



OPEN Differential regulatory effects of the N-terminal region in SYK-fusion kinases reveal unique activation-inducible nuclear translocation of ITK-SYK

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ITK-SYK and TEL-SYK (also known as ETV6-SYK) are human tumor-causing chimeric proteins containing the kinase region of SYK, and the membrane-targeting, N-terminal, PH-TH domain-doublet of ITK or the dimerizing SAM-PNT domain of TEL, respectively. ITK-SYK causes peripheral T cell lymphoma, while TEL-SYK was reported in myelodysplastic syndrome. BTK is a kinase highly related to ITK and to further delineate the role of the N-terminus, we generated the corresponding fusion-kinase BTK-SYK. By generating and analyzing these fusion kinases, we aim to understand the contribution of N-terminal domains to their distinct cellular behavior and oncogenic properties. The fusion kinases were found to behave differently. TEL-SYK showed stronger oncogenic capacity when compared with ITK-SYK and BTK-SYK. Furthermore, ITK-SYK and BTK-SYK triggered IL-3-independent growth of BAF3 pro-B cells. In contrast to BTK-SYK and TEL-SYK, which predominantly localized in perinuclear region and cytoplasm respectively, ITK-SYK exhibits a more diverse cellular distribution, being present in the nucleus, cytoplasm and membrane-bound compartments. Notably, we observed that ITK-SYK undergoes activation-mediated nuclear translocation, a phenomenon that is uncommon among kinases. This unique feature of ITK-SYK is therefore of particular interest due to its potential connection to its transforming capability.

Chromosomal alterations have often resulted in the chimeragenesis and deregulation of various proteins culminating in cancer^{1,2}. Until now, spleen tyrosine kinase (SYK) has been described in two different chromosomal translocation events that give rise to chimeric oncogenes, TEL-SYK and ITK-SYK^{3,4}. ITK-SYK was recurrently identified in a subset of peripheral T cell lymphomas, while translocation leading to TEL-SYK has been documented in 2 patients^{3,5} with myelodysplastic syndrome. Both fusion kinases are potent oncogenes^{3,4,6–10}. Moreover, aberrant/over expression of SYK has also been reported in both T and B cell lymphomas^{11–14}.

SYK comprises N-terminal, tandem SRC homology 2 (SH2) domains connected via the linker-A region, while the linker-B region joins them with the C-terminal kinase domain^{15,16}. SYK is expressed in a wide variety of cells including T and B cells and plays critical roles in immune receptor signaling^{17–19}. Following B and T cell receptor ligation, SYK can be activated either by phosphorylation at key tyrosine residues in the linker-

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regions or by binding to immune receptor tyrosine based activation motifs, ITAMs^{18,20,21}. In resting cells, SYK retains a “closed” conformation, where the tandem SH2 domains fold together with the kinase domain adapting a so-called “linker-kinase sandwich”^{15,18,20}. Fusion-kinases of SYK on the other hand lack tandem SH2 domains altering intramolecular SYK regulatory mechanisms. Thus, in ITK-SYK, the tandem SH2 domains are replaced by the Pleckstrin homology, Tec homology (PH-TH) domain-doublet from ITK. The PH-TH doublet is fused to the major part of the linker-B region⁴.

Based on cell lines and transgenic animal studies, the ITK-SYK chimera is known to be non-auto inhibited and therefore constitutively active^{4,6–9,22,23}. It has a highly efficient phosphorylation capacity for the B- and T cell adapter proteins BLNK (B cell linker), also known as SLP-65, and for SLP-76 (SH2 domain containing leukocyte protein of 76 kDa), respectively. Initially, it was thought that, like TEC family kinases, membrane localization of ITK-SYK leads to phosphorylation and, thus, constitutive kinase activation culminating in oncogenesis. However, intriguingly, a PH domain mutant that lacks membrane-tethering still causes T cell lymphomas in an animal model⁶.

Interestingly, a study in transgenic mice showed that CD19 promoter-driven B cell expression of ITK-SYK caused T cell lymphoma, but no B cell malignancies, presumably through leaky expression in T-cells⁸. To replicate this approach in B cells, we replaced the PH-TH domain doublet of ITK-SYK with the corresponding part of BTK, a kinase highly related to ITK, but whose function instead is crucial for B cell development²⁴. The aim of the current study is to determine whether the BTK-SYK fusion would behave differently from ITK-SYK in terms of transforming capacity, localization and phosphorylation of key tyrosines. The PH-TH domain-doublet of BTK comprises 196 residues and contains two proline-rich regions (PRRs), compared to that of ITK with a single PRR, resulting in a shorter length of 165 amino acids.

ITK-SYK and TEL-SYK fusions share most of the SYK linker-B region and the entire kinase domain. However, the linker-B region is 40 amino acids shorter in ITK-SYK. N-terminal regions in these chimeras are entirely different. In TEL-SYK, the N-terminus is formed by the dimerizing SAM-PNT domain, which is derived from TEL/ETV6^{3,10}. TEL-SYK acquires constitutive activation due to SAM-PNT domain-mediated dimerization and subsequent auto-phosphorylation. STAT5 is the main downstream target of TEL-SYK^{25,26}, which also causes constitutive activation of PI3-kinase and AKT^{3,10}. Furthermore, TEL-SYK can transform pre-B cells resulting in IL-3 independent growth, *in vitro*¹³. For ITK-SYK downstream signaling comprises of STAT3^{27,28} and in the tumor microenvironment IL-6 seems to play a crucial role²⁸.

In this study, we have compared the role of N-terminal region domains in regulating SYK fusion kinases in terms of phosphorylation, activation, stability and localization. Unexpectedly, we found that ITK-SYK has the unique capacity to translocate to the nucleus upon activation.

Materials and methods

Plasmid constructs

The following fusion kinase constructs were analyzed in this study; BTK-SYK, BTK-SYK-KD, BTK-SYK R28C, ITK-SYK, ITK-SYK-KD, ITK-SYK R29C, TEL-SYK, Δ-TEL-SYK and SLP-76. ITK-SYK, ITK-SYK-KD, and SLP-76 have been previously described^{7,23}. BTK-SYK, BTK-SYK-KD and PH-TH domains mutants ITK-SYK-R29C and BTK-SYK-R28C were created by site-directed mutagenesis and cloned in the pCDNA3 mammalian expression vector. The TEL-SYK and Δ-TEL-SYK were kind gifts of Dr. Akihiro Abe, Nagoya University School of Medicine, Japan. All constructs were sequenced and the presence of the corresponding protein was verified.

Cell lines, reagents and transfection

COS-7 (African green monkey fibroblast-like kidney), HEK-293T (Human embryonic kidney cells), NIH3T3 (mouse embryonic fibroblast), SYF (mouse embryonic fibroblast cells deficient in SRC, YES and FYN kinases), Jurkat (human T-cell leukemia cell line) and BAF3 (murine interleukin-3 dependent pro-B cell line) cells were obtained from the American Type Culture Collection (ATCC). COS-7, HEK-293T, SYF and NIH3T3 cells were cultured in DMEM supplemented with 10% heat-inactivated fetal bovine serum. BAF3 and Jurkat cells were cultured in RPMI 1640 medium with supplements. All cells were cultivated at 37 °C in a humidified 5% CO₂ incubator.

COS-7, HEK-293T, NIH3T3 and SYF cells were transfected using Polyethylene imine (PEI) as previously described^{5,23}. BAF3 and Jurkat cells were transfected using the Neon electroporation system²³. IL-3-independent growth of BAF3 cells was evaluated following transfection with ITK-SYK, BTK-SYK or mock pEGFP as control. Cells were washed with cell culture medium, free from IL-3 and electroporated. Nuclear and cytoplasmic fractionation was done by using a fractionation reagent from Thermo Scientific.

Antibodies

Monoclonal mouse anti-SYK (4D10) from Santa Cruz Biotechnology. The phospho-SYK antibodies Tyr-352, and Tyr-525/526, SRC antibody and SLP-76 antibody were purchased from Cell Signaling Technology. Anti-SLP-76 pY128 was from BD Biosciences Pharmingen. Secondary antibodies goat anti-mouse-800CW, goat anti-rabbit-800CW, goat anti-mouse-680LT, and goat anti-rabbit- 680 were from LI-COR Biosciences. Membranes were scanned using Odyssey Imager (LI-COR Biosciences).

Immunofluorescence and microscopy

Cells were seeded overnight at 50% confluence in six-well dishes. The next day, cells were transfected with plasmids and incubated for 48 h. Cells were fixed and permeabilized and immunofluorescence staining was performed as previously described²⁹.

Immunoblotting

Samples were prepared for Western blotting as described previously³⁰. Briefly, 48 h post-transfection, cells were lysed in RIPA buffer containing protease and phosphatase inhibitors, separated by SDS–polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes, followed by incubation with primary and secondary antibodies. The membranes were scanned using Odyssey Imager from LI-COR Biosciences GmbH.

Healing assay

We used μ -Dish 35 mm, high, with a Culture Insert (from ibidi) for wound healing assay. Cells were transfected with the plasmids, and after 24 h, a suspension of the transfected cells was placed in both wells of the silicon-insert, and incubated for additional 24 h at 37 °C and 5% CO₂. Then the silicone-insert was removed using sterile tweezers, and a cell free gap of 500 μ m was created, and cell images were captured at regular intervals using 4X dry objective under a fluorescence microscope.

Statistics

Statistical analysis was performed using GraphPad Prism10 Software. Statistical significance was determined using 1-way ANOVA, followed by the Duncan comparison test. $P \leq 0.05$ was considered statistically significant.

Results

SYK fusion kinases show equal stability and activation

ITK-SYK and BTK-SYK both acquire their N-terminal PH-TH domain from the TEC family kinases (TFKs) ITK and BTK, respectively, while dimerizing SAM-PNT domain forms the N-terminus of TEL-SYK. The C-terminal catalytic domain and the adjacent linker-B region of the fusion kinases are derived from SYK (Fig. 1A). The PH domain is known to play a key role in the activation of TFKs by tethering them onto plasma membrane phospholipids^{31–34}. Despite a high degree of conservation between the N-terminal region of ITK and BTK, subtle characteristics may still result in functional differences^{22,35–37}. To compare the attributes conferred by the PH-TH domains of ITK and BTK, and the SAM-PNT domain of TEL-SYK in fusion kinases, constructs encoding ITK-SYK, BTK-SYK and TEL-SYK were co-expressed with the adapter protein SLP-76. SYK fusion kinases were equally phosphorylated at Tyrosine 352 and 525/526 in the linker-region and activation-loop that play a key role in the activation of SYK, and they potently trans-phosphorylated the SLP-76 substrate in COS-7 cells (Fig. 1B, Supplementary Figure S1A). Moreover, for ITK-SYK, in addition to the 55 kDa full length protein, a shorter, presumably truncated version corresponding to 45 kDa, is generated. In contrast, the expression of BTK-SYK leads to the production of a single detectable molecule, which, as expected, has a slightly higher molecular weight than ITK-SYK. Since SYK has been shown to produce a shorter isoform of 40 kDa, we set out to investigate whether the shorter band of ITK-SYK is due to a second translation initiation site or to a proteolytic event³⁸. Inspection of the primary sequence of ITK-SYK reveals an additional in-frame translation initiation codon in the PH-TH doublet. Substitution of the corresponding methionine for leucine in the form of the ITK-SYK-M126L mutant was generated to abolish this second translation initiation site. Expression of ITK-SYK-M126L in COS-7 cells resulted in the production of only the predicted longer ITK-SYK, suggesting that alternative initiation and not proteolysis is the underlying mechanism (Fig. 1C).

While inspection of the BTK-SYK sequence also revealed potential additional translation initiation codons, we were unable to see shorter isoforms following the expression of the constructs in all tested cell types. Although, we envisaged that a shorter isoform is also produced in cells expressing BTK-SYK, it might be subject to rapid degradation making its detection difficult. To determine whether a shortened isoform is degraded by the ubiquitin proteasome pathway, we expressed constructs encoding ITK-SYK and BTK-SYK in COS7 cells, in the presence, or absence, of the proteasome inhibitor MG132. The two shorter isoforms of BTK-SYK were detected in the presence of MG132, thus suggesting their rapid degradation in the absence of proteasome inhibition (Fig. 1D).

SYK fusion kinases are phosphorylated by SRC and inhibited by SYK and PI3-kinase inhibitors

SRC family kinases (SFK) initiate intracellular signaling in response to different antigen receptor ligation. This is followed by recruitment and binding of SYK to the phosphorylated tyrosine (pY) on ITAM residues, and the activated SYK will subsequently phosphorylate downstream substrates. To investigate the role of SFK in the phosphorylation and activation of ITK-SYK, BTK-SYK and TEL-SYK, we used an SFK-deficient cell line, SYF. In SYF cells, ITK-SYK and BTK-SYK did not show any detectable phosphorylation, while TEL-SYK showed some phosphorylation. However, when expression plasmids were co-transfected with plasmids expressing SRC, cells showed detectable phosphorylation of the fusion constructs. Interestingly, phosphorylation of SLP-76 was seen in the presence or absence of SRC (Fig. 2A, Supplementary Figure S1B).

In the next set of experiments, we compared the phosphorylation capacity of the kinases in the presence or absence of different inhibitors. R406 (the active metabolite of fostamatinib) is a competitive inhibitor of ATP for the SYK kinase. All chimeras were expressed in COS7 cells and R406 was added to compare its potential suppressive effect for SYK fusion kinase activity. Upon the addition of R406, the phosphorylation of the fusion kinases at Y525/526, but not at Y352, was reduced (Fig. 2B, Supplementary Figure S1C).

ITK-SYK is believed, like the native TFKs, to be regulated by the PI3-kinase signaling pathway (5,7,22). To determine whether activation of the BTK-SYK fusion is also PI3-kinase dependent, we employed the PI3-kinase inhibitor LY294002. PI3-kinase inhibition minimized ITK-SYK, BTK-SYK, and to much lower extent, TEL-SYK phosphorylation at Y525/526 (Fig. 2B, Supplementary Figure S1C).

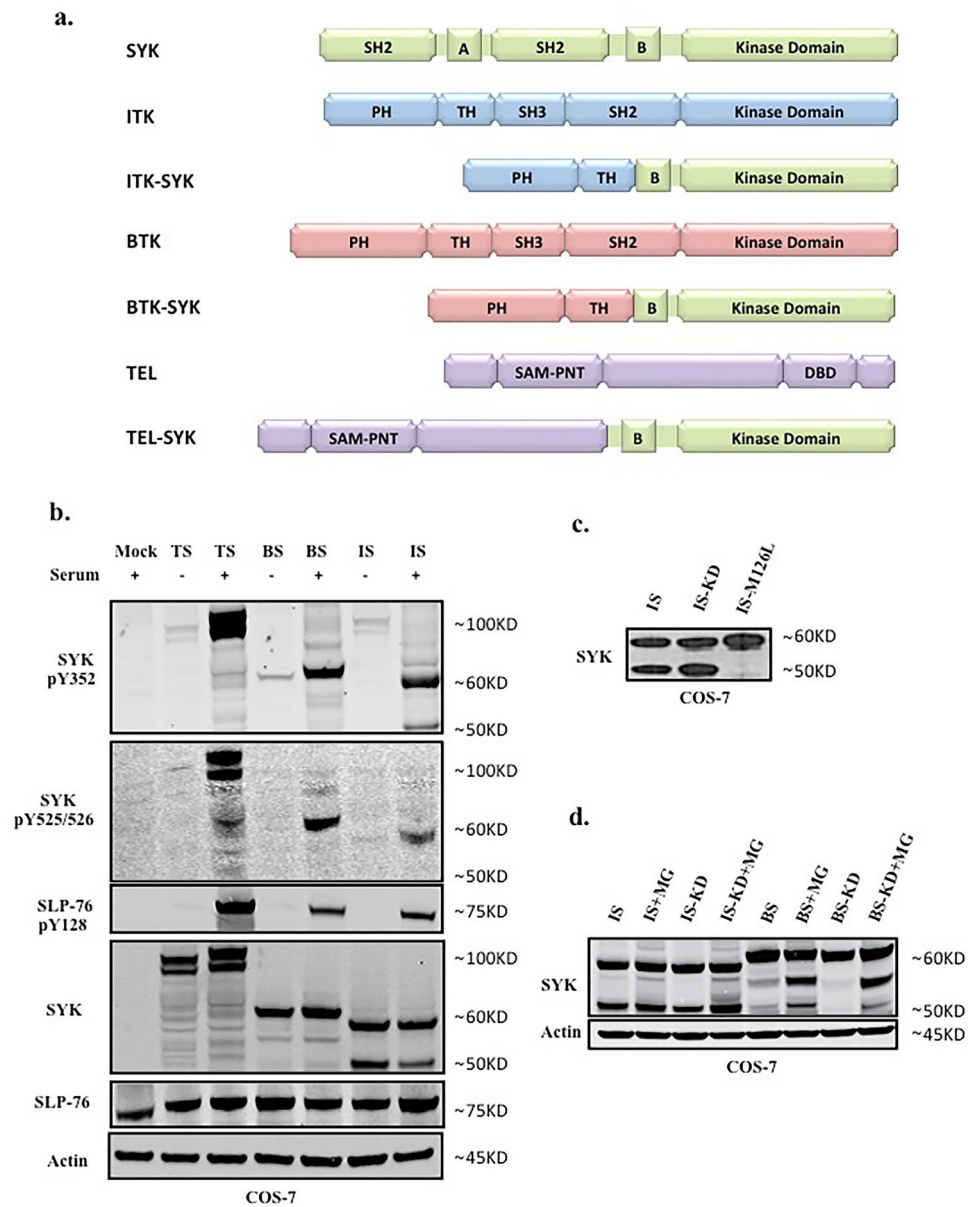


Fig. 1. SYK fusion kinases confer substantial activation. **(A)** Graphic representation of the kinases investigated. **(B)** Whole cell lysates of COS-7 cells expressing IS = ITK-SYK, BS = BTK-SYK, and TS = TEL-SYK along with SLP-76. The cells were starved overnight and then activated with serum for 10 min at room temperature. Detection of native or phosphorylated proteins was done with the corresponding antibodies. Anti-SYK was used to detect native or phosphorylated proteins. Anti-p-Y352 and p-Y525/Y526 were used to detect phosphorylated linker-region and activation-loop tyrosines. Anti-p-SLP-76 antibody was used for the detection of phosphorylated SLP-76. **(C)** Western blot analysis of whole cell lysates of COS7 cells expressing IS = ITK-SYK, IS-KD = ITK-SYK kinase-dead, IS-M126L = ITK-SYK-M126L. Proteins were detected using an anti-SYK antibody. **(D)** Whole cell lysates of COS7 cells expressing the indicated plasmids. Cells were treated overnight with the proteasome inhibitor MG = MG132 (10 μ M) or DMSO (control).

Furthermore, we compared the oncogenic potential of the fusion kinases by using wound-healing assay. Cells expressing TEL-SYK showed stronger oncogenic capacity, as measured by rapid closing of the gap and greater proliferation and migration rate compared to ITK-SYK and BTK-SYK (Supplementary Figure S2A,B).

The BAF3 cell line is often exploited for its property of IL-3-dependent growth³⁹. Several proteins with oncogenic potential have been analyzed using BAF3 as an in vitro model⁴⁰, and many oncogenes induce IL-3-independent growth of these cells. Expression of both ITK-SYK and BTK-SYK rendered proliferation of BAF3 cells IL-3-independent. However, IL-3-independent growth was more pronounced in cells transfected with ITK-SYK (Supplementary Figure S2C).

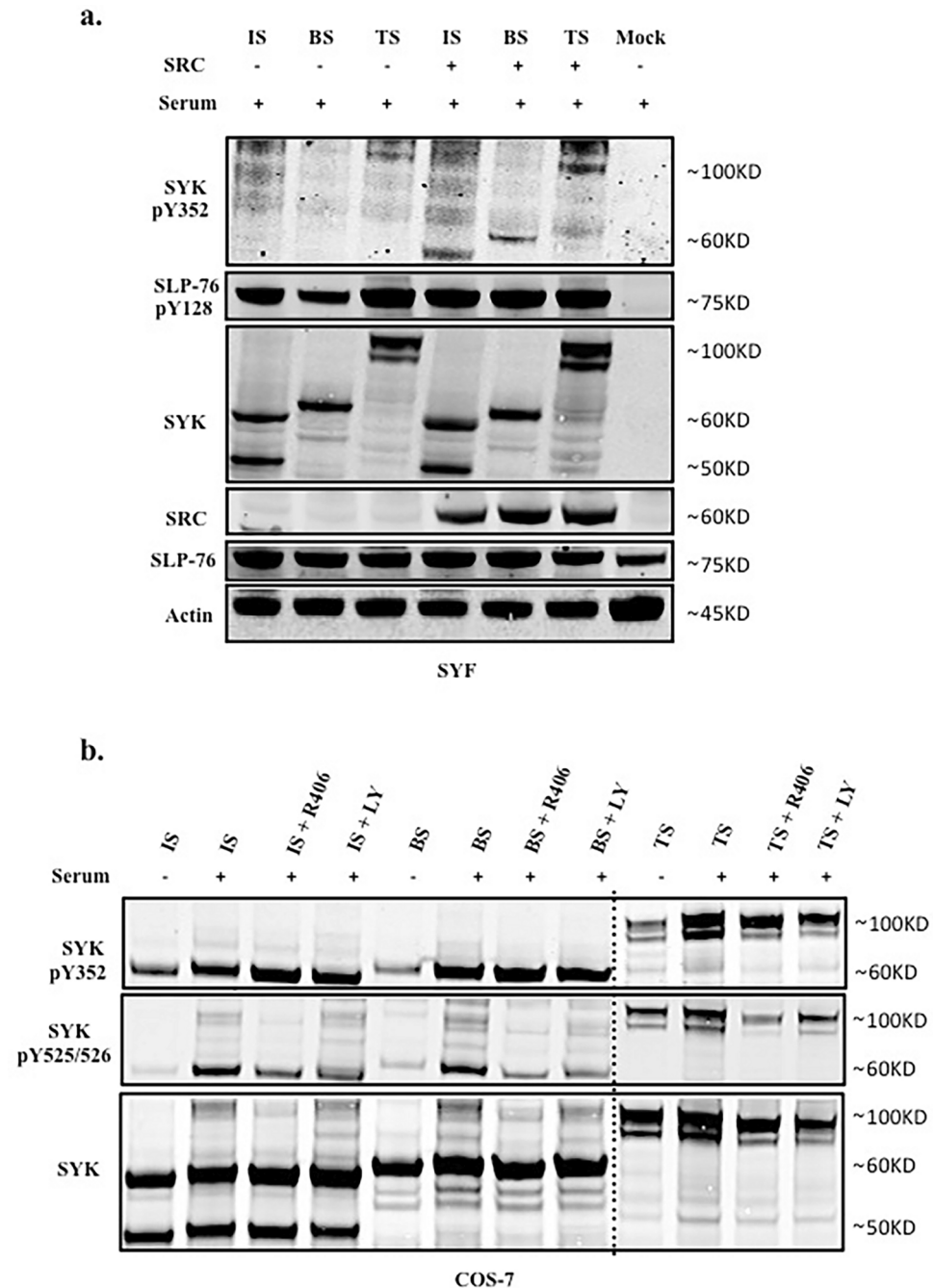


Fig. 2. Phosphorylation and inhibition mechanisms of SYK fusion kinases. **(A)** Western blot analysis of SYF cells transfected with IS, BS or TS along with SLP-76 in the presence or absence of SRC. The cells were serum starved overnight followed by activation with serum for 10 min at room temperature. Cells were processed for western blotting and probed with indicated antibodies. **(B)** Whole cell lysates of COS7 cells expressing the indicated plasmids. Cells were either treated with the SYK inhibitor (R406) (5 μ M) for 2 h or LY294002 (30 μ M) overnight.

ITK-SYK shows robust stimulation-mediated nuclear translocation

To further investigate the role of N-terminal domains of the fusion kinases, we looked into their subcellular localization. We used fluorescence microscopy to study the localization of these proteins in COS-7 cells both in steady state and after activation. In a steady state, ITK-SYK was predominantly in the cytoplasm, and only a small fraction was localized to the nucleus or the membrane (Fig. 3A). Most BTK-SYK appeared perinuclearly, whereas TEL-SYK was predominantly cytoplasmic (Fig. 3A), as previously reported¹⁰. Upon activation of the cells, although, BTK-SYK and TEL-SYK distribution largely remained unaffected, ITK-SYK showed robust nuclear translocation (Fig. 3B). We also found ITK-SYK in the membrane in a subset of cells, but the main part

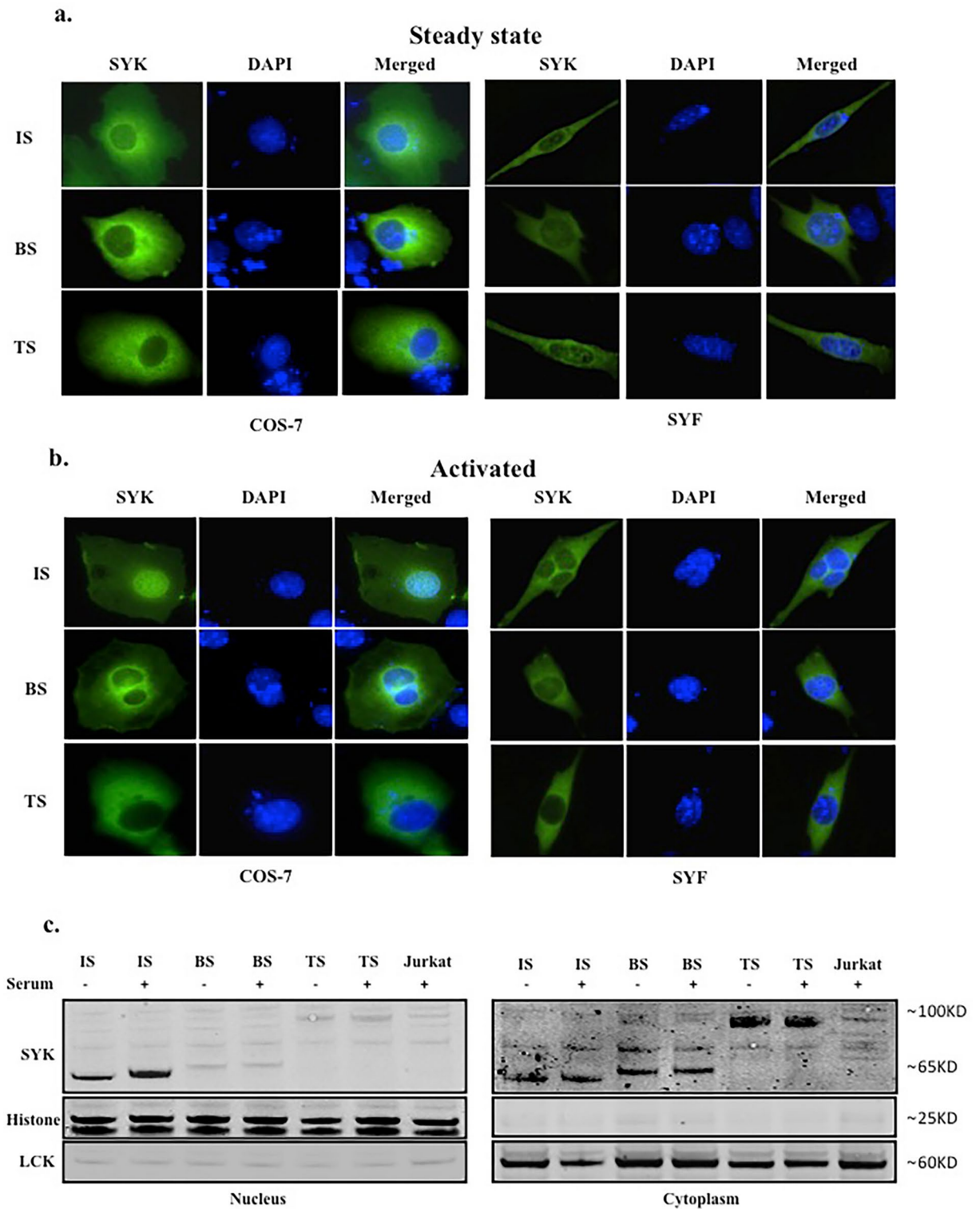


Fig. 3. Subcellular localization of the fusion kinases. **(A,B)** Fluorescence microscopy analysis of COS7 and SYF cells transfected with IS, BS, and TS. 36 h after transfection, cells were either kept unstimulated or serum-starved overnight, then serum activated for 10 min at 37 °C. Following fixation, the cells were decorated with an anti-SYK antibody and images were captured using a fluorescence microscope. **(C)** Nuclear and cytoplasmic fractions of Jurkat cells were transfected with the corresponding plasmids named in 3A. Cells were serum starved overnight, then serum activated for 10 min at room temperature. Subsequently, cells were fractionated into nuclear and cytoplasmic fractions.

accumulated in the nucleus (Table 1). In contrast, when we used SYF cells that completely lack SFKs, we found that all the fusion kinases were localized in the cytoplasm, both in steady state and after activation (Fig. 3A,B). Upon nuclear-cytoplasmic fractionation for Jurkat cells transfected with the fusion kinases, again we found that only ITK-SYK was localized in the nucleus and this nuclear localization increased after activation of the cells (Fig. 3C). Collectively these findings demonstrate that the ITK-SYK-fusion behaves differently compared to both BTK-SYK and TEL-SYK. Since both the PH and the TH domains of BTK and ITK are unique, it is impossible to determine the origin of these differences conclusively. However, given that the native SYK protein has a complex mode of regulation it is not surprising that varying the N-terminal portion of a SYK fusion protein could dramatically affect its function.

Non-membrane binding and non-dimerizing mutants of SYK fusion kinases retain catalytic activity

Idiosyncratic behavior of fusion-kinases like differential phosphorylation, and inhibition upon use of PI3-kinase inhibitors or localization, prompted us to investigate the impact of loss-of-function mutations in the PH-domain (ITK-SYK-R29C and BTK-SYK-R28C), where point mutations into the pleckstrin homology domain could alter the membrane-binding ability. The PH-domain plays a key role in activating TFKs^{22,41–43}. Although a similar loss-of-function mechanism was thought to operate also in the ITK-SYK activation, unexpectedly, ITK-SYK-R29C induced T cell lymphomas in a mouse model⁶. Despite cytoplasmic localization, we hypothesized that the loss-of-function mutant ITK-SYK-R29C may sustain substrate phosphorylation. Likewise, it seemed possible that BTK-SYK-R28C could also be active since even if it did not translocate to the membrane upon activation, it was equally phosphorylated, similar to the non-mutated form. Both fusion-kinases were co-expressed with the adapter protein SLP-76 in COS-7 cells. ITK-SYK-R29C as well as BTK-SYK-R28C potentially phosphorylated SLP-76 (Fig. 4). Moreover, the Δ TEL-SYK mutant, in which the dimerization domain was deleted, also strongly phosphorylated SLP-76 (Fig. 4).

Discussion

The non-receptor tyrosine kinase SYK is considered an “OR” switch, since it can be activated by phosphorylation at key tyrosines or ITAM binding²⁰. Tec family kinases (TFK), on the other hand, have been designated “AND” switches because they are activated in two steps; by PH-TH domain-mediated membrane binding and by SFK-mediated trans-phosphorylation²⁰. Upon chromosomal translocation, SYK contributes its kinase domain into two known fusion-proteins, ITK-SYK and TEL-SYK^{3,4}. Constitutive/deregulated activation of both chimeras leads to oncogenesis. Unexpectedly, B cell expression of ITK-SYK resulted in delayed development of T cell lymphomas instead of B cell malignancies⁸. This observation underlines the uniqueness of transforming events. In order to study this further, we cloned BTK-SYK, as the corresponding fusion protein for B cells and compared it with ITK-SYK.

Domains are functional and structural units of the protein. These fusion kinases possess the same catalytic domain but different N-terminal region domains. In this study, we compared these fusion kinases in order to estimate what influence these N-terminal domains will have when they are introduced in these kinases, also to find out if these domains keep and demonstrate the same characteristics as in their native forms.

We have compared the oncogenic potential of ITK-SYK, BTK-SYK and TEL-SYK by measuring the proliferation and migration rate, using a wound-healing assay. All fusion kinases induced the healing process, and the wound gap filling. The oncogenic capacity was significantly stronger upon TEL-SYK expression compared to ITK-SYK or BTK-SYK expression. This finding, together with the other observations on the activity of TEL-SYK, is of particular interest given the few reports of TEL-SYK mutations that have been reported to date. Thus, in 2001 Japanese researchers presented the first case of TEL-SYK, as a Driver mutation for the Myelodysplastic

Number of cells	Nuclear	Cytoplasmic	Membrane	Cytoplasmic/Nuclear	Nuclear/Membrane
ITK-SYK (Steady state)					
500	17	343	72	61	7
500	22	317	93	57	11
500	14	298	108	72	8
500	12	346	74	64	4
2000	65	1304	347	254	30
Percentage	3.1%	66%	16.7%	12.5%	1.5%
ITK-SYK (Activated)					
500	232	42	94	88	44
500	177	53	108	103	59
500	193	49	88	107	63
500	214	52	91	93	50
2000	816	196	381	391	216
Percentage	40.7%	10.1%	18.5%	19.6%	10.9%

Table 1. Localization of ITK-SYK in different cell compartments in steady state and following activation (from 4 different experiments).

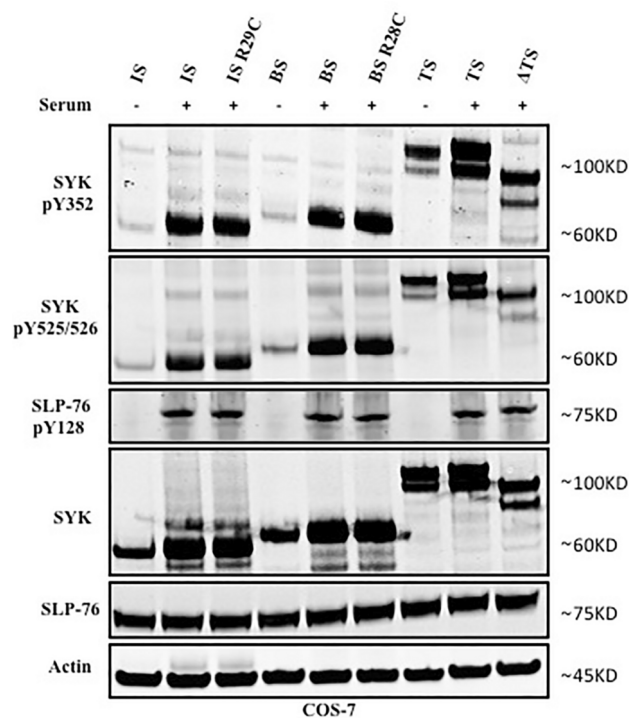


Fig. 4. Structure and function correlation of the fusion kinases. Whole cells lysates of COS7 cells co-expressing the following constructs IS-R29C=ITK-SYK-R29C, BS-R28C=BTK-SYK-R28C and Δ TS = Δ TEL-SYK along with SLP-76. The cells were serum starved overnight followed by activation with serum for 10 min at room temperature. Cells were processed for western blotting and probed with indicated antibodies.

Syndrome (MDS)⁴. Recently, a second patient was documented⁵. Given the enormous amount of sequenced patient samples to date, it is remarkable that only 2 known MDS patients are carrying this Driver mutation. To this end, TEL(ETV6) can fuse with a number of partners; for many of these, there are numerous reports of affected patients^{44,45}.

Considering that ITK-SYK failed to cause lymphomas in B cells in transgenic mice, we hypothesized that the BTK-SYK fusion may show stronger oncogenic potential in a B cell milieu instead. The bone marrow-derived pro-B cell line BAF3 was considered a good candidate for this experiment. These cells are strictly dependent on IL-3 for their survival and proliferation. However, following the expression of many oncogenes, BAF3 cells can grow readily in the absence of IL-3. Both ITK-SYK- and BTK-SYK-encoding plasmids rendered BAF3 cells IL-3-independent. ITK-SYK induced stronger IL-3-independent proliferation, suggesting that BTK-SYK might be less oncogenic even in a B cell setting.

We then compared the inhibitor sensitivity, and the SYK inhibitor (R406) was used to block the kinase activity of the fusion kinases. Although the phosphorylation at Y525/526 was inhibited, R406 did not block Y352 phosphorylation. The insensitivity of Y352 phosphorylation towards the SYK inhibitor could be due to trans-phosphorylation by SRC family kinases, which are insensitive to the SYK inhibitor.

Activation of many different receptors, with PI3-kinase as a common downstream denominator, results in PH-domain-dependent membrane localization of TFKs⁴³. The well-known PI3-kinase inhibitor LY294002 was tested to study the effects of PI3-kinase inhibition on the fusion kinases. The PI3-kinase inhibitor did not completely block the TEL-SYK phosphorylation, although the drug potently inactivated ITK-SYK and BTK-SYK under the same conditions.

These observations prompted us to compare the subcellular localization of the fusion kinases. Unlike BTK-SYK and TEL-SYK, which localized predominantly in the perinuclear region and cytoplasm, respectively, ITK-SYK showed differential cellular localization, and was found in various cellular compartments (nucleus, cytoplasm and membrane) (Table 1). Here, we report the activation-mediated nuclear translocation of ITK-SYK in COS-7 and Jurkat cells, using two different methods, Western blotting and fluorescence microscopy. The robust nuclear translocation of ITK-SYK suggests a specific mechanism for this activation-induced translocation. There are significant numbers of signaling proteins whose nuclear shuttling is induced by activation, like ERK, SMAD3, MEK, and Tet2^{46,47}. Upon stimulation these kinases translocate from the cytoplasm to the nucleus. Importins also have a crucial role in nuclear translocation of proteins that do not contain a bona fide nuclear localization signal (NLS). Therefore we analyzed importin-7, and we found that there is an interaction between ITK-SYK and Importin-7, but when we knocked down importin-7 expression by using specific siRNA, we did not find any detectable effect on ITK-SYK nuclear translocation (data not shown). SFKs have been implicated in stimulation-mediated nuclear translocation of numerous receptor and non-receptor tyrosine kinases and transcription factors⁴⁸⁻⁵¹. Interestingly, when we used SYF cells for localization study, ITK-SYK was mainly

found in the cytoplasm, even after activation. This strongly suggests that SFKs play a role in regulating the stimulated nuclear translocation of ITK-SYK.

This was intriguing, and we therefore decided to investigate the activity of the loss of function PH-domain mutant ITK-SYK-R29C that despite its cytoplasmic localization *in vitro*, leads to lymphomas in a transgenic mouse model. Along with ITK-SYK-R29C, we also generated its counterpart BTK-SYK-R28C. We further investigated the activity of the non-dimerizing Δ TEL-SYK mutant. All fusion kinase mutants potently phosphorylated the adapter protein SLP-76.

Overall, in our study, we found that ITK-SYK, BTK-SYK and TEL-SYK are constitutively active kinases that behave differently from each other in terms of phosphorylation, localization and transformation potential, seemingly reflecting the differences of the N-terminal region, PH-TH domain-doublers of BTK and ITK and the SAM-PNT domain of TEL. Another finding is that the activation of SYK fusion-kinases is not only dependent on the presence of intact PH-TH or SAM-PNT domains, but that the absence of an auto-inhibitory conformation may also be involved in activating such deregulated kinases. While our study provides important insight into the constitutive activity and distinct behavior of SYK fusion kinases, a limitation of the data presented is that they primarily are derived from over expression of these kinases in non-lymphoid cell lines. Hence, while these cell models are potentially valuable for dissecting molecular mechanisms; they may not fully recapitulate the cellular context in which these kinases naturally function. Further studies using physiological expression and relevant models are needed to validate and extend our findings.

Data availability

The dataset generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

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Author contributions

A.H., A.L.H. and D.K.M. performed most of the experiments, analyzed data, and wrote the manuscript; Q.W., and M.O.G. performed some experiments and edited the manuscript; B.F.N., A.J.M., and R.Z. were involved in planning the research and contributed to writing and editing the manuscript; and C.I.E.S. conceived the project, designed the experiments, interpreted data, revised the manuscript, and obtained research funding.

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Declarations

Competing interests

The authors declare no competing interests.

Additional information

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