

# Expression of a Truncated Form of the c-Kit Tyrosine Kinase Receptor and Activation of Src Kinase in Human Prostatic Cancer

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**A truncated form of the c-Kit tyrosine kinase receptor, originally identified in mouse haploid germ cells, is aberrantly expressed in human cancer cell lines of various origin. This alternative transcript originates in the 15th intron of the human *c-kit* gene. We have previously demonstrated that sperm-carried mouse truncated c-Kit (tr-Kit) is a strong activator of the Src-family tyrosine kinases both in transfected cells and in mouse oocytes. In the present work, we report that human tr-Kit mRNA and protein are expressed in LNCaP prostatic cancer cells. We have identified two regions in the 15th and 16th introns of the human *c-kit* gene that show homology with sequences in the spermatid-specific tr-Kit promoter within the 16th intron of mouse *c-kit*. We also show that nuclear factors present in LNCaP cells bind to discrete sequences of the mouse tr-Kit promoter. Moreover, Western blot analysis of 23 primary prostate cancers indicated that tr-Kit was expressed in ~28% of the tumors at less advanced stages (Gleason grade 4 to 6) and in 66% of those at more advanced stages (Gleason grade 7 to 9), whereas it was not expressed in benign prostatic hypertrophies. Sequencing of the cDNA for the truncated c-Kit, amplified from both LNCaP cells and neoplastic tissues, confirmed the existence in prostate cancer cells of a transcript arising from the 15th intron of human *c-kit*. We also show that tr-Kit-expressing LNCaP cells and prostatic tumors have higher levels of phosphorylated/activated Src than tr-Kit-negative PC3 cells or prostatic tumors, and that transfection of tr-Kit in PC3 cells caused a dramatic increase in Src activity. Interestingly, we found that Sam68, a RNA-binding protein phosphorylated by Src in mitosis, is phosphorylated only in prostate tumors expressing tr-Kit. Indeed, both activation of Src and phosphorylation of Sam68 were observed in all of the three grade 7 to 9 tumors analyzed that expressed**

**tr-Kit. Our data describe for the first time the existence of a truncated c-Kit protein in primary tumors and show a correlation between tr-Kit expression and activation of the Src pathway in the advanced stages of the disease. Thus, these results might pave the way for the elucidation of a novel pathway in neoplastic transformation of prostate cells. (Am J Pathol 2004, 164:1243–1251)**

The c-Kit receptor belongs to type III tyrosine kinase receptor, which consists of an extracellular ligand binding domain and an intracellular kinase domain. In the kinase domain, the ATP binding site is separated from the phosphotransferase site by an interkinase sequence required for interaction with signal transduction proteins involved in the c-Kit pathway.<sup>1</sup> The c-Kit receptor is expressed in a wide variety of normal and neoplastic tissues. A positive correlation between misregulation of the *c-kit* gene and malignant transformation of cells has been reported.<sup>2,3</sup> For instance, substitution mutations in exon 11 of the gene, changing amino acids of the juxtamembrane region of the receptor, are associated with gastrointestinal stromal tumors, whereas mutations in exon 17 that substitute Asp816, just downstream of the tyrosine kinase signature, are associated with myeloid leukemias and testicular seminomas.<sup>2,3</sup> These mutations induce ligand-independent dimerization or autophosphorylation of the receptor and they cause constitutive activation of downstream signaling pathways. Moreover, overexpression of c-Kit and its ligand SCF occurs in several tumors and they probably stimulate proliferation in an autocrine or paracrine manner.<sup>2</sup> Recently, *c-kit* expression in various tumors has been revisited because this receptor is a target of the anti-cancer activity of a well described tyrosine kinase inhibitor: imatinib (STI1571).<sup>2,4</sup> Beside mutations affecting the activity of the full-length c-Kit, expression of an alternative transcript of human *c-kit* has been described in several transformed cell lines. The

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alternative transcript encodes for a truncated c-Kit protein that contains only a short sequence of the interkinase segment, the phosphotransferase domain, and the carboxyl-terminal tail of the receptor.<sup>5</sup> Originally cloned from the colon cancer cell line Colo201,<sup>5</sup> expression of this novel mRNA has been detected in ~30% of the gastrointestinal and hematopoietic tumor cell lines examined.<sup>6</sup> To date, no function has been ascribed to this truncated c-Kit protein in transformed human cells. Other receptor tyrosine kinases are expressed as truncated forms in malignant cells, but usually these aberrant proteins are constitutively active kinases in which the catalytic domain is freed by negative constraints present in the full-length receptor.<sup>7-9</sup> By contrast, the truncated c-Kit protein does not contain an ATP binding site and should be catalytically inactive.<sup>5</sup> Our laboratory has previously described that a mouse homologue of the truncated human c-Kit is physiologically expressed only in the postmeiotic haploid cells of the testis;<sup>10</sup> more recently we have detected human tr-Kit also in human mature spermatozoa, indicating a conserved role of this protein in gamete function (Paronetto MP, Geremia R, Rossi P, and Sette C, manuscript in preparation). This alternative c-Kit mRNA encodes for a truncated protein, named tr-Kit, of the same size and structure as the human homolog characterized in cancer cells.<sup>11,12</sup> Mouse tr-Kit also lacks the ATP binding site and it is catalytically inactive; however, we have described that it acts as an activator of the soluble tyrosine kinases Fyn and Src.<sup>13</sup> The direct interaction of tr-Kit with the SH2 domain of Src-like kinases displaces the autoinhibitory constraint caused by binding of this domain to a phosphotyrosine in the carboxyterminal tail of Src-like kinases.<sup>13</sup> Tr-Kit-induced activation of Fyn or Src triggers cell cycle resumption in metaphase-arrested mouse oocyte, indicating the mitogenic potential of this pathway.<sup>13,14</sup> Interestingly, mutations that substitute tyrosine 530 of Src, which abolishes the autoinhibition of the kinase in the same manner as the interaction with tr-Kit, are associated with colorectal cancer tissues and induce cell transformation when aberrantly expressed in cultured cells.<sup>15</sup> More recently, we have demonstrated that activation of Src-kinases by tr-Kit triggers the phosphorylation of the RNA-binding protein Sam68.<sup>16</sup> Functioning as a scaffold, Sam68 promotes the recruitment of Src-kinases and PLC $\gamma$ 1, and the phosphorylation/activation of the phospholipase.<sup>13,16</sup> Because human truncated c-Kit (herein referred as human tr-Kit) contains all of the structural features required for mouse tr-Kit function (see Toyota and colleagues<sup>5</sup> and Rossi and colleagues<sup>10</sup> for comparison), it is possible that the two proteins share the same function and that human tr-Kit is able to promote Src activation in the malignant cells where it is expressed.

Src activation plays an important role both in proliferation and metastatic transformation of androgen-sensitive and -insensitive prostate cancer cells.<sup>17,18</sup> Furthermore, Sam68 is highly expressed in prostate epithelial cells and the involvement of this protein in RNA metabolism during normal growth and neoplastic transformation of prostatic cells has recently been hypothesized.<sup>19</sup> Herein we undertook an analysis of prostate cancer cell lines and primary prostatic tumors for the presence of

human tr-Kit. We found that human LNCaP cells expressed tr-Kit, whereas the more undifferentiated PC3 did not. Furthermore, we observed that human tr-Kit was expressed in ~28% of the less advanced and in 66% of the more advanced primary prostate tumors analyzed. In the latter group, a positive correlation was found between tr-Kit expression in tumors and tyrosine phosphorylation of Src and of the adaptor protein Sam68. Our results suggest that aberrant expression of a truncated c-Kit protein may contribute to cell transformation through the activation of Src kinases.

## Materials and Methods

### Human Tissue Samples

Hypertrophic prostate samples were obtained, after informed consent, from patients who underwent prostatectomy for suspected benign prostatic hyperplasia (serum PSA <4 ng/ml; negative for cancer to the rectal exploration and prostatic ultrasound), confirmed by histological analysis. Prostate cancers were diagnosed by TRU-CUT biopsy. Patients were excluded from the study if they had androgen deprivation therapy or reported a clinical history of repetitive neoplastic lesions at the radionuclide bone scan and at the abdomino-pelvic computed tomographic scan. Prostate cancers were graded using the Gleason grading system. We selected prostates that were positive for cancer to multiple biopsies in just one peripheric area. Samples of tumor and contralateral prostatic tissue (negative to the multiple biopsies analysis and without cancer macroscopy characteristics) from 23 patients, between 58 to 77 years of age, who underwent radical prostatectomy at the Clinical Center located in the Urologic Clinic of University of Rome "Tor Vergata," were immediately frozen in liquid nitrogen (Table 1).

### Immunohistochemistry

Tissues were fixed in formalin and embedded in paraffin immediately after surgery. Antigen retrieval was performed on hydrated sections by three microwaving cycles (5 minutes each) at high power in 1 mmol/L of ethylenediaminetetraacetic acid, pH 8.0. Slides were incubated for 1 hour in blocking solution, phosphate-buffered saline (PBS), pH 7.4, supplemented with 5% bovine serum albumin and 1% goat serum. After two washes in PBS, slides were incubated overnight at 4°C with either rabbit anti-human c-Kit (1:100, SC-168; Santa Cruz Biotechnology) or mouse anti-p416Src (1:50, 05-677; Upstate). Controls using nonimmune IgGs were also performed and gave no signal (data not shown). Horseradish peroxidase-linked secondary antibody incubation was performed for 1 hour at room temperature. Signals were visualized with diaminobenzidine (Sigma) and analyzed by light microscopy.

**Table 1.** Parameters of the 23 Patients Examined for Tr-Kit Expression in Prostatic Tissue

Patient	Age	Gleason sum	Tr-kit expression
1	77	4	-
2	71	4	+
3	62	4	-
4	67	5	+
5	76	5	-
6	70	5	+
7	68	6	-
8	68	6	-
9	71	6	-
10	70	6	-
11	58	6	-
12	67	6	+
13	64	6	-
14	57	6	-
15	76	7	+
16	67	7	+
17	66	7	+
18	70	7	-
19	65	8	-
20	68	8	+
21	71	8	-
22	75	9	+
23	70	9	+

For each patient is reported the Gleason value and age. Tr-Kit expression in the tumor tissue, as assessed by Western blot analysis (see also Figure 3), is also reported.

### Cell Culture and Transfection

LNCaP and PC3 prostatic cancer cells were maintained in Dulbecco's minimal essential medium supplemented with 5% fetal bovine serum (Gibco BRL). Hek293 cells were maintained in Dulbecco's minimal essential medium supplemented with 10% fetal bovine serum (Gibco BRL). PC3 cells were transfected with 10  $\mu$ g of pCMV5-tr-Kit using the CaPO<sub>4</sub> precipitation protocol as described previously.<sup>12</sup>

### Extraction of RNA and Protein from Cultured Cells and Primary Tissue

Total RNA was extracted by homogenization of samples in TRIzol reagent (catalog no. 15596-026; Invitrogen, Life Technologies) and by following the manufacturer's instructions. RNA was resuspended in diethyl pyrocarbonate water and immediately frozen at -80°C for further analysis by reverse transcriptase-polymerase chain reaction (RT-PCR). For protein extraction, LNCaP cells, PC3 cells, and tissue fragments were homogenized in homogenization buffer (20 mmol/L HEPES, pH 7.5, 120 mmol/L KCl, 0.1 mmol/L ethyleneglycol-bis( $\beta$ -aminoethyl ether)-*N,N,N',N'*-tetraacetic acid, 10 mmol/L  $\beta$ -glycerophosphate, 10  $\mu$ g/ml leupeptin, 10  $\mu$ g/ml aprotinin, and 2 mmol/L phenylmethyl sulfonyl fluoride). The extracts were centrifuged for 15 minutes at 12,000  $\times g$  at 4°C before collecting the supernatant for Western blot or immunoprecipitation experiments.

### RT-PCR Analysis

RT-PCR was performed using the RT-PCR kit (catalog no. 28025-021; GibcoBRL, Life Technologies), according to the manufacturer's instructions. To synthesize complementary DNA (cDNA), 4  $\mu$ g of total RNA and 750 ng of random primers were used. Oligonucleotides (0.5  $\mu$ mol/L) used for amplification were: i15A: GCAGTGC-CAATGGTCAATGGCAG (from base 79,786 to 79,808 in the 15th intron of the *c-kit* gene); i15B: AAATCCTCTCT-TCCTCACAGGCT (from base 80,097 to 80,119 in the 15th intron of *c-kit*); i16A: CAAGGCTTGGGGTGAAG-CATAGAC (from base 80,606 to 8629 in the 16th intron of *c-kit*); i16B: TCCGTGTGCCTTGGGAGATGTC (from base 80,917 to 80,940 in the 16th intron of *c-kit*); e18: TGCTTTCAGGTGCCATCCACTTCAC (reverse sequence of *c-kit* exon 18th from base 84,687 to 84,723). PCR reactions were performed in 25  $\mu$ l, using 1.5 mmol/L MgCl<sub>2</sub>, 200  $\mu$ mol/L of each dNTP, and 1  $\mu$ l of cDNA reaction. PCR cycles were as follows: 95°C for 5 minutes for denaturation step, followed by 35 cycles of denaturation at 95°C for 30 seconds, annealing at 58°C for 30 seconds, and extension at 72°C for 1 minute.

### DNA Binding Assays

Nuclear extracts (protein concentration ~7 mg/ml) from mouse spermatids, LNCaP and PC3 cells were prepared as previously described.<sup>11</sup> For each reaction, 10  $\mu$ g of nuclear extracts were used in a final volume of 10  $\mu$ l. DNA restriction fragments for electrophoretic mobility shift assays (EMSA) were labeled at the 5' end with <sup>32</sup>P- $\gamma$ -dATP using sequential alkaline phosphatase and T4 polynucleotide kinase treatment. A *Dral* fragment of 200 bp in the mouse tr-Kit promoter within the 16th intron was chosen because it contained the 82-bp region of homology with the human *c-kit* 16th intron and because it was previously shown to have enhancer-like activity for mouse tr-Kit transcription;<sup>10</sup> a *Nde*I-*Sty*I fragment of 200 bp in the 16th intron mouse tr-Kit promoter was chosen because it contained a 19-bp sequence near the tr-Kit mRNA start site that was essential for transcription and because it showed strong homology with a 15-bp sequence present in the human *c-kit* 15th intron near the presumptive start site of human tr-Kit.<sup>5,6</sup>

### Immunoprecipitation Assay

Tissue extracts (500  $\mu$ g of total proteins) prepared as described above were incubated with 1  $\mu$ g of the anti-phosphotyrosine antibody ( $\alpha$ -PY20, Santa Cruz Biotechnology) for 2 hours at 4°C under constant shaking. Protein A-Sepharose or protein G-Sepharose (Sigma-Aldrich) were preadsorbed with 0.05% bovine serum albumin before the incubation with the immunocomplexes and added to the extracts together with the antibody during the last hour of immunoprecipitation. The Sepharose beads were washed three times with homogenization buffer or lysis buffer. Proteins adsorbed to the

antibody-beads complex were eluted in sodium dodecyl sulfate (SDS)-sample buffer for Western blot analysis.

### Immunokinase Assay of Src Activity

Extracts (500  $\mu$ g of total proteins) from PC3 or LNCaP cells were incubated with 1  $\mu$ g of either monoclonal  $\alpha$ -Src antibody (Ab-1; Oncogene Research Products) or non-immune IgGs for 2 hours at 4°C under constant shaking. Immune complexes were collected by adsorption onto protein A-Sepharose (Sigma-Aldrich). The Sepharose beads were washed three times with kinase buffer (50 mmol/L Hepes, pH 7.4, 10 mmol/L MgCl<sub>2</sub>, 10 mmol/L  $\beta$ -glycerophosphate, 1 mmol/L dithiothreitol, 0.5  $\mu$ mol/L Na-orthovanadate, 50  $\mu$ mol/L ATP). Half of the beads were eluted in SDS-sample buffer for Western blot analysis, the other half was used for the enzymatic reaction. The kinase reactions were performed by incubating the beads with 10  $\mu$ g of tr-kit Y161 peptide, in 25  $\mu$ l of kinase buffer also containing 0.1  $\mu$ Ci of <sup>32</sup>P- $\gamma$ -ATP for 30 minutes at 30°C and terminated by spotting the reaction mixture onto squares of P-81 phosphocellulose paper (Whatman) as previously described.<sup>12</sup> Radioactivity incorporated was determined by scintillation counting of the paper squares or by autoradiography of the dried gels.

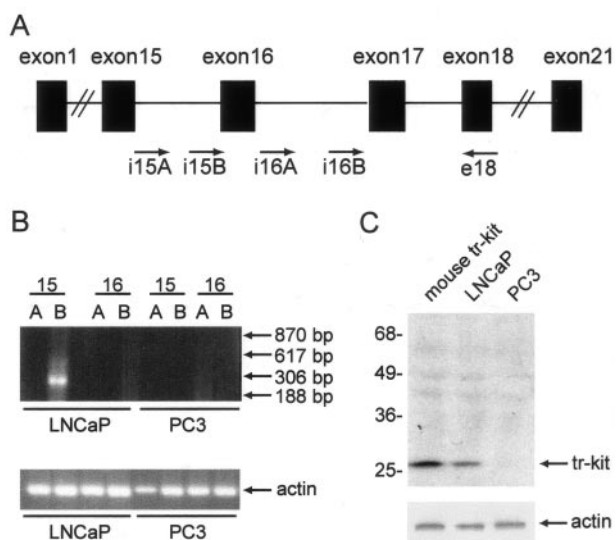
### Western Blot Analysis

Proteins were separated on 10% SDS-polyacrylamide gel electrophoresis gels and transferred to polyvinylidene fluoride Immobilon-P membranes (Millipore) using a semidry blotting apparatus (Bio-Rad). Western blot analysis was performed as previously reported.<sup>13</sup> First antibody (1:1000 dilution) overnight at 4°C: mouse  $\alpha$ -Src (Ab-1, Oncogene Research Products); mouse anti-p416Src (1:50, 05-677; Upstate); rabbit  $\alpha$ -Sam68 (C-20, Santa Cruz Biotechnology); mouse  $\alpha$ -phosphotyrosine (PY20, Santa Cruz Biotechnology); rabbit  $\alpha$ -c-Kit (SC-168, Santa Cruz Biotechnology); goat  $\alpha$ -actin (SC-1616, Santa Cruz Biotechnology). Secondary anti-mouse or anti-rabbit IgGs conjugated to horseradish peroxidase (Amersham) were incubated with the membranes for 1 hour at room temperature at a 1:10,000 dilution in PBS containing 0.1% Tween 20. Immunostained bands were detected by chemiluminescent method (Santa Cruz Biotechnology).

## Results

### Expression of Human tr-Kit in LNCaP Cells

A truncated c-Kit (tr-Kit) transcript and the corresponding protein are expressed in several gastrointestinal and hematopoietic cell lines.<sup>6</sup> To determine whether human tr-Kit is expressed also in prostatic tumors, we first analyzed two well-characterized prostate cancer cell lines: the androgen-sensitive LNCaP and the androgen-insensitive and more undifferentiated PC3. First we undertook a RT-PCR approach using a battery of forward primers spanning introns 15 and 16 of the *c-kit* genomic se-

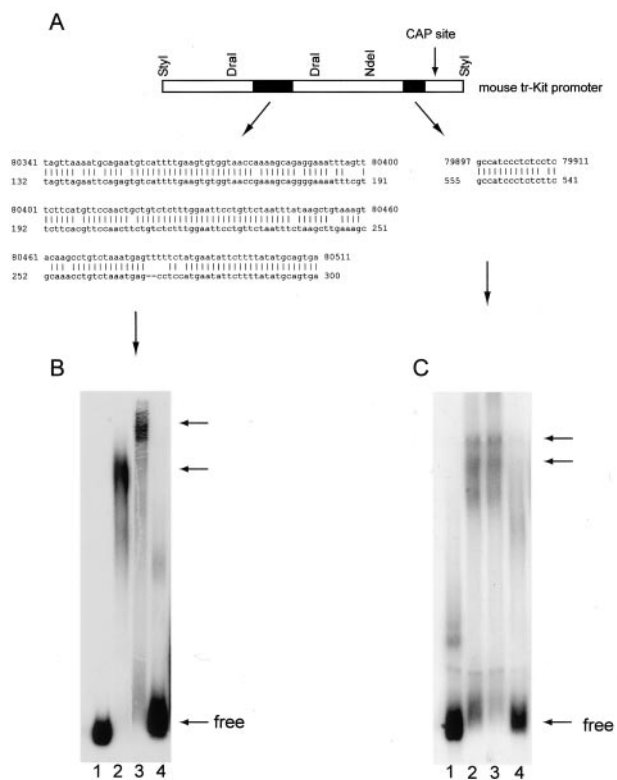


**Figure 1.** Expression of human tr-Kit in LNCaP cells. **A:** Schematic representation of the *c-kit* genomic structure. The approximate position of the oligonucleotides used for RT-PCR analysis of LNCaP and PC3 cells is indicated. **B:** RT-PCR analysis for the expression of human tr-Kit in prostatic cancer cell lines. Oligonucleotides used as forward primers in the PCR reaction were chosen sequences in the 5' (**A**) or 3' (**B**) region of intron 15 and intron 16 of the human *c-kit* gene; as reverse primer was chosen an anti-sense oligonucleotide corresponding to a sequence in the 18th exon of the human *c-kit* gene. **Arrows on the right** show the expected size for the bands amplified by i15A (617 bp), i15B (306 bp), i16A (870 bp), or i16B (188 bp). Shown is a representative experiment that was repeated twice with identical results. **C:** Western blot analysis of cell extracts (40  $\mu$ g) from LNCaP or PC3 cells using either an anti-human c-Kit antibody (**top**) or the anti-actin antibody (**bottom**). A cell extract from Hek293 cells transfected with a mouse tr-Kit expression vector (pCMV5-tr-Kit) was run in the first lane as positive control for the Western blot analysis.

quence, whereas the reverse primer was chosen in exon 18 (Figure 1A). These intron sequences were chosen because the human tr-Kit transcript originates in the 3' region of the 15th intron and is amplified by the i15B oligonucleotide,<sup>5</sup> whereas the mouse tr-Kit transcript begins in the 3' region of the 16th intron<sup>10</sup> and the homologous region in human *c-kit* would be amplified by the i16B oligonucleotide. Furthermore, by using an intronic forward primer we could avoid cross-amplification of full-length c-Kit mRNA, whose expression in some prostate tumors has been reported.<sup>2</sup> As shown in Figure 1B, a band of the correct size (306 bp) was amplified from LNCaP cells only using the primer combination i15B and exon18; primers i15A, corresponding to the 5' region of intron 15, did not amplify any band as well as primers spanning the 16th intron of human *c-kit*. Remarkably, regardless of the primers combination used, no amplification was obtained from the more undifferentiated, androgen-insensitive, PC3 cells. Direct sequencing of the band amplified from LNCaP cells with the i15B-exon18 primer combination indicated that prostatic tr-Kit mRNA was identical to the truncated c-Kit transcript described in Colo1 cells.<sup>5</sup>

Next, we investigated the expression of tr-Kit protein in LNCaP and PC3 cells. In agreement with the RT-PCR data, we found that LNCaP expressed tr-Kit whereas PC3 cells did not (Figure 1C). Human tr-Kit displayed the same apparent molecular weight as recombinant mouse





**Figure 2.** Nuclear factors in LNCaP cells bind to discrete sequences of the mouse tr-Kit promoter. **A:** Schematic structure of the mouse tr-Kit promoter within the 16th intron of *c-kit*. **Black boxes** represent the two regions previously identified as essential for binding of nuclear factors and for promoter activity in mouse spermatids.<sup>11</sup> Above are listed the restriction sites used to isolate these two regions. The homology of these regions with sequences in the human 16th intron (**left**) or 15th intron (**right**) are shown below. Nucleotide coordinates refer to accession number U63834 (human *c-kit* gene genomic sequence, **top lines**) and accession number X65998 (mouse tr-Kit promoter, **bottom lines**). **B:** EMSA experiment using the 200-bp *Dral-Dral* fragment containing the homology with the sequence in the human 16th intron. **C:** EMSA experiment using the 260-bp *NdeI-Syl* fragment containing the homology with the sequence in the human 15th intron. Free-labeled DNA was loaded in **lane 1**; 10  $\mu$ g of nuclear factors from mouse spermatids (**lane 2**), LNCaP cells (**lane 3**), or PC3 cells (**lane 4**) were used for the EMSA. **Arrows** on the **right** show the position of either free DNA or DNA-protein complexes.

tr-Kit, loaded as a control in lane 1 of the SDS-polyacrylamide gel electrophoresis.

### Nuclear Factors Present in LNCaP Recognize the Mouse tr-Kit Promoter

A pairwise BLAST search analysis of the human and mouse *c-kit* genes shows that, beside the obvious homology in the exonic sequences, a strong homology is present in a discrete sequence of the 16th introns (Figure 2A). This region was shown to specifically bind nuclear

factors that recognize a general enhancer element only in mouse spermatids and to be required for maximal activity of mouse tr-Kit promoter during spermiogenesis.<sup>11</sup> No apparent homology in other intronic regions, including the 15th intron of the two genes, was observed. However, a small 15-bp sequence present just upstream of the transcriptional start site in the tr-Kit promoter within the 16th intron of mouse *c-kit*, was found to be present in a reversed orientation, in the presumptive promoter of human tr-Kit within the 15th intron of human *c-kit* (Figure 2A) (ie, between the sequences corresponding to oligonucleotides i15A and i15B). Because we have previously demonstrated that this discrete sequence is essential for transcription of mouse tr-Kit mRNA,<sup>11</sup> we set out to determine whether nuclear factors bind to this sequence in LNCaP cells by EMSA. A 200-bp fragment of the mouse 16th intron centered around this sequence bound nuclear factors from both mouse spermatids and LNCaP cells, but not from PC3 cells that do not express tr-Kit (Figure 2C). Moreover, the migration of the complexes formed by nuclear extracts of spermatids and LNCaP cells was identical, suggesting that similar nuclear factors are present in these cells. In addition, LNCaP cells, but not PC3 cells, expressed nuclear factors that bound also the homologous enhancer-like region shared by the mouse and human *c-kit* genes within the 16th introns (Figure 2B), even though the migration of the complexes was different from that observed with mouse spermatid extracts. Overall, these results suggest that similar genomic elements control the expression of human and mouse tr-Kit and that similar nuclear factors might drive the transcription of tr-Kit mRNA in LNCaP cells and mouse spermatids.

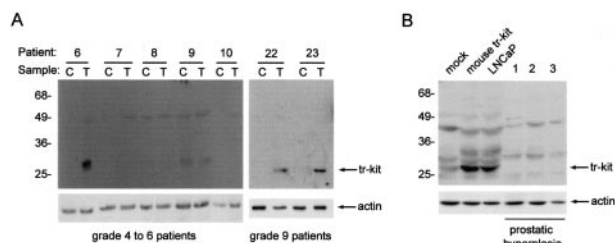
### Expression of Human tr-Kit in Primary Prostate Tumors

Although expressed in cell lines derived from different tumors (gastrointestinal and hematopoietic),<sup>5,6</sup> the expression of human tr-Kit has never been reported in primary tumors. Thus, we tested samples obtained from 26 patients for the expression of human tr-Kit in benign prostatic hyperplasia and prostatic tumors by Western blot analysis (Table 1). Among the 14 patients with low Gleason grade tumors (from 4 to 6), we found that 4 (28%) expressed tr-Kit. Remarkably, when patients with more advanced tumors (Gleason grade from 7 to 9) were examined, we found that 66% (6 of 9) expressed tr-Kit (Table 2). By contrast, none of the three benign prostatic hyperplasia patients examined expressed the truncated form of c-Kit (Table 2 and Figure 3B). For all patients, we examined both the tumor tissue and the contralateral region of prostate, which was not invaded by the tumor.

**Table 2.** Tr-Kit Expression Correlates with Phosphorylation of Src and Sam68 in Prostatic Tumors

Patients	Number	Tr-Kit-positive	Phospho-Src-positive	Phospho-Sam68-positive
BPH	3	0 (0%)	0 (0%)	0 (0%)
Grade 4-6	14	4/14 (28%)	0/6 (0%)	1/6 (16%)
Grade 7-9	9	6/9 (66%)	3/4 (75%)	3/4 (75%)

Sum of the experiments shown in Figure 3, Figure 5, and Figure 6.

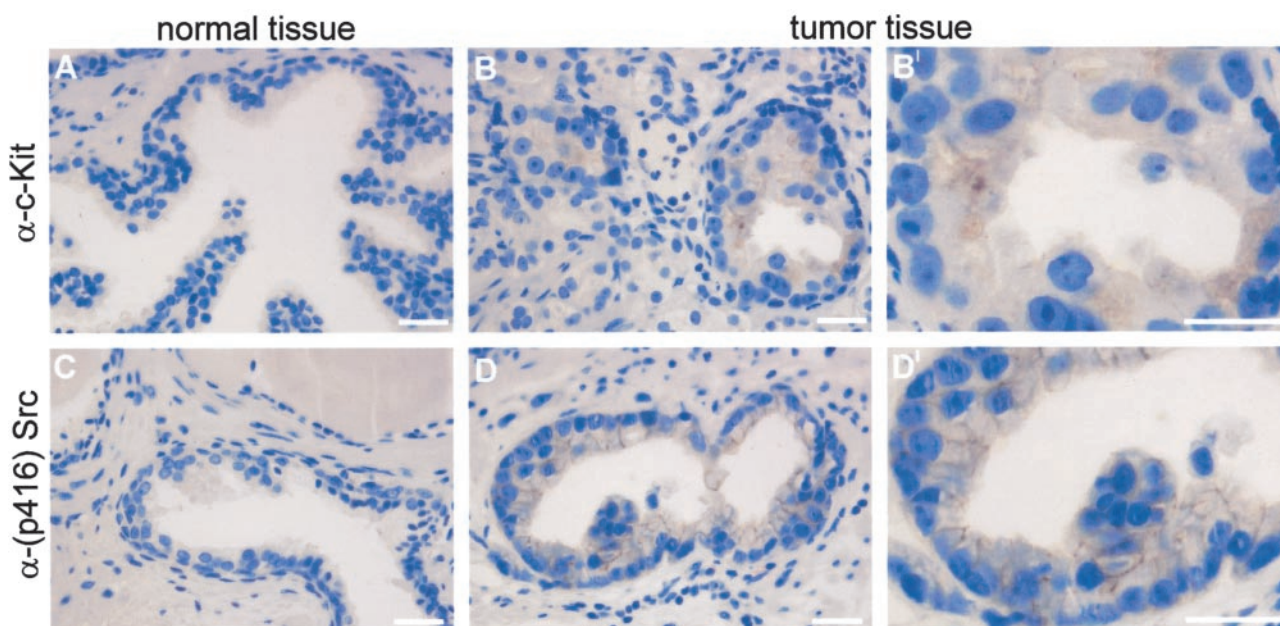


**Figure 3.** Expression of tr-Kit protein in primary prostatic tumors. **A:** Tissues from prostatic tumors (T) or the contralateral, morphologically normal, prostate (C) (see Materials and Methods) from different patients were lysed and analyzed in Western blot using the anti-c-Kit antibody (**top**) or the anti-actin antibody (**bottom**). For each sample were loaded 40  $\mu$ g of total protein. The number of the patient corresponds to that in Table 1; Gleason grades for the patients examined are written below the blot. **B:** Cell extracts (40  $\mu$ g) from mock-transfected and mouse tr-Kit-transfected Hek293 cells, or LNCaP cells, and three benign prostatic hyperplasia patients were analyzed in Western blot using the anti-c-Kit antibody (**top**) or the anti-actin antibody (**bottom**).

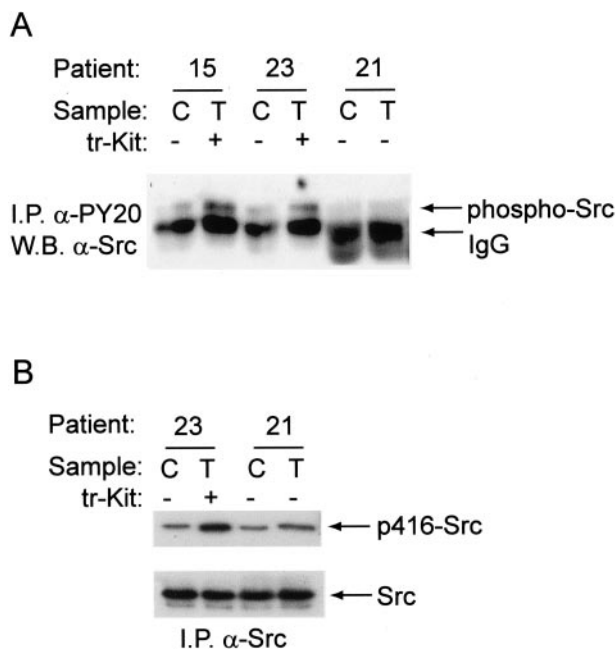
We found that tr-Kit was either not expressed or expressed at lower levels in the contralateral regions of the prostate. Representative examples of Western blot analysis of these samples are shown in Figure 3A. Tr-Kit expression was also confirmed by RT-PCR analysis of available RNA samples, followed by DNA sequencing (data not shown). Immunocytochemistry analysis of patient specimens confirmed that tr-Kit expression was restricted to the cytoplasm of the epithelial cells of neoplastic prostatic glands (Figure 4, B and B'), whereas no staining was observed in the contralateral normal glands (Figure 4A). A representative example of a grade 7 patient is shown in Figure 4 (top). These results suggested that expression of tr-Kit correlates with the more advanced stages of prostatic tumors.

### Activation of Src and Phosphorylation of Sam68 in Prostatic Tumors

We have previously described that overexpression of mouse tr-Kit triggers the activation of Src-like kinases with the consequent phosphorylation of the adaptor protein Sam68 and PLC $\gamma$ 1.<sup>14,16</sup> To test whether activation of this signaling pathway was also a feature of prostatic tumors, we immunoprecipitated tyrosine-phosphorylated proteins from extracts of normal or tumorigenic prostate tissue from 10 patients, and analyzed the immunoprecipitated proteins for the presence of Src or Sam68. We found that among the four patients with advanced prostatic tumors (Gleason grade 7 to 9) examined, three showed an increased level of phosphorylation of Src in the tumor tissue than in the normal tissue (Table 2). Interestingly, the fourth sample was from a patient that did not express tr-Kit (Table 1). A representative Western blot analysis is shown in Figure 5A. To determine whether the phosphorylation of Src was activatory, we immunoprecipitated total Src from normal or tumor prostatic tissues and stained immunoprecipitated proteins with the anti-p416Src antibody, which recognize active Src. The representative example shown in Figure 5B indicates that the signal corresponding to active Src was increased in immunoprecipitates obtained from tr-Kit-expressing tumor prostatic tissues. Furthermore, we observed that the anti-active Src antibody stained the membrane of neoplastic prostatic cells from tr-Kit-expressing tumors (representative sample shown in Figure 4, D and D') whereas it did not stain the epithelial cells of the contralateral prostatic gland (Figure 4C). Interestingly, patients that expressed tr-Kit also showed tyrosine phosphorylation of Sam68



**Figure 4.** Immunohistochemical analysis of prostate tumors. The analysis of a representative grade 7 sample is shown. Sections obtained from the tumor (**B**, **B'**, **D**, and **D'**) or the contralateral, morphologically normal, side of the gland (**A** and **C**) were stained using either the anti-c-Kit antibody (**A**, **B**, and **B'**) or anti-p416Src antibody (**C**, **D**, and **D'**). **B'** and **D'** are enlargements of the neoplastic glands shown in **B** and **D** that allow to better appreciate the cytoplasmic staining obtained with the c-Kit antibody and the membrane staining obtained with the anti-p416Src antibody. Scale bars, 100  $\mu$ m.

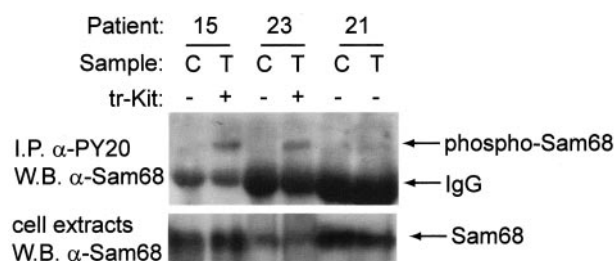


**Figure 5.** Phosphorylation of Src in tr-Kit-expressing prostatic tumors. Cell extracts (500 μg) from prostatic tumors (T) or contralateral prostate (C) were immunoprecipitated for 2 hours using 1 μg of either the anti-phosphotyrosine (α-PY20) antibody (A) or the anti-Src antibody (B). Immunoprecipitated proteins were separated on SDS-polyacrylamide gel electrophoresis and analyzed in Western blot for the presence of Src (A) or active Src (phosphorylated on tyrosine 416, in B). The patient number (as in Table 1) and the positivity for tr-Kit expression are listed above. The Gleason grade of the patients examined was between 7 and 9 (see Table 1).

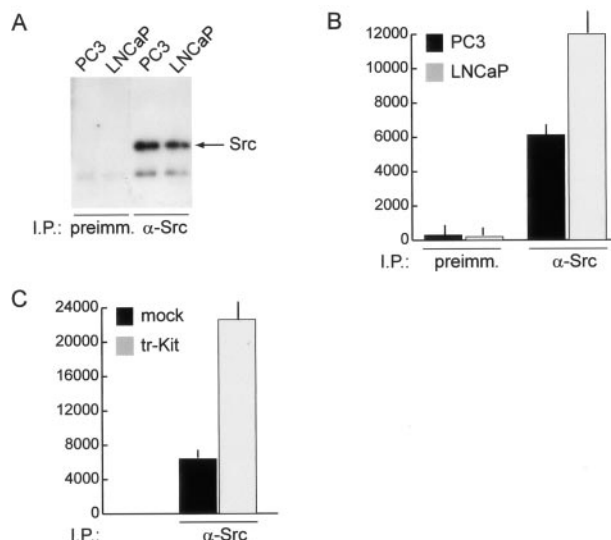
(Figure 6; Table 2), further indicating that Src was activated in these samples. On the other hand, only one among the six patients with low Gleason grade<sup>4-6</sup> examined displayed phosphorylation of Sam68, but not of Src; this patient also expressed tr-Kit (no. 2 in Table 1). Although we found that the expression levels of Sam68 varied from patient to patient, no correlation was found between abundance and phosphorylation of the protein (Figure 6).

### Activation of Src by tr-Kit in PC3 Cells

To test whether expression of tr-Kit in prostate cells correlates with the activation of Src, we performed a kinase assay on α-Src immunoprecipitates from PC3 cells, which do not express tr-Kit, and LNCaP cells, which express tr-Kit. We found that Src was more active in



**Figure 6.** Phosphorylation of Sam68 in tr-Kit-expressing prostatic tumors. Samples were processed as described in Figure 5A and the presence of Sam68 among the tyrosine-phosphorylated proteins immunoprecipitated was determined by Western blot analysis.



**Figure 7.** Assay of Src activity in LNCaP and PC3 cells. Cell extracts (500 μg) from PC3 and LNCaP cells were immunoprecipitated with 1 μg of either preimmune IgGs or α-Src IgGs for 2 hours at 4°C. After washes, samples were divided in two aliquots and either analyzed in Western blot to test the immunoprecipitation of Src (A) or assayed *in vitro* for kinase activity (B) using a specific peptide.<sup>13</sup> C: PC3 were either mock- or tr-Kit-transfected and Src activity was measured by an immunokinase assay as described above. Results in B and C are the mean ± SD of three experiments.

LNCaP than in PC3 cells (Figure 7B), although similar amounts of the kinase were immunoprecipitated from both cell extracts (Figure 7A), indicating a correlation between tr-Kit expression and Src activity. In agreement with this hypothesis, we found that expression of tr-Kit in PC3 cells induced a dramatic increase in Src activity, as measured by immunokinase assay (Figure 7C). Because similar amounts of Src were immunoprecipitated in both samples (data not shown), this result indicates that expression of tr-Kit is sufficient to trigger Src activation in prostatic cancer cells.

### Discussion

Prostate cancer develops as an androgen-dependent hyperproliferation of prostatic gland epithelial cells and it is initially treated with an anti-androgen therapy. However, after initial remission, it often evolves into a highly aggressive and metastatic androgen-independent cancer, for which a successful therapy has not yet been established.<sup>20</sup> Little is known about the molecular mechanisms that render prostatic cancer insensitive to androgen deprivation: amplification of the androgen receptor gene and/or mutations that sensitize the receptor to other steroids are among the possible causes; however, activation of pathways that stimulate prostate cell proliferation even in the absence of androgens have also been proposed.<sup>20</sup> Among these alternative routes, activation of soluble tyrosine kinases of the Src family might play an important role: functional interaction between Src and the estradiol receptor triggers prostate cell proliferation;<sup>17</sup> activation of Src is also required for their neuropeptide-induced androgen independence and for their metastatic potential.<sup>18,21</sup> DOC-2/DAB2, a potent tumor suppressor



in prostate cancer, interacts with and inhibits Src.<sup>22</sup> However, activation of this pathway in primary prostatic tumors was never tested directly.

Our laboratory has recently described a novel strong activator of Src-kinase pathway: tr-Kit.<sup>13,16</sup> Because a homologous truncated c-Kit protein has been detected in several human cancer cell lines,<sup>5,6</sup> and given the role played by Src-kinases in prostate cancer cell lines, we set out to determine whether human tr-Kit could also be expressed in this neoplasia. Here we report that expression of human tr-Kit is frequently observed in prostatic tumors, suggesting that activation of the Src-pathway could also play a role in primary tumors. Two observations support the hypothesis that tr-Kit might be involved in prostatic cancer: in most patients examined, tr-Kit was detected only in specimens from the neoplastic lesions and not in the contralateral tissues, in which tumor formation was not morphologically diagnosed; and tr-Kit expression was more frequent in patients with an advanced stage of the tumor (66% in patients with Gleason grade from 7 to 9) than in those with less advanced stages (28% in patients with Gleason grade from 4 to 6). Thus, our results indicate that tr-Kit expression correlates with prostatic cancer progression.

Although activation of Src has been positively correlated with both cell proliferation and invasiveness of several prostate cancer cell lines,<sup>17,18,21</sup> no data are currently available on the activation of Src in primary prostate tumors. Herein we have investigated activation of Src by assaying the level of tyrosine phosphorylation of the kinase, which is directly correlated to its activity status.<sup>13,18,23</sup> Our results show that Src was activated in 75% (three of four) of the more advanced tumors and in none of the six less advanced tumors tested. Moreover, both tr-Kit and active Src were only observed in the epithelial cells of neoplastic prostate glands by immunohistochemistry. Remarkably, all samples positive for Src phosphorylation also expressed tr-Kit, whereas the only grade 7 to 9 tumor that did not display phosphorylated Src did not express tr-Kit either. These data, although based on a limited number of patients, show a high correlation between the two events and demonstrate for the first time the presence of phosphorylated/activated Src in prostatic tumor tissue. Interestingly, we observed that the activity of Src was higher in tr-Kit-expressing LNCaP cells than in tr-Kit-negative PC3, and that transfection of tr-Kit in PC3 cells dramatically stimulated Src activity. These results further support the correlation between tr-Kit expression and Src activation in prostate cells.

We have previously demonstrated that tr-Kit stimulates Src-kinase activity *in vivo*, as monitored by the phosphorylation status of Src-kinase substrates such as PLC $\gamma$ 1<sup>13,14</sup> and Sam68.<sup>13,16</sup> Tr-Kit stimulates Src activity by interacting with the SH2 domain of the kinase and displacing the intramolecular interaction of this domain with phosphotyrosine 530 in the carboxyterminus of Src.<sup>13,23</sup> Remarkably, mutation and/or deletion of this carboxyterminal phosphotyrosine residue in Src kinases have been detected in human colon cancers.<sup>15</sup> On the other hand, the strong tumor suppressor DOC-2/DAB2

directly interacts with the SH3 domain of Src and inhibits the activity of the kinase and prostate cell proliferation.<sup>22</sup> Thus, taken together these observations suggest that shifting the balance of Src activity toward hyperactive Src may contribute to the advancement of prostate cancer. Whether or not expression of tr-Kit and/or activation of Src is part of the mechanism leading to development of androgen insensitivity in prostate cancer cells remains to be studied. The recent finding that the anti-apoptotic action of androgens in several cell lines is mediated by activation of the Src pathway and independent of the transcriptional activity of the androgen receptor<sup>24</sup> suggests that alternative routes activating the Src pathway might be capable of functioning independently of androgen receptor activity in target cells.

The best substrate found to be phosphorylated by oncogenic variants of Src-like kinases in transformed cells is the RNA-binding protein Sam68 (substrate of Src in mitosis, 68 kd).<sup>25</sup> Sam68 belongs to a class of RNA-binding proteins (STAR, signal transduction and RNA metabolism) that appear to link activation of signal transduction pathways to translational regulation of target mRNAs.<sup>26</sup> Indeed, STAR proteins act as translational repressors and phosphorylation modifies their subcellular localization and RNA affinity.<sup>26</sup> In particular, Sam68 was shown to play a scaffold role in Src kinase-activated pathways,<sup>16,27</sup> and tyrosine phosphorylation of Sam68 by Src kinases triggers the release of bound RNA and might allow translational activation.<sup>28</sup> We have previously shown that activation of Src-like kinases by tr-Kit is required for the efficient localization of Sam68 in the nucleus and that Sam68 acts as a scaffold protein in the tr-Kit-induced signal transduction pathway.<sup>16</sup> A role of Sam68 in the control of cell proliferation was suggested by the transformed phenotype acquired by fibroblast in which the *sam68* gene was ablated.<sup>29</sup> More recently, Sam68 regulation by the Src-related kinase BRK-Sik in prostate cancer development has been hypothesized. The authors suggested that nonregulated phosphorylation of Sam68 might lead to nonregulated release and translation of mRNAs altering the control of cell cycle progression of prostate cells.<sup>19</sup> In agreement with their hypothesis, here we find that tyrosine phosphorylated Sam68 was only immunoprecipitated from tumor tissues derived from the more advanced stages patients (Gleason grade 7 to 9). Remarkably, all these patients also expressed tr-Kit and phosphorylated Src, and Sam68 was not phosphorylated in the contralateral tissue used as internal control. To our knowledge, this is the first report of phosphorylation of Sam68 in primary tumors.

The strong homology found in the 16th introns of human and mouse *c-kit* and between the proximal promoter sequences of human tr-Kit (in the 15th intron) and mouse tr-Kit (in the 16th intron) suggest a conserved functional role for these intronic sequences for tr-Kit transcription. Our work also indicates that nuclear factors expressed in LNCaP are capable to bind to these sequences of the mouse tr-Kit promoter. Interestingly, these factors are not expressed in PC3 cells, which do not express tr-Kit protein either. Because we have found a positive correlation between tr-Kit expression and tumor progression, it is



possible that the aberrant expression of nuclear factors able to bind to the human tr-Kit promoter is an early event in prostate cell transformation. Identification of these nuclear factors, which could be shared by spermatogenic cells, may hence be important to identify novel markers of prostate tumor progression. The observation that the more undifferentiated and aggressive PC3 cells do not express tr-Kit, nor the nuclear factors that bind tr-Kit promoter, might be explained by the fact that PC3 cells are not representative of either normal or transformed prostatic cells.<sup>30</sup>

In conclusion, our study demonstrates that a truncated form of c-Kit, previously shown to activate Src-like kinases,<sup>13,16</sup> is frequently expressed in prostate cancer and that its expression correlates with Src activation and phosphorylation of its substrate Sam68. Thus, our work suggests that activation of this pathway might contribute to the transformation of prostate cells. In this regard, it will be interesting to identify the mRNA molecules bound to Sam68 in prostate cells and follow the expression of the corresponding protein during prostate tumor progression.

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