Oncogenic Tyrosine Kinase of Malignant Hemopathy Targets the Centrosome

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Abstract

Myeloproliferative disorders (MPD) are clonal malignant hematopoietic disorders that affect progenitor cells. Many MPDs result from a chromosomal translocation that creates a fusion gene encoding a chimeric kinase. The fibroblast growth factor receptor 1 (FGFR1)-MPD is characterized by the fusion of the FGFR1 kinase with various partners, including FOP. We show here that both normal FOP and FOP-FGFR1 fusion kinase localize to the centrosome. The fusion kinase encounters substrates at the centrosome where it induces strong phosphorylation on tyrosine residues. Treatment with FGFR1 kinase inhibitor SU5402 abolishes FOP-FGFR1-induced centrosomal phosphorylation and suppresses the proliferative and survival potentials of FOP-FGFR1 Ba/F3 cells. We further show that FOP-FGFR1 allows cells to overcome G1 arrest. Therefore, the FOP-FGFR1 fusion kinase targets the centrosome, activates signaling pathways at this organelle, and sustains continuous entry in the cell cycle. This could represent a potential new mechanism of oncogenic transformation occurring specifically at the centrosome. (Cancer Res 2005; 65(16): 7231-40)

Introduction

Myeloproliferative disorders (MPD) are clonal malignant hematopoietic diseases that affect progenitor cells. MPD cells proliferate continuously but, in contrast to acute leukemia blasts, undergo maturation. The disease progresses towards an acute syndrome. Many MPDs are caused by a chromosome translocation that produces a fusion gene encoding a chimeric, constitutively activated kinase protein. One of these oncogenic events occurs in a rare and aggressive MPD, the fibroblast growth factor receptor 1 (FGFR1)-MPD. This MPD is also called stem cell MPD or 8p12 MPD because both lymphoid and myeloid lineages are affected following activation of the FGFR1 tyrosine kinase, which is encoded by a gene on the p11-12 region of chromosome 8 (1). FGFR1-MPDs are characterized by fusion proteins (hereafter designated X-FGFR1) made of the FGFR1 catalytic domain fused to a protein-protein interaction domain from several possible partner genes. We show here that FOP-FGFR1 is targeted to the centrosome where it activates signaling pathways via tyrosine phosphorylation. This phosphorylation at the centrosome and the proliferative potential of FOP-FGFR1-expressing cells are abolished after treatment with an FGFR1 kinase inhibitor. We also show that FOP-FGFR1 is important during G1-S transition to overcome G1 arrest and allow cells to sustain continuous cell cycle. This led us to hypothesize that FOP-FGFR1 proteins may exert an oncogenic activity through dysregulation of cell processes associated with the centrosome.

Materials and Methods

Plasmids, cells, and reagents. Rat2 cells are fibroblastic cells. Ba/F3 are murine hematopoietic cells that need to be cultured in the presence of interleukin-3 (IL-3). FGFR1 is not expressed in native, nontransfected Ba/F3 cells. FOP, FOP-FGFR1, FOP-FGFR1 kinase-defective (K259A), PLCγ binding site (Y511F) mutants, CEP1-FGFR1, wild-type FGFR1 (FGFR1wt) constructs, and corresponding clones of stably-transfected Rat2 or Ba/F3 cells used in this study have been previously described (5, 6, 9, 10). The largest FOP protein (or FGFR1OP) has 399 amino acid residues; the FOP-FGFR1 fusion protein (568 residues) joins the first 173 NH2-terminal residues of FOP to the intracellular region of FGFR1; the kinase-defective mutation is localized in the first FGFR1 kinase subdomain. The kinase-defective mutant has previously been characterized (10). BCR-FGFR1 construct is described in ref. (7) and was a kind gift from Dr. N.C. Cross. For FGFR1wt, two conditions of stimulation were used: a short stimulation corresponding to 5 minutes of stimulation were used: a short stimulation corresponding to 5 minutes of
stimulation with 10 ng/mL FGF1 (P100-17A from AbCys, Paris, France) and 10 μg/mL heparin (H-0777 from Sigma, Saint Quentin Fallavier, France) and a long stimulation corresponding to a 48-hour culture in the presence of 10 ng/mL FGF1 and 10 μg/mL heparin. For inhibition experiments, concentrations of 0.15, 1.5, and 15 μmol/L of the kinase inhibitor, ATP-competitor, SUS402 (Calbiochem, Merck Biosciences, Darmstadt, Germany) and 0.1, 1, and 10 μmol/L of ST571 (a gift from Dr P. Manley, Novartis), respectively, were used. The EOL-1 cell line, used to study the FIP1L1-PDGFRα fusion (23), was a gift from Dr. B. Papp (Hôpital St Louis, Paris, France).

Antibodies. We used monoclonal anti-myc (9E10), polyclonal anti-FGFR1 (C-15), polyclonal anti-PLCγ (12-C9), anti-phGRB2 (C-23) from Santa Cruz Biotechnology (Santa Cruz, CA), anti-phospho-STAT1 (Y701), anti-phospho-STAT3 (Y705), anti-phospho-STAT5 (Y694) from Cell Signaling Technology (Beverly, MA), anti-p27 (610241) from BD Biosciences (Pont de Clai, France), anti-PI3K (06-195) from Upstate Biotechnology (Mundolsheim, France), anti-γ-tubulin either monoclonal (GTU-88) or polyclonal (T3559) from Sigma, and anti-phosphotyrosine (anti-phosphotyrosine; 4G10; ref. 10).

Immunofluorescence analyses. Immunofluorescence analyses were done as previously described (24). Briefly, Rat2 or Ba/F3 cells either grown on glass coverslips or centrifuged on poly-l-lysine–coated coverslips, respectively, were fixed in cold methanol for 5 minutes. After permeabilization with 0.5% Triton X-100 for 5 minutes, cells were incubated at room temperature for 60 minutes with the first antibody and then for 45 minutes with the secondary antibody. Samples were then stained with the DNA-specific 4′-diamino-2-phenylindole (DAPI; Sigma). Most immunofluorescence images were recorded by a TCS-NT confocal microscope (Leica Microsystems, Mannheim, Germany) controlled by a Leica software. Images shown after confocal acquisitions were pseudocolored with Leica software, corresponding to one confocal section and were not submitted to additional treatment. For immunofluorescence with DAPI staining and on purified centrosomes, acquisitions were done using a Zeiss Axiovolta 200 microscope equipped with Cool Snap HQ camera (Ropper Scientific, Evry, France) controlled by Metamorph software (Universal Imaging, Downingtown, PA). For immunofluorescence images containing DAPI staining, Z stacks were acquired, deconvoluted and analyzed with Metamorph software. Monochrome images were collected for each staining and on purified centrosomes, acquisitions were done using a Zeiss Axiovert 200 microscope equipped with Cool Snap HQ camera (Ropper Scientific, Evry, France) controlled by Metamorph software (Universal Imaging, Downingtown, PA). For immunofluorescence images containing DAPI staining, Z stacks were acquired, deconvoluted and analyzed with Metamorph software. Monochrome images were collected for each staining and on purified centrosomes, acquisitions were done using a Zeiss Axiovert 200 microscope equipped with Cool Snap HQ camera (Ropper Scientific, Evry, France) controlled by Metamorph software (Universal Imaging, Downingtown, PA).

Results

FOP and FOP-FGFR1 are centrosomal proteins. To determine when, during the cell cycle, FOP was present at the centrosome, we did immunofluorescence experiments on stable Rat2 cell clones overexpressing myc-tagged FOP. FOP localizes to the centrosome (Fig. 1A) both in interphase before (Fig. 1Ba and Da) and after centrosome duplication (Fig. 1Ab and Db) and in dividing cells (Fig. 1Ac and Dc). These results suggested that some fusion partners may not only provide dimerization domains but could also determine the subcellular localization of the corresponding oncogenic kinase. Therefore, we wondered if FOP-FGFR1 fusion protein was addressed to the centrosome. In Rat2 clones expressing myc-FOP-FGFR1, the fusion protein was localized exclusively at the centrosome during the whole cell cycle, in interphasic cells before (Fig. 1Ba and De) and after centrosome duplication (Fig. 1Bb and Dj), and also during metaphase (Fig. 1Bc and Dj) and cytokinesis (Fig. 1Bd and Dh). FOP-FGFR1 centrosomal localization is detected with both myc (Fig. 1Bb and Ca) and FGFR1 (Fig. 1Cab) antibodies. When it is not fused to FOP, FGFR1 kinase is not detected at the centrosome (Fig. 1Cd). Thus, an ectopic, activated fusion kinase of FGFR1-MPDs could be targeted to the centrosome.

FOP-FGFR1 proteins can signal at the centrosome during G1/S phase. In subsequent experiments, we used Rat2 cells to allow easy visualization of the centrosome before and after duplication and Ba/F3 cells for functional studies.

To exert its effects at the centrosome, the oncogenic fusion protein must induce tyrosine phosphorylation of downstream substrates at this subcellular site (Fig. 2A). Study of global tyrosine phosphorylation in FOP-FGFR1 expressing Rat2 cells revealed a strong centrosomal staining during interphase before (data not shown) and after (Fig. 2Ba) centrosome duplication. This staining could represent either FOP-FGFR1 auto phosphorylation or the phosphorylation of its downstream substrates or both. The staining was stronger in interphasic cells (Fig. 2Bb) than in mitotic cells (Fig. 2Bd). Because FOP-FGFR1 is present at the centrosome during the whole cell cycle, we think that phosphotyrosine staining at the centrosome in mitosis corresponds
only to FOP-FGFR1 autophosphorylation, whereas phosphotyrosine staining during interphase represents phosphorylation of both FOP-FGFR1 and its substrates. This result shows that FOP-FGFR1 encounters substrates with tyrosine phosphorylation sites at the centrosome. It also suggests that phosphotyrosine signaling is important for G1-S events, when centrosome duplication occurs. Phosphotyrosine staining was absent in cells expressing a kinase-defective FOP-FGFR1 K259A mutant although the mutant protein also localized to the centrosome (Fig. 2C). Because FGFR1wt is not targeted to the centrosome (Fig. 1Cd) vesicular cytoplasmic but not centrosomal phosphotyrosine staining was detected in Rat2 cells overexpressing FGFR1wt (Fig. 2Bc).

A very strong phosphotyrosine staining was similarly detected at the centrosome of interphasic Ba/F3 cells expressing myc-FOP-FGFR1 (Fig. 2Ca) but not in mitotic cells (data not shown) or in cells expressing the kinase-defective mutant (Fig. 2Cb), even if both proteins localized to the centrosome (Fig. 2Ccd). Immunofluorescence on purified centrosomes confirmed this result (Fig. 2Ce-l): phosphotyrosine staining colocalized with pericentriolar material and centrioles stained with γ-tubulin and tyrosylated α-tubulin, respectively.

Expression of FOP-FGFR1 in primary bone marrow cells induced by retroviral transduction generates a fatal MPD in mice characterized by myeloid hyperplasia and hepatosplenomegaly (13). We used cells isolated from the spleen of such transplanted FOP-FGFR1 mice to investigate FOP-FGFR1 activity in conditions close to those of the natural disease. Hematopoietic progenitors (spleen colony-forming unit) from spleens of FOP-FGFR1 mice were collected and maintained in culture without cytokines for >2 months, showing that these cells have a proliferative potential. Immunofluorescence on cultured cells showed strong FOP-FGFR1 (Fig. 2Da) and phosphotyrosine (Fig. 2Db) signals at the centrosome. Thus, FOP-FGFR1 is targeted to the centrosome and signals at this organelle, bringing tyrosine phosphorylation, both in vitro and in vivo.

FOP-FGFR1 protein encounters, recruits, and phosphorylates substrates at the centrosome. We next wondered which were the substrates activated by FOP-FGFR1 at the centrosome. We first focused on known FOP-FGFR1 substrates, which include

**Figure 1.** Both FOP and FOP-FGFR1 chimeric protein are localized at the centrosome during the cell cycle. Immunofluorescence experiment with anti-myc antibody (green) shows the localization of myc-FOP (A) and myc-FOP-FGFR1 (B) in various phases of the cell cycle of Rat2 cells (a-d). Colocalization with γ-tubulin (red) at the centrosome of interphasic cells before (a) and after (b) centrosome duplication, and during metaphase (c) and cytokinesis (d) is shown. Stages of the cell cycle were determined using DAPI staining (blue). C, controls: colocalization of anti-myc (red) and anti-FGFR1 (green) staining on myc-FOP-FGFR1 transfected cells (a), FGFR1 staining (green) at the centrosome (γ-tubulin, red) of myc-FOP-FGFR1 cells (b), no myc staining on nontransfected cells (c), no signal at the centrosome (γ-tubulin, red) but vesicular staining of FGFR1 antibody (green) in FGFR1-transfected cells (d). D, visualization of centrosomal localization of myc-FOP (a-d) and myc-FOP-FGFR1 (a-h) using a confocal microscope. Images were acquired with a Zeiss microscope and treated with Metamorph software (A-C) or with a Leica confocal (D). Bar, 10 μm.
STAT1, -3, -5 proteins, PLCγ, PI3K, AKT and p70S6K proteins (10). We found p70S6K and phospho-AKT at the centrosome in interphasic and mitotic Rat2 cells, respectively (data not shown). PLCγ, which was present at the spindle pole during mitosis in Rat2 cells transfected (Fig. 3Aa) or not with FOP-FGFR1, was recruited to the centrosome in interphase before and after centrosome duplication in FOP-FGFR1 (Fig. 3Abc) but not in FOP-FGFR1 kinase-defective cells (Fig. 3Ad) or in cells expressing FOP-FGFR1 Y511F mutant, which lacks the PLCγ binding site (Fig. 3Ag). In Ba/F3 cells, PLCγ was also recruited to the centrosome in the presence of FOP-FGFR1 (Fig. 3Babc, arrow) but neither in its absence (Fig. 3Babc, arrowhead) nor in the presence of kinase-defective mutant (Fig. 3Bdef). We have previously shown that PLCγ interacts and is phosphorylated by FOP-FGFR1 (10); we show here that this could occur at the centrosome during the G1-S phase of the cell cycle.

PI3K was present at the centrosome of interphasic cells transfected (Fig. 3Bghi, arrow) or not (Fig. 3Bghi, arrowhead) with FOP-FGFR1. PI3K is important for centrosome duplication (28). Because PI3K colocalizes with FOP-FGFR1 (Fig. 3Bghi), we suspect that phosphorylation of PI3K by FOP-FGFR1 occurs at the centrosome. It may play a role when centrosome duplication occurs at the G1-S transition.

Study of phosphotyrosine STAT1, STAT3, and STAT5 subcellular localization in Rat2 cells showed, in addition to their known cytoplasmic and nuclear localizations, a strong phosphotyrosine staining at the centrosome of interphasic cells, before (data not shown) and after (Fig. 3Cabc, arrow) centrosome duplication. This phosphorylation in FOP-FGFR1 but not in kinase-defective mutant cells (Fig. 3Cdef) could facilitate subsequent STAT activation (10). FOP-FGFR1 Ba/F3 cells, in which the STAT3 pathway is activated (10), showed the same result (Fig. 3Cg, arrow). No phospho-STAT3

**Figure 2.** FOP-FGFR1 induces tyrosine phosphorylation at the centrosome during the G1-S transition of the cell cycle. A, schematic representation of FOP-FGFR1 protein. K259 belongs to the ATP-binding site necessary for the kinase activity of the fusion protein (TK, tyrosine kinase subdomains); it is mutated in the kinase-defective mutant (K259A). Y511 is the PLCγ-binding site; it is mutated in the PLCγ-binding mutant. LisH, lissencephaly type-1–like homology motif. Arrow, breakpoint fusion. B, staining using anti-phosphotyrosine (phosphotyrosine, red) and anti-FGFR1 (green) reveals phosphorylation on tyrosine at the centrosome of Rat2 cells expressing FOP-FGFR1 (a and b) but not in Rat2 cells expressing FOP-FGFR1 kinase-defective mutant (c). Phosphotyrosine staining of interphasic cells (a and b) is strong compared with mitotic cells (dM). Staining with anti-phosphotyrosine (red) and anti-γ-tubulin (green) on Rat2 expressing FGFR1wt shows absence of detectable phosphorylation at the centrosome (d). Bar, 10 μm. C, staining using anti-phosphotyrosine (red) and anti-γ-tubulin (green) reveals phosphorylation on tyrosine at the centrosome of Ba/F3 cells expressing FOP-FGFR1 (a) but not kinase-defective mutant (b). Immunofluorescence with anti-myc (red) antibody shows localization of tagged FOP-FGFR1 (c) and FOP-FGFR1 kinase-defective mutant (d) at the centrosome of Ba/F3 cells stained with anti-γ-tubulin (green). Bar, 5 μm. Costaining using anti-γ-tubulin (green), phosphotyrosine (red), and antityrosylated α-tubulin (blue) on purified centrosomes from Ba/F3 cells expressing FOP-FGFR1 (e-h) or kinase-defective mutant (i-l). Scale, 10 μm (3.4 cm). D, presence of FOP-FGFR1 (a) and phosphotyrosine (b) at the centrosome of colony-forming cells isolated from spleens of transplanted FOP-FGFR1 mice revealed by immunofluorescence using anti-myc (green), anti-phosphotyrosine (green) and anti-γ-tubulin (red). Bar, 10 μm.
signal was detected in untransfected (Fig. 3Cg, arrowhead) or kinase-defective mutant (Fig. 3Ch) cells.

To further show that FOP-FGFR1-activated substrates were associated with the centrosome, we immunoprecipitated proteins associated with the pericentriolar material using anti-γ-tubulin antibody. We found many proteins phosphorylated on tyrosine residues bound to γ-tubulin in FOP-FGFR1 Ba/F3 lysates (e.g., red asterisks), which were absent in kinase-defective mutant lysates (Fig. 3D). These proteins can either be signaling molecules phosphorylated at the centrosome or intrinsic centrosomal proteins. Several proteins of high molecular mass in particular (red asterisks) could be centrosomal proteins directly phosphorylated by FOP-FGFR1, however, they remain to be characterized. In conclusion, FOP-FGFR1 protein constitutively activates substrates at the centrosome.

Phosphorylation at the centrosome and proliferation in different clones. We next studied if other kinases were targeted to the centrosome and/or induced phosphorylation at this site. We used proliferating Ba/F3 cells expressing several FGFR1wt or fusion proteins and the EOL-1 cell line (23) expressing FIP1L1-PDGFRα fusion (Fig. 4A). Ba/F3 cells normally need IL-3 for survival and proliferation. Cell proliferation experiments using [3H]thymidine incorporation indicated that FOP-FGFR1 Ba/F3 cells not only survived as previously shown (10), but even proliferated in the absence of IL-3, although to a lesser degree than in the presence of IL-3 (data not shown). Kinase-defective mutant cells did not survive in the absence of IL-3. FGFR1wt cells cultivated in the presence of FGFR1 and heparin were used as controls. To determine if the phosphorylation pattern obtained with FOP-FGFR1 was due to its centrosomal localization we compared (a) phosphorylation profiles on Western blot and (b) global phosphotyrosine localization induced by different fusion proteins and FGFR1wt.

Western blot analysis after IL-3 starvation for 8 hours showed a phosphorylation profile specific for FOP-FGFR1 (Fig. 4A, red asterisk). Some substrates, probably corresponding to the ones immunoprecipitated with γ-tubulin (see Fig. 3D), were detected

Figure 3. FOP-FGFR1 encounters and phosphorylates signaling substrates at the centrosome. A. PLCγ (green) localization during mitosis (a). PLCγ staining (green) shows the recruitment of endogenous PLCγ to the centrosome of Rat2 cells by FOP-FGFR1, before (b) and after (c) centrosome duplication, but neither by kinase-defective (d and e) nor by PLCγ binding site mutant (f and g). Red, anti-γ-tubulin stains the centrosomes. B. PLCγ or PI3K staining (green) in Ba/F3 cells expressing tagged FOP-FGFR1 or kinase-defective mutant (red). Arrows and arrowheads, centrosomes of transfected cells and nontransfected cells, respectively. C. phosphotyrosine Y701-STAT1 (a), Y705-STAT3 (b), and Y694-STAT5 (c) staining (green) detects the phosphorylated protein (P-STAT) at the centrosome (red) of interphasic FOP-FGFR1 but not kinase-defective (d-f) Rat2 cells. STAT5 nuclear staining is also detected after centrosome duplication (c). Phosphotyrosine Y705-STAT3 staining in FOP-FGFR1 (g) Ba/F3 cells but not kinase-defective (h).

Bar, 10 μm. D. Western blot with anti-phosphotyrosine shows phosphorylated FOP-FGFR1 (black asterisk) and its substrates (red asterisk) in FOP-FGFR1 but not kinase-defective mutant lysates or after immunoprecipitation with anti-γ-tubulin.
exclusively after FOP-FGFR1 direct centrosomal activation but not after FGFR1wt activation (whether long or short), which is not directly targeted to the centrosome. This indicates that FOP-FGFR1-specific targeting to the centrosome can directly phosphorylate additional centrosomal proteins. Because of its molecular mass, CEP1-FGFR1, the other fusion protein localizing to the centrosome, did not allow the detection of this pattern. Western blots were also useful to control the expression of the different fusion proteins (Fig. 4A, black asterisk).

Phosphotyrosine staining (Fig. 4B), which allows the detection of the fusion protein and its substrates, showed that FOP-FGFR1 was the most specifically and exclusively targeted to the centrosome where it induces tyrosine phosphorylation (Fig. 4Bd). Ba/F3 cells expressing CEP1-FGFR1 also displayed strong centrosomal staining, suggesting that the fusion protein is also targeted to the centrosome. However, this localization seemed less exclusive than that of FOP-FGFR1 and some cytoplasmic fusion protein could be detected, perhaps due to a high level of expression (Fig. 4A). FGFR1wt, BCR-FGFR1, and FIP1L1-PDGFRA were not directly targeted to the centrosome and the phosphotyrosine signal was detected in the cytoplasm (Fig. 4Bg-j). However, some phosphorylation staining was detected at the centrosome of some proliferating cells (Fig. 4Bg-j, arrow). Similarly, in the presence of IL-3, phosphotyrosine staining was, in some cells, also detected at the centrosome (Fig. 4Ba, arrow). This could correspond to downstream substrates common to both IL-3 and FGFR1 activation pathways (e.g., STAT5). These results suggest that any of these signalings indirectly activate substrates at the centrosome, at least at some specific time of the cell cycle. No centrosomal phosphorylation could be detected in nonproliferating cells, Ba/F3 and kinase-defective mutant cells without IL-3 (Fig. 4Bbc). We propose that bringing phosphorylation directly to the centrosome is sufficient to allow cells to enter a proliferating state.

Centrosomal phosphorylation and proliferation induced by FOP-FGFR1 are inhibited by FGFR1 kinase inhibitor. To further show that centrosomal staining is required for survival and

Figure 4. Phosphotyrosine profiles on Western blot and centrosomal tyrosine phosphorylation localization induced by various oncogenic kinases. A, Western blot analysis shows differential phosphotyrosine profiles of various cells: Ba/F3, FOP-FGFR1, FOP-FGFR1 K259A mutant, FGFR1wt, BCR-FGFR1, CEP1-FGFR1 transfected Ba/F3, or EOL-1 (FIP1L1-PDGFRA) cells. Fusion proteins and substrates are indicated by black and red asterisks, respectively. B, immunofluorescence experiment shows the corresponding localization of tyrosine phosphorylation (red; s. stim and l. stim, short and long stimulation of wild-type FGFR1 by FGF and heparin). Arrows, phosphotyrosine staining at the centrosome. Bar, 10 μm.
proliferation we used the kinase inhibitor SU5402. SU5402 interacts directly with FGFR1 catalytic domain and can inhibit \[^{3}H\]thymidine incorporation of cells stimulated by FGF1 (29). Phosphorylation at the centrosome of FOP-FGFR1 Ba/F3 cells was specifically abolished after SU5402 treatment (Fig. 5A). A strong phosphotyrosine staining was detected at the centrosome of Ba/F3 cells in the presence of a low, inefficient concentration (0.15 μmol/L) of SU5402 (Fig. 5A,ac), or with STI571 (data not shown), an inhibitor with a different specificity known to be inefficient on FGFR1 kinase. In contrast, treatment with 15 μmol/L of SU5402 for 90 minutes reduced phosphorylation staining at the centrosome of Ba/F3 cells expressing FOP-FGFR1 (data not shown). After 15 hours of SU5402 treatment, most cells were dying (data not shown). The remaining cells showed no or very low levels of phosphorysine staining at the centrosome (Fig. 5A,bd). This result indicates that centrosomal phosphorylation is required for survival and proliferation.

Indeed, loss of phosphorylation at the centrosome observed after SU5402 treatment correlated with loss of proliferation measured using \[^{3}H\]thymidine incorporation: FOP-FGFR1-induced proliferation was inhibited by increasing concentrations of SU5402 (Fig. 5B). Because SU5402 is cytotoxic (29) this loss of proliferation was associated with a loss of cell survival verified by cell count after trypan blue exclusion (data not shown). In contrast, proliferation induced by FOP-FGFR1 was not abolished by STI571 (Fig. 5B). SU5402 also showed an inhibitory effect on the proliferation of FGFR1wt, CEP1-FGFR1, BCR-FGFR1 Ba/F3 cells, and EOL-1 cells, but not on untransfected Ba/F3 cells (Fig. 5B). SU5402 is known to inhibit FGFR1 and platelet-derived growth factor receptor tyrosine kinase activity. The results of SU5402 and STI571 treatments were verified in Western blot experiments; in agreement with cell proliferation experiments, SU5402, but not STI571, induced loss of tyrosine phosphorylation in FOP-FGFR1 cells (Fig. 5C).

In conclusion, phosphorylation at the centrosome in the presence of FOP-FGFR1, which is inhibited by SU5402, is essential for survival and proliferation of Ba/F3 FOP-FGFR1.

**FOP-FGFR1 has an effect on the cell cycle.** To reveal FOP-FGFR1 potential, we studied the consequence on the cell cycle of a stable overexpression of FOP-FGFR1 at the centrosome in Ba/F3 cells. Ba/F3 cells transfected with an empty vector or the kinase-defective mutant were used as control. Two different conditions of stress, IL-3 withdrawal and irradiation, were used (Fig. 6A and B).

As expected, Ba/F3 cells transiently arrested in G0/G1 after IL-3 withdrawal, did not enter S phase (Fig. 6A) and died (data not shown). The ratio G1/(S+G2) is used to represent cells blocked at the G1-S checkpoint. The lack of G1 arrest in FOP-FGFR1 cells was revealed by the decrease of this ratio, as compared with control or
kinase-defective mutant cells (Fig. 6A). Accordingly, FOP-FGFR1 cells were protected from cell death and 26% of cells even entered the cell cycle in the absence of IL-3 (Fig. 6Bb) as compared with 9% for kinase-defective mutant cells (Fig. 6Bf). The presence of the constitutively active oncogenic protein at the centrosome was thus enough to bypass a restriction point and sustain continuous entry in S phase in the absence of IL-3.

Similarly, Ba/F3 cells underwent G1 arrest but either survived or rapidly died when irradiated in the presence or absence of IL-3, respectively. FOP-FGFR1 protected irradiated cells from death, although less than the mere presence of IL-3 (Fig. 6Bb) as compared with 9% for kinase-defective mutant cells (Fig. 6Bf). The presence of the constitutively active oncogenic protein at the centrosome was thus enough to bypass a restriction point and sustain continuous entry in S phase in the absence of IL-3.

Figure 6. FOP-FGFR1 induces continuous S phase entry of Ba/F3 cells. A, G0/G1 arrest after IL-3 withdrawal and irradiation (10 Gy) visualized by the following ratio: % of living cells in G0/G1/% of living cells in S-G2-M. Graphical data represent the average of three independent experiments ± mean deviation. B, cell cycle analysis focusing on living cells for FOP-FGFR1 (a-d) and kinase-defective mutant (e-h). The percentage of cells in G0/G1, corresponds to cells with 2 N DNA content. C, Western blot using anti-p27 (top) on total lysates illustrates S phase entry of different proliferating Ba/F3 cells. Total cell lysates were probed with anti-α-tubulin antibody to compare the amount of proteins in the lysates (bottom). D, diagram synthesizing the results. p27 is an inhibitor of the G1-S transition. When cells enter S phase, p27 degradation is induced after CDK2/cyclin E activation (30) and decrease of p27 is observed in proliferating cells (Fig. 6C). Decrease of p27 expression in FOP-FGFR1 Ba/F3 cells revealed the proliferative potential of cells entering S phase (Fig. 6C). SU5402 treatment abolished this effect. This result is in favor of FOP-FGFR1 inducing S phase entry.

Discussion

We have shown that FOP and FOP-FGFR1, the oncogenic fusion kinase of an FGFR1-MPD, are addressed to the centrosome. Centrosomal addressing of the fusion protein occurs not only in transfected cultured cells but also in hematopoietic cells of an MPD mouse model. Our search for FOP partners by two-hybrid screen in yeast identified further centrosomal proteins such as CAP350, confirming these observations. FOP has only one identified domain called LisH, which it shares with other proteins such as PAFAH1B1, TCOF1, and OFD1. LisH proteins are mutated in Miller-Dieker syndrome.
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lissencephaly, Treacher Collins syndrome, oral-facial-digital type I and contiguous syndrome ocular albinism with late onset sensorineural deafness (31). LisH motifs contribute to the regulation of microtubule dynamics, either by mediating dimerization, or else by binding cytoplasmic dynein heavy chain or microtubules directly. However, mutation of the LisH domain of OFD1 did not abrogate centrosomal localization (32). Therefore, the region of FOP-FGFR1 needed for its centrosomal localization remains to be determined.

We have further shown that the oncogenic fusion kinase encounters or recruits substrates (e.g., PLCγ) at the centrosome where it induces strong phosphorylation on tyrosine residues, both in vitro and in vivo. FOP-FGFR1 substrates at the centrosome could either be signaling molecules phosphorylated at the centrosome (e.g., STAT) or intrinsic centrosomal proteins. Comparison of phosphorylation patterns of other fusion proteins or FGFR1 wild-type shows that FOP-FGFR1 and CEP-FGFR1 are the most directly targeted to the centrosome. This further suggests that any of these signaling pathways seem to activate relays at the centrosome, at least at some periods of the cell cycle. Treatment with SU5402 abolishes kinase-induced centrosomal phosphorylation and suppresses the proliferative potential of FOP-FGFR1 Ba/F3 cells. Finally, we have shown that FOP-FGFR1 at the centrosome allows cells to proliferate and overcome G1 arrest. The results are summarized in Fig. 6D.

We therefore propose that targeting an oncogenic, constitutively active kinase to the centrosome is enough to overcome cell cycle arrest, overcome a restriction point during the G1-S transition when centrosome duplication occurs, and force entry in the cell cycle. It is the first time that an oncogenic product of a human disease is shown to be addressed to the centrosome. This raises at least two questions.

How general is the phenomenon? Both FOP-FGFR1 and CEP1-FGFR1 localize to the centrosome. This localization and stability seem sufficient for these kinases to exert their effects. However, centrosomal localization may not be necessary to trigger the disease. Indeed, the study of ZNF198-FGFR1 (3, 9, 11) and BCR-FGFR1 (this work), two other well-characterized fusion kinases of FGFR1-MPDs have not alluded to a potential centrosomal localization. However, we have shown here that even if BCR-FGFR1 is not targeted to the centrosome, some relay of the oncogenic signal may take place at the centrosome. Conversely, FOP-FGFR1 and CEP1-FGFR1 may not be the only oncogenic kinases to target the centrosome. A new FGFR1-MPD with FGFR1OP2-FGFR1 fusion has been described recently (33). FGFR1OP2 has coiled-coil motifs. These motifs are frequently found in centrosomal proteins. Centrosomal targeting of oncogenic kinases may even occur in other malignancies than FGFR1-MPDs. A case of MPD has been described in which the platelet-derived growth factor receptor B kinase is fused to ninein, a centrosomal protein with CEP1-like structure and function (34). Several other cases of MPD have been described with rearrangements involving platelet-derived growth factor receptor B and numerous partners (35–41). Some of these partners may be localized at the centrosome (42, 43). Finally, we and others have evidence for fusion of JAK2 kinase with centrosomal protein PCM1 in MPD with t(8;9) translocation (44).5 Thus, there might be two classes of ectopic oncogenic kinases, those that directly target the centrosome (or at least the Golgi/centrosome area), and those that do not. A non–kinase oncogene may also abnormally function at the centrosome (45).

What are the effects of the oncogenic kinase at the centrosome? It is likely that FOP-FGFR1 exerts its oncogenic activity through dysregulation of cell processes associated with the centrosome. Centrosomes nucleate microtubules and contribute to mitotic spindle organization and function. They also participate in cytokinesis and cell cycle progression. The first type of alteration associated with centrosome defect is aneuploidy. However, the karyotype of X-FGFR1-positive cells, either in patients or after transfection of chimeric genes, does not show a particularly high degree of aneuploidy as compared with other types of hemopathies or cancers. There is no amplification of the centrosomes in cells overexpressing FOP-FGFR1. Experiments in Ba/F3 cells showed that FOP-FGFR1 interferes with the G1 checkpoint. We may have uncovered a mechanism of oncogenic transformation associated with a defect of centrosome function but not of centrosome number; this mechanism will need to be further delineated.

The centrosome is important for the cell cycle; it influences cell shape, polarity, and motility; it is also linked to DNA repair (19–21). Thus, abnormal activation of the FGFR1 tyrosine kinase at the centrosome may affect several processes by disrupting the regulation of various molecular complexes that remain to be identified. We have shown here that components of the FGFR1 cascade which interact with the chimeric X-FGFR1 proteins are localized at the centrosome either during interphase and mitosis or both. The PI3K-AKT/PKB pathway is particularly interesting in this context. It regulates G1 cyclins (D1 and E), is involved in centrosome duplication, and is well-known to be associated with cell survival and cell proliferation, which are two cell processes predominantly affected in MPD (28, 46, 47). Similarly, a recent report has also pointed to the role of STAT3 in centrosome duplication (48). Downstream targets of AKT and STAT in centrosome regulation remain unidentified.

We hypothesize that the centrosome, which is linked to the microtubules, close to the nucleus, and connected to the Golgi apparatus and the proteasome, could be an integrating place for some of the multiple signaling pathways controlling cell division, cell migration, and cell fate (49). In embryogenesis and normal processes of proliferation and differentiation, different types of signaling are associated with centrosome duplication and function and centrosomal activity is linked to cell division (50, 51). Like viral proteins, an oncogenic protein would prey upon these normal signals and use them to its profit. Abnormal kinase activity at the centrosome should be an efficient way to pervert cell division in malignancy.

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5 Murati et al., unpublished.
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