The Src tyrosine kinase Hck is required for Tel-Abl- but not for Tel-Jak2-induced cell transformation

C. Pecquet, R Nyga, V Penard-Lacronique, T Smithgall, H Murakami, A. Regnier, K Lassoued, F. Gouilleux

Oncogene (2007) 26, 1577–1585

www.nature.com/onc

ORIGINAL ARTICLE

The Src tyrosine kinase Hck is required for Tel-Abl- but not for Tel-Jak2-induced cell transformation

C Pecquet[1,5], R Nyga[1,5], V Penard-Lacronique[2], TE Smithgall[3], H Murakami[4], A Régnier[1], K Lassoued[1] and F Gouilleux[1]

[1]*INSERM, E351, 3 rue des Louvels, Université de Picardie Jules Verne, Amiens France;* [2]*INSERM E210, Hôpital Necker-Enfants Malades, 149 rue de Sèvres, Paris, France;* [3]*Department of Molecular Genetics and Biochemistry, University of Pittsburgh School of Medicine, Pittsburgh, PA, USA and* [4]*Department of Biotechnology, Okayama University, Tsushima-Naka, Okayama, Japan*

Tel-Abl and Tel-Jak2 are fusion proteins associated with human haematologic neoplasms. They possess constitutive tyrosine kinase activity and activate common downstream signalling pathways like Stat-5, PI3-K/Akt, Ras/MapK and NF-κB. In this study, we showed the specific requirement of Src family members for the Tel-Abl-mediated cell growth, activation of Stat5, PI3-K/Akt and Ras/MapK while dispensable for Tel-Jak2. Hck was found strongly phosphorylated in Tel-Abl-expressing Ba/F3 cells and sensitive to imatinib mesylate treatment, providing evidence that Hck is a target of Tel-Abl tyrosine kinase activity. Overexpression of a kinase dead form of Hck inhibits the proliferation of Ba/F3 cells expressing Tel-Abl as the phosphorylation of Akt and Erk1/2. These results argue for an important role of Hck in Tel-Abl oncogenic signalling.
Oncogene (2007) **26,** 1577–1585. doi:10.1038/sj.onc.1209949; published online 4 September 2006

Keywords: Tel-Abl; Tel-Jak2; Src kinases; Hck; signal transduction; oncogenes

Introduction

Series of fusion proteins involving Tel, a member of the Ets transcription factor family, and receptor or non-receptor tyrosine kinases have been associated to human leukaemias (Golub *et al*., 1994; Papadopoulos *et al*., 1995; Lacronique *et al*., 1997; Knezevich *et al*., 1998; Cazzaniga *et al*., 1999; Kuno *et al*., 2001). The Tel-Jak2 and Tel-Abl fusions have been reported in T- and pre-B cell acute lymphoblastic leukaemias (ALL) (Papadopoulos *et al*., 1995; Lacronique *et al*., 1997; Peeters *et al*., 1997; Van Limbergen *et al*., 2001) and in various chronic myelogenous leukaemias (CML) (Peeters *et al*., 1997; Van Limbergen *et al*., 2001; Keung *et al*., 2002). These proteins exhibit a constitutive kinase activity, are constitutively tyrosine phosphorylated *in vivo* and harbour transforming capacities in haematopoietic cell lines and mouse models (Golub *et al*., 1996; Lacronique *et al*., 2000). Bone marrow transplantations of Tel-Abl- and Tel-Jak2-transduced cells into irradiated mice induce the development of a CML-like leukaemia (Million *et al*., 2002) and a rapid and fatal myelo- and lymphoproliferative disease respectively (Schwaller *et al*., 1998). Transgenic mouse models have also illustrated the leukaemogenic properties of Tel-Jak2 in the B- and T- cell lineages (Carron *et al*., 2000; Dos Santos and Ghysdael, 2005). Expression of Tel-Abl and Tel-Jak2 in haematopoietic cells results in the constitutive activation of effectors and/or pathways associated to cellular growth and survival, in particular the two adapters Gab2 and Shc, the transcription factors Stat3 and Stat5 and effectors of the NF-κB, PI3-K/Akt and Ras/Erk1/2 pathways (Voss *et al*., 2000; Santos *et al*., 2001; Ho *et al*., 2002; Spiekermann *et al*., 2002; Malinge *et al*., 2006). Tel-Jak2 also activates effectors of the SAPK/JNK and p38 pathways (Ho *et al*., 2002). Activation of the PI3-K/Akt and Ras/Erk1/2 signalling is partly mediated by the binding of the Grb2-Sos complex to a phosphorylated tyrosine residue (Y^{314}) present in the Tel moiety of the fusions. Following Grb2 binding, recruitment and activation of Gab2 by the oncogenes allows in turn the recruitment of the PI3-K kinase and SHP-2 phosphatase, leading to the activation of their downstream effectors Akt and Erk1/2. A Tel-Abl mutant lacking the Y^{314} residue exhibited decreased Gab2 activation, impaired Akt and Erk1/2 phosphorylation and attenuated the induction of a CML-like disease in mice (Million *et al*., 2004). Introduction of a similar substitution in the Tel-Jak2 protein fails to fully abolish the activation of the Ras/MapK pathway, arguing for alternative mechanisms of activation. In keeping, binding sites for Shc and Ship1 have been identified in Tel-Jak2 and expression of Tel-Jak2 in Ba/F3 cells results in the constitutive phosphorylation of Shc and SHP-1, recruitment of Grb2 to Tel-Jak2 and consequently activation of the Ras/MapK pathway (Ho *et al*., 2002).

It remains unclear whether Tel-Jak2 and Tel-Abl fusions may activate other nonreceptor tyrosine kinases

Correspondence: Dr F Gouilleux, INSERM, E351, 3 rue des Louvels, Université de Picardie Jules Verne, 80036 Amiens, France.
E-Mail: fabrice.gouilleux@sa.u-picardie.fr
[5]These two authors contributed equally to the work.
Received 6 January 2006; revised 17 July 2006; accepted 25 July 2006; published online 4 September 2006

that may participate to their leukaemogenic activity. The CML-associated oncoprotein Bcr-Abl, activates members of Fps/Fes and Src families (Ernst *et al*., 1994; Danhauser-Riedl *et al*., 1996) and part of its oncogenic activity in haematopoietic cells is mediated by Src kinases (Lionberger *et al*., 2000; Klejman *et al*., 2002; Hu *et al*., 2004), In particular, Hck is responsible for the phosphorylation of a critical Y^{177} residue present in the fusion and associated to the recruitment of the Grb2-Sos complex and further induction of the Ras/MapK pathway (Warmuth *et al*., 1997). We therefore investigated whether Src tyrosine kinases may be required for the Tel-Abl- and Tel-Jak2-mediated transforming properties. By using the Src inhibitors PP1 and PP2, we demonstrated herein that inhibition of Src activities completely suppressed Tel-Abl proliferative signals in the IL-3-dependent lymphoid Ba/F3 cells but had no effect on Tel-Jak2 signalling. We showed that Hck is heavily phosphorylated in Tel-Abl-expressing Ba/F3 cells on the Y^{411} residue known to play a critical role in Hck activity. Inhibition of Tel-Abl tyrosine kinase activity by imatinib mesylate/STI 571 blocks the phosphorylation of Hck, suggesting that this Src kinase is a downstream effector of Tel-Abl activity. We also found that expression of a kinase inactive Hck mutant inhibits the cellular growth and the phosphorylation of Akt and Erk1/2. These results indicate that Hck is involved in the Tel-Abl-mediated oncogenic signalling but dispensable for the transforming properties of Tel-Jak2.

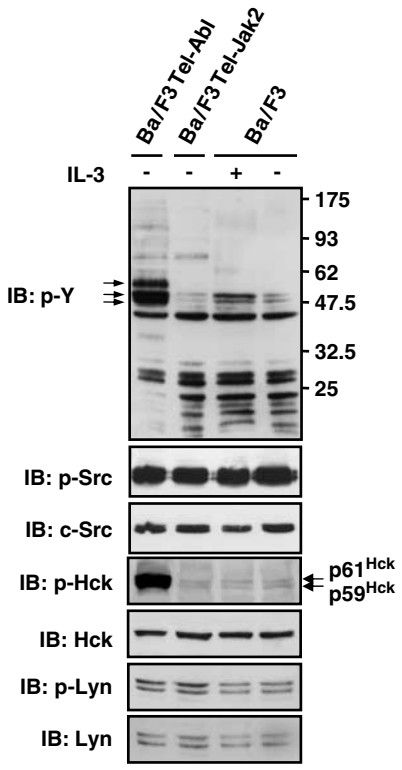

Figure 1 The Src family member Hck is strongly phosphorylated in Tel-Abl-expressing cells. Extracts from Tel-Abl- Tel-Jak2-expressing Ba/F3 cells and parental Ba/F3 cells stimulated or not with IL-3 were prepared and analysed by Western blot with an antiphospho-tyrosine (4G10) antibody. Lysates were also analysed by immunoblot with antiphospho-Src, antiphospho-Hck and antiphospho-Lyn antibodies (**b**). Membrane was reprobed with anti-c-Src, anti-Hck and anti-Lyn antibodies.

Results

The Src kinase Hck is (hyper)phosphorylated in Tel-Abl-expressing cells

To evaluate the involvement of the Src kinases in the transforming properties of Tel-Abl and Tel-Jak2 fusion proteins, we first analysed the levels of Src kinase activities in Ba/F3 cells stably expressing the two oncoproteins (Lacronique *et al*., 2000). We first compared patterns of phosphotyrosine proteins in these transformed cells and in parental Ba/F3 cells by Western blot using an antiphosphotyrosine antibody (4G10) (Figure 1). Prominent accumulations of phosphotyrosine proteins, whose molecular weights ranged from 50 to 60 kDa, were evidenced in Tel-Abl-expressing cell lysates. Among the Src kinase family members, Hck and Lyn are specifically associated with the leukaemogenic activity of Bcr-Abl and constitutively activated in haematopoietic cells transformed by this oncoprotein (Lionberger *et al*., 2000; Klejman *et al*., 2002; Hu *et al*., 2004). To analyse whether Hck and Lyn activities may be affected in Tel-Abl-expressing Ba/F3 cells compared to parental or Tel-Jak2-expressing cells, cell lysates were incubated with antiphospho-Hck, antiphospho-Lyn and antiphospho-Src antibodies, which recognize the active forms of these kinases. Results clearly showed that Hck (p61Hck and p59Hck) is strongly phosphorylated in Tel-Abl- but not in Tel-Jak2-expressing cells. In contrast, the phosphorylated state of Lyn and c-Src was found similar in all samples, suggesting that Lyn and c-Src activities are not affected by the expression of Tel-Abl.

The Src kinase inhibitors PP1 and PP2 suppress the growth and survival of Ba/F3 cells transformed by Tel-Abl but have no effect on Tel-Jak2-expressing cells

To evaluate the role of Hck, Ba/F3 cells expressing Tel-Abl or Tel-Jak2 were treated with two concentrations of the pyrazolo-pyrimidine Src kinase inhibitors PP1 and PP2. PP2 was initially described as a potent inhibitor of $p56^{Lck}$, $p59^{Fyn}$ and $p59/61^{Hck}$ while PP1 was reported to be selective for Fyn and Lck over other members of the Src kinases family (Hanke *et al*., 1996). Incubation with PP2 and, to a lesser extent, with PP1 resulted in a dose-dependent inhibition of Tel-Abl-expressing cells growth while Ba/F3 cells expressing Tel-Jak2 remained unaffected (Figure 2a). As a control, treatment of the two cell lines with PP3, an inactive analogue of PP2, showed no effect on cellular proliferation. Cells treated with PP2 were then stained by AnnexinV/propidium iodide (PI) to estimate the percentage of apoptosis in these cultures (Figure 2b). While almost negligible on

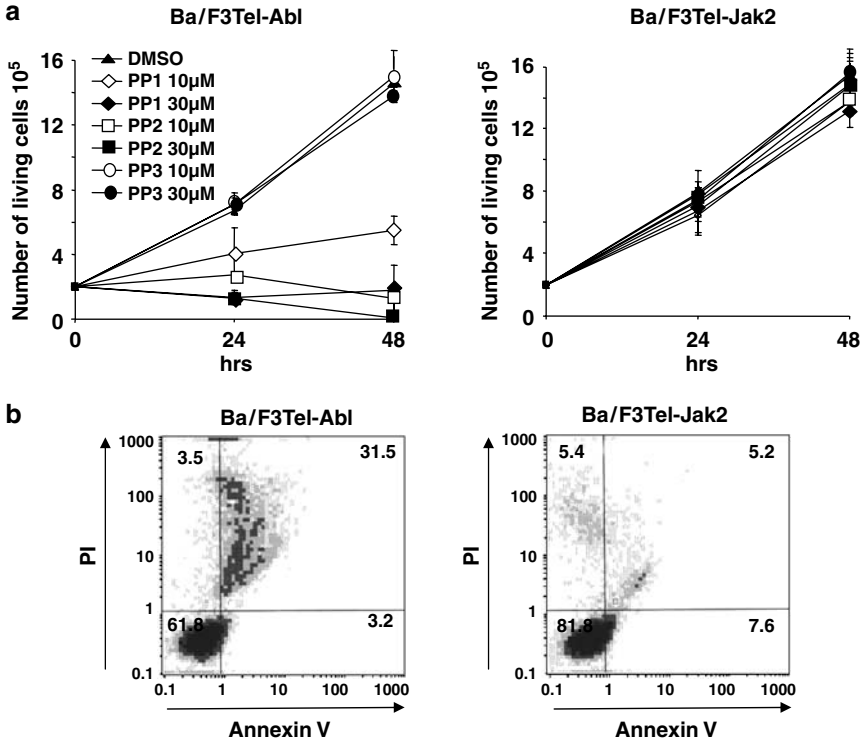

Figure 2 The Src inhibitors PP1 and PP2 abrogate the growth and induce apoptosis of Tel-Abl-expressing cells. (**a**) Tel-Abl- and Tel-Jak2-expressing Ba/F3 cells were treated with the indicated concentrations of PP1, PP2, PP3 and DMSO for 48 h and the number of viable cells was determined daily using the Trypan Blue dye exclusion method. Results shown are representative of three experiments. (**b**) Tel-Jak2- and Tel-Abl-expressing cells were treated for 48 h with 10 μM of PP2 and then labelled with AnnexinV-PE and PI. The percentage of double-positive cells was determined by flow cytometry.

Tel-Jak2-expressing cells viability, PP2 treatment was shown to induce a strong increase in apoptotic cells number in the Tel-Abl-Ba/F3 cell line 24 h after addition of the compound. In addition, we also observed that PP2 treatment induced a G1 arrest in the cell cycle (data not shown). Taken together, these data reveal the requirement of Src kinase activity for the Tel-Abl-mediated growth signaling.

PP2 inhibits Stat5, Erk1/2 and Akt activation in Tel-Abl-expressing cells

We next examined the contribution of Src kinases activities on the phosphorylated state of some effectors of growth and survival pathways like Stat-5, Akt and Erk1/2 (Supplementary Figure 1). As shown in Figure 3a, Tel-Abl and Tel-Jak2 induce the phosphorylation of these proteins when compared to IL-3-starved Ba/F3 cells. These data are consistent with previously published works (Lacronique *et al*, 2000; Santos *et al*., 2001; Spiekermann *et al*., 2002). Treatment of Tel-Abl-expressing Ba/F3 cells with 10 μM of PP2 and, to a lesser extent with PP1, induced a strong decrease in the accumulation of phosphorylated Stat5, Akt and Erk1/2 species, while not in the Tel-Jak2 Ba/F3 cellular context (Figure 3b). Reprobing the membranes with antibodies raised against these proteins confirmed the absence of PP1 and PP2 impacts on protein expressions.

The Src kinase inhibitors PP2 and SU6656 inhibit Tel-Abl activity

The inhibitory effect of PP1 and PP2 on Src and Hck phosphorylation was confirmed in the two cell lines by Western blot using antiphospho-Src and antiphospho-Hck antibodies (Figure 4a). We also determined the effect of PP2 on Tel-Jak2 and Tel-Abl activities by Western blot analysis with anti P-Y^{1007}-Jak2 and anti P-Y^{245}-Abl, two tyrosine residues critical for the activity of both kinases and observed that PP2 inhibits Tel-Abl but not Tel-Jak2 phosphorylation. We also used the Src kinase inhibitor SU6656 which was previously shown to be more specific (Blake *et al*., 2000). Treatment of Tel-Abl-expressing Ba/F3 cells with SU6656 induced growth arrest (Figure 4b) and inhibited the phosphorylation of Tel-Abl, Hck and c-Src kinases (Figure 4c) thereby mimicking the effects of PP2.

Imatinib mesylate suppresses Hck, Stat5, Akt, Erk1/2 phosphorylations

As the Src inhibitors PP2 and SU6656 affected the phosphorylation of Tel-Abl, we therefore determined

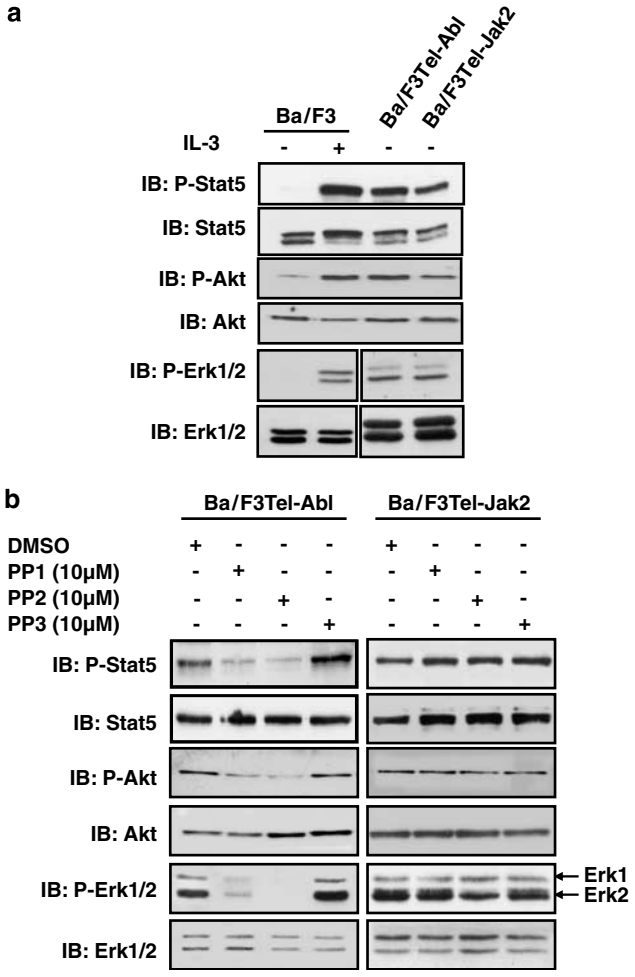

Figure 3 PP2 inhibits Tel-Abl but not Tel-Jak2 oncogenic signalling. (**a**) Extracts from Tel-Abl-, Tel-Jak2-expressing Ba/F3 cells and parental Ba/F3 cells stimulated or not with IL-3 were prepared and analysed by Western blot with the indicated antibodies. (**b**) Tel-Abl- and Tel-Jak2-expressing cells were incubated with 10 μM of PP1, PP2, PP3 or DMSO for 24 h. Lysates were subjected to SDS–PAGE and then immunoblotted with the indicated antibodies.

whether Tel-Abl targets Hck by using the Abl kinase inhibitor imatinib mesylate (STI-571, Gleevec) (Schindler *et al*., 2000). Effects of imatinib mesylate on the Tel-Abl activity based on Western blot and biological analysis were determined (Figure 5). A complete inhibition of cell growth was already observed with 1 μM of inhibitor, a concentration needed to inhibit Tel-Abl activity (Figure 5a and b). Inhibition of Hck was also observed after treatment with 1 μM of inhibitor indicating that phosphorylation of Hck is dependent on the tyrosine kinase activity of Tel-Abl (Figure 5b). As expected, the phosphorylations of Stat5, Akt and Erk1/2 were completely suppressed after imatinib mesylate treatment. To determine whether Tel-Abl interacts with Hck in Ba/F3 cells, Tel-Abl was immunoprecipitated from Tel-Abl-expressing cell lysates and the presence of Hck was assessed by immunoblotting with antiphosphotyrosine (4G10) and anti-Hck antibodies. As shown Figure 5c, Hck was found associated with Tel-Abl in Ba/F3 cells. Collectively, these data indicate that Hck is a downstream signalling effector of Tel-Abl.

Hck is required for Tel-Abl-induced cell growth

To evaluate the role of Hck in Tel-Abl-induced mitogenic and antiapoptotic signals, we transiently expressed a Flag-tagged kinase inactive form of Hck (Hck K269E, Hck KE), its wild-type counterpart (Hck WT) or the empty vector (mock) and we measured proliferation rates and viability of the cells (Figure 6). We used a bicistronic expression vector allowing the coexpression of both the kinase and the hrGFP indicator (pIREShrGFP) to sort by flow cytometry and analyse 24 h post-transfection the behaviour of green fluorescent protein (GFP)-expressing cells only. In these experiments, a Flag-tagged kinase inactive form of Lyn (Lyn K275R, Lyn KR) and the WT kinase (Lyn WT) were used as controls. Rates of growth of GFP-positive cells were determined in a time course experiment using Trypan Blue dye exclusion method. As shown Figure 6, expression of the Hck KE mutant but not of Hck WT or Lyn KR induced the Tel-Abl Ba/F3 cells growth inhibition, while Tel-Jak2-expressing cells remained insensitive. These data indicate that Hck but not Lyn activity is required for Tel-Abl-induced cell growth.

Hck is required for Tel-Abl-induced Akt and Erk1/2 phosphorylation

The phosphorylation states of Akt, Stat5 and Erk1/2 were assessed by Western blot in lysates of Tel-Abl- and Tel-Jak2-expressing cells transiently transfected with the above Hck expression vectors (Figure 7). Interestingly, we observed the disappearance of phosphorylated Akt and Erk1/2 proteins while not of phosphotyrosine Stat-5 suggesting that Hck is required in the Tel-Abl-induced activation of PI3-K/Akt and the Ras/Erks pathways.

Discussion

The Src family of tyrosine kinases consists of eight members (Src, Lyn, Lck, Fyn, Yes, Hck, Blk, Fgr) implicated in a wide variety of intracellular signalling pathways associated with cell growth, differentiation, cell shape, migration and survival as in development of cancer (Tatosyan and Mizenina, 2000). A number of human primary tumours and tumour-derived cell lines including leukaemias possess elevated Src kinase activities (Rosen *et al*., 1986; Ottenhoff-Kalff *et al*., 1992; Talamonti *et al*., 1993; Mao *et al*., 1997; Donato *et al*., 2003). For instance, Lyn and Hck have been implicated in the oncogenic activity of Bcr-Abl in haematopoietic cells (Danhauser-Riedl *et al*., 1996; Hu, Nature genetics, 2004) and with the use of dominant-negative src mutants or pharmacological inhibitors have confirmed that these two kinases contribute to the Bcr-Abl-induced

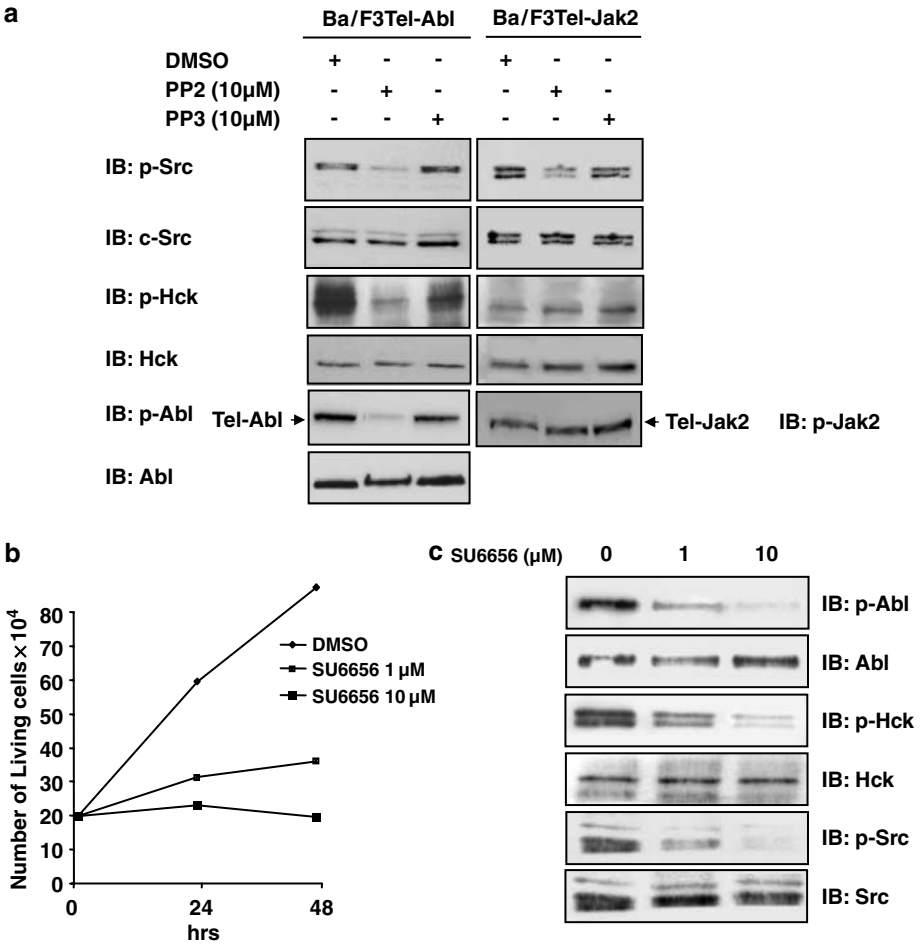

Figure 4 The Src kinase inhibitors PP2 and SU6656 inhibit Hck and Tel-Abl phosphorylations. (**a**) Tel-Abl- and Tel-Jak2-expressing cells were treated with the indicated concentrations of PP1, PP2, PP3 and DMSO for 24 h. Lysates were subjected to SDS–PAGE and immunoblotted with antiphospho-Src and antiphospho-Hck antibodies. Phosphorylation of Tel-Jak2 and Tel-Abl was also evaluated with antiphospho-Y^{1007}Jak2 and antiphospho-Y^{245}-Abl antibodies. Membranes were reprobed with anti-c-Src and anti-Hck and anti-Abl antibodies. (**b**) Tel-Abl-expressing cells were also treated with the indicated concentrations of SU6656 for 48 h and viable cells were enumerated daily using the Trypan Blue dye exclusion assay. (**c**) Extracts from Tel-Abl-expressing cells treated or not with the indicated concentrations of SU6656 were analysed by Western blot with the indicated antibodies.

mitogenic and antiapoptotic signals (Lionberger *et al*., 2000; Wilson *et al*., 2002). Here, we showed that Src kinases, and primarily Hck, is also a target of the oncogenic Tel-Abl fusion protein. Hck was found strongly phosphorylated on Y^{411} residue in Tel-Abl-expressing Ba/F3 cells and treatment of the cells with imatinib mesylate, which suppresses Tel-Abl activity, inhibits Hck phosphorylation indicating that Hck is directly activated by Tel-Abl. Hck binds to four independent regions of Bcr-Abl: one region present in the Bcr portion of the fusion and three regions comprising the SH2 and SH3 domains, the kinase domain and the carboxy-terminal region of Abl (Wilson *et al*., 2002; Stanglmaier *et al*., 2003). As the last three regions are present in the Tel-Abl fusion it is likely that Hck also binds to Tel-Abl through same domains. Accordingly, we found by immunoprecipitation experiments that Hck interacts with Tel-Abl in Ba/F3 cells. Binding of Hck to Bcr-Abl results in the phosphorylation of Y^{177} residue on the Bcr moiety, allowing in turn the recruitment of the Grb2 adapter and activation of the Ras/Map pathway. Mutation of this residue abrogates the leukaemogenic potential of Bcr-Abl (Warmuth *et al*., 1997). A Grb2 binding site (e.g Y^{314}) has been identified in the Tel part of Tel-Abl and a Tel-Abl mutant lacking this residue exhibits decreased Gab2 recruitment and phosphorylation, impaired activation of Akt and Erk1/2 and is unable to transform Rat1 fibroblast nor to efficiently induce a myeloproliferative disease in mice (Million *et al*., 2004). One can thus imagine that Hck or a Src kinase may be responsible for the phosphorylation of the Y^{314} residue of Tel-Abl. This notion is also supported by the findings that expression of the kinase inactive form of Hck abrogated the phosphorylation of Akt and Erk1/2 in Tel-Abl-expressing cells (Figure 7). Regarding the known Tel-Abl effectors Stat5, expression of HckK269E does not inhibit the phosphorylation of these factors in Tel-Abl-expressing Ba/F3 cells while Hck was demonstrated to interact with and induce the activation of Stat5 in

Bcr-Abl-transformed haematopoietic cells. Interestingly, this interaction does not require the phosphorylation of the partners (Klejman *et al*., 2002) and our unpublished data also argue for a constitutive Stat5/Hck interaction in Ba/F3 cells expressing Tel-Abl that does not require the phosphorylation of Stat5 or Hck. However, since treatment with imatinib mesylate or PP2 which has been reported to inhibit also Bcr-Abl (Tatton *et al*., 2003; Warmuth *et al*., 2003) and herein Tel-Abl, suppresses Stat5 phosphorylation, Tel-Abl itself or another Src kinase may activate these factors. Although the Lyn kinase was shown to physically interact with and activate Stat5 factors (Danhauser-Riedl *et al*., 1996; Chin *et al*., 1998; Tilbrook *et al*., 2001), the absence of any effect of the dominant-negative form of the kinase on Stat5 phosphorylation in Tel-Abl-expressing cells do not argue for a role of Lyn in this process (data not shown).

Tel-Abl induces a CML-like disease that is very similar to that induced by Bcr-Abl, but with a different latency (Million *et al*., 2002). The Src kinases Lyn, Hck and Fgr have been demonstrated to be required for the induction of B-ALL but are dispensable for a CML-like disease (Hu *et al*., 2004). The role of the Src kinases in the leukaemogenic potential of Tel-Abl remains therefore questionable. However, the situation may be different in the development of CML in human because Bcr-Abl-expressing cell lines or CD34+ from CML patients resistant or not to imatinib mesylate treatment are sensitive to Src inhibitors or to downregulation of Src expression (Donato *et al*., 2003; Dai *et al*., 2004). Our results support the use of Src inhibitors in the treatment of leukaemia associated with oncogenic activation of c-Abl.

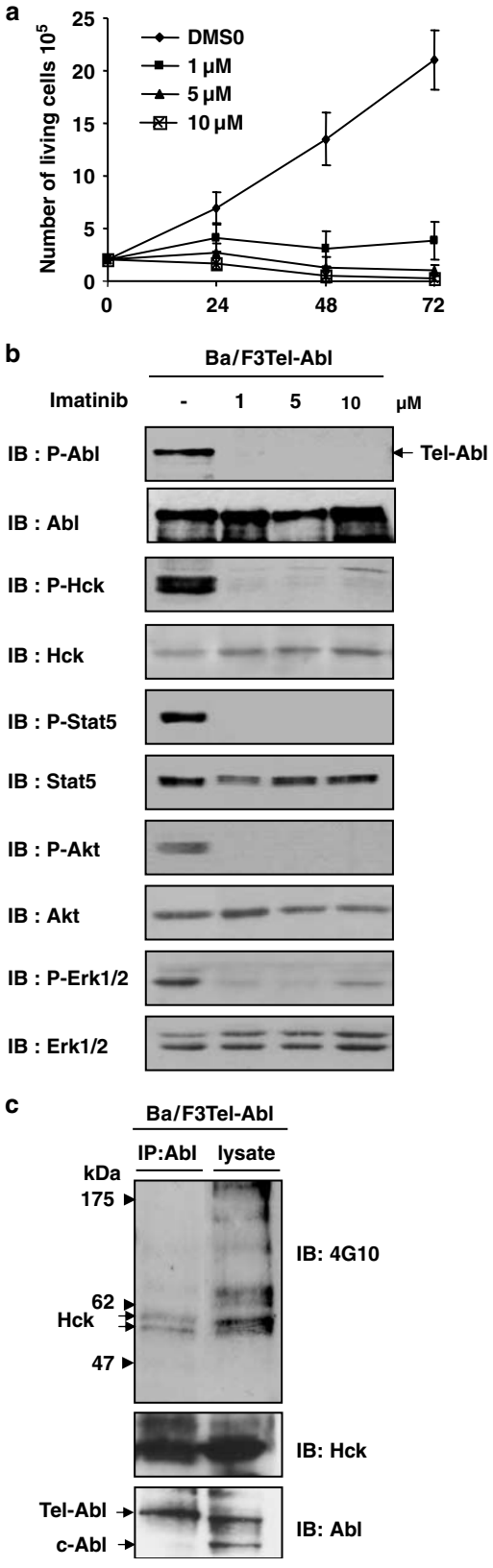

Figure 5 Imatinib mesylate inhibits Hck and Tel-Abl phosphorylation and suppresses cell growth and activation of downstream signalling pathways induced by Tel-Abl. (**a**) Cells were treated with various concentrations of imatinib mesylate as indicated or DMSO and the number of living cells was determined daily by using the Trypan Blue dye exclusion method. (**b**) Extracts from Tel-Abl-expressing Ba/F3 cells treated with imatinib mesylate or DMSO were also analysed by Western blot with the indicated antibodies. (**c**) Tel-Abl was immunoprecipitated from Tel-Abl-expressing cell lysates and the presence of co-precipitated Hck was assessed by immunoblotting with anti-P-tyr (4G10) and anti-Hck antibodies. Membrane was reproved with anti-c-Abl antibody.

Materials and methods

Cell culture and reagents

The parental Ba/F3 cells were grown in Rosewell's Park Memorial Institute media (RPMI) 1640 medium with 10% foetal calf serum and 4% WEHI conditioned medium (WCM) as a source of IL-3. Stimulation experiments were performed with recombinant murine IL-3 (Valbiotech, Paris, France) Ba/F3 cells expressing Tel-Jak2 and Tel-Abl fusions have been described elsewhere (Lacronique *et al*., 2000).

Imatinib mesylate (Gleevec, STI-571) was a generous gift of Novartis Pharma AG, Basel, Switzerland. The pyrazolopyrimidine Src inhibitors PP1 and PP2, the inactive analogue PP3 and SU6656 were purchased from Calbiochem-Novabiochem Co. (San Diego, CA, USA).

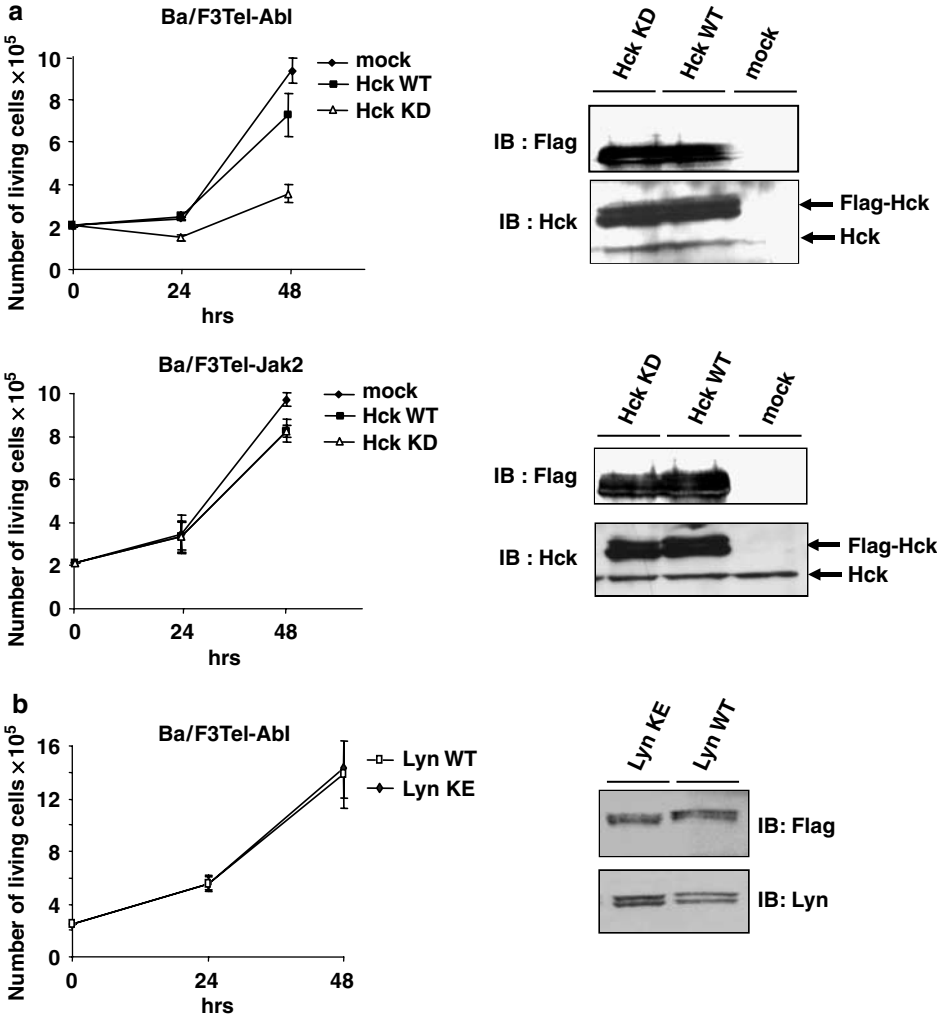

Figure 6 Overexpression of the mutant HckK269E inhibits the growth of Ba/F3 cells transformed by Tel-Abl. Ba/F3 cells expressing Tel-Abl were electroporated with the empty vector (pIREShrGFP) (mock) and the different Lyn and Hck constructs. The following day, GFP+ cells were sorted by flow cytometry and analysed for growth by cell counting using the Trypan Blue dye exclusion method. Results shown are representative of three different transfection assays (top and middle panels). A similar experiment was conducted in Tel-Jak2-expressing cells with the Hck constructs (bottom panel). Expression of HckWT, HckK269E, LynWT and LynK275R in transfected cells was verified by Western blot with anti-Flag or as indicated with anti-Hck or anti-Lyn antibodies.

Plasmids and transfections

LynWT, LynK275R, HckWT and HckK269E cDNAs which have been described elsewhere (Briggs *et al*., 1995; Omura *et al*., 2002) were amplified by polymerase chain reaction (PCR) reaction and subcloned at *XhoI* and *EcoRI* sites of the pIRES-hrGFP-1a vector (Stratagene, La Jolla, CA, USA). For transient transfections assays, cells were electroporated (300 V, 960 μF) with the different constructs or the empty vector (40 μg). Transfected cells were expanded for 24 h in medium and the (GFP)+ cells were sorted by flow cytometry (Elite, Becton-Dickinson). Proliferation and viability were next examined by counting viable cells using the Trypan Blue dye exclusion method.

Apoptosis studies

Twenty four hours after treatments with dimethylsulphoxide (DMSO), PP1, PP2 and PP3 at 10 μM and 30 μM, cells were washed with phosphate-buffered saline (PBS) and resuspended in 10 mM Hepes (pH 7,4), 140 mM NaCl, 2,5 mM CaCl2. Cells were then labelled with AnnexinV-FITC, PI-PE and the percentage of AnnexinV+, PI+ (apoptotic cells) cells was next determined by flow cytometry (EPICS Elite Cytometer, Beckman Coulter).

Western blotting and immunoprecipitation studies

Immunoprecipitation studies using NP40 cell lysates were performed as previously described (Rosa Santos *et al*., 2000). For Western blot experiments, lysates were separated by electrophoresis on sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) and blotted onto cellulose membrane (Hybond-C super membrane, Amersham Life Science). Blots were incubated with antibodies specific for: Stat5 (BD Biosciences, San Diego, CA, USA), c-Src (B-12), Lyn (44), Hck (M-28), Akt (H-136), Jak2 (C-20), actin (C-11) (Santa Cruz Biotechnology, Santa Cruz, CA, USA), Erk1/2, c-Abl (Cell Signaling Technology, Beverly, MA). We also used

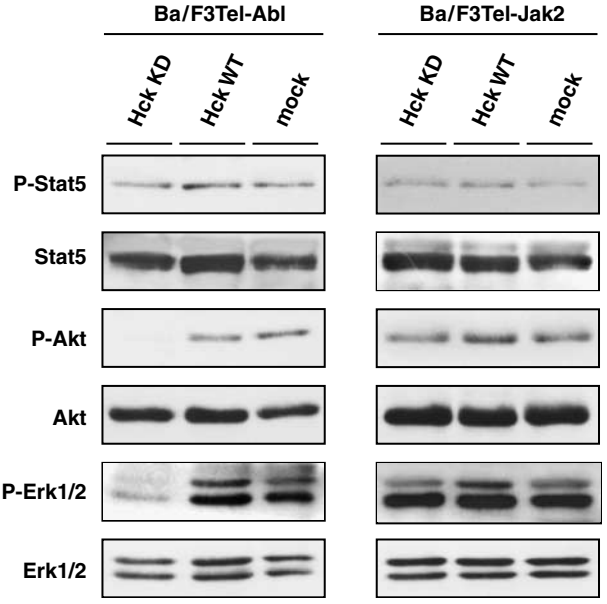

Figure 7 Expression of the kinase inactive Hck inhibits activation of Akt and Erk1/2 in Tel-Abl-expressing cells. Ba/F3 cells expressing Tel-Abl or Tel-Jak2 were transfected with HckWT and HckK269E constructs or the empty vector (mock). GFP+ cells were sorted 24 h later by flow cytometry and the levels of phosphorylated Stat5, Akt and Erk1/2 in transfected cells were determined by immunoblotting with the indicated antibodies.

antibodies raised against the following phosphorylated proteins: phospho-Stat5 (Y^{694}), phospho-Akt (ser^{473}), phospho-Erk1/2 (thr^{202}/Y^{204}) phospho-c-Abl (Y^{245}), phospho-Src (Y^{416}), phospho-Lyn (Y^{507}) (Cell Signaling Technology), phospho-Hck (Y^{411}) and phospho-Jak2 (Y^{1007}) (Santa Cruz). The antiphospho-tyrosine (4G10) monoclonal antibody was purchased from Upstate Biotechnology (Lake Placid, NY, USA).

Acknowledgements

This work was supported by grants from ARC (Association de la Recherche contre le Cancer), Ligue contre le Cancer (Comité du Nord-Pas de Calais), Conseil Régional de Picardie, Fondation de France, Cent pour Sang la Vie and National Institutes of Health (grant CA101828 to TES). CP is supported by ARERS-Verre Espoir and Fondation pour la Recherche Médicale and R N by the 'Ministere de la Recherche'.

References

Blake RA, Broome MA, Liu X, Wu J, M, Sun L *et al*. (2000). SU6656, a selective Src family kinase inhibitor, used to probe growth factor signaling. *Mol Cell Biol* **20**: 9018–9027.

Briggs SD, Bryant SS, Jove R, Sanderson SD, Smithgall TE. (1995). The Ras GTPase-activating protein (GAP) is an SH3 domain-binding protein and substrate for the Src-related tyrosine kinase, Hck. *J Biol Chem* **270**: 14718–14724.

Carron C, Cormier F, Janin A, Lacronique V, Giovannini M, Daniel MT *et al*. (2000). TEL-JAK2 transgenic mice develop T-cell leukaemia. *Blood* **95**: 3891–3899.

Cazzaniga G, Tosi S, Aloisi A, Giudici G, Daniotti M, Pioltelli P *et al*. (1999). The tyrosine kinase abl-related gene ARG is fused to ETV6 in an AML-M4Eo patient with a t(1;12)(q25;p13): molecular cloning of both reciprocal transcripts. *Blood* **94**: 4370–4373.

Chin H, Arai A, Wakao H, Kamiyama R, Miyasaka N, Miura O. (1998). Lyn physically associates with the erythropoietin receptor and may play a role in activation of the Stat5 pathway. *Blood* **91**: 3734–3745.

Dai Y, Rahmani M, Corey SJ, Dent P, Grant S. (2004). A Bcr/Abl-independent, Lyn-dependent form of imatinib mesylate (STI-571) resistance is associated with altered expression of Bcl-2. *J Biol Chem* **279**: 34227–34239.

Danhauser-Riedl S, Warmuth M, Druker BJ, Emmerich B, Hallek M. (1996). Activation of Src kinases p53/ 56lyn and p59hck by p210bcr-abl in myeloid cells. *Cancer Res* **56**: 3589–3596.

Donato NJ, Wu JY, Stapley J, Gallick G, Lin H, Arlinghaus R *et al*. (2003). BCR-ABL independence and LYN kinase overexpression in chronic myelogenous leukemia cells selected for resistance to STI571. *Blood* **101**: 690–698.

Dos Santos NR, Ghysdael J. (2005). A transgenic mouse model for TEL-JAK2-induced B-cell lymphoma/leukaemia. *Leukemia* **20**: 182–185.

Ernst TJ, Slattery KE, Griffin JD. (1994). p210Bcr/Abl and p160v-Abl induce an increase in the tyrosine phosphorylation of p93c-Fes. *J Biol Chem* **269**: 5764–5769.

Golub TR, Barker GF, Lovett M, Gilliland DG. (1994). Fusion of PDGF receptor β to a novel ets-like gene, *tel*, in chronic myelomonocytic leukemia with t(5;12) chromosomal translocation. *Cell* **77**: 307–316.

Golub TR, Goga A, Barker GF, Afar DE, McLaughlin J, Bohlander SK *et al*. (1996). Oligomerization of the ABL tyrosine kinase by the Ets protein TEL in human leukemia. *Mol Cell Biol* **16**: 4107–4116.

Hanke JH, Gardner JP, Dow RL, Changelian PS, Brissette WH, Weringer EJ *et al*. (1996). Discovery of a novel, potent, and Src family selective tyrosine kinase inhibitor. Study of Lck- and FynT-dependentT cell activation. *J Biol Chem* **271**: 695–701.

Ho JM, Nguyen MH, Dierov JK, Badger KM, Beattie BK, Tartaro P *et al*. (2002). TEL-JAK2 constitutively activates the extracellular signal-regulated kinase (ERK), stress-activated protein/Jun kinase (SAPK/JNK), and p38 signaling pathways. *Blood* **100**: 1438–1448.

Hu Y, Liu Y, Pelletier S, Buchdunger E, Warmuth M, Fabbro D *et al*. (2004). Requirement of Src kinases Lyn, Hck and Fgr for *Bcr-Abl1*-induced B-lymphoblastic leukaemia but not chronic myeloid leukaemia. *Nat Genet* **36**: 453–461.

Keung YK, Beaty M, Steward W, Jackle B, Pettnati M. (2002). Chronic myelocytic leukemia with eosinophilia, t(9;12)(q34;p13), and ETV6-ABL gene rearrangement: case report and review of the literature. *Cancer Genet Cytogenet* **138**: 139–142.

Klejman A, Schreiner SJ, Nieborowska-Skorska M, Slupianek A, Wilson M, Smithgall TE *et al*. (2002). The Src family kinase Hck couples BCR/ABL to STAT5 activation in myeloid leukemia cells. *EMBO J* **21**: 5766–5774.

Knezevich SR, McFadden DE, Tao W, Lim JF, Sorensen PH. (1998). A novel ETV6-NTRK3 gene fusion in congenital fibrosarcoma. *Nat Genet* **18**: 184–187.

Kuno Y, Abe A, Emi N, Iida M, Yokozawa T, Towatari M *et al*. (2001). Constitutive kinase activation of the TEL-Syk fusion gene in myelodysplastic syndrome with t(9;12) (q22;p12). *Blood* **97**: 1050–1055.

Lacronique V, Boureux A, Monni R, Dumon S, Mauchauffe M, Mayeux P *et al*. (2000). Transforming properties of chimeric TEL-JAK proteins in Ba/F3 cells. *Blood* **95**: 2076–2083.

Lacronique V, Boureux A, Valle VD, Poirel H, Quang CT, Mauchauffe M *et al*. (1997). A TEL-JAK2 fusion protein with constitutive kinase activity in human leukemia. *Science* **278**: 1309–1312.

Lionberger JM, Wilson MB, Smithgall TE. (2000). Transformation of myeloid leukemia cells to cytokine independence by Bcr-Abl Is uppressed by kinase-defective Hck. *J Biol Chem* **275**: 18581–18585.

Mao W, Irby R, Coppola D, Fu L, Wloch M, Turner J *et al*. (1997). Activation of c-Src by receptor tyrosine kinases in human colon cancer cells with high metastatic potential. *Oncogene* **15**: 3083–3090.

Malinge S, Monni R, Bernard O, Penard-Lacronique V. (2006). Activation of the NF-κB pathway by the leukemogenic TEL-Jak2 and TEL-Abl fusion proteins leads to the accumulation of anti-apoptotic IAP proteins and involves IKKα. *Oncogene* **25**: 3589–3597.

Million RP, Aster J, Gilliland DG, Van Etten RA. (2002). The Tel-Abl (ETV6-Abl) tyrosine kinase, product of complex (9;12) translocations in human leukemia, induces distinct myeloproliferative disease in mice. *Blood* **99**: 4568–4577.

Million RP, Harakawa N, Roumiantsev S, Varticovski L, Van Etten RA. (2004). A Direct binding site for Grb2 contributes to transformation and leukemogenesis by the Tel-Abl (ETV6-Abl) tyrosine kinase. *Mol Cell Biol* **24**: 4685–4695.

Omura T, Sakai H, Murakami H. (2002). Acceleration of granulocyte colony-stimulating factor-induced neutrophilic nuclear lobulation by overexpression of Lyn tyrosine kinase. *Eur J Biochem* **269**: 381–389.

Ottenhoff-Kalff AE, Rijksen G, van Beurden EA, Hennipman A, Michels AA, Staal GE. (1992). Characterization of protein tyrosine kinases from human breast cancer: involvement of the c-src oncogene product. *Cancer Res* **52**: 4773–4778.

Papadopoulos P, Ridge SA, Boucher CA, Stocking C, Wiedemann LM. (1995). The novel activation of *ABL* by fusion to an *ets*-related gene. *TEL Cancer Res* **55**: 34–38.

Peeters P, Raynaud SD, Cools J, Wlodarska I, Grosgeorge J, Philip P *et al*. (1997). Fusion of TEL, the ETS-variant gene 6 (ETV6), to the receptor-associated kinase JAK2 as a result of t(9; 12) in a lymphoid and t(9; 15; 12) in a myeloid leukemia. *Blood* **90**: 2535–2540.

Rosa Santos SC, Dumon S, Mayeux P, Gisselbrecht S, Gouilleux F. (2000). Cooperation between STAT5 and phosphatidylinositol 3-kinase in the IL-3-dependent survival of a bone marrow derived cell line. *Oncogene* **19**: 1164–1172.

Rosen N, Bolen JB, Schwartz AM, Cohen P, DeSeau V, Israel MA. (1986). Analysis of pp60c-src protein kinase activity in human tumor cell lines and tissues. *J Biol Chem* **261**: 13754–13759.

Santos SC, Monni R, Bouchaert I, Bernard O, Gisselbrecht S, Gouilleux F *et al*. (2001). Involvement of the NF-KB pathway in the transforming properties of the TEL-Jak2 leukemogenic fusion protein. *FEBS Lett* **497**: 148–152.

Schindler T, Bornmann W, Pellicena P, Miller WT, Clarkson B, Kuriyan J. (2000). Structural mechanism for STI-571 inhibition of Abelson tyrosine kinase. *Science* **289**: 1938–1942.

Schwaller J, Frantsve J, Aster J, Williams IR, Tomasson MH, Ross TS *et al*. (1998). Transformation of hematopoietic cell lines to growth-factor independence and induction of a fatal myelo- and lymphoproliferative disease in mice by retrovirally transduced TEL/JAK2 fusion genes. *EMBO J* **17**: 5321–5333.

Spiekermann K, Pau M, Schwab R, Schmieja K, Franzrahe S, Hiddemann W. (2002). Constitutive activation of STAT3 and STAT5 is induced by leukemic fusion proteins with protein tyrosine kinase activity and is sufficient for transformation of hematopoietic precursor cells. *Exp Hematol* **30**: 262–271.

Stanglmaier M, Warmuth M, Kleinlein I, Reis S, Hallek M. (2003). The interaction of the Bcr-Abl tyrosine kinase with the Src kinase Hck is mediated by multiple binding domains. *Leukemia* **17**: 283–289.

Talamonti MS, Roh MS, Curley SA, Gallick GE. (1993). Increase in activity and level of pp60c-src in progressive stages of human colorectal cancer. *J Clin Invest* **91**: 53–60.

Tatton L, Morley GM, Chopra R, Khwaja A. (2003). The Src-selective kinase knhibitor PP1 also Inhibits Kit and Bcr-Abl tyrosine kinases. *J Biol Chem* **278**: 4847–4853.

Tatosyan AG, Mizenina OA. (2000). Kinases of the Src family: structure and functions. *Biochemistry (Moscow)* **65**: 49–58.

Tilbrook PA, Palmer GA, Bittorf T, McCarthy DJ, Wright MJ, Sarna MK *et al*. (2001). Maturation of erythroid cells and erythroleukemia development are affected by the kinase activity of Lyn. *Cancer Res* **61**: 2453–2458.

Van Limbergen H, Beverloo HB, van Drunen E, Janssens A, Hahlen K, Poppe B *et al*. (2001). Molecular cytogenetic and clinical findings in ETV6/ABL1-positive leukemia. *Genes Chromosomes Cancer* **30**: 274–282.

Voss J, Posern G, Hannemann JR, Wiedemann LM, Turhan AG, Poirel H *et al*. (2000). The Leukaemic Bcr-Abl et Tel-Abl (ETV6/Abl) have altered substrate preferences and activate similar intracellular signalling pathways. *Oncogene* **19**: 1684–1690.

Warmuth M, Bergmann M, Priess A, Hauslmann K, Emmerich B, Hallek M. (1997). The Src Family Kinase Hck Interacts with Bcr-Abl by a Kinase-independent Mechanism and Phosphorylates the Grb2-binding Site of Bcr. *J Biol Chem* **272**: 33260–33270.

Warmuth M, Simon N, Mitina O, Mathes R, Fabbro D, Manley PW *et al*. (2003). Dual-specific Src and Abl kinase inhibitors, PP1 and CGP76030, inhibit growth and survival of cells expressing imatinib mesylate-resistant Bcr-Abl kinases. *Blood* **101**: 664–672.

Wilson MB, Schreiner SJ, Choi HJ, Kamens J, Smithgall TE. (2002). Selective pyrrolo-pyrimidine inhibitors reveal a necessary role for Src family kinases in Bcr-Abl signal transduction and oncogenesis. *Oncogene* **21**: 8075–8088.

Supplementary Information accompanies the paper on the Oncogene website (http://www.nature.com/onc).