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c-Src activation through a TrkA and c-Src interaction is essential for cell proliferation and hematological malignancies

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ABSTRACT

Although the kinase receptor TrkA may play an important role in acute myeloid leukemia (AML), its involvement in other types of leukemia has not been reported. Furthermore, how it contributes to leukemogenesis is unknown. Here, we describe a molecular network that is important for TrkA function in leukemogenesis. We found that TrkA is frequently overexpressed in other types of leukemia such as acute lymphoblastic leukemia (ALL), chronic myelogenous leukemia (CML), and myelodysplastic syndrome (MDS) including AML. In addition, TrkA was overexpressed in patients with MDS or secondary AML evolving from MDS. TrkA induced significant hematological malignancies by inducing PLK-1 and Twist-1, and enhanced survival and proliferation of leukemia, which was correlated with activation of the phosphatidylinositol 3-kinase/Akt/mTOR pathway. Moreover, endogenous TrkA associated with c-Src complexes was detected in leukemia. Suppression of c-Src activation by TrkA resulted in markedly decreased expression of PLK-1 and Twist-1 via suppressed activation of Akt/mTOR cascades. These data suggest that TrkA plays a key role in leukemogenesis and reveal an unexpected physiological role for TrkA in the pathogenesis of leukemia. These data have important implications for understanding various hematological malignancies.

1. Introduction

TrkA, a neurotrophic tyrosine kinase receptor (NTKR), and its neurotrophin ligand, nerve growth factor (NGF), primarily regulate the growth, differentiation, and survival of neurons [1,2]. Inactivation of TrkA mutations leads to severe congenital neuronal disorders. TrkA mutations have been identified as the cause of congenital insensitivity to pain and anhidrosis in humans [3,4]. It is also important for tumor formation and progression. Fusion of the TrkA receptor tyrosine kinase region with other proteins that homodimerize can lead to an oncogenic tyrosine kinase gene and thyroid cancer [5,6]. Furthermore, TrkA is involved in tumor growth and the progression of other non-neuronal cancers, including lung [7], pancreatic [8], prostatic [9], ovarian [10], and breast cancers [11]. Importantly, there is increasing evidence of its involvement in leukemogenesis. TrkA is overexpressed in patients with AML, its overexpression in 32D cells induces leukemia, and its activation by NGF efficiently rescues 32D cells from irradiation-induced apoptosis [12]. Moreover, a deleted form of TrkA (ΔTrkA), in which 75 amino acids are lacking in the extracellular domain, was identified in a patient with AML, and its overexpression in 32D cells induced cell transformation mainly via phosphatidylinositol 3-kinase (PI3K) and mTOR [13].

Although that study suggested that TrkA may play an important role in AML, the involvement and functional role of TrkA in other types of leukemia have not been reported. Moreover, the mechanisms of oncogenesis mediated by TrkA in hematological malignancies are poorly understood. In this study, we identified a molecular and functional network present in hematological malignancies that regulate and coordinate with TrkA. Surprisingly, TrkA was mainly present in other types of leukemia including AML and acted as a key regulator of PLK-1/Twist-1-mediated leukemogenesis and survival through c-Src activation. These results have important implications for hematological malignancies including AML.

2. Materials and methods

2.1. Cell culture and reagents

Human leukemia cell lines (KG-1, HL-60, Meg01, KU812, CCRF-HSB2, NB4, K252, CCRF-SB, K-562, Jurkat, ML-1, and U937) were

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maintained in RPMI 1640 (Gibco, Grand Island, NY, USA) supplemented with 10% heat-inactivated fetal bovine serum. The protein kinase inhibitor K252a was purchased from Calbiochem (Gibbstown, NJ, USA).

2.2. Human samples

RNA from human bone marrow samples was used after written informed consent was obtained from each healthy donor or patient. This study was approved by the institutional review board and the ethics committee of Seoul St. Mary's Hospital.

2.3. Plasmids

Each of the two shRNA-encoding oligonucleotides against human TrkA were designed and verified to be specific to TrkA by a BLAST search against the human genome (Supplementary Table 1). The human TrkA–shRNA insert was subcloned into the pLKO lentiviral vector. A control shRNA, which does not match any known human coding cDNA, was used as a control.

2.4. Antibodies and Western blotting

We performed Western blotting as described previously [14]. The primary antibodies used (and their manufacturers) were as follows: mTOR, PLK-1, Twist-1, and phospho-mTOR were from Abcam (Cambridge, MA, USA); poly ADP ribose polymerase (PARP), caspase-3, phospho-c-Src, Src, and phospho-Akt were obtained from Cell Signaling Technology (Danvers, MA, USA); and β-actin was purchased from Sigma–Aldrich (St. Louis, MO, USA).

2.5. Reverse transcription-polymerase chain reaction (RT-PCR) and quantitative RT-PCR

The primer sequences are listed in Supplementary Table 2. Total RNA was isolated using RNeasy Mini Kits (Qiagen, Valencia, CA, USA) and were reverse-transcribed with the hexa-nucleotide mix (Roche, Manheim, Germany). The PCR and data collection were performed using the 7900HT Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). All quantitations were normalized to the 18S RNA endogenous control. Specific TrkA (Hs01008225_m1) and 18S (Hs99999901_s1) quantitative probes for Taqman RT-PCR were obtained from Applied Biosystems.

2.6. Cell viability assay

K562 control-shRNA, K562 TrkA–shRNAs cells, or K562 cells treated with/without K252a were plated in six-well plates (1×10^5 cells/well), cultured, and counted. Each data point represents the mean of cells counted in three dishes. Cell viability was assessed using the MTT assay.

2.7. Analysis of apoptosis by Annexin-V staining

K562 control-shRNA, K562 TrkA–shRNA, and K562 cells treated with K252a were seeded at a density of 1×10^6 cells/mL. Apoptosis was determined using an Annexin-V apoptosis detection kit (BD Pharmingen, San Diego, CA, USA) according to the manufacturer's protocol.

2.8. Microarray data analysis

To compare TrkA expression levels in various hematological malignancies, the correlations between the gene expression profiles of TrkA and those of tumors from patients with leukemia in the Queen's University datasets were evaluated. Analysis of Variance was performed and Boxplot graphs of gene expression were plotted using GraphPad Prism v 5.0 (GraphPad Software, Inc., San Diego, CA, USA).

3. Results

3.1. Leukemia cells aberrantly express TrkA

TrkA expression patterns have not been well characterized in leukemia types other than AML. To determine its potential involvement and role in other leukemia types, we examined its transcription levels in 2143 patients with hematological malignancies through published microarray database mining. We compared the TrkA expression values against the whole-genome expression profiles of these patients and found that TrkA was highly expressed in the majority of hematological malignancies. Strikingly, TrkA expression was significantly upregulated in patients with acute lymphoblastic leukemia (ALL), AML, chronic myelogenous leukemia (CML), and myelodysplastic syndrome (MDS) compared to that in non-leukemic samples. However, its expression in patients with chronic lymphocytic leukemia was lower than that in non-leukemic samples (Fig. 1A). Interestingly, TrkA expression differed according to ALL subtype, being significantly upregulated in B-cell ALL and pro-B ALL, but downregulated in B-cell childhood ALL and T-cell ALL (Fig. 1B). Based on these observations, we evaluated TrkA expression using bone marrow samples from patients with MDS ($n = 5$) or secondary AML evolving from MDS ($n = 5$). TrkA expression was elevated in four of five (80%) samples from patients with MDS compared to those with normal bone marrow. In addition, 100% of the AML samples evolving from MDS contained a level of TrkA mRNA above that of normal bone marrow, suggesting a crucial role for TrkA in the development of MDS rather than MDS progression (Fig. 1C).

Next, we evaluated TrkA expression in a panel of established AML, ALL, and CML cell lines using the TrkA probes for Taqman quantitative RT-PCR. TrkA was highly expressed in a variety of human leukemic cell lines, including AML cell lines (Jurket, HL-60, ML-1, and NB-4), CML cell lines (K562, KU-812, and Meg01), and an ALL cell line (CCRF-HSB2) (Fig. 1D). The expression patterns in these cell lines were consistent with that in the microarray database (Fig. 1A). Together, our findings show that TrkA expression is upregulated in the vast majority of clinical leukemia types (AML, ALL, CML, and MDS), suggesting that TrkA may play an important role in hematological malignancies including AML.

3.2. TrkA induces PLK-1 and Twist-1 expression

We set out to determine how TrkA contributes to leukemogenesis by suppressing its expression in CML cell lines (K562, KU-812, and Meg01). As shown in Fig. S1, TrkA–shRNAs suppressed the expression of endogenous TrkA in the CML cell lines. To gauge the contribution of TrkA to leukemogenesis, we evaluated whether it regulates PLK-1 and Twist-1. PLK-1 is a key effector of cell division and its overexpression in several cancers is linked with a negative prognosis [15]. Another novel key prognostic factor in leukemogenesis, Twist-1, is overexpressed in patients with CML who later develop cytogenetic resistance to imatinib, where Twist-1 overexpression is involved in the resistance phenotype [16]. We found that stable knockdown of TrkA in K562, KU812, and Meg01 cells led to systematic decreased expression of PLK-1 and Twist-1 at both the RNA and protein levels (Figs. 2A and S2A). In addition, to determine whether activated TrkA is an important mediator of the tumorigenic potential of leukemia, we examined whether pharmacologic inhibition of TrkA with K252a, an inhibitor of Trk tyrosine kinases, would influence the ability of

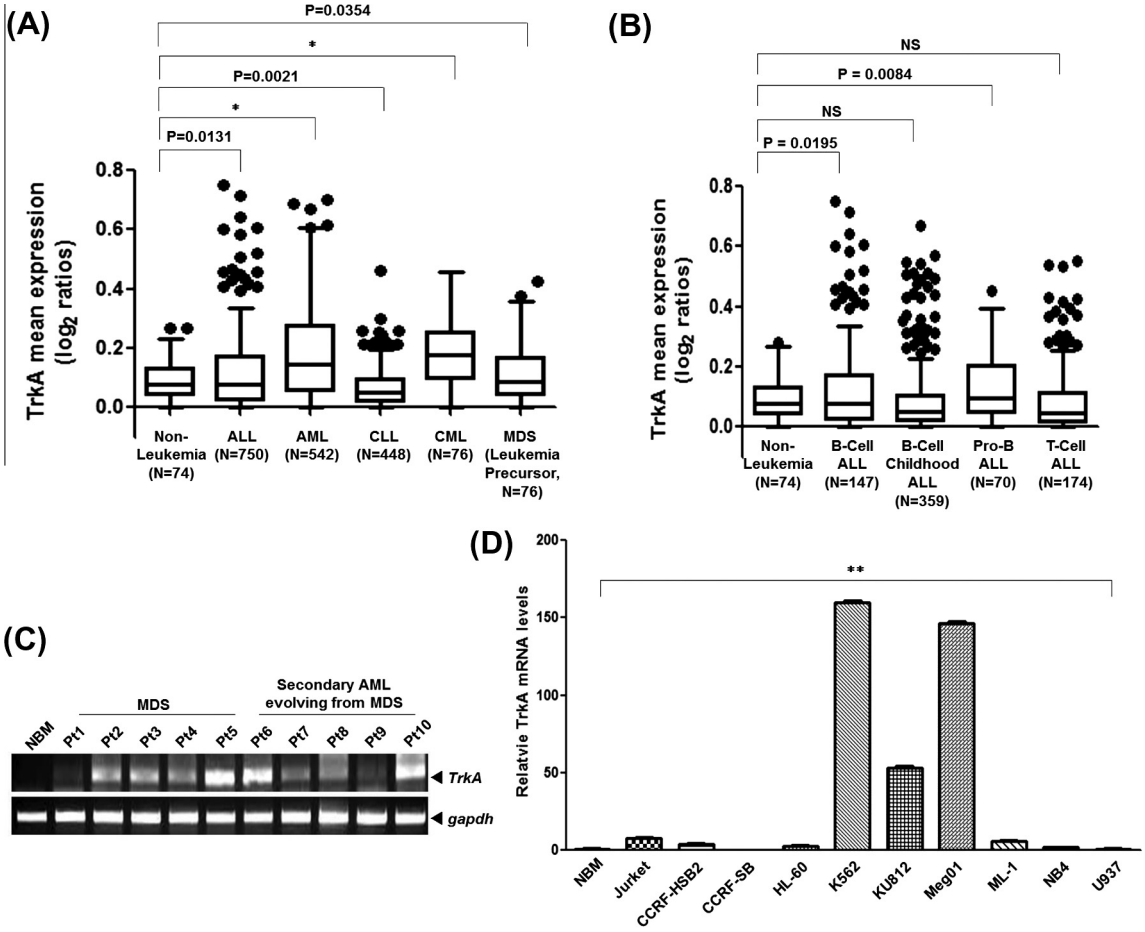

Fig. 1. Expression pattern of TrkA in human leukemia cells and human leukemia samples. (A) *TrkA* gene expression is correlated with leukemia subtypes. Box-and-whisker (Tukey) plots for mean expression of *TrkA* in samples from human patients with leukemia compared to those without leukemia. *TrkA* gene level was extracted from the dataset and averaged for each tumor. Points below and above the whiskers are drawn as individual dots. $^{*}P < 0.0001$ by Student's *t*-test. (B) *TrkA* gene expression is correlated with acute lymphoblastic leukemia (ALL) subtypes. Box-and-whisker (Tukey) plots for mean *TrkA* expression in samples of human patients with ALL compared to those without leukemia. The *TrkA* gene level was extracted from the dataset and averaged for each tumor. Points below and above the whiskers are drawn as individual dots. $^{*}P < 0.0001$ by Student's *t*-test. NS, not significant. (C) *TrkA* expression in 10 patients with myelodysplastic syndrome (MDS). Five patients with MDS (pt. 1–5) and 5 with secondary AML evolving from MDS (pt. 6–10) were compared to normal bone marrow (NBM). The endogenous *gapdh* mRNA level was measured as an internal control (D) *TrkA* mRNA expression in a panel of human normal cord blood (NBM) and leukemia cell lines was examined via TaqMan real-time quantitative polymerase chain reaction analysis. The endogenous 18S mRNA level was measured as an internal control. Data are presented as mean ± standard deviation. $^{**}P < 0.05$ by Student's *t*-test.

leukemic cells to decrease PLK-1 and Twist-1 expression. The inhibition of TrkA activation by K252a treatment significantly reduced PLK-1 and Twist-1 expression (Figs. 2B and S2B). These results show that the activation of TrkA contributes significantly to hematological malignancies.

3.3. TrkA induces a pervasive and sustained progression of leukemia and survival through activation of Akt

Twist-1 transcriptionally upregulates AKT2 in breast cancer, leading to increased migration and invasion [17]. Moreover, protein kinase B (PKB) (PKB/AKT) phosphorylates one of the anti-apoptotic proteins, transcription factor Twist-1, at Ser42. In addition, cells expressing Twist-1 display inefficient p53 upregulation in response to DNA damage induced by γ-irradiation or the genotoxic drug adriamycin and this influences activation of p53 target genes, such as $p21^{Waf1}$ and *Bax*, leading to aberrant regulation of the cell cycle and inhibited apoptosis [18]. Inhibiting PLK-1 selectively reduces proliferation and induces apoptosis in leukemic cells. In addition, inhibiting or depleting PLK-1 using pharmacological and siRNA approaches decreases phosphorylation of two mTOR substrates in leukemic cells [15].

Therefore, we speculated that the TrkA and Akt/mTOR pathways may be functionally linked to regulate hematological malignancies and the survival of leukemia. To better understand the mechanism underlying the regulation of PLK-1 and Twist-1 by TrkA, we examined whether inhibiting Akt activity in the presence of TrkA would regulate PLK-1 or Twist-1. As shown in Figs. 3A and S3A, PLK-1 and Twist-1 expression levels decreased markedly after treating K562, KU812, and Meg01 leukemia cells with LY294002, an inhibitor of Akt kinase. Next, we examined whether TrkA is involved in the activation of the AKT/mTOR pathway. As shown in Figs. 3B and S3B, TrkA knockdown in K562, KU812, and Meg01 cells led to markedly decreased Akt and mTOR phosphorylation levels compared to those in control shRNA cells. Interestingly, mTOR protein level was also markedly reduced after TrkA knockdown, suggesting that TrkA regulates mTOR activation by inducing mTOR expression. Moreover, K562, KU812, and Meg01 cells treated with the Trk tyrosine kinase inhibitor K252a significantly reduced P-mTOR and P-Akt expression levels (Figs. 3C and S3C). Furthermore, K562 cells that were not treated with K252a or K562 control shRNA cells proliferated well, whereas K562 cells treated with K252a or K562 TrkA shRNA cells had significantly less growth (Fig. S4A and B). To further identify the TrkA inhibition mechanism of apoptosis, we investigated the activation of caspase-3 and

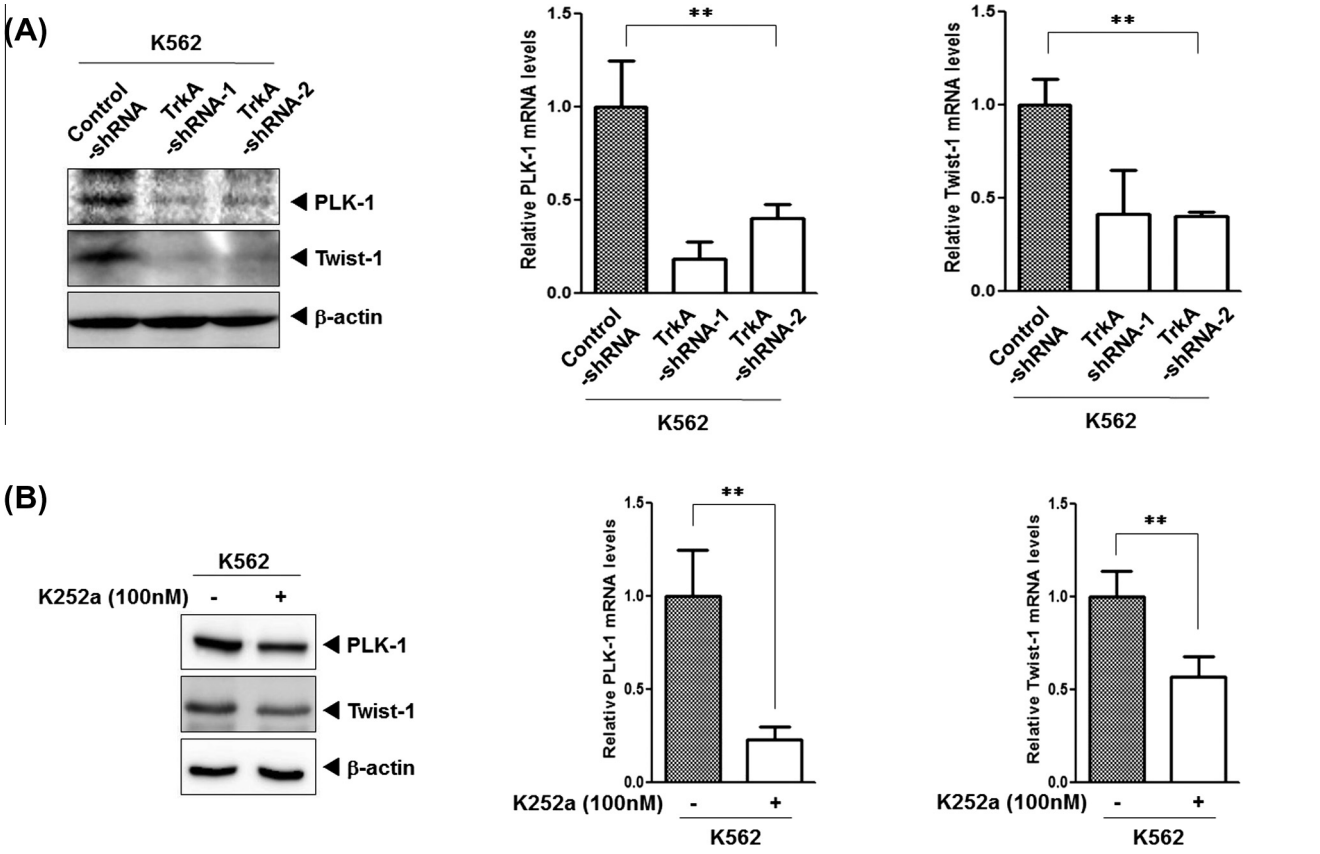

Fig. 2. TrkA induces leukemogenesis by upregulating PLK-1 and Twist-1 as downstream targets. (A) Relative expression levels of PLK-1, and Twist-1 were examined with immunoblotting and quantitative RT-PCR in K562, KU812, and Meg01 control-shRNA or TrkA–shRNA cells. Data are presented as mean ± standard error (SE). $^{**}P < 0.05$ as determined by the Student's *t*-test. (B) Relative expression levels of PLK-1 and Twist-1 were examined with immunoblotting and quantitative RT-PCR in K562, KU812, and Meg01 cells after K252a treatment. Data are presented as mean ± SE. $^{**}P < 0.05$ as determined by the Student's *t*-test.

cleavage of PARP. As shown in Fig. S4C and D, K562 TrkA shRNA cells, and pharmacologically inhibiting TrkA with K252a, significantly increased activation of caspase-3 and cleavage of PARP (an endogenous substrate of caspase-3) compared to K562 control shRNA cells and untreated K252a. Moreover, we examined whether pharmacologically inhibiting TrkA with K252a (an inhibitor of Trk tyrosine kinases) would influence the ability of K562 cells to survive and proliferate. Indeed, K562 cells treated with K252a grew more slowly than parental cells (Fig. S4E). To further characterize the inhibition of apoptosis by TrkA, we assessed the translocation of phosphatidylserine using Annexin V. As shown in Fig. S4F and G, the number of early apoptosis cells (Annexin V-positive) increased up to 1.5-fold in K562 cells treated with K252a and fivefold in K562 TrkA shRNA-expressing cells but was markedly decreased in K562 cells. This result suggests that the activation of Akt and mTOR, as well as total mTOR, by TrkA inhibits apoptosis. These results further demonstrate the important role of endogenous TrkA in the regulation of leukemia proliferation and progression.

3.4. c-Src is required to induce PLK-1 and Twist-1 through TrkA-induced Akt activation

Src kinase is a proto-oncogene that plays a prominent role in leukemogenesis [19]. In addition, c-Src is directly associated with Akt [20]. However, the correlation between TrkA and c-Src remains unknown in leukemia. These results prompted us to examine whether TrkA induces PLK-1 and Twist-1 by activating PI3K/Akt cascades via activation of c-Src.

To investigate whether TrkA modulates c-Src expression, we examined the expression of c-Src in TrkA knockdown cells and after K562a treatment. c-Src expression was not affected by TrkA. However, c-Src phosphorylation levels decreased markedly in TrkA knockdown cells and following K562a treatment, suggesting that TrkA activates c-Src in leukemic cells (Figs. 4A and B, S5A and B). Next, we assessed whether TrkA and c-Src are physically associated. An interaction between these two proteins was readily detected (Figs. 4C and S5C). We used a series of c-Src deletion constructs to identify the functional domain of c-Src responsible for the interaction with TrkA. The C-terminal 275–360 amino acids of c-Src, which include the ATP binding domain, are required for TrkC interaction (Fig. 4D). These data demonstrate that the activation of c-Src by TrkA depends on the interaction between c-Src and TrkA. To test the role of c-Src in the induction of PLK-1 and Twist-1 in TrkA signaling, we examined whether inhibiting c-Src activity in the presence of TrkA would regulate PLK-1 or Twist-1. As shown in Figs. 4E and S5D, c-Src, Akt, and mTOR phosphorylation levels decreased markedly following SU6656 treatment. In addition, PLK-1 and Twist-1 expression levels decreased markedly after SU6656 treatment. These data demonstrate that activating the Akt/mTOR cascade and inducing PLK-1 or Twist-1 by c-Src activity depends on the direct interaction between TrkA and c-Src.

4. Discussion

A TrkA mutation conferring ligand-independent progrowth and prosurvival activity has been documented in patients with AML. However, the functional role and involvement of TrkA in other leukemia types remains largely unknown. In the present study,

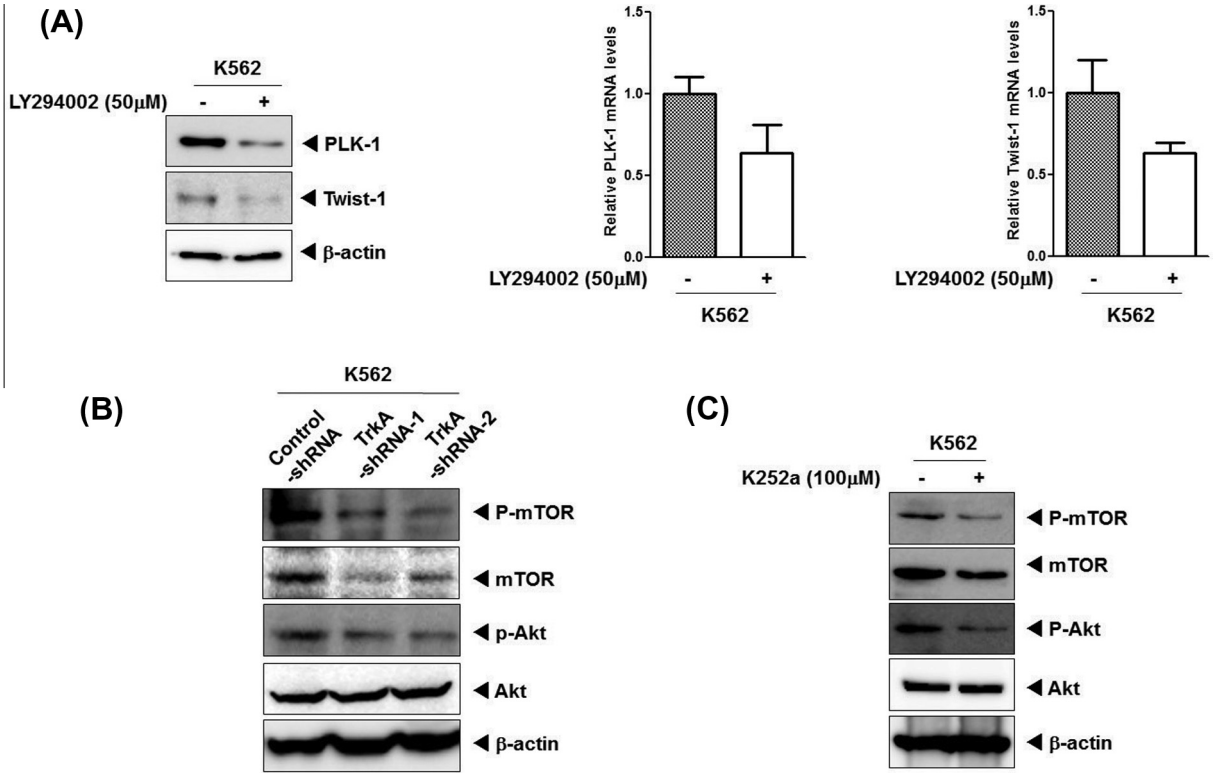

Fig. 3. TrkA regulates PLK-1 and Twist-1 expression by activating the Akt/mTOR pathway. (A) Relative PLK-1 and Twist-1 expression levels were examined with immunoblotting and quantitative RT-PCR in K562 cells after LY294002 treatment. Data are presented as mean ± standard error. $^{**}P < 0.05$ as determined by the Student's *t*-test. (B) Cell extracts of K562 control-shRNA, or TrkA–shRNA cells were analyzed by immunoblotting with antibodies against the indicated proteins. (C) K562 cell lysates were analyzed by immunoblotting with antibodies against the indicated proteins after treatment with K252a.

we demonstrated that TrkA expression is elevated in the vast majority of leukemia subclasses (AML, CML, and, ALL). In addition, upregulation of TrkA occurred in MDS, which is a clonal hematologic disorder that frequently represents an intermediate disease stage before progression to AML. Our observations are consistent with other published results demonstrating that several genes promote the metastatic behaviors and poor prognosis of aggressive cancers; these include *Slug*, *HOXB13*, *HER2/neu*, and *Goosecoid*, which are expressed in clinical specimens before the appearance of the malignant tumor phenotype [21]. Thus, it is possible that TrkA primes cells to express aggressive phenotypes in human leukemia, which manifests in the context of subsequent changes. These results suggest that TrkA plays an important role in the vast majority of leukemia types.

The function of TrkA in leukemogenesis remains unclear. We delineated its role in hematological malignancies. Knockdown of TrkA and treatment with a Trk kinase inhibitor or PI3K/Akt kinase inhibitor significantly inhibited PLK-1 and Twist-1 expression, both of which are activated at the beginning of leukemogenesis. PLK-1 inhibitors counteract cell cycle progression and growth in solid tumors or hematological malignancies [22,23]. In addition, Twist-1 is a powerful biomarker for early detection of TKI resistance and is overexpressed in CML diagnostic samples of patients who later developed cytogenetic resistance to imatinib through a yet-unknown mechanism [16]. Moreover, our results indicate that TrkA is a crucial positive regulator of the growth and survival of leukemic cells. In addition, TrkA tyrosine kinase activity leads to the continuous proliferation of leukemic cells. Adding to our recent observation that TrkA inhibits apoptosis of leukemic cells, we further dissected the role of TrkA in the survival of leukemic cells by gain-of-function knockdown of TrkA. Knockdown of TrkA decreased activation of Akt and mTOR expression, and TrkA tyrosine kinase activity played an important role in the induction of PLK-1 and Twist-1 function through activation of Akt and mTOR.

Our study further uncovered c-Src as an integral component in the induction of PLK-1 and Twist-1 by activating Akt/mTOR cascades through TrkA in patients with hematological malignancies. TrkA markedly increased c-Src phosphorylation levels, which promoted cell survival and leukemogenesis through induction of PLK-1 and Twist-1 function via activation of the Akt/mTOR cascade (Fig. S6). These findings represent an unexpected convergence of two lines of recent research. Previous work in other cell systems suggests that Erk and Akt in the presence of TrkA are phosphorylated to a much greater degree in cells treated with imatinib (which only inhibits BCR–ABL) than in untreated cells [24]. In addition, the Src kinases (Lyn, Hck, and Fgr) remain active following imatinib inhibition of BCR–ABL kinase activity in leukemic cells. This result indicates that Src kinase activation by BCR–ABL is independent of BCR–ABL kinase activity and provides an explanation for the reduced effectiveness of the BCR–ABL kinase activity inhibitors in Philadelphia chromosome-positive acute lymphoblastic leukemia [25]. However, the correlation between TrkA and c-Src in leukemia had remained unknown and none of these findings hinted at a link between these two sets of phenomena. Our results suggest that inducing PLK-1 and Twist-1 through c-Src activation by TrkA may promote resistance to imatinib.

In addition, activation of nuclear factor-κB by Akt upregulates Snail expression, which is involved in leukemia formation and/or progression in gain-of-function mouse models [26,27]. In summary, our results provide important molecular insights into how TrkA-controlled hematological malignancies are modulated and coordinated in leukemia. We conclude that TrkA serves as a master enforcer of leukemia cell fate and leukemogenesis, highlighting TrkA as a potential target for early therapeutic intervention in hematological malignancies.

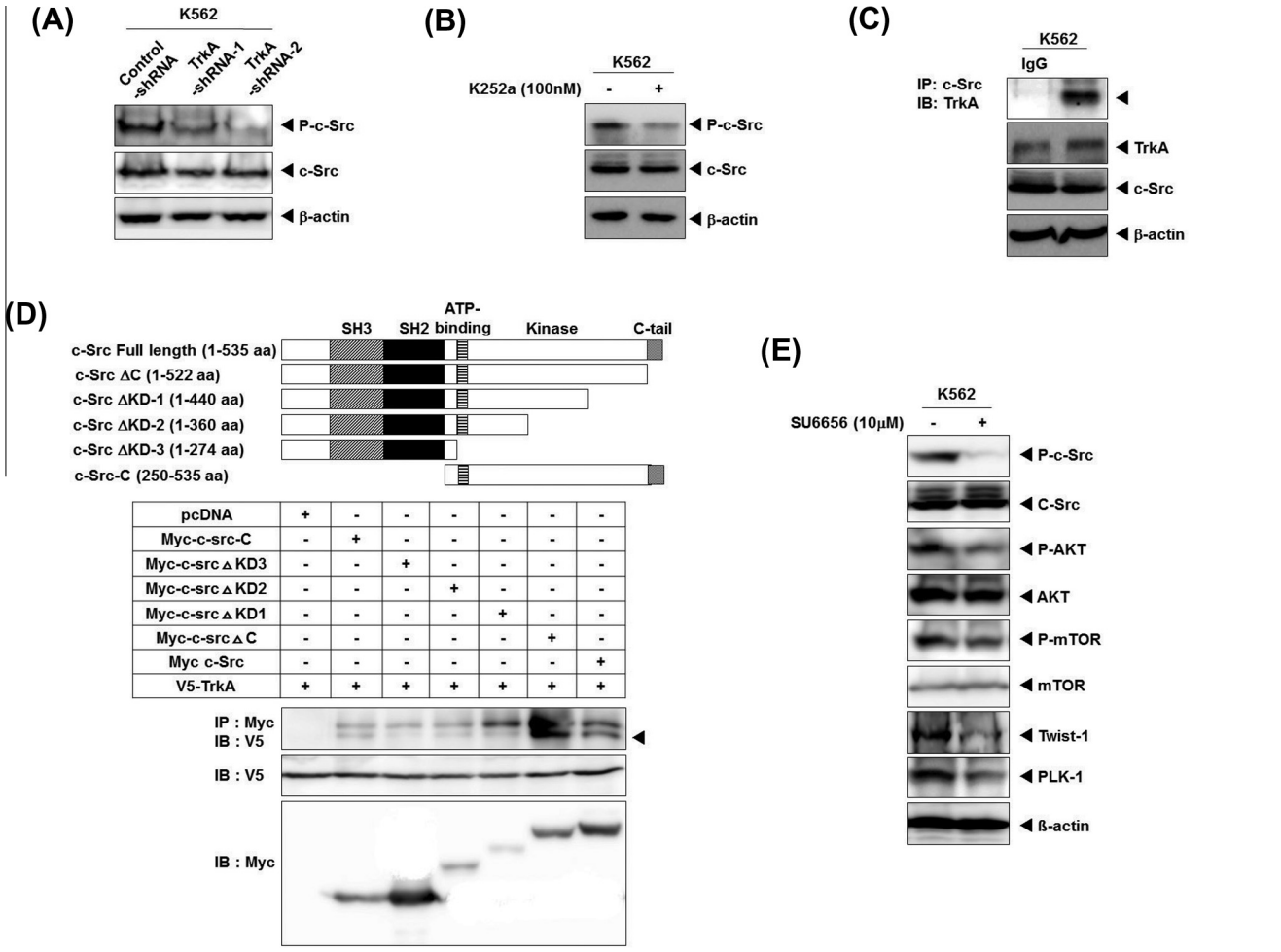

Fig. 4. TrkA-mediated c-Src activation induces PLK-1 and Twist-1 through the Akt/mTOR cascade. (A) Expression of phospho-c-Src and c-Src was examined in K562 control-shRNA or TrkA–shRNA cells. (B) Expression of phospho-c-Src, and c-Src was examined with immunoblotting in K562 cells after K252a treatment. (C) TrkA associated with c-Src. K562 cell lysates were immunoprecipitated with anti-c-Src or IgG antibody, followed by immunoblot analysis of TrkA. (D) Identification of the c-Src region responsible for the interaction with TrkA. Immunoprecipitation analyses of V5-TrkA and Myc-tagged c-Src proteins in 293T cells. (E) K562 cell lysates were analyzed by immunoblotting with antibodies against the indicated proteins after treatment with SU6656.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.10.082.

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