyn (75). Thus, the coordinated recruitment of Fyn and FES to the juxtamembrane of activated KIT may facilitate FES activation by allowing Fyn to phosphorylate the FES activation loop tyrosine (Y713; Figure 3), as reported in co-transfections with FES and Hck (52). Mutations of juxtamembrane sites in KIT disrupt mast cell migration and survival signals *in vitro* (111, 112). Interestingly, our studies of mast cells lacking Fyn, FES, or FER, show that all of these proteins promote migration of mast cells (46, 75, 113). The importance of KIT juxtamembrane signaling for mast cell development in mice (114, 115) also warrants characterization of mast cell numbers in FES- and FER-deficient mice. Studies of compound FES/FER-deficient mice and cultured mast cells will also be required to address potential overlapping functions of FES and FER in the KIT juxtamembrane signaling axis.

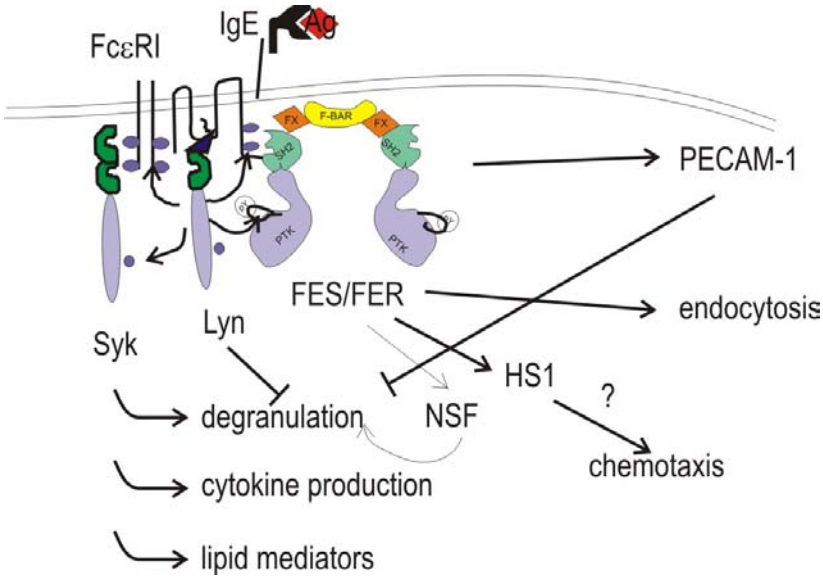

Figure 4. FcεRI signaling to FES/FER in mast cells. FcεRI aggregation via IgE/Ag leads to rapid phosphorylation of receptor chains via Lyn PTK, and recruitment of additional PTKs, including Syk and FES/FER (see text for details). Syk signaling is required to initiate downstream signals required for degranulation, production of lipid mediators or cytokines. Through inhibitory pathways (not shown here), Lyn can also suppress mast cell degranulation and mediator release by recruiting key inhibitory proteins (eg. Csk, SHIP, SHP1). FES/FER are recruited to FcεRI via their SH2 domains, and their activation proceeds via a Lyn-dependent pathway. FES/FER signal downstream to PECAM-1 (an ITIM receptor that recruits SHP1) and represses mediator release.

Another important aspect for future studies will include the identification of FES/FER substrates in mast cells. Given the importance of the FES SH2 domain interaction with ligands for FES activation (65), a comprehensive analysis of SH2 ligands in mast cells should reveal at least one class of FES/FER substrates, those that are "primed" by phosphorylation on a site that binds the SH2 domains of FES/FER. Cortactin and HS1 are potential examples of FES/FER substrates that are primed by Src or Syk kinases (116, 117), and further phosphorylated by FES/FER in fibroblasts and mast cells, respectively (12, 34). Although the function of HS1 in mast cells remains unknown, it has been linked to F-actin regulation in the T cell synapse (118) and NK cell migration and cytolysis (119). The latter study linked HS1 phosphorylation at Y378 to chemotaxis, and Y397 to integrin signaling and cell adhesion. We showed that HS1 is an SH2 domain ligand of FES, that FES/FER phosphorylate HS1 primarily at Y378 (Y388 in mouse HS1) *in vitro*, and FES/FER promote HS1 phosphorylation in mast cells activated via IgE/antigen (12). HS1 also participates in KIT signaling, since KIT inhibitor treatment of HMC-1 cells showed reduced tyrosine phosphorylation of HS1, with similar kinetics to that observed for FES and FER (105). Another recent study links HS1 in regulating F-actin reorganization and migration of B cells, and for homing of normal and leukemic B cells to bone marrow (120). Taken together, we predict that defects in HS1 phosphorylation in FES/FER-deficient mast cells contribute to the observed defects in mast cell adhesion and migration (46, 75). For oncogenic KIT receptor (D816V), FES was shown to be a key mediator of proliferation signaling, possibly via promoting activation of p70 S6 kinase (p70S6K) (106). This implicates FES in regulating the PI3K/AKT/mTOR pathway downstream of KITD816V. Interestingly, Rapamycin treatment of mast cells, which blocks mTORC1-dependent p70S6K phosphorylation, disrupts chemotaxis via the SCF/KIT axis, and also impairs growth/survival of KIT-driven leukemias (121-123). Future studies defining whether FES is a valid therapeutic target in KIT-driven cancers, and more understanding of direct substrates of FES are needed.

7. FES/FER SIGNALING DOWNSTREAM OF HIGH AFFINITY IgE RECEPTOR IN MAST CELLS

FES and FER are also activated following IgE receptor (FcεRI) crosslinking by multivalent antigens (Ag) in mast cells (46, 95). In this system, FcεRI signaling is initiated by Src family kinases Lyn and Fyn that phosphorylate immunoreceptor tyrosine-based activation motifs (ITAMs) within FcεRI β and γ chains to recruit SH2 domain-containing proteins (102). We showed that the FES SH2 domain can interact with phosphorylated FcεRI β chains (Figure 4) (12), and this likely coincides with the rapid activation of FES/FER in this pathway (46, 73). Membrane targeting via the phosphoinositide-binding site within the F-BAR domain of FES was also required for FES activation in RBL-2H3 cells treated with IgE/Ag (12). Given that PA binding to FER can promote FER activation (11), these results suggest that FES/FER activation may be maximal when bound to membrane phospholipids and phosphorylated SH2 ligands (eg. FcεRI). We previously showed that Lyn, but not Syk, is required for FER

phosphorylation downstream of FcεRI (124). It is still unclear whether Lyn phosphorylates FER in this pathway, or whether the delayed activation kinetics of FER in Lyn-null mast cells reflects reduced phosphorylation of FcεRI chains, leading to delayed FER recruitment. We know that an upstream PTK acts on FER and FES, since mast cells expressing kinase-dead FER and FES are phosphorylated upon IgE/Ag treatment (95)(C. Udell & A. Craig, unpublished data). We hypothesize that coupling of FER/FES to the FcεRI pathway involves membrane localization via F-BAR domains, their recruitment to ITAMs within FcεRI β or γ chains, and phosphorylation by Lyn or a Lyn-dependent tyrosine kinase. This leads to an active pool of FER/FES at the membrane, likely within the lipid rafts where FcεRI/Lyn signaling occurs (125, 126). Once activated, FES and FER may dampen mast cell activation (eg. degranulation) via phosphorylation of immunoreceptor tyrosine-based inhibitory motifs (ITIMs) within Platelet-Endothelial Cell Adhesion Molecule-1 (PECAM-1) (124). In Lyn-deficient mast cells, FER and PECAM-1 phosphorylation were greatly reduced, along with recruitment of SHP1 or SHP2 to PECAM-1 (124)(C. Udell & A. Craig, unpublished data). Mast cells lacking Lyn, PECAM-1, or SHP1 show elevated degranulation and release of inflammatory mediators following IgE/Ag treatment (127-129). Whereas, SHP2-deficient mast cells have no defects in degranulation, defects in signaling to Erk and Jnk MAPKs and TNFα release were observed (130).

Another potential substrate of FES/FER with relevance to membrane trafficking in mast cells is N-ethylmaleimide-sensitive factor (NSF) that regulates vesicle fusion and exocytosis (131). NSF is itself regulated by phosphorylation at Y83, that inhibits NSF binding to soluble NSF attachment protein (SNAP) and vesicle fusion. Huynh and co-workers identified FER and FES as potential kinases that trigger NSF phosphorylation, and this is reversed by the phosphatase PTP-MEG2 that localizes to secretory vesicles that must undergo vesicle fusion during exocytosis. Hypophosphorylation of NSF due to overexpression of PTP-MEG leads to extensive fusion of secretory vesicles in T cells (132), whether this also occurs in FES/FER-deficient mast cells remains to be determined. This could relate to the modulatory effects of FES overexpression or FES/FER-deficiency in mast cell degranulation (12, 124). This may also relate to potential effects on F-actin reorganization in mast cells, which must be coordinated with vesicle trafficking and exocytosis (133, 134).

Given the link between FES/FER and regulation of the innate immune response (90, 96, 97), future studies characterizing allergic inflammation in FES/FER-deficient mice could lead to a better understanding of their roles in allergic disease.

8. EMERGING ROLES OF FES/FER IN LEUKEMIA

Like the Janus kinase JAK2, FES signals downstream of several cytokine and colony stimulating factor receptors implicated in hematopoiesis, and promotes terminal differentiation of myeloid and erythroid cells (135). While mutations and translocations causing elevated JAK2 kinase activity have been found in leukemias (136), to date there are no reported cases of FES mutations or gene translocations in human leukemias. However, recent studies identify FES and FER as potential downstream effectors of growth or survival signaling in leukemias. As mentioned above, silencing of FES expression in leukemias driven by oncogenic KIT receptors leads to reduced cell growth (106). Another recent study of acute myeloid leukemias (AML) driven by constitutively activated internal tandem duplication (ITD) mutants of FLT3 receptor tyrosine kinase depend on both FER and FES for growth and survival, respectively (137). In this paper, silencing of FER or FES led to striking defects in FLT3 signaling to Src family kinases, STAT5, and the PI3K/mTOR/AKT pathway. The authors did not observe any defects in survival upon silencing of FES or FER in another AML cell line expressing wild-type FLT3 (137). This study implicates that AML blasts driven by FLT3-ITD signaling become acutely dependent on the downstream signals conferred by FER and FES. What is unclear is why there is no apparent functional redundancy built in to this system, despite a very similar profile of defects with individual FER or FES silencing. Although the mechanisms by which FER and FES function in AML awaits discovery, these studies identify FES and FER as potential new therapeutic targets in AML driven by FLT3-ITD, or oncogenic KIT-driven cancers (AML, mastocytosis, and gastrointestinal stromal tumors). Another study used a kinome-wide RNA interference approach to identify potential kinase targets in multiple myeloma, and found that FES is one of fifteen potential therapeutic kinase targets in multiple myeloma (138). Also, a kinase-based proteomics study of signaling proteins downstream of the BCR-ABL oncoprotein in chronic myelogenous leukemia identified FER as a potential target (139).

9. SUMMARY

The FES/FER kinases propagate signals from cytokines, growth factors, and immune receptors within the hematopoietic system that controls differentiation of myeloid progenitors, and activation of mature immune cells. Signaling by FES/FER kinases is normally tightly controlled within cells, and recent studies support a model for FES/FER activation whereby conformational changes induced by membrane targeting via their F-BAR domains, and SH2 domain binding to phosphorylated receptors or adaptor proteins. Using knock-out and RNA interference approaches, we are beginning to see evidence that FES and FER are potential targets for blocking growth or survival of some leukemias driven by activating mutations in KIT or FLT3 receptors. Further understanding of the functions of FES/FER in regulating innate immunity will be required to understand their protective roles against inflammatory responses. Mast cells are an excellent model to define FES/FER functions since upon activation they undergo dramatic changes in their cytoskeleton, membrane trafficking, mediator release, and gene expression.

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Send correspondence to: Andrew W.B. Craig, Cancer Biology and Genetics, CRI315, Queen's University, Kingston, Ontario K7L 3N6, Canada, Tel: 613.533.2496, Fax: 613.533.6830, E-mail: andrew.craig@queensu.ca

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