Research Article

The Tyrosine Phosphatase Shp2 Interacts with NPM-ALK and Regulates Anaplastic Lymphoma Cell Growth and Migration

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Abstract

Anaplastic large cell lymphomas (ALCL) are mainly characterized by the reciprocal translocation t(2;5)(p23;q35) that involves the *anaplastic lymphoma kinase* (*ALK*) gene and generates the fusion protein NPM-ALK with intrinsic tyrosine kinase activity. NPM-ALK triggers several signaling cascades, leading to increased cell growth, resistance to apoptosis, and changes in morphology and migration of transformed cells. To search for new NPM-ALK interacting molecules, we developed a mass spectrometry–based proteomic approach in HEK293 cells expressing an inducible NPM-ALK and identified the tyrosine phosphatase Shp2 as a candidate substrate. We found that NPM-ALK was able to bind Shp2 in coprecipitation experiments and to induce its phosphorylation in the tyrosine residues Y542 and Y580 both in HEK293 cells and ALCL cell lines. In primary lymphomas, antibodies against the phosphorylated tyrosine Y542 of Shp2 mainly stained ALK-positive cells. In ALCL cell lines, Shp2-constitutive phosphorylation was dependent on NPM-ALK, as it significantly decreased after short hairpin RNA (shRNA)–mediated NPM-ALK knock down. In addition, only the constitutively active NPM-ALK, but not the kinase dead NPM-ALK^{K210R}, formed a complex with Shp2, Gab2, and growth factor receptor binding protein 2 (Grb2), where Grb2 bound to the phosphorylated Shp2 through its SH2 domain. Shp2 knock down by specific shRNA decreased the phosphorylation of extracellular signal-regulated kinase 1/2 and of the tyrosine residue Y416 in the activation loop of Src, resulting in impaired ALCL cell proliferation and growth disadvantage. Finally, migration of ALCL cells was reduced by Shp2 shRNA. These findings show a direct involvement of Shp2 in NPM-ALK lymphomagenesis, highlighting its critical role in lymphoma cell proliferation and migration. [Cancer Res 2007;67(9):4278–86]

Introduction

Anaplastic large cell lymphomas (ALCL) represent a subgroup of non–Hodgkin's lymphomas characterized by the frequent chromosomal translocation t(2;5)(p23;q35). As a consequence, the *anaplastic lymphoma kinase* (*ALK*) gene fuses to *nucleophosmin* (*NPM*) gene, resulting in the aberrant expression of the chimeric protein NPM-ALK (1). The NPM-ALK fusion protein is composed of the entire catalytic domain of ALK fused to the NPM oligodimerization motif that leads to the dimerization of the protein and to its constitutive autophosphorylation (2). NPM-ALK plays a key role in ALCL lymphomagenesis and it has been shown to cause lymphocyte and fibroblast transformation *in vitro* and *in vivo*, to induce cellular proliferation, to inhibit apoptosis, to modify the cytoskeleton, and to deregulate cell-cell adhesion and migration (3–6). NPM-ALK kinase is essential for the survival and growth of ALK-positive ALCLs *in vitro* and *in vivo* (7, 8) and exerts its transforming capacity through phosphorylation and activation of several cellular pathways such as Ras/extracellular signal-regulated kinase (Erk), PLC-γ, phosphatidylinositol 3-kinase (PI3K)/Akt and the Jak3/signal transducers and activators of transcription 3 (Stat3; refs. 9–13). Activation of Ras/Erk pathway through the recruitment of different adaptor proteins such as SHC and IRS1 leads to increased ALK-positive ALCL cell growth (14), whereas phosphorylation of Stat3 and stimulation of PI3K pathway prevent cell death and result in increased cell survival (10, 11). As the molecular mechanisms mediating the oncogenic properties of NPM-ALK remain in part unclear, the identification of new interacting molecules and substrates could further elucidate NPM-ALK–mediated transformation and, eventually, reveal new potential targets for drug therapy in lymphomas. Mass spectrometry (MS)–based proteomics is a powerful tool to study protein-protein interactions and, in general, to identify molecules involved in intracellular signaling (15–17). We used a proteomic approach based on immunoaffinity purification followed by nanoflow reverse-phase liquid chromatography-tandem MS (LC-MS/MS) to identify NPM-ALK interacting molecules and substrates. The same approach has allowed the identification of p130Cas as a new NPM-ALK downstream molecule required for NPM-ALK cytoskeleton reorganization and transformation (6). In this study, we report the identification of the SH2 domain–containing tyrosine phosphatase Shp2 and elucidate its role in NPM-ALK–mediated transformation. We show that Shp2 undergoes phosphorylation after association with NPM-ALK in human ALCL cells and that its activation is dependent on ALK tyrosine kinase activity. More importantly, our findings identify Shp2 as a mediator of NPM-ALK oncogenic properties by promoting ALCL cell proliferation and migration.

Materials and Methods

Cell lines, cell cultures, and reagents. For the *in vitro* experiments, the following cell lines were used: Karpas 299, SU-DHL-1, and TS (a subclone of SUP-M2; NPM-ALK–positive human lymphoid cells), K562 (chronic myeloid leukemia cell line), CEM, and Jurkat (T lymphoblastic cell lines), all obtained

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from New York University and maintained in RPMI 1640 (BioWhittaker) containing 10% FCS (7).

Human embryonal kidney (HEK) cells 293T and 293T-Rex Tet-On (Invitrogen) were cultured in DMEM supplemented with 10% FCS. In 293 Tet systems, the working concentration of tetracycline in the medium was 1 μg/mL.

Inducible ALK short hairpin (shRNA) SU-DHL-1 and TS (SU-DHL-1 TTA A5 and TS TTA A5, respectively) cells were obtained by cotransduction with pLV-tTRKRAB (TTA) vector (18) and pLVTHM vector containing the H1 promoter ALK-shRNA (A5) cassette (7). These cells undergo NPM-ALK silencing when 1 μg/mL doxycycline is added to the medium for at least 72 h.

Immunoprecipitation, trypsin digestion, and MS analysis. Anti–phosphotyrosine immunoprecipitation before MS analysis and protein identification was done as previously described (6). Briefly, HEK 293T-Rex Tet-On cells transfected with NPM-ALK or a kinase dead mutant control NPM-ALK^{K210R} were grown to confluency and induced with 1 μg/mL tetracycline for 24 h. Cells were lysed in 20 mmol/L Tris (pH 7.4), 150 mmol/L NaCl, 5 mmol/L EDTA, 0.1% Triton X-100 in the presence of protease and phosphatase inhibitors. Ten milligrams of cleared cell lysate were immunoprecipitated with 50 μg of agarose-conjugated monoclonal 4G10 (Upstate Biotechnology) and 10 μg monoclonal PY20 (Transduction Laboratories) and incubated rotating at 4°C overnight. Immunoprecipitated samples were resolved on SDS-PAGE and silver stained. Bands of interest were excised from the gel and subjected to in-gel reduction, alkylation, and digestion with trypsin (Promega). Samples were analyzed by LC-MS/MS using a nanoflow high performance liquid chromatography system (Ultimate; LC Packings) interfaced to electrospray quadrupole time-of-flight (Q-TOF) tandem mass spectrometers (QTOF Ultima or QTOF Micro, Waters/Micromass). Protein identification was achieved by analyzing MS/MS spectra using the Mascot software for searching the National Center for Biotechnology Information nonredundant protein database.

Cell lysis, coimmunoprecipitation, and Western blot analysis. Total cellular proteins were extracted with 20 mmol/L Tris-HCl (pH 7.4), 150 mmol/L NaCl, 5 mmol/L EDTA, 1% Triton X-100, 1 mmol/L phenylmethylsulfonyl fluoride, 10 mmol/L NaF, 1 mmol/L Na_3VO_4, and protease inhibitors (Roche). Samples were resuspended in the lysis solution and incubated at 4°C for 30 min. Cell lysates were clarified by centrifugation at 10,000 × *g* (13,000 rpm) for 10 min at 4°C and the supernatants were collected and assayed for protein concentration using the Bio-Rad protein assay method. Thirty micrograms of proteins were run on SDS-PAGE under reducing conditions.

For immunoprecipitation experiments, 0.5 to 2 mg whole-cell lysates were precleared with 30 μL protein A–Sepharose beads (Amersham) for 1 h at 4°C, then incubated with 1 to 5 μg of monoclonal antibody or 5 to 10 μg of polyclonal antibody at 4°C for 1 h or overnight. Immune complexes were collected on 40 μL protein A–Sepharose beads, and the beads were washed thrice with lysis buffer. Bound proteins were recovered by boiling in Laemmli sample buffer and resolved on SDS-PAGE.

For immunoblotting, proteins were transferred to nitrocellulose, incubated with the specific antibody, and detected with peroxidase-conjugated secondary antibodies and enhanced chemiluminescent reagent (Amersham).

The following antibodies were used: anti-ALK (1:2,000; Zymed), monoclonal anti-ALK (1:4,000; Zymed), anti–phospho-Tyr (PY20; 1:1,000; Transduction Laboratories), agarose-conjugated 4G10 (Upstate Biotechnology), polyclonal anti–growth factor receptor binding protein 2 (Grb2; 1:2,000; Santa Cruz Biotechnology), anti–phospho-ALK Y1604 (1:1,000; Cell Signaling Technology), anti-Shp2 (1:1,000; Cell Signaling Technology), anti–phospho-Shp2 Y542 (1:1,000; Cell Signaling Technology), anti–phospho-Shp2 Y580 (1:1,000; Cell Signaling Technology), anti-Gab2 (1:2,000; Upstate Biotechnology), anti–phospho-Gab2 (1:1,000; Cell Signaling Technology), anti-Stat3 (1:1,000; Cell Signaling Technology), anti–phospho-Stat3 (1:1,000; Cell Signaling Technology), anti–phospho-eIF2α (1:1,000; Cell Signaling Technology), anti–phospho-Erk1/2 (1:500; Cell Signaling Technology), anti-Erk1/2 (1:1,000; Santa Cruz Biotechnology), anti–phospho-Src Y416 (1:1,000; Cell Signaling Technology), anti-Src (1:1,000; Santa Cruz Biotechnology), and anti-tubulin (1:10,000; Santa Cruz Biotechnology).

Immunohistochemical and immunofluorescence stainings. Three primary cases of ALK-positive ALCLs were retrieved from the archives of the Department of Pathology of the University of Torino. Immunohistochemical stainings were done on formalin-fixed, paraffin-embedded or frozen tissues of ALCLs. All diagnosis were confirmed by CD30 and ALK stainings. Sections were incubated with primary antibodies anti-ALK (1:1,000; Zymed), anti-CD30 (clone BerH2, 1:100, DAKO), and against the phosphotyrosine Y542 of Shp2 (1:100, Cell Signaling Technology). Bound complexes were revealed by the DAKO's Envision+ system on a semi-automated immunostainer. For immunofluorescence stainings, 293T cells were grown for 12 h on glass coverslips and transiently transfected with GFP-NPM-ALK or the kinase dead mutant GFP-NPM-ALK^{K210R} for 24 h. Samples were fixed in PBS containing 4% paraformaldehyde at room temperature for 10 min and permeabilized with PBS containing 0.3% Triton X-100 for 5 min. Coverslips were incubated with PBS containing 3% bovine serum albumin for 1 h at room temperature and stained with primary antibody for 1 h followed by tetramethyl rhodamine isothiocyanate–conjugated secondary antibody (1:400; Sigma) for 1 h at room temperature.

Primary antibodies anti-ALK (Zymed), anti–phospho-ALK Y1604 (Cell Signaling Technology), anti-Shp2 (Cell Signaling Technology), and anti–phospho-Shp2 Y542 (Cell Signaling Technology) were diluted 1:50 before use. Nuclei were stained 10 min at room temperature with HOECHST (300 ng/mL; Sigma). Coverslips were mounted on slides and images were acquired on a Leica DM LA upright microscope equipped with a DC300F camera and were analyzed with IM 50 software.

shRNA sequences, DNA constructs, and mutagenesis. NPM-ALK or the kinase dead mutant NPM-ALK^{K210R} were cloned into pcDNA5TO vector (Invitrogen) at *Hin*dIII/*Xho*I sites and stably transfected into 293T-Rex Tet-On cells using Effectene reagents (Qiagen) as previously described (6).

To obtain the Shp2 construct, total cDNA from ALCL cell lines was amplified using specific primers flanking the Shp2 coding region. Primers were as follows: Shp2 forward 5′-CGGAGCCTGAGCAAGGA-3′ and Shp2 reverse 5′-CTGAAGTTTTGGCAGGTTTTC-3′. PCR products were cloned into PCRII vector using the TA-cloning system (Invitrogen) and sequenced. For transient transfection in HEK 293T cells, Shp2 was digested to obtain a *Bam*HI/*Eco*RV fragment and subcloned in the plasmid vector pcDNA3 at the same cloning sites (Invitrogen). For lentiviral vectors, Shp2 construct were digested to obtain a *Bam*HI/*Eco*RV fragment, blunted with Klenow enzyme (Roche), and cloned into the *Sal*I-*Sma*I–blunted sites of the bidirectional lentiviral vector pCCL.sin.cPPT.polyA.CTE.eGFP (described in ref. 19) and kindly provided by Dr. Luigi Naldini (San Raffaele Scientific Institute, Milan, Italy).

Grb2^{wt} and the dominant-negative Grb2^{P49L} (mutated in the NH_2-terminal SH3 domain) and Grb2^{R86K} (mutated in the SH2 domain) were kindly provided by Dr. A. Pellicer (New York University, New York, NY).

Human Shp2-specific shRNA sequences were designed and cloned into pLVTHM vector containing the H1 promoter kindly provided by Dr. Trono [School of Life Sciences, National Center for Competence in Research, École Polytechnique Fédérale de Lausanne (EPFL), Lausanne, Switzerland; ref. 18] as previously described (7). The sense strand of Shp2-shRNA that we used is 5′-GGGCCAGAGCAGTCAGTAA-3′.

Transfection, lentivirus production, and cell infection. shRNA Shp2 lentiviruses were obtained by cotransfection of HEK 293T with pLVTHM vector and pMD$_2$VSV-G, pCMV8.74 and pRSV.REV plasmid vectors (18) into human 293T cell line (Invitrogen). Transfections were done using Effectene reagent (Qiagen). High-titer lentiviral vector stocks were produced in HEK 293T as previously described (7). Briefly, aliquots of virus, supplemented with 8 μg/mL polybrene (Sigma), were used to infect 10×10^4 TS and SU-DHL-1 cells and after 72 h the percentages of transduced cells were analyzed for GFP expression by fluorescence-activated cell sorting (FACS).

Inducible Shp2-shRNA interference SU-DHL-1 and TS cells were obtained by cotransduction with pLVTHM vector containing the H1 promoter Shp2-shRNA cassette and pLV-tTRKRAB vector, as described above.

For cell sorting, cells were resuspended at a concentration of 10×10^6/mL in basic sorting buffer and then sorted for enhanced green fluorescent protein (EGFP) expression on a MoFlo High-Performance Cell Sorter (DAKO Cytomation) as described (6).

Cell cycle analysis and cell apoptosis assay. Cell cycle analysis was carried out by flow cytometry. Cells were washed in PBS and fixed for 1 h in 70% ethanol at 4°C. After washing, cells were treated with RNase (0.25 mg/mL) and stained with propidium iodide (50 μg/mL). The G_1-G_0 phase fraction was calculated using the CellQuest program (BD Pharmigen Biosciences; ref. 7).

Apoptosis was measured by flow cytometry after staining with the mitochondrion-permeable, voltage-sensitive dye tetramethylrhodamine methyl ester (TMRM; Molecular Probes). Cells were washed once in PBS and incubated for 15 min at 37°C in HEPES buffer solution [10 mmol/L HEPES (pH 7.4), 140 mmol/L NaCl, 2.5 mmol/L CaCl] with 200 nmol/L TMRM. Cells were analyzed by FACScan using the CellQuest program (BD Pharmigen Biosciences; ref. 7).

Cell proliferation assay. For cell proliferation assay, 5×10^4 cells/mL were grown in triplicate in 24-well plates in complete RPMI. The number of viable cells was determined each day up to 6 days by trypan blue exclusion.

Chemotaxis assay. Cell migration was evaluated using a 24-well, 8-μm pore size Transwell system plates (Costar). Purified cells were washed once in RPMI 1640 (BioWhittaker) containing 0.1% FCS and then adjusted to 9×10^5 cells in 100 μL in the same medium. Cells (9×10^5) in 100 μL medium were placed in triplicate on the top of the Transwell. Stromal-derived factor-1α (SDF-1α; 100 ng/mL; R&D Systems) in 600 μL of the same RPMI medium was added to the bottom of the Transwell system. After 3-h incubation at 37°C in a 5% CO_2 atmosphere, the inserts of the transwell chambers were removed and the cells that had migrated to the bottom well were collected and counted on a hemacytometer (6).

Coculture and flow cytometry analysis. For coculture experiments, ALCL cells and ALCL cells infected with Shp2 shRNA and control shRNA, respectively, were mixed in a 1:1 ratio and cultured in standard conditions for 3 weeks. The same experiment was carried out for control cells CEM and K562 infected with Shp2 shRNA. GFP expression was checked over time by FACS and normalized against the initial time point (day 0).

Results

Identification of Shp2 by MS. We developed a MS-based proteomic approach to identify new substrates of NPM-ALK. We stably transfected NPM-ALK or the kinase dead mutant NPM-ALK^{K210R} as a control into human 293T-Rex Tet-On cell line. NPM-ALK expression was induced by the addition of tetracycline to the medium (Fig. 1*A*). We did immunoprecipitations with a mixture of anti-phosphotyrosine antibodies and compared the band patterns of NPM-ALK and NPM-ALK^{K210R} samples. The bands present only in the NPM-ALK sample were digested and analyzed by LC-MS/MS. LC-MS/MS analysis allowed the identification of previously known NPM-ALK interacting molecules (PLC-γ, PI3K, Stat3, and p130Cas) and new potential substrates (Fig. 1*B*). The most abundant protein at 76 kDa corresponded to NPM-ALK as confirmed by MS analysis. Duplicate MS/MS analysis of the digested bands resulted in the identification of 46 unique proteins belonging to different Gene Ontology functional groups (data not shown). Among phosphatases, we identified four peptides corresponding to the SH2 domain–containing phosphatase Shp2 with a 6% sequence

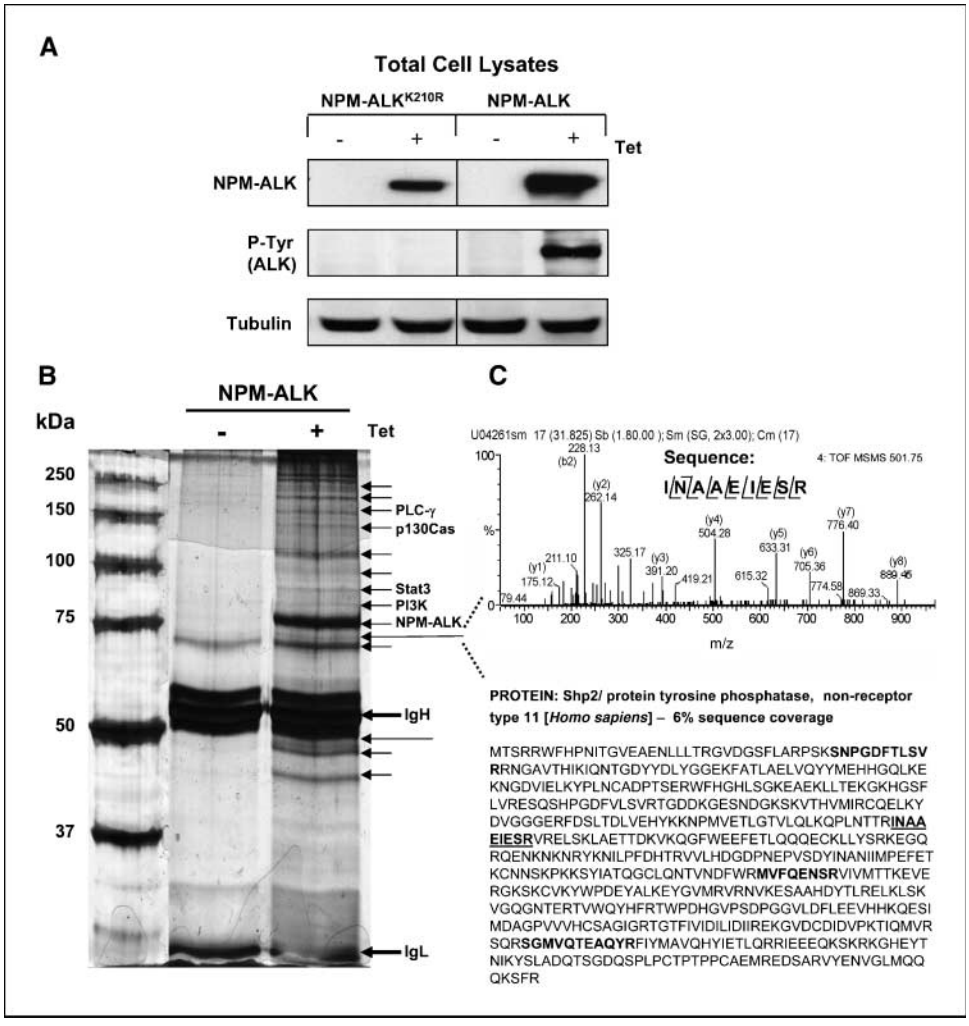

Figure 1. Anti–phosphotyrosine immunoprecipitation and Shp2 identification by MS. *A,* T-REx-293 Tet-On were stably transfected with NPM-ALK or NPM-ALK^{K210R} as control. NPM-ALK expression was induced by adding 1 μg/mL tetracycline to the medium. Total cell lysates were blotted with anti-ALK and anti-phosphotyrosine antibodies. Anti-phosphotyrosine Western blot shows that NPM-ALK was phosphorylated, whereas the dead mutant control NPM-ALK^{K210R} was not. *B,* T-REx-293 Tet-On cells transfected with NPM-ALK were grown in the presence or absence of tetracycline for 24 h. Total cell lysates were immunoprecipitated with a mixture of anti-phosphotyrosine antibodies, run on SDS-PAGE, and silver stained. *Arrows,* bands that were excised from the gel and analyzed by LC-MS/MS. NPM-ALK and known interacting molecules identified by LC-MS/MS are indicated. *C,* MS/MS spectrum of Shp2 peptide 221-INAAEIESR-229. Shp2 was identified by the MS/MS sequencing of four peptides. Percentage coverage of 6% for Shp2 identification is represented by showing the identified peptides (*bold*) in the total protein sequence.

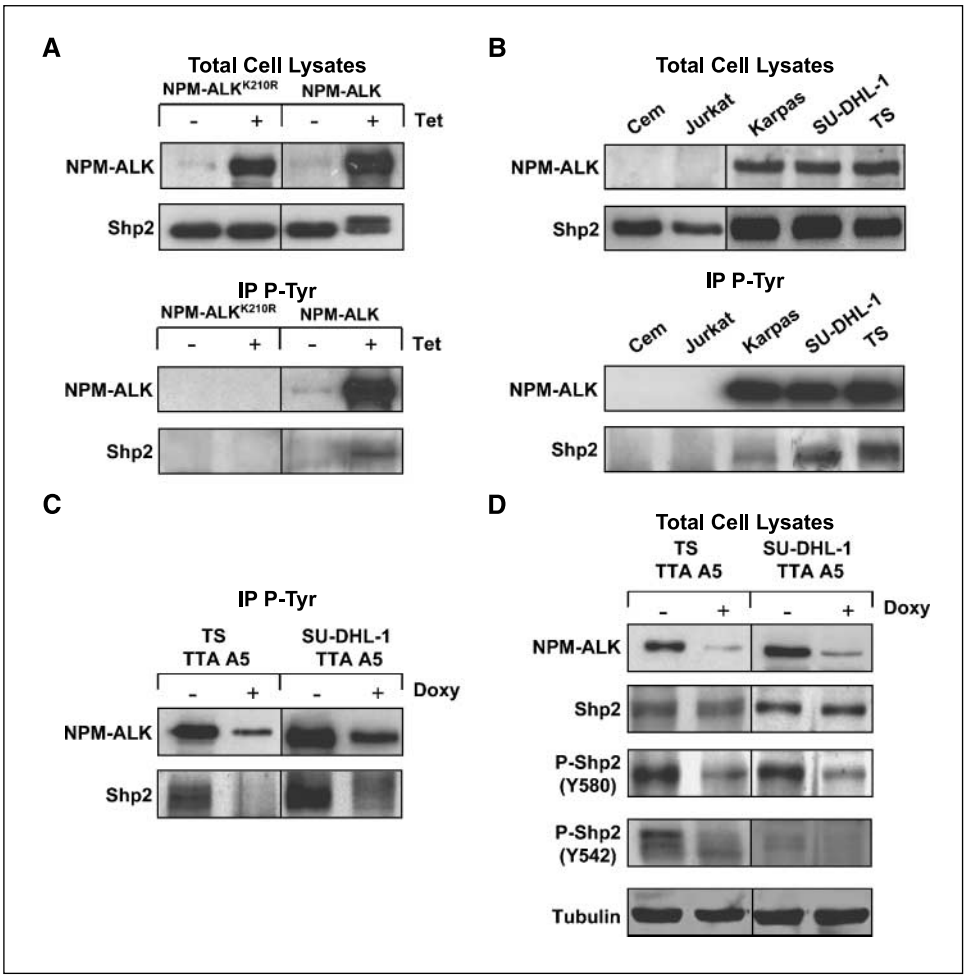

Figure 2. Shp2 phosphorylation is dependent on NPM-ALK tyrosine kinase activity. *A,* Shp2 is phosphorylated in the presence of NPM-ALK but not NPM-ALK^{K210R}. T-REx-293 Tet-On cells were induced with 1 μg/mL tetracycline for 24 h. Total cell lysates were immunoprecipitated (*IP*) with a mixture of monoclonal anti-phosphotyrosine antibodies and blotted with anti-Shp2 antibody. *B,* Shp2 phosphorylation was clearly detectable in ALK-positive lymphoma cell lines (Karpas 299, SU-DHL-1, and TS) compared with controls (CEM and Jurkat). Lymphoid cell lines were immunoprecipitated as described in *A. C* and *D,* NPM-ALK silencing by shRNA decreases Shp2 phosphorylation. TS TTA A5 and SU-DHL-1 TTA A5 were grown in the presence of 1 μg/mL doxycycline and harvested after 72 h. Total cell lysates were immunoprecipitated with a mixture of monoclonal anti-phosphotyrosine antibodies and blotted with an anti-Shp2 and anti-ALK antibodies (*C*), and were immunoblotted with specific anti–phospho-Shp2 antibodies (Y542 and Y580; *D*).

coverage. MS/MS spectrum of the peptide 221-INAAEIESR-229 is shown in Fig. 1*C*. As Shp2 has been described to be involved in the transformation of other hematologic malignancies (20, 21), we decided to further elucidate its role in NPM-ALK–mediated lymphomagenesis.

Shp2 phosphorylation is dependent on NPM-ALK tyrosine kinase activity. Because Shp2 is mutated in a subset of patients with hematologic malignancies (22, 23), we first ruled out the presence of activating mutations of Shp2 in ALCL cell lines by cDNA cloning and sequencing (data not shown). Immunoblotting total cell lysates from 293T cells with anti-Shp2 antibody showed the presence of two protein species, suggesting posttranscriptional modified forms (Fig. 2*A*). Immunoprecipitation assays with anti-phosphotyrosine antibody confirmed that Shp2 was phosphorylated only in the presence of NPM-ALK and not in cells expressing the kinase dead mutant NPM-ALK^{K210R}, indicating that an active kinase domain is required for a functional activation (Fig. 2*A*). Moreover, Shp2 phosphorylation was detectable in ALCL cells compared with ALK-negative cell lines (Fig. 2*B*).

To investigate whether Shp2 phosphorylation was dependent on NPM-ALK tyrosine kinase activity in ALK-positive ALCL cells, we generated a doxycycline-dependent anti-ALK shRNA inducible system to abrogate NPM-ALK expression. TS TTA A5 and SU-DHL-1 TTA A5 cells were cultured in the presence of doxycycline for 72 h to knock down NPM-ALK expression and anti-phosphotyrosine immunoprecipitations showed a marked reduction of Shp2 phosphorylation in both cell lines (Fig. 2*C*). Accordingly, the phosphorylation of both the C-tail tyrosine residues (Y542 and Y580) of Shp2 was significantly reduced after ALK silencing, as detected by immunoblotting with specific antibodies (Fig. 2*D*). We concluded that in ALCL cell lines, Shp2 phosphorylation was strictly correlated to NPM-ALK kinase activity.

By immunofluorescence stainings, only 293T cells transfected with NPM-ALK, but not untransfected or NPM-ALK^{K210R} kinase dead mutant, showed a strong signal for phosphorylated Shp2 (Y542; Fig. 3*A*).

To confirm the findings obtained on ALCL cell lines in primary tissues, we, then, analyzed ALK-positive lymphomas and found that Shp2 was strongly phosphorylated in anaplastic cells also expressing ALK and CD30, whereas surrounding reactive lymphocytes showed a weak to negative staining (Fig. 3*B*).

NPM-ALK forms a complex with Shp2, Gab2, and Grb2. To explore whether Shp2 associates with NPM-ALK, we did NPM-ALK immunoprecipitations in 293T-Rex cells. As shown in Fig. 4*A*, Shp2 associated only with active NPM-ALK, as the binding did not occur in the presence of the kinase dead mutant NPM-ALK^{K210R}. Significantly, the association between the two molecules was also confirmed in ALK-positive cell lines (Karpas 299, SU-DHL-1, and TS; Fig. 4*A*). ALK-negative Jurkat and CEM cells were used as controls. The same results were obtained with anti-Shp2 immunoprecipitations (data not shown).

Previous studies reported that the association of Shp2 to the scaffolding adapter protein Gab2 is a frequent event in response to

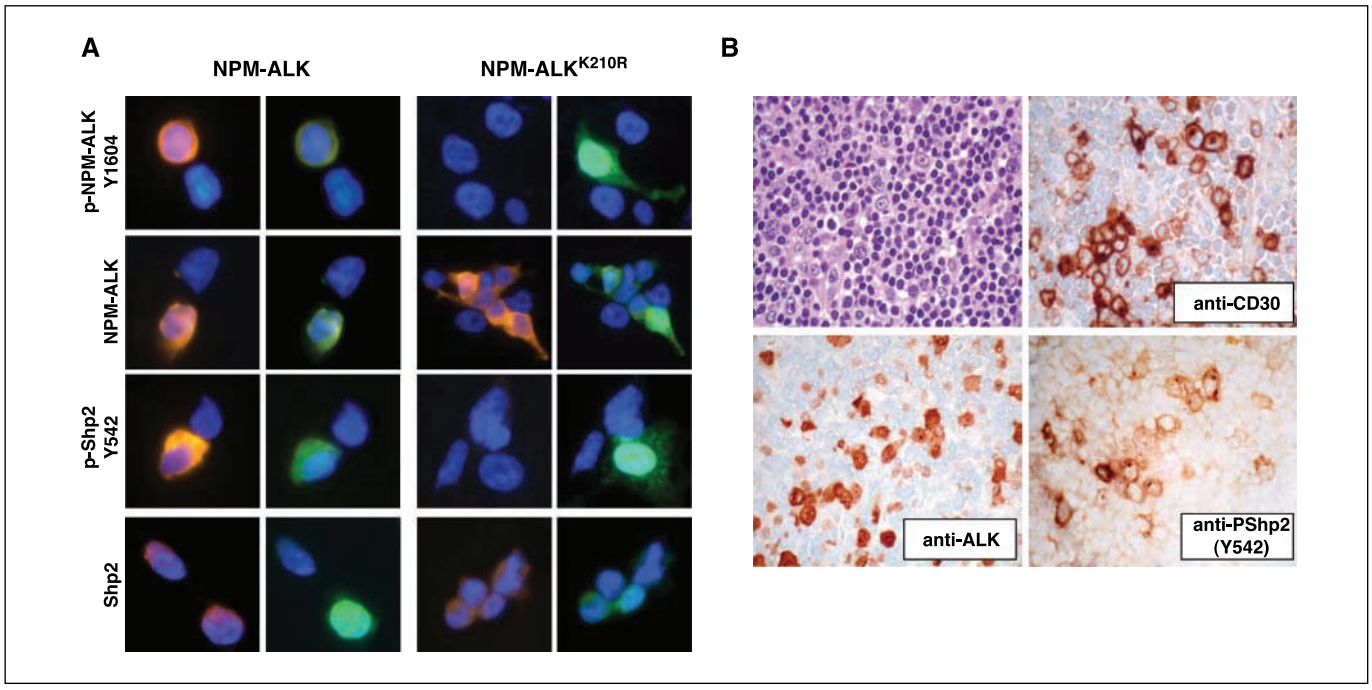

Figure 3. Shp2 is phosphorylated in NPM-ALK–expressing 293T cells and in primary ALK-positive human lymphomas. *A,* immunofluorescence stainings of 293T cells transfected with NPM-ALK (*left*) or NPM-ALK^{K210R} (*right*) were done by the indicated specific antibodies (*red fluorescence*). *Green fluorescence,* EGFP reporter protein encoded by the transfection vectors. All images were taken with a ×100 oil-immersion objective. *B,* formalin-fixed, paraffin-embedded sections from a representative case of NPM-ALK–positive primary ALCL were stained with H&E (*top left*) and with antibodies against CD30 (*top right*) and ALK (*lower left*) by immunohistochemistry. A corresponding frozen section from the same case was stained with the phosphospecific antibody that recognizes the Y542 phosphotyrosine of Shp2 (*lower right*). All images were taken with a ×40 objective.

growth factor stimulation (20, 21, 24) and that Gab2 precipitated with NPM-ALK in ALCL cell lines (11). Therefore, we investigated whether Gab2 is phosphorylated in a complex with NPM-ALK. In total cell lysates from 293T-Rex cells, Gab2 was phosphorylated only in the presence of NPM-ALK (Fig. 4*B*). Anti-Gab2 immunoprecipitation in 293T-Rex cells showed that Gab2 precipitated only with the active NPM-ALK, but not with the kinase dead mutant (Fig. 4*C*). The same results were observed in ALCL cell lines (Fig. 4*C*). Reciprocal anti-ALK immunoprecipitation in both HEK293 and ALCL cells confirmed the above-described association (data not shown).

Because Grb2 has been described to interact with NPM-ALK (14) and to mediate the recruitment of Shp2 through Gab2 in Bcr-Abl–transformed leukemia cells (20), we hypothesized that Grb2 was involved in the interaction of Shp2 with NPM-ALK. Transient cotransfection experiments in 293T cells showed that Grb2 was able to bind Shp2 only in the presence of active NPM-ALK (Fig. 4*D*). In addition, the use of two dominant-negative forms of Grb2, mutated, respectively, in the NH_2-terminal SH3 domain (P49L) and in the SH2 domain (R86K), showed that the Shp2 binding to Grb2 was mediated by the SH2 domain of Grb2 as Shp2 did not precipitate in the presence of R86K Grb2-mutant, whereas the binding of Shp2 to NPM-ALK was not abrogated (Fig. 4*D*). This finding suggests that Grb2 is involved as a downstream molecule of Shp2 rather than a mediator of the binding to NPM-ALK.

Shp2 activation regulates cell growth in ALK-positive lymphoma cells. To assess the biological role of Shp2 in human lymphoma cells, we designed a specific Shp2 shRNA to down-modulate Shp2 expression. TS and SU-DHL-1 cells were transduced with Shp2 shRNA and control shRNA lentiviral vectors. Shp2 knock down was confirmed by immunoblotting with anti-Shp2 antibody and the unchanged expression level of P-eIF2α excluded unspecific regulation via IFN-mediated responses (Fig. 5*A*). To compare the growth rate of ALCL cells and ALCL cells transduced with Shp2 shRNA, a coculture experiment was done. We found that TS and SU-DHL-1 cells transiently transduced with Shp2 shRNA had a significant growth disadvantage compared with untransduced counterparts, whereas no effect was found in TS and SU-DHL-1 infected with a control shRNA or in a control cell line (CEM) infected with Shp2 shRNA (Fig. 5*B*). As expected, K562 infected with Shp2 shRNA displayed a similar slower rate of proliferation (25, 26; data not shown). To verify that the proliferative disadvantage was not derived from off-target effects of the Shp2 shRNA, we did a functional rescue experiment. As Shp2 shRNA targets a sequence in the 3′-untranslated region of Shp2, we cotransduced TS cells with Shp2 shRNA and a Shp2-EGFP-lentiviral construct that does not contain the shRNA target sequence. In coculture, TS cells ectopically expressing Shp2 and Shp2 shRNA were almost completely rescued to a normal growth (data not shown).

Shp2 down-modulation affects lymphoma cell proliferation but not apoptosis. To further study the involvement of Shp2 on ALCL cell proliferation, we generated cell lines carrying a doxycycline-dependent inducible Shp2 shRNA silencing. TS and SU-DHL-1 were cotransduced with pLV-tTRKRAB (TTA) and pLVTHM Shp2 shRNA. Cotransduced cells grown in the presence of doxycycline efficiently down-regulated Shp2 expression as shown in Fig. 5*C*. TS and SU-DHL-1 transduced with the pLV-tTRKRAB vector only were used as control. The abrogation of Shp2 in both cell lines significantly impaired cellular proliferation over time compared with cells grown without doxycycline (Fig. 5*D*) or to the control cell lines (with or without doxycycline; data not shown). To understand whether the observed low proliferation rate was due to cell cycle arrest and/or increased apoptosis, we did cell cycle

analysis with propidium iodide staining and TMRM apoptosis assay. Cell cycle analysis revealed that Shp2 knock down led to a significant increase in the percentage of TS cells in the G_1 phase and a corresponding decrease in the S and G_2-M phases, without changes in the apoptotic fraction as indicated by the sub–G_0-G_1 populations (Fig. 6*A*). Furthermore, the absence of apoptosis was confirmed by TMRM staining (data not shown). We showed that, in TS cells, Shp2 knock down led to a decrease in Erk1/2 phosphorylation (Fig. 6*B*). A similar decrease was observed in the SU-DHL-1 cell line (Supplementary Fig. S1). Concomitantly, we observed a decrease in the phosphorylation of the tyrosine residue Y416 in the activation loop of Src, whereas Stat3 and Akt phosphorylations were not affected (Fig. 6*B*). Notably, NPM-ALK total amount and phosphorylation did not change after Shp2 silencing, thus excluding major effects of Shp2 on NPM-ALK stability, contrary to what has been described for Bcr-Abl (Fig. 6*B*; ref. 27). These findings indicate that the activation of Shp2 in ALCL cells regulates the Ras/Erk signaling pathway and the activation of Src, thus influencing ALCL cell proliferation.

Shp2 knock down reduces spontaneous and SDF-1α–induced migration of ALCL cells. Our group recently described that NPM-ALK increases ALCL spontaneous and SDF-1α–induced cell migration (6). As Shp2 is required for integrin-driven cell spreading and migration (28, 29) and participates in α-chemokine receptor CXCR-4–mediated cell signaling in T lymphocytes (30), we investigated its role in ALCL cell migration. We did a chemotaxis assay in the inducible TS TTA shShp2 shRNA cells. Shp2 knock down reduced both the spontaneous migration and the SDF-1α–induced migration of lymphoma cells, suggesting its role in ALCL cell migration (Fig. 6*C*).

Discussion

In this study, we used a functional proteomic approach to identify new NPM-ALK phosphorylated and/or interacting proteins by immunoaffinity purification and MS by using 293T-Rex cells ectopically expressing NPM-ALK under an inducible tetracycline promoter. In addition to known NPM-ALK substrates, our analysis

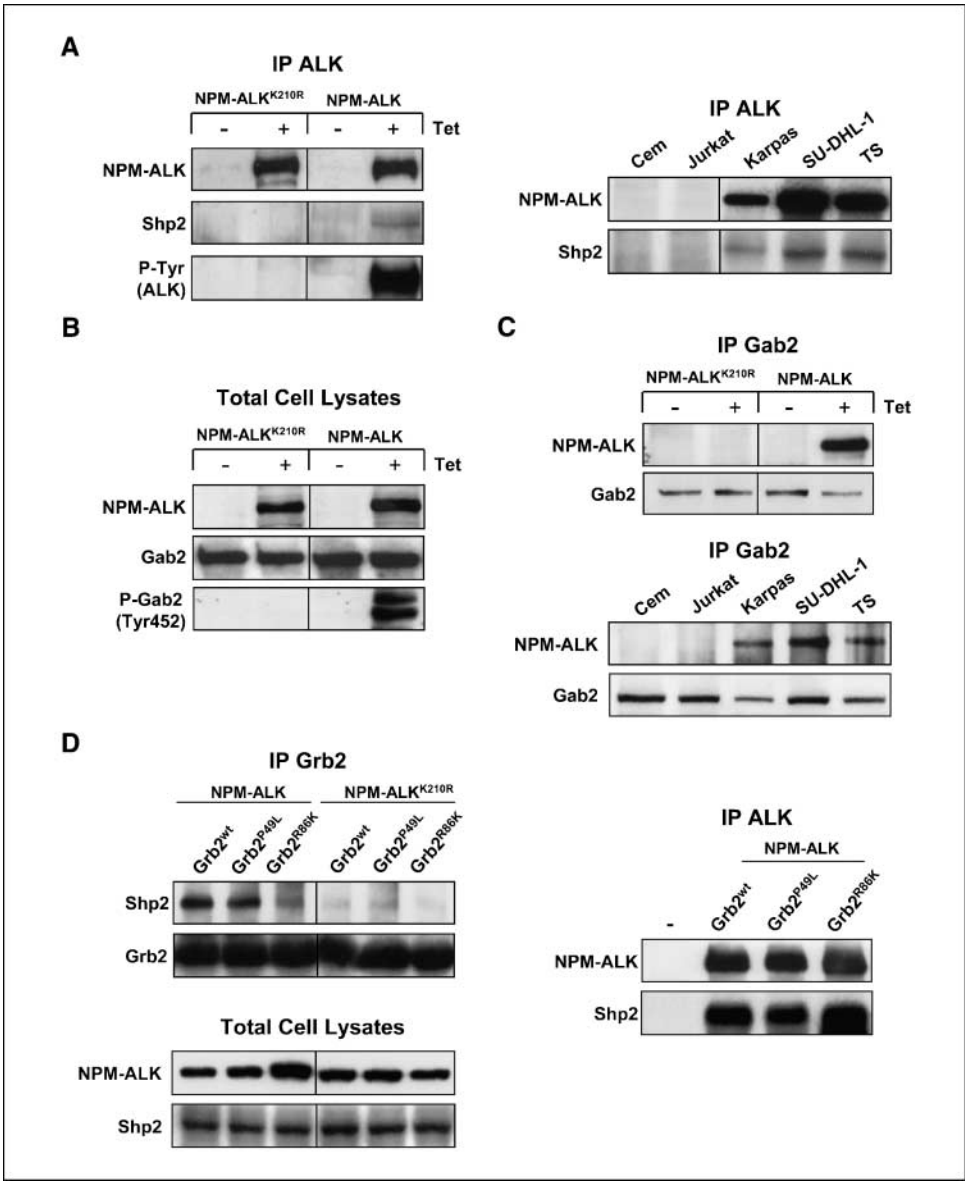

Figure 4. NPM-ALK coprecipitates with Shp2, Gab2, and Grb2. *A,* Shp2 coprecipitates with NPM-ALK in T-REx-293 and in lymphoma cell lines (Karpas 299, SU-DHL-1, and TS). Total cell lysates from T-REx-293 (*left*) and lymphoid cell lines (*right*) were immunoprecipitated with anti-ALK antibody and blotted with anti-Shp2. *B,* Gab2 is phosphorylated only in the presence of NPM-ALK. Total cell lysates from T-REx-293 were immunoblotted with a specific anti–phospho-Gab2 antibody and anti-Gab2 antibody as control. *C,* Gab2 coprecipitates with NPM-ALK in T-REx-293 and in ALK-positive ALCL cells. Total cell lysates from T-REx-293 (*top*) and lymphoid cell lines (*bottom*) were immunoprecipitated with anti-Gab2 antibody and blotted with the indicated antibodies. *D,* HEK 293T cells were cotransfected with $Shp2^{wt}$ and the indicated constructs. As detected by anti-Grb2 immunoprecipitation and anti-Shp2 immunoblotting (*top left*), Grb2 was able to bind Shp2 only in the presence of NPM-ALK, and the binding was disrupted in the presence of $Grb2^{R86K}$. Shp2 binding to NPM-ALK was not abrogated in the presence of both Grb2 dominant-negative mutants (*right*).

revealed novel potential targets. Among the identified proteins, we found the ubiquitously expressed SH2 domain–containing tyrosine phosphatase Shp2. We showed that Shp2 forms a complex with NPM-ALK both in 293T-Rex cells and in ALK-positive ALCL cells TS, SU-DHL-1, and Karpas 299. In ALCL cells, Shp2 phosphorylation was higher compared with other cell lines, such as K562 and Jurkat (data not shown), in which it is reported to be overexpressed and constitutively phosphorylated (25). More importantly, in ALCL cells, Shp2 phosphorylation is dependent on NPM-ALK tyrosine kinase activity as it is almost completely abrogated when NPM-ALK expression is knocked down by shRNA. As it is reported for other transforming tyrosine kinases, this phosphorylation status is indicative of an activation state of Shp2 (20).

It has been shown that Shp2 can bind to receptor tyrosine kinases (RTK) either directly or through adaptor/scaffolding molecules such as IRS1 and Gab family molecules (31, 32), and here we report that NPM-ALK precipitates both Gab2 and Shp2. Therefore, we can suppose that NPM-ALK forms a multimeric complex with Gab2 and Shp2 as described for Bcr-Abl, although we cannot exclude that Shp2 binds directly to NPM-ALK. The Gab2-Shp2 complex with other tyrosine kinases such as Bcr-Abl very often involves the adaptor protein Grb2 through the binding of its SH3 domain to Gab2 or its SH2 domain to the phosphotyrosine Y542 of Shp2 (20, 33–35). Previous studies showed that Grb2 coprecipitates with NPM-ALK (14), and we show that Shp2-Grb2 interaction is dependent on the catalytic activity of NPM-ALK as there is no association in the presence of the kinase dead mutant NPM-ALK^{K210R}. Moreover, the association is mediated by the Grb2-SH2 domain as its dominant-negative SH2 mutant R86K disrupts the binding. On the contrary, Shp2 association to NPM-ALK is not abrogated. As previously described in fibroblasts stimulated with platelet-derived growth factor or fibroblast growth factor (FGF), tyrosyl-phosphorylated Shp2 functions as an adaptor protein for Grb2 although it is controversial whether this association promotes the full Erk activation (33, 35). In fact, Shp2 is commonly required for the activation of the Ras/Erk signaling pathway in response to several cytokines and RTKs (31, 32). Our results reveal that Shp2 knocked down by specific shRNA in ALCL cells results in ~50% reduction of Erk phosphorylation compared with ALCL cells still expressing the phosphatase. Thus, in ALCL cells, our findings suggest that Shp2 acts upstream of the Ras pathway, possibly by recruiting a Grb2/Sos complex. Also, Shp2 could regulate the Ras pathway activation through the Src family protein-tyrosine kinases as we observed a strong decrease in the phosphorylation of the

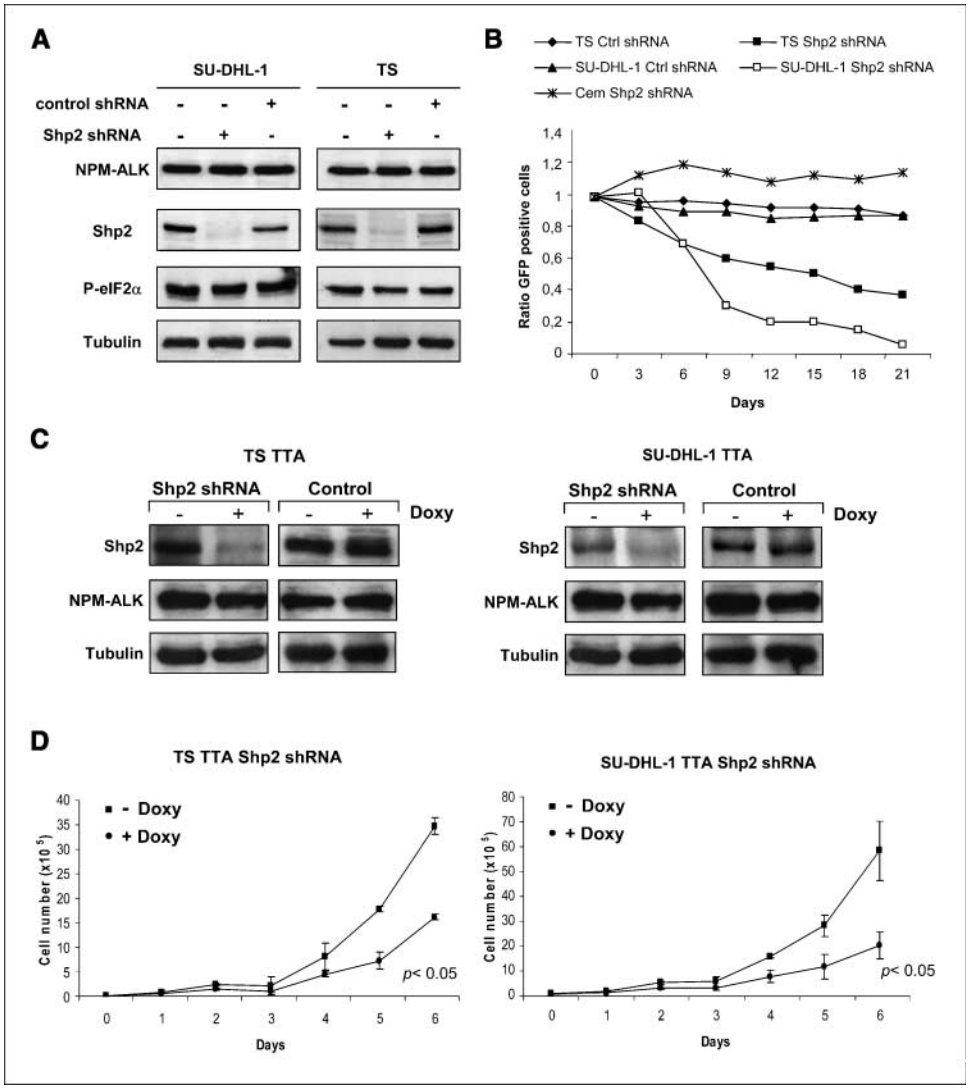

Figure 5. Shp2 regulates cell growth of ALCL cells. *A,* Shp2 expression is abrogated by specific shRNA. TS and SU-DHL-1 cells were transduced with Shp2 shRNA and control shRNA lentiviral vectors. After 72 h, protein expression was knocked down as verified by Western blot. No up-regulation of nonspecific IFN-mediated responses was observed as shown by blotting with anti–P-eIF2α. *B,* SU-DHL-1 and TS transduced with Shp2 shRNA and cocultured with the untransduced counterpart showed a significant growth disadvantage according to Student's *t* test ($P = 0.001$ and $P = 0.0036$, respectively). TS and SU-DHL-1 cells infected with a control shRNA and the T lymphoblastoid control cell line CEM infected with Shp2 shRNA did not change over time. Results are representative of three different experiments. *C,* TS TTA Shp2 shRNA and SU-DHL-1 TTA Shp2 shRNA were grown in the presence of 1 μg/mL doxycycline for at least 72 h to knock down Shp2 expression as shown in the Western blot with anti-Shp2 antibody. TS TTA and SU-DHL-1 TTA transduced with pLV-tTRKRAB were only used as controls. *D,* Shp2 silencing by shRNA affects lymphoma cell proliferation. TS TTA and SU-DHL-1 TTA infected with Shp2 shRNA were induced with 1 μg/mL doxycycline (*Doxy*) for 96 h and then plated at 5×10^4/mL in a 24-well plate. Cells were counted each day for 6 d by trypan blue exclusion. *Points,* mean of triplicates; *bars,* SE. One representative experiment from two independent assays.

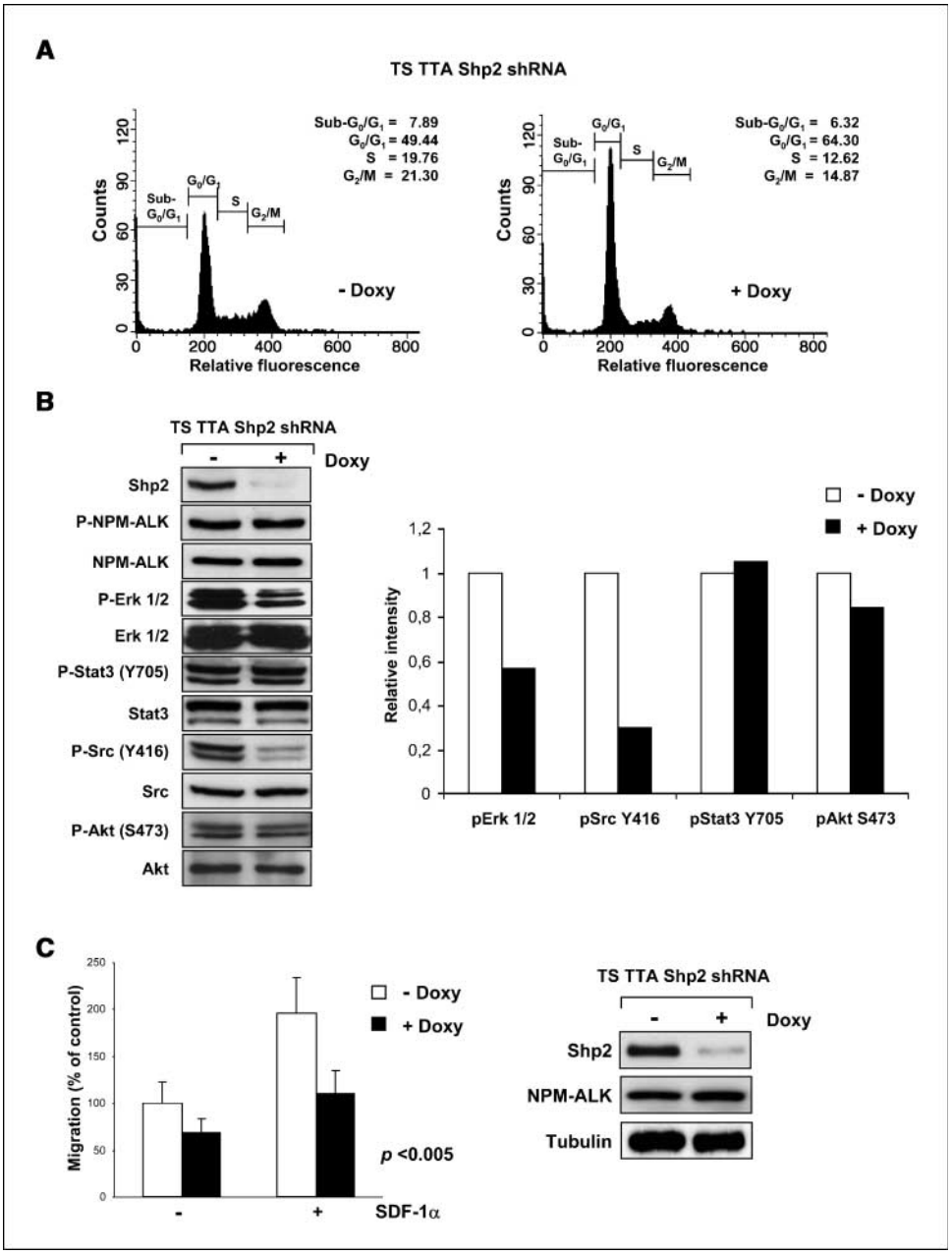

Figure 6. Shp2 knock down leads to ALCL cell accumulation in G_1 by reducing Ras/Erk pathway activation and decreases lymphoma cell migration. *A,* flow cytometry profiles of propidium iodide–stained TS TTA Shp2 shRNA not induced or induced with 1 μg/mL doxycycline for 96 h. In the absence of Shp2, ALCL cells showed a significant cell cycle arrest in G_1, but not increased sub–G_0-G_1 populations. One of four independent experiments. *B,* TS TTA Shp2 shRNA were induced with 1 μg/mL doxycycline for 96 h. In Shp2 knocked down cells, the phosphorylation of Erk1/2 and of the tyrosine residue Y416 of Src were decreased, whereas Stat3 and Akt phosphorylations were not affected. Samples were incubated with the indicated antibodies. Phospho–Src Y416, phospho–Stat3 Y705, Akt, and Erk1/2 phosphorylations were measured by normalizing the absorbance of the phosphorylated bands to the total amount of the indicated proteins (*right*). These findings are representative of two independent experiments. *C,* TS TTA Shp2 shRNA cells were induced with 1 μg/mL doxycycline for 96 h followed by a Transwell migration assay in the presence or absence of the chemokine SDF-1α (100 ng/mL). *Columns,* mean of triplicates for each experiment; *bars,* SE. One representative experiment from two independent assays. *Right,* Western blot analysis of Shp2 knock down by shRNA in the same cell line.

activating tyrosine Y416 of Src after Shp2 silencing by specific shRNA. This is a conceivable parallel pathway given that upon RTK stimulation Shp2 regulates PAG/Cbp tyrosyl phosphorylation, thereby controlling Csk recruitment to Src (36), and Src is activated by NPM-ALK in ALCL cells (37).

Shp2 has been previously described to be involved in the transformation of some hematologic malignancies, either acting as an oncogene or as an effector molecule (20, 21, 25, 26). Moreover, Shp2 is implicated both in growth factor receptor and integrin signaling usually with a positive regulatory role, thus promoting mitogenic and proliferative signals (31, 32) and its phosphatase activity seems to mediate the transforming capacity of constitutively active forms of some tyrosine kinase receptors such as epidermal growth factor receptor, FGF receptor 3, and RET (38–40). ALCL cells transduced with Shp2 shRNA showed a significant growth disadvantage and a lower proliferation rate compared with control cell lines. These effects are not related to a decrease in the NPM-ALK stability in ALCL cells, in contrast to what it has been described for Bcr-Abl–transformed cells in which Shp2 stabilizes the protein through an interaction with HSP90 (27). The impaired proliferation coincided with the reduced activation of Src and of the Ras/Erk pathway. Interestingly, in ALCL cell lines, knocking down Erk1/2 by specific shRNA leads to a similar impairment of cell proliferation as the knock down of Shp2 (41). Taken together, our results show that in ALCL cells Shp2 is a critical mediator of NPM-ALK–driven cell growth.

Shp2 has been described to transduce promigratory signals from various type of receptors (31, 42), including a CXCR4-mediated signaling pathways in T cells (30). In ALCL cells, our group recently showed that NPM-ALK is able to influence both spontaneous and SDF-1α/CXCR4–induced migration (6). Here, we found that Shp2 down-modulation by shRNA decreased significantly the migration

rate of ALK-positive ALCL cells. It is likely that NPM-ALK might promote ALCL cell migration through Shp2 engagement, although further studies are required to better elucidate the mechanism.

In conclusion, our work provides another clue in the molecular mechanisms underlying NPM-ALK transformation. Our findings show that Shp2 associates to NPM-ALK and forms a multimeric complex with Gab2 and Grb2, resulting in the activation of multiple downstream effectors such as the Ras/Erk pathway and show that Shp2 plays a critical role in ALK-evoked cell proliferation and migration.

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References

1. Morris SW, Kirstein MN, Valentine MB, et al. Fusion of a kinase gene, ALK, to a nucleolar protein gene, NPM, in non-Hodgkin's lymphoma. Science 1994;263:1281–4. Erratum in: Science 1995 Jan 20;267:316–7.

2. Bischof D, Pulford K, Mason DY, Morris SW. Role of the nucleophosmin (NPM) portion of the non-Hodgkin's lymphoma-associated NPM-anaplastic lymphoma kinase fusion protein in oncogenesis. Mol Cell Biol 1997; 17:2312–25.

3. Wellmann A, Doseeva V, Butscher W, et al. The activated anaplastic lymphoma kinase increases cellular proliferation and oncogene up-regulation in rat 1a fibroblasts. FASEB J 1997;11:965–72.

4. Kuefer MU, Look AT, Pulford K, et al. Retrovirus-mediated gene transfer of NPM-ALK causes lymphoid malignancy in mice. Blood 1997;90:2901–10.

5. Chiarle R, Gong JZ, Guasparri I, et al. NPM-ALK transgenic mice spontaneously develop T-cell lymphomas and plasma cell tumors. Blood 2003;101:1919–27.

6. Ambrogio C, Voena C, Manazza AD, et al. p130Cas mediates the transforming properties of the anaplastic lymphoma kinase. Blood 2005;106:3907–16.

7. Piva R, Chiarle R, Manazza AD, et al. Ablation of oncogenic ALK is a viable therapeutic approach for anaplastic large-cell lymphomas. Blood 2006;107:689–97.

8. Wan W, Albom MS, Lu L, et al. Anaplastic lymphoma kinase activity is essential for the proliferation and survival of anaplastic large-cell lymphoma cells. Blood 2006;107:1617–23.

9. Bai RY, Dieter P, Peschel C, Morris SW, Duyster J. Nucleophosmin-anaplastic lymphoma kinase of large-cell anaplastic lymphoma is a constitutively active tyrosine kinase that utilizes phospholipase C-γ to mediate its mitogenicity. Mol Cell Biol 1998;18:6951–61.

10. Zamo A, Chiarle R, Piva R, et al. Anaplastic lymphoma kinase (ALK) activates Stat3 and protects hematopoietic cells from cell death. Oncogene 2002;21: 1038–47.

11. Bai RY, Ouyang T, Miething C, Morris SW, Peschel C, Duyster J. Nucleophosmin-anaplastic lymphoma kinase associated with anaplastic large-cell lymphoma activates the phosphatidylinositol 3-kinase/Akt antiapoptotic signaling pathway. Blood 2000;96:4319–27.

12. Rassidakis GZ, Feretzaki M, Atwell C, et al. Inhibition of Akt increases p27Kip1 levels and induces cell cycle arrest in anaplastic large cell lymphoma. Blood 2005;105: 827–9.

13. Chiarle R, Simmons WJ, Cai H, et al. Stat3 is required for ALK-mediated lymphomagenesis and provides a possible therapeutic target. Nat Med 2005;11:623–9.

14. Fujimoto J, Shiota M, Iwahara T, et al. Characterization of the transforming activity of p80, a hyperphosphorylated protein in a Ki-1 lymphoma cell line with chromosomal translocation t(2;5). Proc Natl Acad Sci U S A 1996;93:4181–6.

15. Pandey A, Podtelejnikov AV, Blagoev B, Bustelo XR, Mann M, Lodish HF. Analysis of receptor signaling pathways by mass spectrometry: identification of vav-2 as a substrate of the epidermal and platelet-derived growth factor receptors. Proc Natl Acad Sci U S A 2000; 97:179–84.

16. Blagoev B, Kratchmarova I, Ong SE, Nielsen M, Foster LJ, Mann M. A proteomics strategy to elucidate functional protein-protein interactions applied to EGF signaling. Nat Biotechnol 2003;21:315–8.

17. Kratchmarova I, Blagoev B, Haack-Sorensen M, Kassem M, Mann M. Mechanism of divergent growth factor effects in mesenchymal stem cell differentiation. Science 2005;308:1472–7.

18. Wiznerowicz M,Trono D. Conditional suppression of cellular genes: lentivirus vector-mediated drug-inducible RNA interference. J Virol 2003;77:8957–61.

19. Amendola M, Venneri MA, Biffi A, Vigna E, Naldini L. Coordinate dual-gene transgenesis by lentiviral vectors carrying synthetic bidirectional promoters. Nat Biotechnol 2005;23:108–16.

20. Sattler M, Mohi MG, Pride YB, et al. Critical role for Gab2 in transformation by BCR/ABL. Cancer Cell 2002;1: 479–92.

21. Mohi MG, Williams IR, Dearolf CR, et al. Prognostic, therapeutic, and mechanistic implications of a mouse model of leukemia evoked by Shp2 (PTPN11) mutations. Cancer Cell 2005;7:179–91.

22. Tartaglia M, Niemeyer CM, Fragale A, et al. Somatic mutations in PTPN11 in juvenile myelomonocytic leukemia, myelodysplastic syndromes and acute myeloid leukemia. Nat Genet 2003;34:148–50.

23. Loh ML, Vattikuti S, Schubbert S, et al. Mutations in PTPN11 implicate the SHP-2 phosphatase in leukemogenesis. Blood 2004;103:2325–31.

24. Gu H, Griffin JD, Neel BG. Characterization of two SHP-2-associated binding proteins and potential substrates in hematopoietic cells. J Biol Chem 1997;272: 16421–30.

25. Xu R, Yu Y, Zheng S, et al. Overexpression of Shp2 tyrosine phosphatase is implicated in leukemogenesis in adult human leukemia. Blood 2005;106:3142–9.

26. Scherr M, Chaturvedi A, Battmer K, et al. Enhanced sensitivity to inhibition of SHP2, STAT5, and Gab2 expression in chronic myeloid leukemia (CML). Blood 2006;107:3279–87.

27. Chen J, Yu WM, Daino H, Broxmeyer HE, Druker BJ, Qu CK. SHP-2 phosphatase is required for hematopoietic cell transformation by Bcr-Abl. Blood 2007;109:778–85.

28. Yu DH, Qu CK, Henegariu O, Lu X, Feng GS. Protein-tyrosine phosphatase Shp-2 regulates cell spreading, migration, and focal adhesion. J Biol Chem 1998;273: 21125–31.

29. Inagaki K, Noguchi T, Matozaki T, et al. Roles for the protein tyrosine phosphatase SHP-2 in cytoskeletal organization, cell adhesion and cell migration revealed by overexpression of a dominant negative mutant. Oncogene 2000;19:75–84.

30. Chernock RD, Cherla RP, Ganju RK. SHP2 and cbl participate in α-chemokine receptor CXCR4-mediated signaling pathways. Blood 2001;97:608–15.

31. Feng GS. Shp-2 tyrosine phosphatase: signaling one cell or many. Exp Cell Res 1999;253:47–54.

32. Neel BG, Gu H, Pao L. The 'Shp'ing news: SH2 domain-containing tyrosine phosphatases in cell signaling. Trends Biochem Sci 2003;28:284–93.

33. Bennett AM, Tang TL, Sugimoto S, Walsh CT, Neel BG. Protein-tyrosine-phosphatase SHPTP2 couples platelet-derived growth factor receptor β to Ras. Proc Natl Acad Sci U S A 1994;91:7335–9.

34. Keegan K, Cooper JA. Use of the two hybrid system to detect the association of the protein-tyrosine-phosphatase, SHPTP2, with another SH2-containing protein, Grb7. Oncogene 1996;12:1537–44.

35. Araki T, Nawa H, Neel BG. Tyrosyl phosphorylation of Shp2 is required for normal ERK activation in response to some, but not all, growth factors. J Biol Chem 2003; 278:41677–84.

36. Zhang SQ, Yang W, Kontaridis MI, et al. Shp2 regulates SRC family kinase activity and Ras/Erk activation by controlling Csk recruitment. Mol Cell 2004;13:341–55.

37. Cussac D, Greenland C, Roche S, et al. Nucleophosmin-anaplastic lymphoma kinase of anaplastic large-cell lymphoma recruits, activates, and uses pp60c-src to mediate its mitogenicity. Blood 2004;103:1464–71.

38. Zhan Y, O'Rourke DM. SHP-2-dependent mitogen-activated protein kinase activation regulates EGFRvIII but not wild-type epidermal growth factor receptor phosphorylation and glioblastoma cell survival. Cancer Res 2004;64:8292–8.

39. D'Alessio A, Califano D, Incoronato M, et al. The tyrosine phosphatase Shp-2 mediates intracellular signaling initiated by Ret mutants. Endocrinology 2003;144:4298–305.

40. Agazie YM, Movilla N, Ischenko I, Hayman MJ. The phosphotyrosine phosphatase SHP2 is a critical mediator of transformation induced by the oncogenic fibroblast growth factor receptor 3. Oncogene 2003;22:6909–18.

41. Marzec M, Kasprzycka M, Liu X, Raghunath PN, Wlodarski P, Wasik MA. Oncogenic tyrosine kinase NPM/ALK induces activation of the MEK/ERK signaling pathway independently of c-Raf. Oncogene 2007; 26:813–21.

42. Qi JH, Ito N, Claesson-Welsh L. Tyrosine phosphatase SHP-2 is involved in regulation of platelet-derived growth factor-induced migration. J Biol Chem 1999; 274:14455–63.