Cell growth, global phosphotyrosine elevation, and c-Met phosphorylation through Src family kinases in colorectal cancer cells

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The heterogeneity of cancer cell signaling is a significant obstacle for the effective development and clinical use of molecularly targeted therapies. As a contribution to a better understanding of the diversity of signaling activities in colorectal cancers (CRCs), we have analyzed the activity of Src family kinases (SFKs), which are implicated in human cancer development, in 64 CRC cell lines. A striking diversity of SFK activity was observed within this panel. Importantly, all CRC lines tested depend on SFK activity for their growth. In addition, SFK activity levels strongly correlated with global levels of tyrosine-phosphorylated (pTyr) proteins in CRC lines. SFK inhibition substantially reduced these pTyr levels, suggesting that SFKs may function as signal integration points and master controllers for the pTyr protein status in CRC lines. The majority of analyzed CRC lines with high-SFK activity express activated c-Met (pYpY1234/1235), a receptor tyrosine kinase contributing to the regulation of cell proliferation, migration, and invasion. Inhibition of SFKs reduced c-Met phosphorylation in most cases, indicating a reversed signal flow from SFK to c-Met. We conclude that SFK activity is important for the growth of CRC lines, although only low activity levels are required. If this also is true for CRC patients, tumors with low-SFK activity may be particularly sensitive to SFK inhibitors, and such patients should be targeted in clinical trials testing SFK inhibitors.

colon | therapy | kinase inhibition | molecular heterogeneity

Genetic and epigenetic changes found in cancer cell genomes lead to the deregulation of physiological-signaling pathways, as well as partial rewiring (signal spillover) and degradation (signal loss) of cellular-signaling networks. Moreover, individual tumors derived from the same cell type may have very distinct sets of molecular lesions (1–5). This diversity makes it desirable to develop molecularly targeted therapies that are optimized with respect to the particular genetic and epigenetic constitution of a patient's cancer. Because cancers are heterogeneous in this respect, many individual cases need to be studied to get an overall picture of the underlying pathological signals. The functional consequences of genetic and epigenetic changes are, however, sometimes not immediately apparent from DNA-based or transcriptional studies but may be revealed by in-depth analyses of signaling pathway activities and biological assays.

This study focuses on Src family kinases (SFKs) in a panel of colorectal cancer (CRC)-derived cell lines. CRC is a frequent disease often diagnosed at a relatively late stage, when currently available therapies are rarely curative (6). Molecularly targeted therapies for CRC have recently entered into clinical use or are currently in clinical trials (7). These therapies include kinase inhibitors, such as dasatinib, that target SFKs.

Src, the first known and one of the most studied oncogenes, has been implicated in CRC development (8) and poor clinical prognosis (9). Overexpression and hyperactivation of Src, not apparently associated with mutations in the Src gene, appears to be predominant in CRC, although occasional mutations have been reported (10, 11). In addition to Src, other SFK members, such as Yes and Lck, also are activated or aberrantly expressed in some CRC (12, 13).

Gene expression microarray data from 30 CRC lines have provided evidence for the expression of mRNAs for the SFK members Src, Yes, and Lyn in all cases and Fyn and Lck in some CRC lines, but no evidence for the expression of Fgr, Hck, and Blk (J. Wilding, D.C.B., and W.F.B., unpublished data; see also ref. 14). Furthermore, some SFKs are known or suspected to exist in several splice forms (15, 16), creating an additional layer of complexity. This has led us to study the overall activity of SFK proteins in CRC lines, rather than focus on a single SFK member or isoform, as has been done in most previous studies. The simultaneous analysis of all SFKs expressed in CRC is possible because a key regulatory epitope of SFKs in the activation loop of their catalytic domains is well conserved (Fig. 1*A*), and antibodies highly specific for this phosphoepitope [pY419 in human c-Sc (UniProtKB/Swiss-Prot entry P12931) and pY416 in chicken] have been generated. A genome-wide sequence search of human DNA revealed that, apart from the tyrosine kinases Btk (*ca.* 77 kDa) and Abl (*ca.* 145 kDa), which differ substantially in size from all known SFK forms, no other proteins have high homology to the sequence of the Y419 epitope.

Our results document not only a striking variability in the activity levels of SFKs in the CRC lines, but also show that some SFK activity is required for the growth of all lines analyzed. The results also indicate that SFKs play an important role in controlling the overall tyrosine-phosphorylated (pTyr) levels in CRC cells and that they may act as signal integration points for multiple pathways. In addition, we show that SFK signals often lead to the activation of c-Met, a multipotent receptor tyrosine kinase implicated in a variety of cancers.

Results

Total cell protein extracts from 64 CRC lines [see supporting information (SI) Table 1 for further details] were made 48 h after the last medium change and were analyzed by Western blotting for phosphorylation of the conserved key regulatory epitope of human SFKs, designated pY419Src throughout this article (Fig. 1*A*). The results shown in Fig. 1*B* reveal a striking heterogeneity of SFK activity not only in the absolute signal levels, but also in the pattern of bands detected. The multiple bands observed in some CRC lines could be due to differences in the SFKs activated, but also may result from different splice variants or phosphorylation states. Signals for pY419Src were seen in

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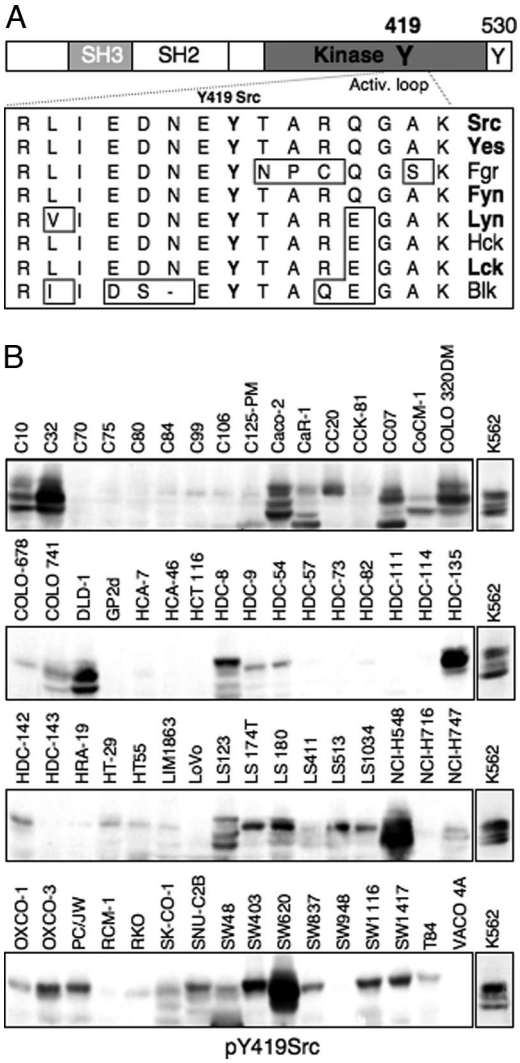


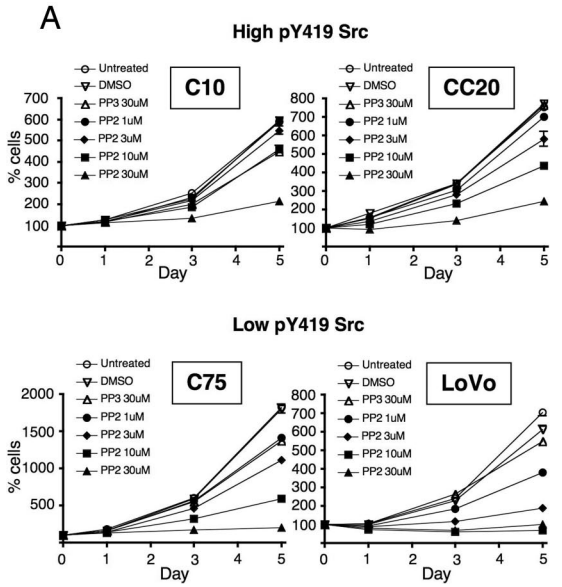
Fig. 1. Heterogeneity of steady-state SFK activity in a panel of 64 human CRC cell lines. (*A*) (*Upper*) Schematic representation of the human c-Src domain structure. Phosphorylation of Tyr-419 in the activation loop of the catalytic domain (corresponds to Tyr-416 in chicken c-Src) is crucial for tyrosine kinase activity. (*Lower*) Sequence alignment of all human SFK regions corresponding to the Tyr-419 epitope in human c-Src. Names of SFKs with detectable mRNA expression in CRC lines are in bold. Divergent amino acids in this conserved epitope are boxed. Note the high degree of sequence identity in this key regulatory epitope for all SFKs expressed in the CRC lines. (*B*) Western blots of total cell protein extracts from 64 human CRC lines grown as described in *Methods* were separated by SDS/PAGE and immunoblotted with the anti-phosphoTyr419Src antibody that recognizes the SFK members expressed in the CRC lines. Membranes were exposed to x-ray film for 1–2 min. Standardization of signal intensities between the four panels was done by using a single batch of lysate from K562 CML cells, which are known to contain activated SFKs. There is a striking degree of diversity in signal intensity and detectable band sizes. For longer exposures of the same blots, see SI Fig. 6*E*.

virtually all CRC lines after prolonged exposure (SI Fig. 6*E*), raising the question as to whether all of the CRC lines depend, at least to some extent, on basal SFK activity. To address this issue, 16 CRC lines with either very high- or low-SFK activity were selected and exposed to different doses of PP2, a well characterized SFK inhibitor with a good selectivity profile (17). The lines also were exposed to a control compound void of SFK inhibitory activity (PP3) or to solvent alone (DMSO). Representative data for four CRC lines are shown in Fig. 2*A*, and the IC_{50} values for all of the lines studied are given in Fig. 2*B*. All of the tested CRC lines are sensitive to PP2, as measured by growth inhibition. However, the fact that even at high concentrations of PP2 there was no apparent reduction in cell number suggested that there was no cell-killing effect of the inhibitor. This lack of cell killing was confirmed by light microscopic examination of treated and untreated cells. Cells with low-SFK activity are on average significantly more sensitive to PP2 than cells with high-SFK activity (mean IC_{50} value = 4.64 μM $\pm$ 1.22 vs. 17.38 μM $\pm$ 2.12, respectively; $P = 0.0017$). These results were not anticipated and would seem to indicate that a relatively low threshold level of SFK activity is sufficient to maintain the growth of the CRC lines. Some cell lines also were analyzed with a different SFK inhibitor, SU6656, and again were found to be growth-inhibited (SI Fig. 7).

It is well known that oncogenic SFKs such as v-Src in fibroblasts cause hyperphosphorylation of tyrosyl residues on multiple cellular proteins (18). To investigate whether high-SFK activity also impacts on the global steady-state pTyr levels in the CRC cells, a subset of 16 lines was analyzed by Western blotting total cell proteins with anti-pTyr mAb (4G10). The results (see Fig. 3) show that this antibody detects substantially more Tyr phosphorylation in CRC lines with high- compared with low-SFK activity. Similar results also were obtained from further CRC lines and with another pTyr mAb (SI Fig. 8). The patterns of pTyr bands observed in the CRC lines with high SFK are very variable probably because of the different genetic and epigenetic changes in the cell lines, reflecting the heterogeneity of CRCs. However, in some cell lines, similarly sized pTyr bands are detected. Whether these are indeed identical proteins remains to be investigated. To determine whether the constitutively elevated pTyr levels in CRC lines with high-SFK activity are a consequence of these kinase activities, such cells were treated with PP2 or a compound solvent (DMSO) and analyzed by Western blot with anti-pTyr mAb (P-Tyr-100) and anti-pY419Src. The results (see Fig. 4) document a substantial reduction of pTyr protein levels in each case. Similar data were obtained with SU6656 (SI Fig. 9). This finding raises the interesting possibility that SFKs function as signal integration points and master regulators of pTyr levels in the CRC lines.

Activation of SFKs by a variety of signaling pathways and in a wide range of different cell types has been described. For example, receptor tyrosine kinases such as EGFR, IGF1R, and c-Met (11, 19, 20) have been reported as important SFK activity regulators when ligand-activated, activated by mutations, overexpressed, or trans-activated through interactions with other signaling proteins (refs. 21–23 and references therein). To determine whether these receptors may be important for the maintenance of constitutively elevated SFK activities, EGFR, IGFR, and c-Met were immunoprecipitated (IP) and analyzed with phospho-specific antibodies recognizing important, activity-regulating tyrosine residues, namely, pYpY1135/1136 for IGF1R and pYpY1234/1235 for c-Met, or with a pTyr mAb for EGFR (P-Tyr-100). c-Met was found to be phosphorylated in five of the six high-SFK activity cell lines analyzed (Fig. 5 *Upper*), whereas little phosphorylation was detected on EGFR and IGF1R tyrosines (SI Fig. 10). C32 cells have high-SFK activity, but apparently do not express c-Met. No clear correlation between c-Met protein abundance and its pYpY1234/1235 phosphorylation was observed, which is suggestive of multiple deregulating mechanisms for this kinase, in addition to gene expression changes.

To investigate whether c-Met activates SFKs or whether SFKs affect c-Met through a reversed signal flow, cells with detectable c-Met pYpY1234/1235 were treated with PP2 to inhibit SFK, or with a c-Met inhibitor, SU11274, and then analyzed for the effects on c-Met or SFK activity, respectively. Treatment with PP2 led to a significant reduction of c-Met pYpY1234/1235 in four of five CRC lines analyzed (Fig. 5 *Lower*). COLO 320DM cells, however, showed no reduction of c-Met phosphorylation



B

Cell line	High pY419 Src	IC_{50} (μM PP2)
C10	+	**21.9**
C32	+	**27.8**
Caco-2	+	**7.1**
CC20	+	**12.1**
COLO 320DM	+	**17.7**
DLD-1	+	**14.3**
HDC-8	+	**11.0**
OXCO-3	+	**>30.0**
SW403	+	**19.7**
SW620	+	**16.1**
SW1116	+	**13.5**
C70	-	7.2
C75	-	4.8
HCA-7	-	2.4
HDC-111	-	7.4
LoVo	-	1.4

$P = 0.0017$

Fig. 2. SFK activity is required for the growth of CRC lines: Enhanced sensitivity of low-SFK activity CRC lines to an SFK inhibitor. For growth assays, CRC lines were incubated with 0.3% DMSO or the indicated concentrations of the SFK inhibitor PP2 (μM, micromolar) or the control compound PP3. Cell growth at the indicated times was measured by crystal violet staining (shown as arbitrary units with cells at day zero as 100%). (*A*) Representative results of growth assays from four CRC lines, two (C10 and CC20) with high-SFK activity and two (C75 and LoVo) with low-SFK activity are shown. Error bars (SE) are indicated but are usually smaller than symbol size ($n \geq 6$). (*B*) The IC_{50} values of PP2 in growth assays for 16 CRC lines on day 5 of incubation with compound. High-SFK activity is indicated by a plus symbol; 11 of these lines were selected to represent different patterns observed with the pY419Src mAb (also see Fig. 1*B*). Statistical analysis of the determined IC_{50} values indicates that CRC with low-SFK activity (mean value = 4.64 μM $\pm$ 1.22) is, on average, significantly more sensitive to PP2 than CRC with high-SFK activity (mean value = 17.38 μM $\pm$ 2.12; $P = 0.0017$).

on this epitope. The reason for this insensitivity is currently unclear, but one of several possible explanations would be the presence of an activating c-Met mutation. No detectable effect of SU11274 on global pTyr levels or SFK activity in any of the five high-SFK activity lines with c-Met expression lines was observed (SI Fig. 11). These results led us to conclude that retrograde signaling occurs from SFK to c-Met, identifying c-Met as a frequent effector protein in CRC lines with high steady-state SFK activity.

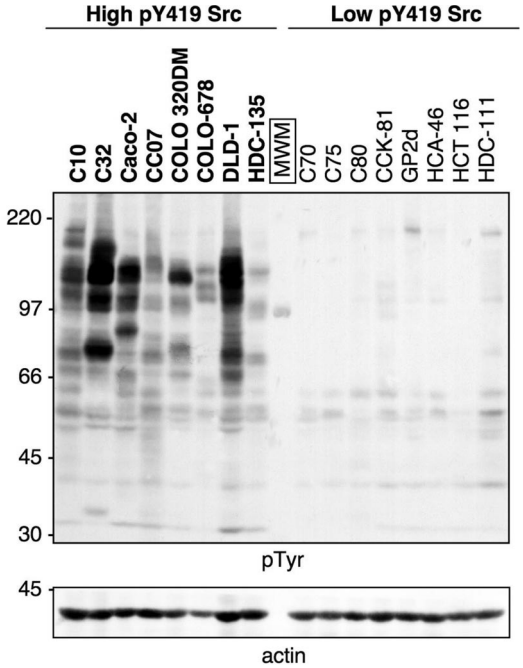


Fig. 3. CRC lines with high-SFK activity show elevated global levels of cellular tyrosine phosphorylation. (*Upper*) Total cell lysates from eight CRC lines with either high- or low-SFK activity, as indicated, were immunoblotted, after normalization for protein concentration, with anti-pTyr mAb (clone 4G10). MWM, molecular weight marker. (*Lower*) The membrane was then stripped and reprobed for actin (see SI Fig. 8 for a similar experiment).

Discussion

The main hope for improved therapies for patients with more advanced disease must come from novel molecularly targeted drugs, such as those currently undergoing clinical evaluation. In addition to the SFK inhibitor, dasatinib, which is already approved for some advanced forms of chronic myelogenous leukemia (CML) (24), other SFK inhibitors with activity in preclinical animal models are currently being tested in patients. Therefore, the routine clinical use of SFK inhibitors for several solid cancers may be only a few years away. However, it is probable that most of these drugs will not be highly specific for one particular SFK, but will affect multiple, if not all, SFK family members. To make optimal use of SFK inhibitors in the clinic, it will thus be important to obtain a better understanding of the role of SFK activities in human cancer development. The large CRC panel accumulated in the Cancer and Immunogenetics Laboratory (Weatherall Institute of Molecular Medicine) over the last two decades (25–27) is a valuable resource for such studies and allows detailed investigation of the molecular heterogeneity in CRC signaling protein activities. Based on our results, we suggest that studying the overall SFK activity may be more productive than focusing on a single SFK member. In support of this suggestion, we have found that overall SFK activity levels do not correlate well with c-Src activity (SI Fig. 12). Our results show that many, if not all, CRC lines require SFK activity, although even quite low levels seem to be sufficient for cell growth.

The dependence of the CRC lines on basal activity levels of SFK may have significant clinical implications. Thus, if low-SFK activity is sufficient for the proliferation of tumor cells in patients, individuals who have low tumor SFK activity could be

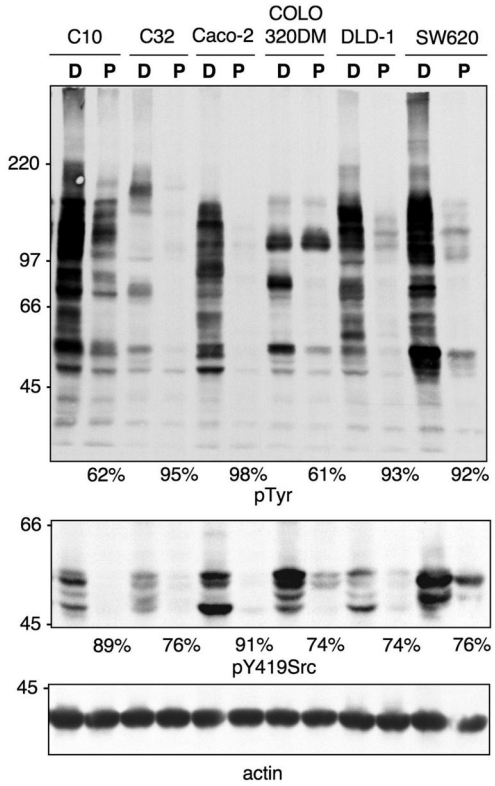


Fig. 4. Elevated global phosphotyrosine levels in CRC lines with high-SFK activity are greatly reduced by SFK inhibition. Six CRC lines with high-SFK activity were grown for 24 h after splitting and then treated with either 0.3% DMSO (D) or 30 μM SFK inhibitor PP2 (P), which were added into the medium for a further 24 h. Total cell lysates were separated by SDS/PAGE and then immunoblotted with anti-pTyr mAb (P-Tyr-100), anti-pY419Src, or anti-actin as indicated. The signals were quantified as described in *Methods*, and observed signal reductions upon PP2 treatment of cells are indicated in percentage below the corresponding lanes. A similar experiment conducted with the SFK inhibitor SU6656 is shown in SI Fig. 9.

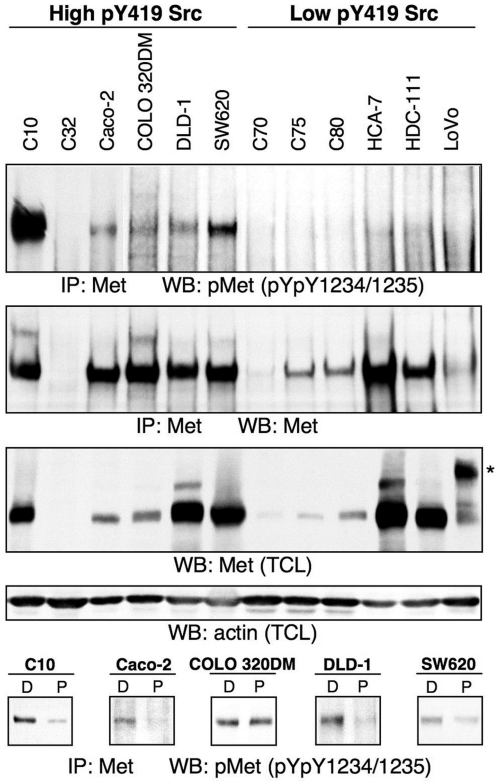


Fig. 5. High-SFK activity increases c-Met receptor tyrosine kinase phosphorylation at the key regulatory site Y1234/Y1235. Six cell lines each with either high- or low-SFK activity were analyzed for the level of protein expression and persisting phosphorylation of the c-Met receptor 48 h after their last feed with fresh medium. For phospho-status analysis at the c-Met key regulatory site, Y1234/Y1235, c-Met was immunoprecipitated from 1 mg of total cell lysate and blotted with the phosphoepitope-specific antibody (uppermost) or anti-c-Met to control for total c-Met in the IPs (second from top). Protein expression levels of c-Met in total cell lysates (TCL) are shown (third from top). The asterisk indicates partially processed c-Met protein in LoVo cells as described previously (32). Total cell lysates also were blotted for actin as a loading control. The five CRC lines with detectable c-Met phosphorylation on the Y1234/Y1235 epitope were subsequently treated with PP2 inhibitor (P) or solvent control (DMSO; D). Four of the five lines showed a substantial reduction of c-Met phosphorylation at the Y1234/Y1235 epitope after incubation with PP2 (bottom). No changes were detected in COLO 320DM cells. Actin was also analyzed in TCL (second from bottom).

more sensitive to SFK inhibitors and so have a better therapeutic window for the drug. It may therefore be quite inappropriate to include only patients with elevated SFK activity in clinical trials of SFK inhibitors.

The identification of a reversed signal flow from SFK to c-Met is not entirely surprising because c-Met has long been known to form a complex with c-Src (28). However, other SFK members are not well studied in this respect. c-Met is a multifunctional receptor tyrosine kinase that can drive various biological processes, so the actual consequences of c-Met activation by SFKs in different CRC lines still need to be elucidated. Many questions remain to be answered. For example, what are the underlying genetic, epigenetic, and signaling protein activity changes that are responsible for the constitutively elevated SFK activities in a substantial proportion of the CRC lines? It could be that other receptor or nonreceptor tyrosine kinases, which we have not analyzed so far, are responsible. Suppression of certain phosphatases, either directly or indirectly by mutation or epigenetic changes, also may lead to steady-state activation of SFKs (29).

Another unexpected finding that warrants further studies is the predominant link between SFK and c-Met. Both EGFR and IGF1R are known to physically and functionally interact with SFK in various contexts. Furthermore, although c-Met is clearly affected by SFK activity, it is by no means certain that this receptor kinase is a key player in driving SFK-mediated CRC cell proliferation. For example, Colo 320DM cells are sensitive to SFK inhibition, but this sensitivity does not appear to affect the c-Met pYpY1234/1235 phosphorylation. However, because c-Met is a multipotent receptor, other functional consequences of the SFK–c-Met interaction remain to be explored. Whether c-Met is a direct target of SFK also is not certain. Although Src and c-Met are known to form a complex in different cells, thereby leading to a high local concentration of both and potentially driving a direct phosphorylation, an involvement of other tyrosine kinases cannot be formally excluded at this point. Many of the proteins hyperphosphorylated in CRC lines with high-SFK activity remain unidentified, and corresponding mass spectrometric analyses, which may unravel important new SFK targets, are urgently needed.

In this study, we have solely relied on inhibitor compounds to analyze the roles of SFK in part because of the observation that CRC cells are difficult to transfect with oligonucleotide siRNAs, but also because a simultaneous knockdown of multiple SFK members, if technically feasible (for example with multicistronic viral vectors), is likely to elicit some off-target effects that are not dissimilar to those seen with many small-molecule inhibitors. Hence, additional investigations are warranted into some of the intriguing findings made in the course of this study. Further improved understanding of the SFK-regulated signaling path-

ways in CRC will undoubtedly increase the range of novel molecular strategies and targets for the development of improved therapies that have greater specificity for a given cancer and fewer side effects.

Methods

CRC Lines and Cell Lysates. The 64 lines in the panel have been previously HLA-typed and characterized for other genetic changes to determine whether they are derived from cancers of different donors. They are derived from 63 different patients; LS 174T and LS 180 originate from the same individual (30). Information about line origins is given in SI Table 1. OXCO-1 and OXCO-3 lines are a gift from Khoon Lin Ling and Vincenzo Cerundolo (Weatherall Institute of Molecular Medicine). CRC lines were grown in DMEM supplemented with 2 mM L-Glu, antibiotics, and 10% FBS at 37°C in humidified atmosphere with 10% CO_2. Cells were cultured for 48 h from the last feeding before lysis, washed three times with chilled PBS, and lysed in a RIPA-type buffer [20 mM Tris·HCl (pH 7.5), 100 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.5% deoxycholic acid, and 0.1% SDS] supplemented with 2x complete protease inhibitor mix (11697498001; Roche Diagnostics) and phosphatase inhibitor cocktails 1 and 2 (P2850 and P5726; Sigma–Aldrich) at 4°C for 30 min on a nutator. Lysates were then clarified by centrifugation at 22,000 × *g* for 30 min at 4°C. Protein concentrations were determined by the Bradford method (31), and Coomassie blue staining of SDS/PAGE-separated proteins was used to further ensure approximately equal loading of samples (SI Fig. 6 *A–D*).

Western Blot Analyses and IPs. Total lysates or IPs were separated by SDS/PAGE and transferred onto Hybond-P (Amersham). Membranes were blocked overnight at 4°C with BSA or nonfat dry milk in TBST [20 mM Tris·HCl (pH 7.5), 100 mM NaCl, and 0.1% Tween 20] as recommended by the antibody manufacturer, followed by incubation with primary antibody for 4–8 h at room temperature. After washing extensively with TBST, bound antibodies were detected by using peroxidase-conjugated anti-mouse or anti-rabbit IgG secondary antibody and ECL.

The pY419Src (pAb, 2101; Cell Signaling Technology) recognizes most members of the SFK family (see Fig. 1 for details). Two different anti-pTyr mAbs, 4G10 and P-Tyr-100 (9411; Cell Signaling Technology), were used as indicated. Actin expression was analyzed by using a mAb (clone AC-40; Sigma–Aldrich). IPs were carried out with 1 mg of total cell lysate by using anti-c-Met mAb (clone DO-24; Upstate), anti-IGF1R pAb (3027; Cell Signaling Technology), or anti-EGFR mAb (clone 528; Calbiochem). Western blots of IPs were performed with anti-c-Met mAb (clone DO-21; Upstate), anti-phospho-c-Met (pYpY1234/1235), pAb (3126; Cell Signaling Technology), anti-phospho-IGF1R (pYpY1135/1136), pAb (3024; Cell Signaling Technology), anti-IGF1R pAb (3027; Cell Signaling Technology), anti-EGFR pAb (2232; Cell Signaling Technology), or P-Tyr-100 as indicated.

Cell Growth Assays. Briefly, 1,000–2,000 cells were seeded into 96-well plates, allowed to adhere for 1 day, and either left in medium alone or incubated in medium with DMSO (inhibitor solvent; D2650; Sigma) or different concentrations of SFK inhibitor PP2 (529573; Calbiochem) or the structural analogue PP3, which lacks SFK inhibitory activity (529574; Calbiochem). Cell growth at the indicated times was assessed by a colorimetric assay using crystal violet (C3886; Sigma–Aldrich). Cells were stained and fixed in 0.1% crystal violet dissolved in 50% methanol for 15 min at room temperature before being washed three times with PBS and once with H_2O and then air dried. Bound dye was solubilized in 10% acetic acid for 30 min at room temperature, and absorbance was measured at 560 nm by using a μ-Quant microplate reader (Bio-Tek). Cell growth was expressed as a percentage of control cells (in medium alone at day 0). Similar experiments were conducted with the SFK inhibitor SU6656 (572635; Calbiochem) for some cell lines (SI Fig. 7).

Inhibition of SFK and c-Met for Phospho-Protein Analyses. CRC lines were incubated as indicated with PP2 or SU11274 (448101; 1 μM for 24 h; Calbiochem) for c-Met inhibition, lysed, and analyzed by Western blotting as described in *Western Blot Analyses and IPs*. Note that DMSO concentrations of >0.06% affected the phospho-status of c-Met at least in some CRC lines. Therefore, in all experiments analyzing c-Met phosphorylation, DMSO concentrations were kept at ≤0.06%.

Quantification of Inhibitor Effects on Cell Growth or Protein Phosphorylations. IC_{50} values were calculated by using Prism software (GraphPad) by comparing PP2-treated cells to DMSO-treated controls. Levels of protein phosphorylation were determined by using Alpha Scan (Alpha Innotech), and data were analyzed with ImageQuant (Molecular Dynamics). The *P* value for the difference between the mean IC_{50} values for the lines with high- versus low-SFK activity was determined by Student's *t* test.

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