Characterization of FIM-FGFR1, the Fusion Product of the Myeloproliferative Disorder-associated t(8;13) Translocation*

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The t(8;13) translocation found in a rare type of stem cell myeloproliferative disorder generates a constitutively activated tyrosine kinase containing N-terminal sequence encoded by the FIM gene linked to the FGFR1 kinase domain. Here we have further characterized FIM and FIM-FGFR1 proteins. Firstly, we have studied their respective subcellular localization. We show that FIM has nuclear and nucleolar localization, whereas FIM-FGFR1 is mainly cytoplasmic. Within the nucleolus, FIM colocalizes with the upstream binding factor in interphase cells, indicating that FIM may be involved in the regulation of rRNA transcription. We demonstrate that the targeting of FIM to the nucleus depends upon its C-terminal region, which is absent in the cytoplasmic FIM-FGFR1 protein. Secondly, we demonstrate that FIM-FGFR1 has constitutive dimerization capability mediated by the FIM N-terminal sequences. Finally, we show that FIM-FGFR1 promotes survival of pro-B Ba/F3 cells after interleukin-3 withdrawal, whereas ligand-activated FGFR1 induced not only cell survival but also interleukin-3 independence. Taken together, these results indicate that FIM-FGFR1 is activated by dimerization as a cytoplasmic kinase and suggest that FIM-FGFR1 partially signals through the FGFR1 pathways.

A stem cell myeloproliferative disorder with a multilineage involvement that suggests transformation of a primitive hematopoietic stem cell is associated with three different translocations with a breakpoint in region p11–12 of chromosome 8: t(6;8)(q27;p11), t(8;9)(p11;q33), and t(8;13)(p12;q12), respectively (1). On chromosome arm 8p, it involves in each case the rearrangement of the FGFR1 gene (2), which encodes a transmembrane tyrosine kinase receptor for members of the fibroblast growth factor family (3). We have cloned the partner genes of FGFR1 on chromosomes 6q27, 9q33, and 13q12. They are novel and unrelated genes named FOP (4), CEP110,¹ and FIM (fused in myeloproliferative disorders) (5). The 13q12 breakpoint gene has also been partially characterized by others and named ZNF198 (6, 7) and RAMP (8).

FIM ubiquitous transcript encodes a protein of 1379 amino acid residues (5) that shows several motifs: a N-terminal cysteine-rich region containing 10 repeats with the consensus sequence C-X₂₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁₋₃₋₁-
Cells and Culture Conditions—Cos-1 cells were maintained in Dulbecco’s modified Eagle’s medium with 10% new born calf serum in a 5% CO₂ incubator at 37 °C. Murine Ba/F3 cells from a lymphoid pro-B-cell line dependent on IL-3 for survival and proliferation (15, 16) were maintained in RPMI 1640 medium with 10% fetal bovine serum supplemented with IL-3 in a 5% CO₂ incubator at 37 °C. DNA plasmid constructs were derived from the wild type FIM (pFIM) and fusion FIM-FGFR1 (chIM) cDNAs inserted in the pcDNA3 expression vector (Invitrogen) as described in a previous work (5) and corresponding to an exon splicing from nucleotides 492 to 753 of the FIM long form (EMBL accession number Y13472). The respective positions of nucleotides and amino acids for all the constructs mentioned below (either wild type or chimeric) correspond to the FIM sequence from ATG minus the 261-base pair alternatively spliced. A sequence encoding the Myc epitope tag, MEQKLISEEDL, (17, 18), was derived from L-CHIM (425–1218) by the corresponding region in L-CHIMKD (425–1218) by swapping a 1.65-kb EcoRI fragment from L-CHIM (24–1218) and inserted in the pVP16-A vector. Alternatively spliced region of both pBTM116 and pVP16 vectors has been remodeled to derive from L-CHIM (24–1292), corresponding to a 1.2-kb NotI fragment containing the HindIII filled in site of pBTM116 and V-FIM (24–1292), corresponding to a 3.8-kb NcoI/NotI fragment derived from mycFIM and inserted in pBTM-C cut by BamHI and filled in with the Klence polymerase; V-FIM (24–1292), corresponding to a NotI/SalI fragment derived from L-FIM (24–1292) and ligated in the NotI/SalI sites of pBTM-C; L-FIM (314–1292), corresponding to a NotI/SalI fragment derived from pFIM (full-length cloned in pBluescript SK) and ligated in the EcoRI site of pBTM-A; V-FIM (314–1292), corresponding to a 3-kb BamHI/SalI fragment from L-FIM (314–1292) inserted in the BamHI/SalI sites of pVPl6-A; L-FIM (24–425), corresponding to a 1.2-kb HindIII filled in/NotI fragment, which contains the sequences encoding the N-terminal sequence of FIM, derived from L-FIM (24–1292), and ligated into the EcoRI filled in/NotI sites of pBTM-C and V-FIM (24–425), corresponding to a 1.2-kb NotI/SalI fragment derived from L-FIM (24–425), and introduced in pVP16-C. Seven FIM-FGFR1 plasmids were constructed as follows: L-CHIM (24–1218), a 4.2-kb NotI/NotI fragment containing the full-length coding sequence of FIM-FGFR1 was derived from mycFIM filled in with Klenow and ligated in BamHI filled in site of pBTM-C; V-CHIM (24–1218), the 4.2-kb NotI/SalI fragment from L-CHIM (24–1218) cloned in pBluescript SK; L-CHIMKD (24–1218) (kinase dead), because the FIM-FGFR1 fused to LexA (construct L-CHIM (24–1218) constitutively transactivated the reporter genes of the L40 yeast strain, a kinase-defective mutant FIM-FGFR1 fused to LexA was made by site-directed mutagenesis using Quickchange kit (Stratagene) according to the manufacturer’s recommendations changing lysine 910 (lysine 514 in the FGFR1 sequence) to alanine (20) in the L-CHIM (24–1218); L-CHIM (314–1218), a 3-kb EcoRI fragment was derived from L-CHIM (24–1218) and inserted in the EcoRI site of pBTM-A; V-CHIM (314–1218), a 3-kb NotI/SalI fragment derived from L-CHIM (314–1218) was inserted in pVP16-A; L-CHIM (425–1218), a 2.7-kb blunt end HindIII/NotI fragment was derived from mycFIM and inserted into pBTM-A and V-CHIM (425–1218), the 2.7-kb NotI EcoRI fragment was inserted in BamHI filled in site of pVPl6-A. The LexA and VP16 CHIM (425–1218) fusion constructs are deleted of the N-terminal FIM region and retain only 6 of the 10 zinc fingers motifs present in FIM. For CHIMKD (425–1218), as with the previously described construct L-CHIM (24–1218), L-CHIM (425–1218) activated constitutively the reporter genes in yeast. To prevent this we made a L-CHIMKD (425–1218) by swapping a 1.65-kb NotI/EcoRI fragment from the CHIM sequence (24–1218) by the construct DN1 and DN2, similarly, a Myc-tagged construct retaining C-terminal sequences including both NLSs was made by inserting a 1200-base pair FIM restriction fragment from EcoRV (nucleotide position 3021) and XhoI (polynucleotide) in the plasmid RKS myc cut by EcoRI and filled in with Klenow.

Characterization of t(8;13) Fusion Protein

HA-tagged FIM-FGFR1 expression vectors were cloned in pcDNA3 in frame with three repeats of the flexible HA epitope tag (DHFR/His) (20) for HACH. HACH, a near full-long coding sequence of 4.3-kb fragment (fragment NcoI filled in NotI (polynucleotide) derived from the mycFIM was cloned into pcDNA3HA vector cut by XhoI and blunt ended. For HAΔR1CHIM, a 3-kb EcoRI (nucleotide position 1200/XhoI) (polynucleotide) fragment from pmic-FGFR1 and retaining the sequence coding for 8 of the 10 zinc fingers was subcloned in pcDNA3HA vector. For HAΔHIICCHIM, similarly, a 2.7-kb HindIII/NotI (polynucleotide) filled in fragment from myc-FGFR1 containing FIM sequences coding for 6 of the 10 zinc finger motifs was inserted in the pcDNA3HA vector cut with EcoRV. For pFGR1A, the full-length FGR1 cDNA was excised from pFgl16 (22) by digestion with ApaI and NotI and was inserted in the Apal-EcoRV sites of pcDNA3 by blunt end ligation.

Transmission—Cos-1 cells were transiently transfected using 2 μg of plasmid DNA and 3 μl of FuGENE 6 transfection reagent (Boehringer Diagnostics, Meylan, France) following the manufacturer’s recommendations. Ba/F3 cells were electroporated as follows. 1 x 10⁶ cells were washed in phosphate-buffered saline (PBS) and incubated for 10 min at room temperature with 20 μg of plasmid DNA at 350 mV/960 microfarad in a Bio-Rad apparatus. Following a 10-min incubation at room temperature, cells were settled in plates.

Selection of Stable Transfected Clones—After electroporation, Ba/F3 cells were plated in 10 ml of IL-3 medium for 24 h and then selected in IL-3 medium plus 1 mg of G418/ml. Neomycin-resistant cells were subcloned by limiting dilution. FGFR1 positive cells were selected in G418 medium containing 10 ng/ml of FGFR1 plus 10 μg/ml of heparin (22) and refeed every 2 days. Stably transfected clones were selected 15 days later culture.

Antibodies—The DSKTTPSKELASKQ peptide, corresponding to amino acids 96–111 of FIM sequence, was chosen for chemical synthesis (Neosystem, Strasbourg, France) owing to its predicted antigenicity. This peptide was coupled to keyhole limpet hemocyanin as hapten, suspended in PBS, and used to immunize rabbits by intramuscular and subcutaneous injections. The antibody generated against this peptide was designated anti-N-FIM.

The mouse monoclonal anti-Myc (9E10) (17) and anti-phosphory-rose 4G10 antibodies were purchased from Santa Cruz Biotechnology, Inc. and Upstate Biotechnology, Inc. (Lake Placid, NY), respectively. The anti-Myc was revealed by an Alexa-conjugated anti-mouse antibody (Molecular Probes, Oregon, WA).

Immunofluorescence Analysis—Cos-1 cells were grown on coverslips 1 day before transfection (1 to 2 x 10⁶ cells/60-mm plate). On the day of transfection, the coverslips were rinsed once in PBS and fixed at room temperature in 1 x paraformaldehyde for 15 min at room temperature. After extensive PBS washes, cells were permeabilized and blocked in 5% fetal calf serum/PBS, 0.1% Triton X-100 for 15 min. Cells were incubated with anti-Myc antibody used at a final concentration of 1 μg/ml for 1 h at room temperature, rinsed several times in 1 x PBS, and then incubated with 2 μg/ml of the Alexa-conjugated anti-Mouse secondary antibody.
To detect the endogenous FIM protein, transfected Cos-1 cells were incubated with the antipetide serum anti-N-FIM (dilution, 1:1000 or 1:5000) for at least 1 h. This antibody was revealed either by an Alexa-conjugated anti-rabbit (Molecular Probes) or Texas Red-conjugated goat anti-rabbit antibodies (Molecular Probes). Controls were made by pretreatment of the FIM antibody with the corresponding 24-mer of total peptide (50 μg/ml) or by incubating the cells with a preimmune serum rather than the anti-N-FIM serum. In these instances, the immunofluorescence staining was abolished, confirming the specificity of anti-N-FIM antiserum.

To detect UBF, cells were incubated with anti-UBF serum used at the same dilution of 1:100 or 1:200 as described in Nicoletti et al. (30). Briefly, after cell culture of the selected clones as described above, 100% ethanol fixation, RNase treatment, and propidium iodide staining, the DNA content of cell nuclei was determined using a FACScan cytometer (Becton Dickinson FACScan). Subdiploid cells were considered apoptotic cells.

RESULTS

FIM-FGFR1 Is a Cytoplasmic Protein, Whereas FIM Is Nuclear—The fusion between two proteins resulting from a chromosomal translocation event often creates an aberrantly located protein. This abnormal subcellular localization can be, at least in part, the source of its oncogenic effect. Based on sequence analyses, we predicted the cytoplasmic localization of FIM-FGFR1 fusion protein because it lacks the FGFR1 transmembrane domain and the two putative NLSs present in the C terminus of the wild type FIM (5). To test this prediction, we compared the localization of both fusion FIM-FGFR1 and wild type FIM proteins by immunofluorescence in Cos-1 cells transiently transfected with the corresponding Myc-tagged constructs. As suspected, the FIM-FGFR1 fusion protein was mostly located in the cytoplasm (Fig. 1A, panels a and c). In contrast, the FIM protein was found exclusively located within the cell nucleus, in the nucleoplasm, and in discrete nucleolar areas (Fig. 1B, panels a, c, d, and f). One nuclear pattern recurrently observed is shown in detail (Fig. 1B, panels d–f). Therefore the FIM-FGFR1 fusion protein resulting from the t(8;13) translocation is mainly translocated to a subcellular compartment different from both FIM and FGFR1 wild type proteins, which are nuclear and plasma membrane bound, respectively.

FIM C-terminal Region Contains Two Functional NLSs—FIM C-terminal sequence displays two putative nuclear localization signals (Fig. 1, dark and light blue boxes), the more C-terminal one being bipartite (5). To demonstrate that these nuclear localization sequences are actually important, we studied the localization of different FIM proteins (Fig. 2). Two short C-terminal deletion constructs, named DC1 and DC2, were first studied. The respective truncated proteins lack one and two putative nuclear localization signals, respectively (Fig. 2, A and B). Eliminating the FIM C-terminal region containing the putative bipartite NLS greatly affected the localization of the protein, which showed a cytoplasmic pattern in most cells (Fig. 2A, panels a–c). However, in some cells the localization of this truncated protein was partially nuclear, suggesting that another signal localization could still be functional (Fig. 2A, panels d–f). Indeed, the localization of the DC2 protein, which lacks the two putative NLSs, was exclusively cytoplasmic (Fig. 2B), either remaining diffuse (Fig. 2B, panels d–f) or concentrated in aggregates (Fig. 2B, panels a–c). These results suggest that the two FIM NLSs are functional.

We then studied the localization of reciprocal proteins, DN1 and DN2, containing FIM C-terminal sequences with either one or two of the NLSs, respectively. DN1 protein displayed a mixed nuclear and cytoplasmic localization (Fig. 2C), indicating that this region alone containing the bipartite NLS was able to target the protein to the nuclear compartment although quite imperfectly. In contrast, the localization of the protein bearing the FIM C terminus including the two NLSs (DN2) was
exclusively nuclear in most cells (Fig. 2D). This result suggests that the other NLS, not examined here in isolation, is capable of directing nuclear localization of FIM. Altogether, these results indicate that two functional NLSs are present in the C-terminal region of FIM and that they are likely to cooperate to target FIM to the nucleus.

**Fig. 1. Immunolocalization of mycFIM-FGFR1 and mycFIM.** mycFIM-FGFR1 (A) and mycFIM (B) expression constructs are shown above each panel; both contain a Myc epitope tag at their N terminus (green box). The black bars represent the 10 putative zinc fingers of the N-terminal FIM region. The FGFR1 kinase is shown in gray, and the two putative nuclear localization signals present in the C-terminal region of FIM are represented as dark and light blue boxes, respectively. Transfected Cos-1 cells were grown on coverslips and subjected to double staining immunofluorescence with anti-Myc antibody, revealed by Alexa-conjugated anti-mouse secondary antibody and ethidium acridine to visualize mycFIM-FGFR1 and mycFIM (in green) and the DNA (in red), respectively. Magnifications: A, 630×; B, panels a–c, 1000×; panels d–f, 3000×.

**Fig. 2. Mapping of the nuclear localization signal of FIM by immunofluorescence.** The immunolocalization of four Myc-tagged FIM deletions constructs were determined by immunofluorescence staining, as described in legend to Fig. 1. DC1 (A) and DC2 (B) are truncated FIM proteins deleted in their C termini of one or two putative NLSs, respectively. DN1 (C) and DN2 (D) are truncated FIM proteins deleted of a large portion of the molecule but retaining one and two putative NLS, respectively. Magnifications: 1000×.
**Endogenous FIM Exhibits a Nuclear and a Nucleolar Localization**—To confirm the localization of FIM within the cell nucleus, we studied the distribution of the endogenous FIM by immunofluorescence with a polyclonal anti-N-FIM antibody (Fig. 1B). Endogenous FIM was found in the same localization as transfected mycFIM (Fig. 3A, panel b), i.e. not only throughout the nucleoplasm but also as concentrated dots in the nucleoli.

To precisely define the location of endogenous FIM in nucleoli, we studied its potential colocalization with the UBF, one of the elements of the multimeric protein complex required for rDNA transcription (for review see Ref. 31). During interphase, UBF was detected in discrete foci arranged in a necklace-like pattern (Fig. 3A, panel a), as described previously by others (23, 32). Colocalization of UBF and FIM proteins was visualized as overlapping nucleoli dots in yellow (Fig. 3A, panel c). However, during mitosis the localization of the two proteins was different. From early prophase to anaphase, endogenous FIM was diffuse in the cytoplasm, excluded from the condensed DNA (Fig. 3B, panels a–d, respectively). In contrast and as expected, UBF remained associated with the condensed chromosomes at all phases of the mitosis (Fig. 3B, panels e–h). Therefore, endogenous FIM and UBF colocalized only during interphase.

**FIM N-terminal Motifs Are Able to Trigger Dimerization**—To establish whether or not the FIM N-terminal region is responsible for dimerization and subsequent activation of the FGFR1 kinase, two types of approaches were used, i.e. in vitro using the two-hybrid system in yeast and in vivo using Cos-1 cotransfection experiments with FIM-FGFR1 constructs bearing two different N-terminal epitope tags.

We first used the two-hybrid system. For this purpose, we made several constructs with either FIM or FIM-FGFR1, fused to either the LexA DNA binding domain or the VP16 activation domain. Following cotransformation in the L40 yeast strain containing two integrated reporter genes (HIS3 and LACZ), the interactions between a LexA fusion construct and a VP16 fusion construct were determined by testing several independent clones on plates depleted of histidine (see “Experimental Procedures”). Full-length FIM protein as a LexA fusion protein interacted with itself as a VP16 fusion (L-FIM (24–1292)/V-FIM (24–1292)) showing that FIM is able to dimerize (Fig. 4A). A series of two-hybrid constructs was made to delineate the region necessary for dimerization. A strong two-hybrid interaction was observed whenever the N terminus was present (L-FIM (24–1292)/V-FIM (24–1292), L-FIM (24–1292)/V-FIM (24–1292), L-FIM (24–1292)/V-FIM (24–1292), L-FIM (24–1292)/V-FIM (24–1292)), demonstrating that this region, which contains 4 of the 10 zinc finger motifs, is sufficient to observe an interaction between two FIM proteins. In contrast, deleting this N-terminal region (L-FIM (314–1292)) either abrogated (L-FIM (314–1292)/V-FIM (314–1292)) or severely reduced the interaction between two FIM proteins (L-FIM (314–1292)/V-FIM (24–1292)), confirming that the interaction between two FIM proteins takes place within the N-terminal FIM region (amino acids 24–425). In summary, these results indicate that two FIM proteins can interact through their respective N-terminal region.

**FIM-FGFR1 Is Able to Dimerize**—Because FIM-FGFR1 contains the N-terminal region of FIM that triggers the dimerization, several FIM-FGFR1 constructs (called CHIM) were similarly analyzed by the two-hybrid system (Fig. 4B). As predicted, two CHIM proteins containing the N-terminal region of FIM were able to dimerize (L-CHIMKD (24–1218)/V-CHIM (24–1218) and V-CHIM (425–1218)). Like for the FIM proteins, this dimerization was essentially dependent on the presence of N-terminal sequences because a deletion of this region inhibited the two-hybrid interaction with N-terminal deletions (V-CHIM (314–1218) and V-CHIM (425–1218)). However, a weak interaction is still...
Gray boxes parentheses amino acid limits of either FIM or FIM-FGFR1 (CHIM) proteins (in Procedures), are indicated as L- or V-, respectively, followed by the different constructs, fused to either LexA or VP16 (see “Experimental Procedures”).

The precise subcellular localization of FIM was also examined in vivo to gather some insights about its unknown function. Our immunofluorescence data on either transfected or untransfected FIM-FGFR1 was found in mutant clones.

One important issue in characterizing the functional properties of a translocation product is to determine its subcellular localization. Many chromosomal translocations that generate constitutively activated kinases lead to a delocalization of the fusion protein compared with its normal counterpart. We have shown here that the same occurs with FIM-FGFR1; whereas the wild type FIM protein is nuclear and nucleolar, the FIM-FGFR1 protein localizes to the cytoplasm. Thus, FIM-FGFR1 may affect cell growth through two combined dysregulations, i.e. continuous kinase stimulus and recruitment of signaling molecules not normally involved in FGFR1 signaling. Lack of recruitment of normal FGFR1 substrates, such as SNTs, may also be important to FIM-FGFR1 activity, in switching off the RAS pathway.

A FIM FUSED TO LEXA FUSED TO VP16

L-FIM (24–1292) V-FIM (24–1292)
L-FIM (214–1292) V-FIM (214–1292)
L-FIM (24–425) V-FIM (24–425)
V-FIM (24–1292)
V-FIM (214–1292)
V-FIM (24–425)

B FIM-FGFR1 FUSED TO LEXA FUSED TO VP16

L-CHIMKD (24–1218) V-CHIM (24–1218)
L-CHIMKD (314–1218) V-CHIM (314–1218)
L-CHIMKD (425–1218) V-CHIM (425–1218)
V-CHIM (314–1218)
V-CHIM (425–1218)

Fig. 4. Yeast two-hybrid assays detect FIM/FIM and FIM-FGFR1/FIM-FGFR1 interactions. Human FIM and FIM-FGFR1 cDNAs were used to derive all constructs seen in A and B, respectively. Gray boxes represent the positions of the FIM zinc finger motifs. The different constructs, fused to either LexA or VP16 (see “Experimental Procedures”), are indicated as L- or V-, respectively, followed by the amino acid limits of either FIM or FIM-FGFR1 (CHIM) proteins (in parentheses). The S. cerevisiae strain L40 was cotransformed with a combination of LexA and VP16 fusions, and individual colonies were tested for growth on minus histidine plates containing 10 mM 3-amino-triazol. β-Galactosidase activity was also qualitatively checked. The results of the two-hybrid FIM/FIM interactions (A) and FIM-FGFR1/FIM-FGFR1 (CHIM/CHIM) interactions (B) are summarized in the tables. N/A, not applicable.

detectable between L-CHIMKD (425–1248) and the full-length FIM-FGFR1 (V-CHIM (24–1218)) (Fig. 4B), indicating that in the absence of the N-terminal region the remaining zinc fingers are still capable of triggering dimerization. In conclusion, the two-hybrid results demonstrate that, as demonstrated for the FIM wild type proteins, the FIM-FGFR1 proteins are able to dimerize and that the N-terminal region is mainly responsible for this dimerization. FIM-FGFR1 Fusion Proteins Dimerize in Vivo—To further establish that FIM-FGFR1 is able to dimerize, we cotransfected Cos-1 cells with constructs tagged with either Myc or HA epitope tags (Fig. 5B). As seen in the two-hybrid analysis, a FIM-FGFR1 protein deleted in its N-terminal portion is still able to interact weakly with a full-length FIM-FGFR1 protein (Fig. 5B, lanes 5 and 6). As seen in the two-hybrid analysis, a FIM-FGFR1 protein deleted in its N-terminal portion is still able to interact weakly with a full-length FIM-FGFR1 protein (Fig. 5B, lane 4). N-terminal deletions of HAPIM-FGFR1 protein diminished its ability to dimerize with a full-length mycFIM-FGFR1 protein (Fig. 5B, lanes 5 and 6). As seen in the two-hybrid analysis, a FIM-FGFR1 protein deleted in its N-terminal portion is still able to interact weakly with a full-length FIM-FGFR1 protein (Fig. 5B, lane 4). N-terminal deletions of HAPIM-FGFR1 protein diminished its ability to dimerize with a full-length mycFIM-FGFR1 protein (Fig. 5B, lanes 5 and 6).

DISCUSSION

FIM-FGFR1 is the chimeric product of the t(8;13) translocation associated with a stem cell myeloproliferative disorder. This fusion protein contains the FIM zinc finger motifs and the catalytic domain of the tyrosine kinase receptor FGFR1.

FIM-FGFR1 Is Cytoplasmic, and FIM Is Nuclear—One important issue in characterizing the functional properties of a translocation product is to determine its subcellular localization. Many chromosomal translocations that generate constitutively activated kinases lead to a delocalization of the fusion protein compared with its normal counterpart. We have shown here that the same occurs with FIM-FGFR1; whereas the wild type FIM protein is nuclear and nucleolar, the FIM-FGFR1 protein localizes to the cytoplasm. Thus, FIM-FGFR1 may affect cell growth through two combined dysregulations, i.e. continuous kinase stimulus and recruitment of signaling molecules not normally involved in FGFR1 signaling. Lack of recruitment of normal FGFR1 substrates, such as SNTs, may also be important to FIM-FGFR1 activity, in switching off the RAS pathway.

FIM Colocalizes with UBF in the Nucleolus at Interphase—The precise subcellular localization of FIM was also examined to gather some insights about its unknown function. Our immuno-fluorescence data on either transfected or untransfected cells show that FIM is localized in the nucleus and the nucle-
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Thus, suggesting that it may play some role in transcription or gene regulation. Moreover, we observed a colocalization of nucleolar FIM with the upstream binding factor, one of the trans-acting factors required for efficient transcription of rDNA by RNA polymerase I (31). This colocalization was seen during interphase but not in mitotic cells when transcription of rRNA genes is shut off (24), indicating that FIM proteins could have some regulatory role on either rRNA synthesis or maturation. We showed that FIM sequences containing four zinc finger motifs can mediate efficient dimerization. Therefore, the FIM N-terminal region present in the chimeric protein is able, in theory, to dimerize in vivo. However, because we have shown that they are localized in different subcellular compartments, it is unlikely that FIM-

![Figure 5](#)

**FIG. 5.** Dimerization of FIM-FGFR1 in Cos-1 cells. Two differently tagged FIM-FGFR1 constructs (Myc and HA) were made, and dimerization between a Myc-tagged and an HA-tagged FIM-FGFR1 protein was studied following overexpression in Cos-1 cells and immunoprecipitation with anti-Myc antibody. A shows schematically the different constructs that were made in the pcDNA3 expression vector: a full-length mycFIM-FGFR1, a full-length HAFIM-FGFR1, and two N-terminal deletions, HAFIM-FGFR1ΔR1 and HAFIM-FGFR1ΔHd3, maintaining eight and six zinc fingers of the FIM region, respectively. These constructs were transfected in Cos-1 cells in different combinations. 24 h after transfection, total cell lysates (B, left) or anti-Myc immunoprecipitates (B, right) were analyzed by SDS-gel electrophoresis followed by immunoblotting with either anti-HA or anti-Myc antibody. B shows the results of Western blot from Cos-1 cells transfected with 10 μg of mycFIM-FGFR1 (lane 1), 10 μg of HAFIM-FGFR1 (lane 2), 10 μg of empty vector pcDNA3 (lane 3), 5 μg of mycFIM-FGFR1 + 5 μg of HAFIM-FGFR1 (lane 4), 5 μg of mycFIM-FGFR1 + 5 μg of HAFIM-FGFR1ΔR1 (lane 5) and 5 μg of mycFIM-FGFR1 + 5 μg of HAFIM-FGFR1ΔHd3 (lane 6).

![Figure 6](#)

**FIG. 6.** Expression pattern of stable transfected Ba/F3 clones. Cell lysates from $2 \times 10^6$ Ba/F3 cells were immunoprecipitated with anti-C-FGFR1 antibody and analyzed by Western blotting with either anti-phosphotyrosine or anti-C-FGFR1 antibodies as indicated. Lysates were prepared from different Ba/F3 clones: untransfected Ba/F3 (lane 1) or Ba/F3 cells from clones stably transfected with pcDNA3 vector (lane 2), FIM-FGFR1 (four different clones, lanes 3–6), or wild type FGFR1 (lane 7).

![Table 1](#)

**TABLE 1**

| Clones           | 6 h | 24 h | 48 h | 72 h |
|------------------|-----|------|------|------|
| pcDNA3           | 86  | 85   | 76   | 54   |
| FIM-FGFR1 (1)    | 75  | 71   | 77   | 79   |
| FIM-FGFR1 (2)    | 88  | 70   | 71   | 67   |
| FIM-FGFR1 (3)    | 74  | 73   | 74   | 69   |
| FIM-FGFR1 (4)    | 68  | 69   | 72   | 74   |
| FGFR1            | 67  | 72   | 68   | 71   |

Therefore, the FIM N-terminal region present in the chimeric protein is able to induce its dimerization leading to the constitutive activation of the FGFR1 kinase. It is likely that such a mechanism of activation is also involved for the two other chimeric proteins found in the 8p11 myeloproliferative disorder. Indeed, in this disorder, the nonkinase partners of FGFR1, FOP (4), and CEP1101 contain in their respective N-terminal region leucine-rich repeats and leucine zippers motifs known to be capable of mediating dimerization. Therefore, the fusion partners of FGFR1 in these translocations appear to be required to juxtapose a dimerization domain N-terminal of the FGFR1 kinase, inducing in this manner its constitutive activity. This phenomenon has been shown to be involved in a number of neoplasia-associated tyrosine kinase (37, 38). It has also been shown that the ligand-independent activation of FGFR1 leads to a constitutively active form responsible for oncogenic transformation (39).
FGFR1 oncogenic property is mediated through such an heterodimerization.

**FIM-FGFR1 Expression Induces Cell Survival**—Based on the knowledge that FGFR1 activation leads to cell survival and growth in the pro-B Ba/F3 cell line (29), we explored the cell growth properties of Ba/F3 cells expressing FIM-FGFR1. Our results show that FIM-FGFR1 supports cell survival following IL-3 withdrawal. However, FIM-FGFR1-expressing Ba/F3 cells did not proliferate in the absence of IL-3, suggesting that the fusion protein is only able to activate a partial FGFR1 response. Similar results have been recently reported in skeletal muscle cells in which the FGFR1 kinase domain regulates myogenesis differentiation but does not stimulate cell proliferation (40).

Factors such as IL-3 not only stimulate cell growth but are also necessary for survival of hematopoietic cells (41). IL-3-dependent survival is known to rely on the activity of multiple signaling pathways leading to activation of phosphoinositide 3-kinase and the protein kinase AKT (42), an important component of a cell survival pathway (43). Effect on cell survival rather than cell proliferation has been well documented for the product of E2A-HLF, the fusion gene formed by the t(17;19) chromosomal translocation involved in the leukemic transformation of early B-cell precursors (44). We cannot rule out that a stronger Ba/F3 response could be obtained with a higher expression of FIM-FGFR1. However, we were able to isolate only low expressing FIM-FGFR1 clones; this may reflect a toxic effect of the fusion protein. Alternatively, the limited effect of FIM-FGFR1 may signify that Ba/F3 cells, despite being hematopoietic cells, are not a truly relevant cell culture system for assaying its potential. The FIM-FGFR1 oncogenic effect could be restricted to permissive cells, which may be the hematopoietic stem cells only, as demonstrated for other fusion proteins (45).

In conclusion, FIM-FGFR1 may participate in the malignant process through two combined dysregulations, i.e. continuous kinase stimulus and abnormal recruitment of signaling molecules because of both its cytoplasmic localization and modified structure, and this may result in uncoupling apoptosis from other cell regulatory signals.

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**FIG. 7. Flow cytometric analyses of Ba/F3 transfected cells.** The flow cytometry profiles show DNA fluorescence of propidium iodide-stained Ba/F3 cell nuclei cultured 48 h in the presence (A) or absence (B) of IL-3. Cells were stably transfected with pcDNA3 vector (top panels), FIM-FGFR1 (middle panels), or FGFR1 (bottom panels). The cell cycle phase distribution was calculated from flow cytometric measurements of the DNA content. This is a representative experiment of three, all of which gave similar results.
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