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Stimulation of hTAF$_{II}$68 (NTD)-mediated transactivation by v-Src

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Abstract The three genes *hTAF$_{II}$68*, *EWS*, and *TLS* (called the TET family) encode related RNA binding proteins containing an RNA recognition motif and three glycine-, arginine-, and proline-rich regions in the C-terminus and a degenerated repeat containing the consensus sequence Ser-Tyr-Gly-Gln-Ser in the N-terminus. In many human cancers, the N-terminal portion of hTAF$_{II}$68, EWS, or TLS is fused to the DNA binding domain of one of several transcription factors including Fli-1, ERG, ETV1, E1AF, WT1, ATF-1, CHOP, or TEC. We have recognized the presence of several potential tyrosine phosphorylation sites within the amino-terminal domain of hTAF$_{II}$68 and have investigated the potential effects of cytoplasmic signaling on hTAF$_{II}$68 function. Herein, we find that hTAF$_{II}$68 is phosphorylated on tyrosine residue(s) by ectopic expression of v-Src protein tyrosine kinase in vitro and in vivo. The hTAF$_{II}$68 protein can associated with the SH3 domains of several cell signaling proteins, including v-Src protein tyrosine kinase. We also document that full-length v-Src can stimulate hTAF$_{II}$68-mediated transcriptional activation, whereas deletion mutants of v-Src are unable to exert this effect. In addition, cellular Src activity appears important for hTAF$_{II}$68 function since hTAF$_{II}$68-mediated transactivation is reduced in a dose-dependent fashion by ectopic overexpression of a dominant-negative mutant of Src. Taken together, our results suggest that the biological activities of hTAF$_{II}$68 are linked to the cytoplasmic Src signal transduction pathway.

Key words: hTAF$_{II}$68; Transcription factor; v-Src; Protein–protein interaction; Tyrosine kinase

1. Introduction

Molecular characterization of specific chromosome abnormalities associated with human tumors has led to the discovery of new mechanisms involved in neoplastic transformation. Chromosomal rearrangements are very common molecular abnormalities in hematologic malignancies. These rearrangements consist mainly of chromosomal translocations and, less frequently, chromosomal inversions which can lead to neoplastic malignancy by two different mechanisms: (i) the transcriptional activation of proto-oncogenes, or (ii) the creation of fusion genes [1,2]. In some leukemias and lymphomas, the juxtaposition of promoter or enhancer elements from immunoglobulin genes with the intact coding region of other genes, such as c-*myc*, *bcl-2*, *bcl-6* etc., determines the neoplastic phenotype by altering cell cycle progression, halting the physiologic process of apoptosis, or modifying lineage-specific transcription [3]. In chronic myeloid leukemia, *bcr* fuses with c-*abl* to generate the *bcr-abl* chimeric transcript. This transcript encodes a fusion protein of 210 kDa with increased tyrosine kinase activity and abnormal cellular localization [4,5].

Unlike in hematologic malignancies, however, recurrent chromosome rearrangements are relatively uncommon in solid tumors. A rare exception is the Ewing's sarcoma gene (*EWS*), which is translocated in a wide variety of human solid tumors. In each translocation case, fusions of the N-terminal domain (NTD) of EWS to novel DNA binding domains create chimeric proteins with altered transactivation potential [6–16]. The *EWS* gene encodes a 656 amino acid protein that contains three arginine-rich tracts and an 85 amino acid RNA recognition motif at its C-terminus. The EWS (NTD) is composed almost exclusively of tyrosine, glutamine, glycine, alanine, serine, threonine, and proline residues organized in a repeated and degenerated polypeptide motif having the consensus NSYGQQS. Interestingly, this domain shares distant homology with the C-terminal domain of eukaryotic RNA polymerase II [7].

Human TATA binding protein (TBP)-associated factor 68 (hTAF$_{II}$68) encodes a putative RNA/ssDNA binding protein and was originally identified due to its homology to the proto-oncogenes EWS and TLS (another member of the *EWS* gene family) [17,18]. Recently, a proportion of extraskeletal myxoid chondrosarcomas have been shown to harbor a characteristic translocation t(9;17)(q22;q11.2) involving the *hTAF$_{II}$68* gene at 17q11.2 and the *TEC* (Translocated in Extraskeletal Chondrosarcoma) gene at 9q22 [19,20]. *TEC* (also called *CHN* and *MINOR*) is the human homologue of the rat *NOR-1* receptor [21] and encodes a novel orphan nuclear receptor belonging to the steroid/thyroid receptor gene superfamily [22,23].

The Src family of tyrosine kinases is composed of nine members in higher eukaryotes of which three, Src, Yes, and Fgr, have viral counterparts. v-Src, the first viral form identified, is the transforming product of the chicken retrovirus, Rous sarcoma virus [24,25]. The Src family of non-receptor protein tyrosine kinases plays critical roles in a variety of cellular signal transduction pathways, regulating such diverse processes as cell division, motility, adhesion, angiogenesis, and survival. Constitutively activated variants of Src family kinases, including the viral oncoproteins v-Src and v-Yes, are capable of inducing malignant transformation in a variety of cell types. Although exactly how Src family tyrosine kinases contribute to individual tumors remains to be defined com-

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pletely, they appear to be important for multiple aspects of tumor progression, including proliferation, disruption of cell–cell contacts, migration, invasiveness, resistance to apoptosis, and angiogenesis [26].

Signal transduction pathways can regulate gene expression by modulating the activity of nuclear transcription factors [27,28]. We have previously shown that ectopic expression of v-Src phosphorylates EWS/WT1 in vivo, as well as enhancing the transactivation ability of the EWS NTD [29]. However, tyrosine phosphorylation of EWS/WT1 by c-Abl negatively regulates its DNA binding properties [30]. Interestingly, it has been reported that many signaling proteins, such as Ras, Btk, RET/PTC, and v-Src, enhance the transcriptional activity of several transcription factors, such as AP-1, TFII-I, and STAT3 [31–39]. We show herein that hTAF$_{II}$68 (NTD) is phosphorylated on tyrosine residue(s) by ectopic expression of v-Src protein tyrosine kinase in vitro and in vivo. We also define the interaction between hTAF$_{II}$68 (NTD) and v-Src in vivo. Furthermore, the transactivation activity of hTAF$_{II}$68 (NTD) is positively regulated by v-Src, but negatively modulated by a dominant-negative form of v-Src. These data implicate the Src signaling pathway in hTAF$_{II}$68 (NTD)-mediated transcriptional activation.

2. Materials and methods

2.1. Materials and general methods

Restriction endonucleases, polynucleotide kinase, calf intestinal alkaline phosphatase, the Klenow fragment of DNA polymerase I, and T4 DNA ligase were purchased from New England Biolabs. [γ-^{32}P]ATP (3000 Ci/mmol) and [^{35}S]methionine (1000 Ci/mmol) were obtained from Amersham. Preparation of plasmid DNA, restriction enzyme digestion, agarose gel electrophoresis of DNA, DNA ligation, bacterial transformations, and sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) of proteins were carried out using standard methods [40].

2.2. Plasmid constructions

To generate glutathione *S*-transferase (GST)-hTAF$_{II}$68 (NTD), the NTD of hTAF$_{II}$68 (obtained from Dr. L. Tora, Institut de Génétique et de Biologie Moléculaire et Cellulaire, CU de Strasbourg, France) was amplified by polymerase chain reaction (PCR) using primer 5′-TAF$_{II}$68-1A (5′-GATCGGATCCATGTCGGATTCTGGAAGT-3′) and primer 3′-TAF$_{II}$68-477 (5′-GATCCTCGAGTCAATCTTGTGTGTGGTGGCT-3′), digested with *Bam*HI and *Xho*I, and cloned into the same sites of pGEX(4T-1) (Pharmacia Biotech). To construct pTRC·HisA-v-Src, the *Bam*HI fragment of pcDNA3/v-Src was subcloned into the *Bam*HI site of pTRC·HisA vector (Invitrogen). For His$_6$-Flag-hTAF$_{II}$68 (NTD), the NTD of hTAF$_{II}$68 was generated by PCR using primer 5′-TAF$_{II}$68-1B (5′-GATCGGATCCCATGTCGGATTCTGGAAGT-3′) and primer 5′-TAF$_{II}$68-476 (5′-GATCCTGCAGTCTTGTGTGTGGTGGCT-3′), digested with *Bam*HI and *Pst*I, and cloned into the same sites of pCMV-Tag2A (Stratagene) to generate pCMV-Tag2A-hTAF$_{II}$68 (NTD). Then, the *Not*I-*Hin*dIII fragment of pCMV-Tag2A-hTAF$_{II}$68 (NTD) was subcloned into the *Not*I and *Hin*dIII sites of pcDNA3.1 myc·his B(−) (Invitrogen). Details on the construction of GST-EWS (NTD) have been described previously [30]. To generate GST-TLS (NTD), the NTD of TLS (kindly provided by Dr. T. Okamoto, Nagoya City University Medical School, Nagoya, Japan) was amplified by PCR using primers 5′-TLS-1 (5′-GATCGGATCCATGGCCTCAAACGATTAT-3′) and 3′-TLS-265 (5′-CTAGCTCGAGTCAACCAAATTTATTGAAGCC-3′), digested with *Bam*HI and *Xho*I, and cloned into the same sites of pGEX(4T-1). The construction of pcDNA3/GST has been previously described [30]. pcDNA3/GST-hTAF$_{II}$68 (NTD) was made by isolating an *Eco*NI and *Xho*I fragment from pGEX(4T-1)-hTAF$_{II}$68 (NTD) and cloned into the same sites of pcDNA3/GST.

GST fusion plasmids GST-hTAF$_{II}$68 (1–72), GST-hTAF$_{II}$68 (29–105), and GST-hTAF$_{II}$68 (75–159) were generated by the following strategies. (A) For GST-hTAF$_{II}$68 (1–72), plasmid pGEX(4T-1)-hTAF$_{II}$68 (NTD) was digested with *Sac*I and *Xho*I, blunted with the Klenow fragment, and then self-ligated. This construct was digested with *Not*I, treated with the Klenow fragment, and self-ligated to generate an in-frame stop codon. (B) For GST-hTAF$_{II}$68 (29–105), plasmid pGal4-hTAF$_{II}$68 (NTD) was digested with *Bam*HI and *Bst*XI and self-ligated. This construct was then re-digested with *Xcm*I and *Pst*I and self-ligated. The newly generated construct was then digested with *Eco*RI, the excised fragment blunted with Klenow fragment, and ligated into the blunted *Bam*HI and *Xho*I sites of pGEX(4T-1). (C) For GST-hTAF$_{II}$68 (75–159), plasmid pGal4-hTAF$_{II}$68 (NTD) was digested with *Bam*HI and *Sac*I, repaired with the Klenow fragment, and self-ligated. This new construct was digested with *Eco*RI and *Xba*I, the excised fragment blunted with the Klenow fragment, and then ligated into the blunted *Bam*HI and *Xho*I sites of pGEX(4T-1).

To generate Gal4-hTAF$_{II}$68 (NTD), the NTD of hTAF$_{II}$68 was amplified by PCR using primer 5′-TAF$_{II}$68-1C (5′-GATCGGATCCGAATGTCGGATTCTGGAAGT-3′) and primer 3′-TAF$_{II}$68-476 (5′-GATCCTGCAGTCTTGTGTGTGGTGGCT-3′), digested with *Bam*HI and *Pst*I, and cloned into the same sites of the pM vector (Clontech). Descriptions of plasmids encoding GST fusions containing SH3 domains from various kinases and adapter molecules, as well as of the mammalian expression v-Src constructs (pcDNA3-v-Src, Δkinase, and R295) have been previously described [29].

2.3. Purification of His$_6$-tagged v-Src

The recombinant pTRC·HisA-v-Src plasmid was transformed into *Escherichia coli*, the expression of His$_6$-v-Src was induced by isopropyl-β-D-thiogalactopyranoside, and the recombinant protein was purified by chromatography on Ni-nitrilotriacetic acid (NTA)-agarose according to the supplier's protocol (Qiagen). Briefly, the cell pellet from a culture was resuspended in lysis/wash buffer (50 mM Tris–HCl pH 8.0, 300 mM NaCl, 10 mM imidazole), clarified by centrifugation (16 000 rpm for 15 min) at 4°C, and the supernatant was incubated with NTA-agarose resin for 1 h at 4°C on a slowly rotating wheel. The protein-bound NTA-agarose resin was then packed into a Micro Bio-Spin chromatography column (Bio-Rad) and washed extensively with lysis/wash buffer. The protein was eluted with elution buffer (50 mM Tris–HCl pH 8.0, 300 mM NaCl, 20 mM imidazole). Purified v-Src kinase activity was assessed using myelin basic protein (Sigma) as a substrate in an in vitro kinase assay.

2.4. In vitro kinase assay and phosphoamino acid analysis

Bacterially produced GST or GST-hTAF$_{II}$68 (NTD) were incubated with recombinant His$_6$-v-Src protein with 50 μM ATP and 10 μCi of [γ-^{32}P]ATP (3000 Ci/mmol) in a reaction mixture for 20 min at 30°C. Reaction mixtures were resolved on a 15% SDS–PAGE and labeled proteins were visualized by exposing the dried gel to X-ray film (Kodak). For phosphoamino acid analysis of phosphorylated hTAF$_{II}$68 (NTD) by v-Src, acid hydrolysates of phosphorylated GST-hTAF$_{II}$68 (NTD) were prepared and fractionated by electrophoresis on thin layer cellulose at pH 1.9 (0.58 M formic acid, 1.36 M acetic acid) and pH 3.5 (0.87 M acetic acid, 0.5% v/v pyridine, 0.5 mM EDTA) using the Hunter Thin Layer Peptide Mapping Electrophoresis System™ (C.B.S. Scientific) according to the manufacturer's instruction. Non-radioactive standards were visualized by staining with 0.25% ninhydrin in acetone and labeled amino acids were detected by autoradiography.

2.5. In vitro transcription and translation

In vitro transcriptions and translations were performed in the presence of [^{35}S]methionine in rabbit reticulocyte lysates using the TNT T7 coupled reticulocyte system, essentially as described by the manufacturer (Promega).

2.6. GST pull-down assays

GST or GST fusion proteins were expressed in bacteria and adsorbed onto glutathione-agarose. The resin was then incubated at 4°C for 1 h with [^{35}S]methionine-labeled hTAF$_{II}$68 (NTD) or v-Src or recombinant His$_6$-tagged proteins. Agarose beads were washed three times with wash buffer (50 mM Tris–HCl pH 8.0, 150 mM NaCl, 2 mM EDTA, 0.1% NP-40, 10% glycerol), and the bound proteins were eluted with SDS loading buffer (62.5 mM Tris–HCl pH 6.9, 10% glycerol, 2% SDS, 5% β-mercaptoethanol) and resolved by SDS–PAGE. Bound proteins were detected using autoradiography or immunoblotting as previously described [29].

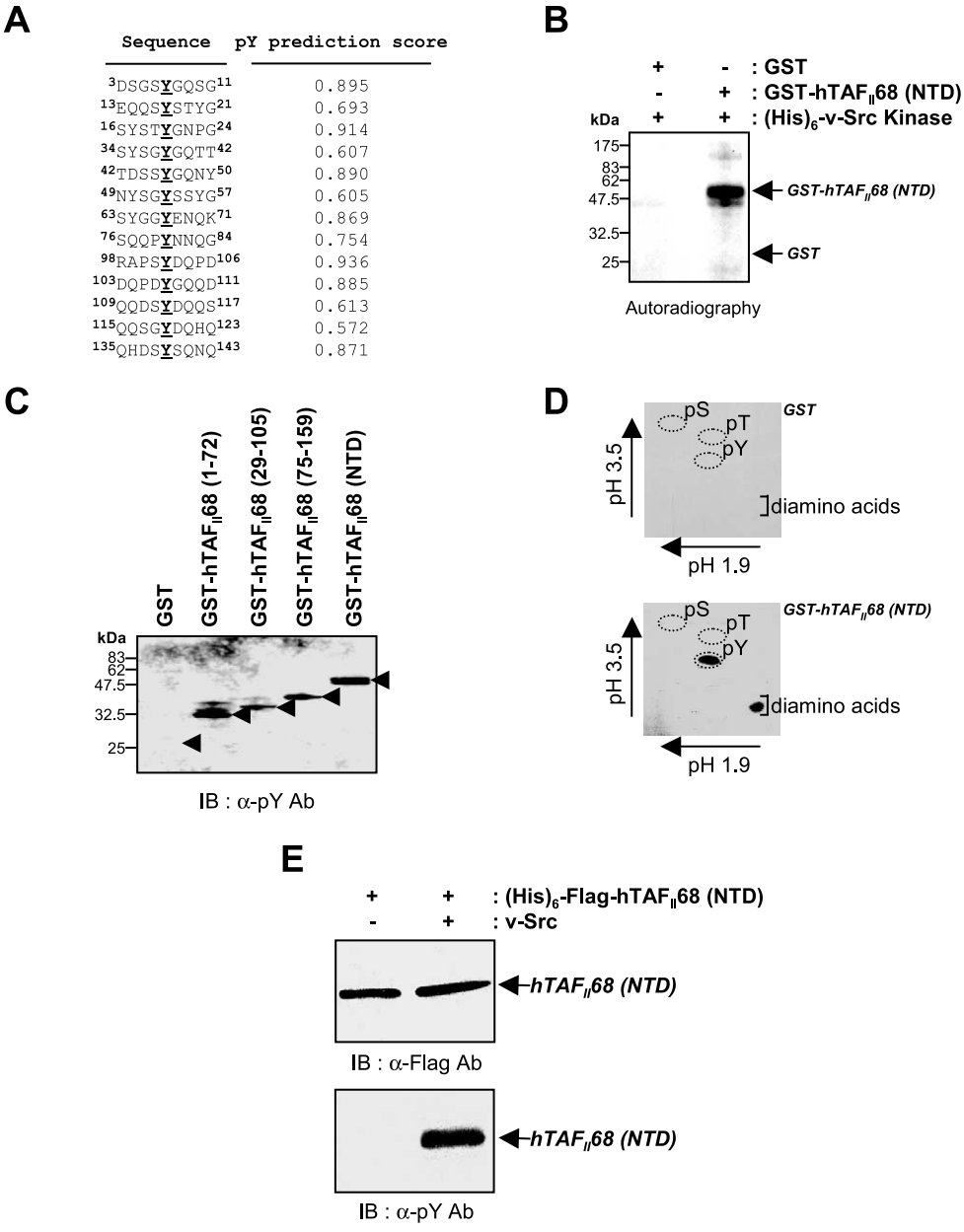

Fig. 1. Tyrosine phosphorylation of hTAF$_{II}$68 (NTD). A: Putative tyrosine phosphorylation sites in hTAF$_{II}$68 (NTD). The presence of 13 potential tyrosine phosphorylation sites is indicated by a bold character and underscores within the sequence. The pY prediction score is the output score from the ensemble of the neural networks trained on that acceptor residue type (performed at http://www.cbs.dtu.dk/services/NetPhos/). The sequence shows the context of the acceptor residue flanked on each side by four residues. B: In vitro phosphorylation of hTAF$_{II}$68 (NTD) by v-Src. One microgram of GST or GST-hTAF$_{II}$68 (NTD) was incubated with recombinant His$_6$-v-Src protein with 50 μM ATP and 10 μCi of [γ-^{32}P]ATP (3000 Ci/mmol) in a reaction mixture for 20 min at 30°C. Reaction mixtures were resolved on 15% SDS–PAGE and labeled proteins were visualized by exposing the dried gel to X-ray film (Kodak). The presence of v-Src, GST, or GST-hTAF$_{II}$68 (NTD) in each incubation is indicated above the lanes. The position of migration of the GST-hTAF$_{II}$68 (NTD) or GST is indicated. Molecular mass markers are provided to the left and are derived from prestained protein standards (New England Biolabs). C: In vitro kinase assay with a series of hTAF$_{II}$68 (NTD) deletion mutants. Reaction mixtures were resolved on 15% SDS–PAGE and labeled proteins were visualized by exposing the dried gel to X-ray film (Kodak). The presence of v-Src, GST, or GST-hTAF$_{II}$68 (NTD) deletion mutants in each incubation is indicated above the lanes. The presence of tyrosine phosphorylation was detected with an anti-phosphotyrosine antibody (4G10). The position of migration of the GST-hTAF$_{II}$68 (NTD) deletion mutants or GST is indicated by arrowheads. Molecular mass markers are provided to the left and are derived from prestained protein standards (New England Biolabs). D: Phosphoamino acid analysis of in vitro phosphorylated hTAF$_{II}$68 (NTD) by v-Src. Phosphorylated GST-hTAF$_{II}$68 (NTD) was separated by 15% SDS–PAGE, transferred onto Immobilon PVDF membrane, and GST-hTAF$_{II}$68 (NTD) identified by autoradiography. Hydrolysis was performed in 6 M HCl at 110°C for 1 h, after which time 1 ml of water was added and the samples lyophilized. Samples were analyzed using Hunter Thin Layer Peptide Mapping Electrophoresis System™ (bottom panel). The same molecular mass region was also excised from the PVDF membrane, which contained extract prepared from GST alone (top panel). E: In vivo tyrosine phosphorylation of hTAF$_{II}$68 (NTD) by v-Src. A mammalian expression plasmid encoding His$_6$-tagged Flag-hTAF$_{II}$68 (NTD) and either v-Src or the empty vector (pcDNA3) was cotransfected into COS-7 cells. Forty-eight hours after transfection, His$_6$-Flag-hTAF$_{II}$68 (NTD) protein was purified using Ni-NTA-agarose, separated by 10% SDS–PAGE, and transferred onto Immobilon PVDF membrane. The presence of hTAF$_{II}$68 (NTD) protein was confirmed with an anti-Flag antibody (top panel) and tyrosine phosphorylation was detected with an anti-phosphotyrosine antibody (4G10) (bottom panel).

2.7. Co-affinity precipitations

Plasmid pcDNA3/v-Src was cotransfected into mammalian cells with either pcDNA3/GST or pcDNA3/GST-hTAF$_{II}$68 (NTD). After 48 h, cells were harvested in resuspension buffer (50 mM Tris–HCl pH 8.0, 2 mM EDTA, 1% NP-40, 1×Complete Protease Inhibitor Cocktail Solution (Roche Diagnostics)) and lysed by sonication. Supernatants were collected by centrifugation at 16 000×*g* for 15 min at 4°C and incubated with glutathione beads (Amersham Biosciences) for 1 h. The beads were collected by brief centrifugation and washed with resuspension buffer four times. The affinity-selected proteins were eluted in SDS loading buffer (62.5 mM Tris–HCl pH 6.9, 10% glycerol, 2% SDS, 5% β-mercaptoethanol) and fractionated by SDS–PAGE.

2.8. Western blot analysis

COS-7 cells were lysed in lysis buffer (50 mM sodium phosphate pH 8.0, 300 mM NaCl, 10 mM imidazole, 1×Complete Protease Inhibitor Cocktail Solution), lysates were heated to 95–100°C for 5 min, and equal amounts of protein were loaded onto SDS–polyacrylamide gels. Gels were electrotransferred onto polyvinylidene difluoride (PVDF) membranes, treated with a blocking buffer (20 mM Tris–HCl pH 7.5, 137 mM NaCl, 0.1% Tween-20, 5% w/v non-fat dried milk) for 1 h at room temperature, and incubated with the primary antibody (anti-flag M2 monoclonal antibody (Sigma)) for 1 h at room temperature. After three washes in TBST (20 mM Tris–HCl pH 7.5, 137 mM NaCl, 0.1% Tween-20), membranes were incubated with the horseradish peroxidase-conjugated secondary antibody (goat anti-mouse IgG (Santa Cruz Biotechnology)) in blocking buffer for 1 h at room temperature. Detection was performed by chemiluminescence using Western Lightening (Perkin-Elmer Life Sciences).

2.9. Cell culture, electroporation and reporter assays

C33A or 293T cells were maintained in Dulbecco's modified Eagle's medium supplied with 10% heat-inactivated fetal calf serum (Gibco BRL), penicillin, and streptomycin. C33A or 293T cells were transiently transfected with plasmids by electroporation using the Gene Pulser II RF module system according to the manufacturer's instruction (Bio-Rad). Luciferase assays were performed with the Promega Luciferase Assay System or Dual-luciferase Assay System according to the supplier's protocol (Promega). β-Galactosidase or *Renilla* luciferase activities were used to normalize the transfection efficiency.

2.10. Subcellular localization experiment

Immunocytochemical analyses were performed as previously described [41]. Briefly, COS-7 cells were plated on glass coverslips and transfected with the indicated DNA plasmids using FuGene (Roche Diagnostics) following the manufacturer's instructions. At 48 h post transfection, the cells were washed in phosphate-buffered saline (PBS) and fixed for 10 min at −20°C with acetone/methanol (1:1, v/v). For hTAF$_{II}$68-TEC detection, anti-Flag antibody and secondary antibody conjugated with rhodamine (Sigma) were used. The coverslips were mounted with 50% glycerol/PBS. Fluorescence was detected with a fluorescence microscope (Olympus, 1X51) equipped with a CoolSNAP digital camera (Olympus).

3. Results

3.1. hTAF$_{II}$68 is a phosphotyrosine protein

The NTD of human hTAF$_{II}$68 is fused to the orphan family nuclear receptor TEC in extraskeletal myxoid chondrosarcoma and the encoded product likely acts as a transcriptional activator [19,42]. This domain is composed almost exclusively of tyrosine, glutamine, alanine, serine, threonine, glycine, and proline residues and shares homology with the amino-terminal domain of the EWS proto-oncoprotein [17,43]. Because the NTD of EWS has been previously shown to contain several potential tyrosine phosphorylation sites [29,30], we assessed if this was also the case for hTAF$_{II}$68. Interestingly, analysis of the hTAF$_{II}$68 (NTD) protein sequence (performed at http://www.cbs.dtu.dk/services/NetPhos/) revealed the presence of 13 potential tyrosine phosphorylation sites (Fig. 1A).

To assess the functional significance of these sites, we performed in vitro kinase assays utilizing recombinant v-Src and found that this caused phosphorylation of GST-hTAF$_{II}$68 (NTD) (Fig. 1B). Recombinant GST was not a substrate for v-Src phosphorylation (Fig. 1B), establishing that phosphorylation occurs within the hTAF$_{II}$68 (NTD) part of the fusion protein. These results demonstrate that GST-hTAF$_{II}$68 (NTD) is a substrate of Src kinase in vitro.

In order to better define the phosphorylation site(s) of v-Src on the hTAF$_{II}$68 (NTD), we also carried out an in vitro kinase assay with a series of hTAF$_{II}$68 (NTD) deletion mutants. Consistent with the prediction from the analysis of the putative tyrosine phosphorylation sites in hTAF$_{II}$68 (NTD) (Fig. 1A), in vitro incubation of GST-hTAF$_{II}$68 (1–72), hTAF$_{II}$68 (29–105), and hTAF$_{II}$68 (75–159) with recombinant v-Src protein kinase resulted in the phosphorylation of all three hTAF$_{II}$68 (NTD) truncation mutants (Fig. 1C).

To identify the amino acid acceptor of hTAF$_{II}$68, we performed phosphoamino acid analyses. Acid-hydrolyzed samples were spotted onto a TLC plate and the components separated by two-dimensional electrophoresis utilizing pH 1.9 in the first dimension and pH 3.5 in the second dimension. This analysis revealed that the site(s) phosphorylated by v-Src is/are tyrosine residue(s) (Fig. 1C).

To determine whether hTAF$_{II}$68 (NTD) is a substrate of the Src protein tyrosine kinase in vivo, a plasmid encoding His$_6$-Flag-tagged hTAF$_{II}$68 (NTD) was cotransfected into COS-7 cells with an expression plasmid driving synthesis of v-Src. Forty-eight hours after transfection, cells were lysed and His$_6$-Flag-tagged hTAF$_{II}$68 (NTD) proteins were affin-

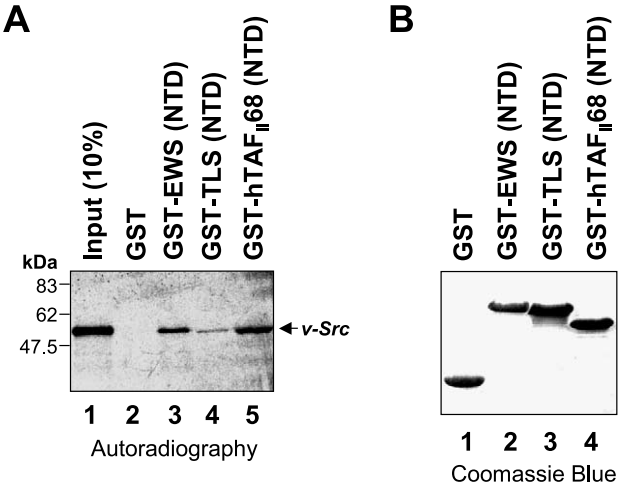

Fig. 2. Interaction between hTAF$_{II}$68 (NTD) and v-Src in vitro. A: Association of v-Src with the NTDs of EWS, TLS, or hTAF$_{II}$68. [^{35}S]Methionine-labeled v-Src was incubated with GST fusion proteins containing the NTDs of EWS, TLS, or hTAF$_{II}$68. An aliquot of input (10%) and the pellet from the various GST-TET (NTD) pull-downs were analyzed on a 10% SDS–polyacrylamide gel. The bound v-Src protein was visualized by fluorography. The nature of the GST fusion proteins is indicated above the panel. The positions of migration of the molecular weight markers is indicated to the left. v-Src protein is indicated by an arrow to the right. Lane 1, 10% input; lane 2, GST alone; lane 3, GST-EWS NTD; lane 4, GST-TLS NTD; lane 5, GST-hTAF$_{II}$68 NTD. B: Quantitation of the amounts of GST fusion proteins used in the GST pull-down assay. The GST fusion proteins utilized in the pull-down assays were fractionated on 15% SDS–PAGE and visualized by Coomassie blue staining. Lane 1, GST alone; lane 2, GST fusion EWS NTD; lane 3, GST fusion TLS NTD; lane 4, GST fusion hTAF$_{II}$68 NTD.

ity-precipitated with Ni^{2+}-NTA resin. Affinity-purified $hTAF_{II}68$ (NTD) proteins were fractionated by SDS–PAGE, transferred to PVDF membrane, and immunoblotted with an anti-Flag antibody to quantitate the amount of protein in each sample (Fig. 1D, top panel). To detect tyrosine phosphorylation, the anti-phosphotyrosine antibody 4G10 was used as a probe. Significant amounts of tyrosine phosphorylation were detected on $hTAF_{II}68$ (NTD) protein isolated from cells that had received the v-Src cDNA (Fig. 1D, bottom panel), but not in cells in which an empty expression vector had been introduced. These results demonstrate that v-Src is capable of modifying $hTAF_{II}68$ (NTD) by tyrosine phosphorylation in vitro and in vivo.

3.2. $hTAF_{II}68$ (NTD) interacts with v-Src in vitro and in vivo

Given that EWS (NTD) contains interaction motif(s) for v-Src SH3 and SH2 domains [29,30], the relatedness of the EWS and $hTAF_{II}68$ NTDs, and the results presented above indicating that $hTAF_{II}68$ is a substrate for Src, we assessed whether $hTAF_{II}68$ could also interact with v-Src. To test for the potential interaction between $hTAF_{II}68$ (NTD) and v-Src, in vitro pull-down assays were performed using bacterially expressed GST fusion $hTAF_{II}68$ (NTD), TLS (NTD), and EWS (NTD) proteins and radiolabeled full-length v-Src generated in an in vitro translation reaction. In this assay, GST did not show any non-specific association with v-Src (Fig. 2A, lane 2). In addition, in vitro translated luciferase did not bind to immobilized GST, GST-EWS (NTD), GST-TLS (NTD), or GST-$hTAF_{II}68$ (NTD), indicating that the interaction between v-Src and $hTAF_{II}68$ (NTD) is specific (H.J. Lee and J. Kim, data not shown). The NTD of EWS pulled down v-Src confirming this previously reported interaction [29] (lane 3). Both other members of the TET family, TLS and $hTAF_{II}68$, also pulled down v-Src, albeit with different efficiencies (compare lanes 4 and 5 to lane 3). For the purpose of the current study, we focused on better defining and charac-

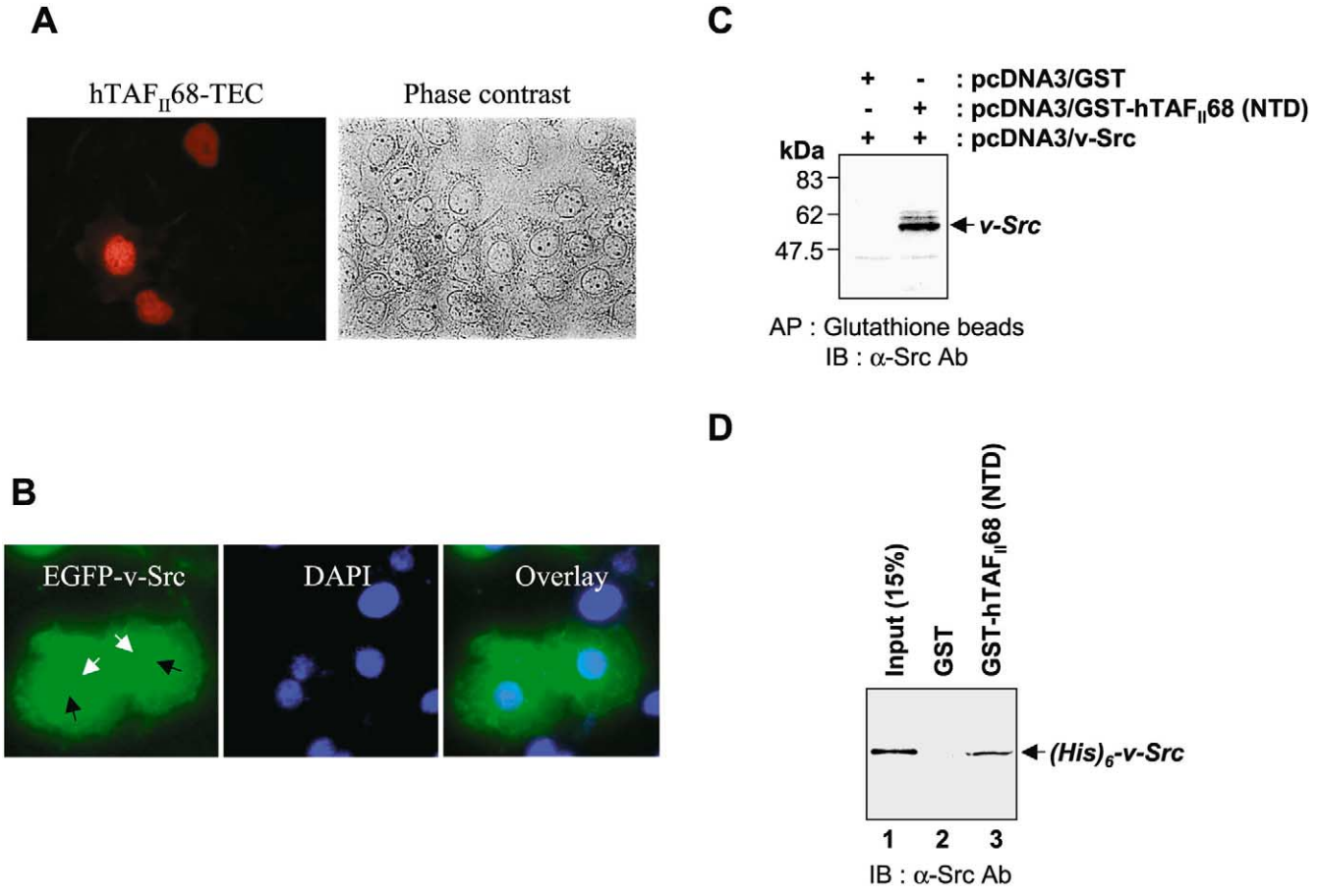

Fig. 3. Association of $hTAF_{II}68$ (NTD) with v-Src in vivo. A: Subcellular localization of $hTAF_{II}68$-TEC in cells. COS-7 cells grown on coverslips were transfected with mammalian expression vectors encoding Flag-tagged $hTAF_{II}68$-TEC. Subcellular distribution of $hTAF_{II}68$-TEC was analyzed with anti-Flag antibody. B: Subcellular localization of v-Src in cells. COS-7 cells grown on coverslips were transfected with mammalian expression vectors encoding GFP-tagged v-Src. Subcellular distribution of GFP-tagged v-Src was analyzed by fluorescent microscopy for green fluorescence. The white arrowheads show perinuclear regions with high concentrations of v-Src. The black arrowheads point out the fluorescent nuclei indicating the possible presence of v-Src in nucleus. C: Co-affinity purification of $hTAF_{II}68$ (NTD) with v-Src from 293T cells. Forty-eight hours after cotransfection of 293T cells with 10 μg of pcDNA3/v-Src and either 10 μg of pcDNA3/GST or pcDNA3/GST-$hTAF_{II}68$ (NTD), cell extracts were prepared as described in Section 2 and affinity-precipitated with glutathione-Sepharose beads. After fractionation on 10% SDS–PAGE, the affinity precipitates were analyzed for the presence or absence of v-Src protein by Western blotting utilizing an anti-Src antibody (H-12, Santa Cruz Biotechnology). The positions of the molecular weight markers are indicated to the left and the position of migration of v-Src is indicated by an arrow to the right. D: The interaction between $hTAF_{II}68$ (NTD) and v-Src is direct. Bacterially produced His_6-tagged v-Src protein was incubated with GST alone or GST-$hTAF_{II}68$ (NTD) at 4°C for 1 h. After extensive washing, bound v-Src was assessed by SDS–PAGE and Western blot analysis with an anti-v-Src antibody (H-12, Santa Cruz Biotechnology). Lane 1, 15% input; lane 2, GST alone; lane 3, GST-fusion $hTAF_{II}68$ (NTD).

terizing the interaction between hTAF$_{II}$68 (NTD) and v-Src. The amounts of GST fusion proteins utilized in this assays were fractionated on 15% SDS–PAGE and visualized by Coomassie blue staining and revealed that similar amounts of protein had been used in the pull-down assay (Fig. 2B).

The existence of hTAF$_{II}$68 (NTD)-v-Src complex in vitro was further supported by the intracellular localization of hTAF$_{II}$68 and v-Src in COS-7 cells. For this purpose, we constructed plasmid expressing Flag-tagged hTAF$_{II}$68-TEC fusion protein. A pCMV Tag2a-Flag-hTAF$_{II}$68-TEC was introduced into COS-7 cells and the subcellular localization of the protein was detected by immunocytochemistry. In transiently transfected cells, the Flag-tagged hTAF$_{II}$68-TEC protein was clearly localized in the nucleus (Fig. 3A). In addition, we also transfected hTAF$_{II}$68 or TEC constructs into COS-7 cells and detected both proteins in the nucleus (H.J. Lee and J. Kim, data not shown and manuscript in preparation). To further examine a possible colocalization of v-Src and hTAF$_{II}$68-fusion protein, we transfected pEGFP(N1)/v-Src (a mammalian expression vector encoding a fusion of v-Src and EGFP, in which the v-Src coding region was cloned into the 5′ end of EGFP) into COS-7 cells to determine its intracellular localization by using green fluorescence. Consistent with previous reports [44–46], the majority of v-Src proteins localize in the cytoplasm (Fig. 3B). In addition, significant amounts of myristoylated v-Src proteins were associated with the perinuclear membrane, as reported previously [47] (Fig. 3B, white arrowheads). Furthermore, consistent with another report [48], non-membrane-anchored v-Src was also detected in the nucleus, indicating the possible presence of myristoylation-deficient v-Src proteins in this cellular compartment (Fig. 3B, black arrowheads). Thus, these results demonstrate that hTAF$_{II}$68 and v-Src proteins partially colocalized in cells.

To determine whether the interaction between hTAF$_{II}$68 (NTD) and v-Src occurred in vivo, we performed co-affinity precipitations following transfection of 293T cells with expression vectors driving the synthesis of both proteins. For these assays, two mammalian expression vectors containing the NTD of hTAF$_{II}$68 fused to GST (pcDNA3/GST-hTAF$_{II}$68 (NTD)) and the GST domain alone (pcDNA3/GST) were cotransfected with pcDNA3/v-Src into 293T cells. Cells were lysed 48 h after transfection and GST or GST-hTAF$_{II}$68 (NTD) fusions were affinity-precipitated with glutathione beads. Immunoblottings performed on the eluents using an anti-Src antibody revealed that v-Src is specifically coprecipitated with GST-hTAF$_{II}$68 (NTD), but not with GST alone (Fig. 3A). These results demonstrate that hTAF$_{II}$68 (NTD) and v-Src can associate in vivo.

However, neither the in vivo interaction in mammalian cells nor the GST pull-down assay using in vitro translated v-Src addresses whether this interaction is direct or occurs via an intermediate bridging protein. To investigate this, v-Src was expressed as a His$_6$-containing fusion protein in *E. coli* and purified by Ni^{2+}-NTA-agarose resin. A GST pull-down assay was then performed using recombinant His$_6$-v-Src with GST or GST-hTAF$_{II}$68 fusion protein which had also been produced in *E. coli*. Following extensive washing, the amount of v-Src retained was determined by Western blotting using an anti-v-Src antibody. As shown in Fig. 3B, bacterially produced GST-hTAF$_{II}$68 (NTD) protein interacts with bacterially produced v-Src, indicating that the hTAF$_{II}$68 (NTD)-v-Src interaction appears not to require an adapter protein.

3.3. The C-terminal part of hTAF$_{II}$68 (NTD) is involved in v-Src interaction

To define the domain within hTAF$_{II}$68 (NTD) required for interacting with v-Src, in vitro binding experiments were performed with a series of hTAF$_{II}$68 (NTD) deletion mutants. The structures of the hTAF$_{II}$68 (NTD) deletion mutants used in this study are shown schematically in Fig. 4A. We first performed GST pull-down assays using in vitro translated [^{35}S]methionine-labeled v-Src protein, together with deletion mutants of hTAF$_{II}$68 (NTD). As shown in Fig. 4B, v-Src bound strongly to GST-hTAF$_{II}$68 (75–159), but interacted weakly with GST-hTAF$_{II}$68 (1–72) and GST-hTAF$_{II}$68 (29–105). The GST fusion hTAF$_{II}$68 (NTD) showed a similar

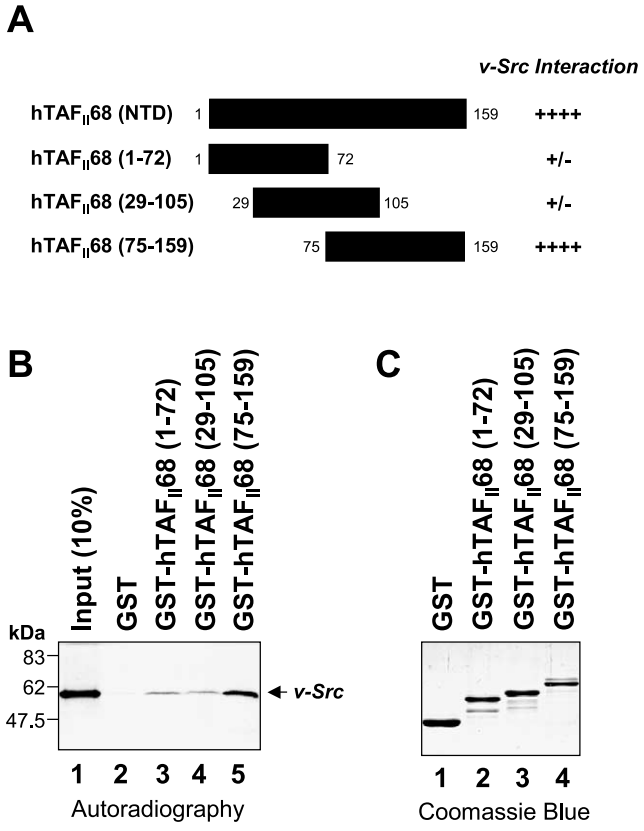

Fig. 4. Mapping the hTAF$_{II}$68 (NTD) region that interacts with v-Src. A: Schematic representation of the GST-hTAF$_{II}$68 (NTD) fusion proteins and their ability to bind to v-Src. Numbers refer to amino acid residues. Strength of binding is indicated in a semi-quantitative fashion (++++, strong binding; +/−, weak binding; −, no binding). B: Strong binding of v-Src to the GST-hTAF$_{II}$68 (75–159). Recombinant GST-hTAF$_{II}$68 (NTD) deletion mutants were incubated with [^{35}S]methionine-labeled v-Src protein. Following GST pull-down assays, the bound proteins were eluted with SDS loading buffer and analyzed by 15% SDS–PAGE. The positions of migration of molecular weight markers and of v-Src are indicated. Lane 1, 10% input; lane 2, GST alone; lane 3, GST fusion hTAF$_{II}$68 (1–72); lane 4, GST fusion hTAF$_{II}$68 (29–105); lane 5, GST fusion hTAF$_{II}$68 (75–159). C: Coomassie blue staining of various GST fusion hTAF$_{II}$68 deletions. The amounts of GST fusion proteins utilized in this assays were fractionated on a 15% SDS–polyacrylamide gel and visualized by Coomassie blue staining. Lane 1, GST alone; lane 2, GST fusion hTAF$_{II}$68 (1–72); lane 3, GST fusion hTAF$_{II}$68 (29–105); lane 4, GST fusion hTAF$_{II}$68 (75–159).

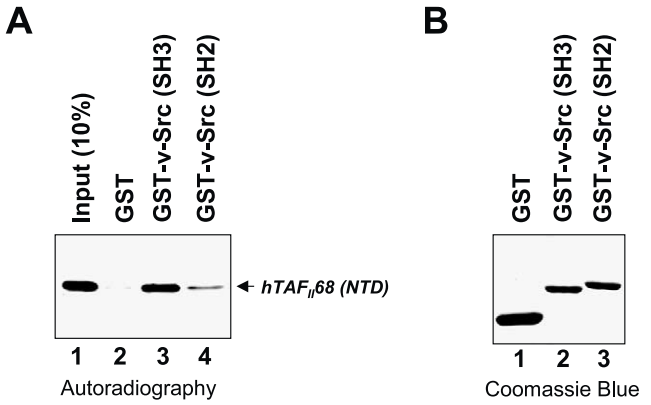

Fig. 5. Involvement of the SH3 and SH2 domains of v-Src in the interaction of $hTAF_{II}68$ (NTD). A: Binding of $hTAF_{II}68$ (NTD) to the v-Src SH3 and SH2 domains. In vitro generated [^{35}S]methionine-labeled $hTAF_{II}68$ (NTD) protein was incubated with GST, GST fusion SH3, or GST fusion SH2 domains bound to glutathione-Sepharose beads. An aliquot of the input and the pellets from GST pull-down assays were analyzed by 10% SDS–PAGE, and the bound $hTAF_{II}68$ (NTD) proteins were detected by fluorography. The position of migration of $hTAF_{II}68$ (NTD) is indicated by an arrow to the right. Lane 1, 10% input; lane 2, GST alone; lane 3, GST fusion v-Src SH3 domain; lane 4, GST fusion v-Src SH2 domain. B: Coomassie blue staining of GST, GST fusion v-Src SH3, and v-Src SH2 domains. The amounts of GST fusion proteins utilized in this assays were fractionated on 10% SDS–PAGE and visualized by Coomassie blue staining. Lane 1, GST alone; lane 2, GST fusion v-Src SH3 domain; lane 3, GST fusion v-Src SH2 domain.

binding profile as GST-$hTAF_{II}68$ (75–159), indicating that amino acids 75–159 of the $hTAF_{II}68$ (NTD) are sufficient for v-Src interaction (H.J. Lee and J. Kim, unpublished data). Our experiments do not address whether the observed in vitro interaction between $hTAF_{II}68$ (NTD) and v-Src is direct or indirect. As shown in Fig. 4C, the amounts of individual GST fusion proteins utilized in this assays were similar.

3.4. The SH3 and SH2 domains of v-Src interact with $hTAF_{II}68$ (NTD)

To determine whether the SH3 and/or SH2 domains of v-Src were required to bind to $hTAF_{II}68$ (NTD), a series of GST pull-down assays were performed with GST fusions of v-Src SH2 and SH3 domains. The important v-Src functional regions for protein–protein interaction, such as the SH3 and SH2 domains, were individually expressed as GST fusion proteins in *E. coli*, purified and coupled to glutathione-Sepharose beads. Following incubation with [^{35}S]methionine-labeled $hTAF_{II}68$ (NTD) protein and extensive washing, the amount of $hTAF_{II}68$ (NTD) retained was determined by SDS–PAGE and autoradiography. As shown in Fig. 5A, the GST fusion protein containing the v-Src SH3 domain was able to retain $hTAF_{II}68$ (NTD). The GST fusion protein containing the SH2 domain also bound to $hTAF_{II}68$ (NTD) but retained much less protein (Fig. 5A, compare lanes 3 and 4). However, $hTAF_{II}68$ (NTD) did not interact with GST alone (lane 2). These results suggest that the association of $hTAF_{II}68$ (NTD) with v-Src can be mediated independently through the SH3 or SH2 domains albeit the SH3 domain appears to have a higher affinity. The amounts of GST fusion proteins utilized in these assays were similar (Fig. 5B).

3.5. Differential binding of $hTAF_{II}68$ (NTD) to SH3 domains

Since $hTAF_{II}68$ (NTD) can associate with the SH3 domain of v-Src (Fig. 5A), we assessed whether $hTAF_{II}68$ (NTD) could interact with other SH3 domain-containing proteins. [^{35}S]Methionine-labeled $hTAF_{II}68$ (NTD) was prepared by in vitro transcription and translation, and GST pull-down assays were performed with GST fusion proteins containing the SH3 regions of various protein tyrosine kinases or adapter proteins. According to previous reports, EWS/WT1 protein was tightly associated with fusion proteins containing the c-Abl and v-Src SH3 domains [29,30]. In addition, a small amount of binding was also noted with the SH3 domain of

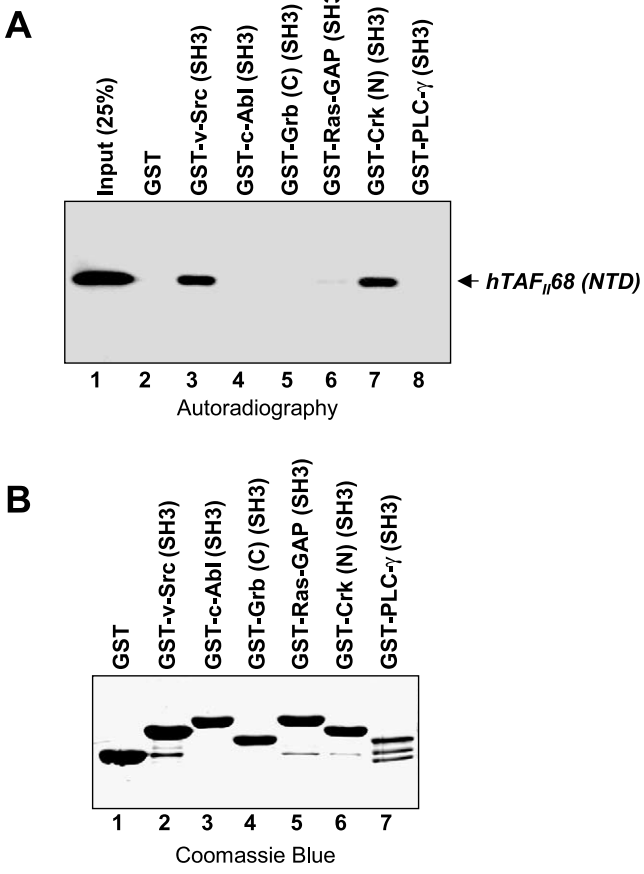

Fig. 6. The Crk(N) SH3 domain is capable of recognizing $hTAF_{II}68$ (NTD). A: Binding analysis of $hTAF_{II}68$ (NTD) to various SH3 domains. A number of GST-SH3 fusion proteins were expressed in *E. coli*, affinity purified from bacterial lysates, and coupled to glutathione-Sepharose beads. [^{35}S]Methionine-labeled $hTAF_{II}68$ (NTD) was incubated with GST fusion proteins containing the SH3 regions of v-Src, c-Abl, Grb(C), Ras-GAP, Crk(N), or PLC-γ. The proteins binding to the SH3 fusion proteins were eluted with SDS sample buffer, fractionated on 10% SDS–PAGE, and visualized by fluorography. The GST-SH3 domains from various proteins are indicated at the top of the panel. The position of migration of $hTAF_{II}68$ (NTD) is indicated to the right. Lane 1, 25% input; lane 2, GST alone; lane 3, GST-v-Src SH3 domain; lane 4, GST-c-Abl SH3 domain; lane 5, GST-Grb(C) SH3 domain; lane 6, GST-Ras-GAP SH3 domain; lane 7, GST-Crk(N) SH3 domain; lane 8, GST-PLC-γ SH3 domain. B: Coomassie blue staining of various GST fusion SH3 domains. The amounts of GST fusion proteins utilized in these assays were fractionated on 15% SDS–PAGE and visualized by Coomassie blue staining. Lane 1, GST alone; lane 2, GST-v-Src SH3 domain; lane 3, GST-c-Abl SH3 domain; lane 4, GST-Grb(C) SH3 domain; lane 5, GST-Ras-GAP SH3 domain; lane 6, GST-Crk(N) SH3 domain; lane 7, GST-PLC-γ SH3 domain.

Crk(N) [29,30]. We assessed the ability of hTAF$_{II}$68 (NTD) to interact with a number of SH3 domains isolated from various sources. The hTAF$_{II}$68 (NTD) polypeptide was retained by fusion proteins containing the v-Src and Crk(N) SH3 domains, but not significantly to the c-Abl, Grb(C), Ras-GAP, or PLC-γ SH3 domains (Fig. 6A, lanes 3–8). Thus, hTAF$_{II}$68 binds differentially to the SH3 domains tested and provides a molecular selectivity that can distinguish among SH3 domains. The lack of reactivity of several different SH3 domains with hTAF$_{II}$68 suggests that the hTAF$_{II}$68 interaction is specific and that the different types of SH3 domains are not equivalent with respect to hTAF$_{II}$68 binding activity. The amounts of GST fusion proteins utilized in this assays were equivalent (Fig. 6B).

3.6. v-Src activates hTAF$_{II}$68 (NTD)-mediated transactivation

Given the physical interaction between hTAF$_{II}$68 (NTD) and v-Src in vivo (Fig. 3A) and having identified hTAF$_{II}$68 (NTD) as an in vivo target of v-Src (Fig. 1D), we next measured the effect of v-Src expression on the transcriptional activation property of hTAF$_{II}$68 (NTD). This was done by examining gene expression from a luciferase-based reporter plasmid cotransfected with pcDNA3/Gal4-hTAF$_{II}$68 (NTD) with or without cotransfection of pcDNA3/v-Src into C33A cells. Consistent with previously reported data [42], introduction of Gal4-hTAF$_{II}$68 (NTD) into C33A cells significantly activated transcription (~20-fold) from pG5 luc, a reporter plasmid containing the E1B minimal promoter harboring five copies of Gal4 DNA binding sites and driving synthesis of *Renilla* luciferase (Fig. 7A, compare bar 2 to bar 1). Interestingly, the presence of v-Src augmented hTAF$_{II}$68 (NTD)-mediated transactivation five-fold (Fig. 7A, compare bars 3–5 to bar 2). v-Src had no significant effect on the transcriptional response from pG5 luc when introduced in the absence of hTAF$_{II}$68 (NTD) (H.J. Lee and J. Kim, data not shown).

To investigate the effect of v-Src on hTAF$_{II}$68 (NTD)-mediated transcription, two kinase-inactivated v-Src mutants, v-Src Δkinase and v-Src R295K, were used in the transfection

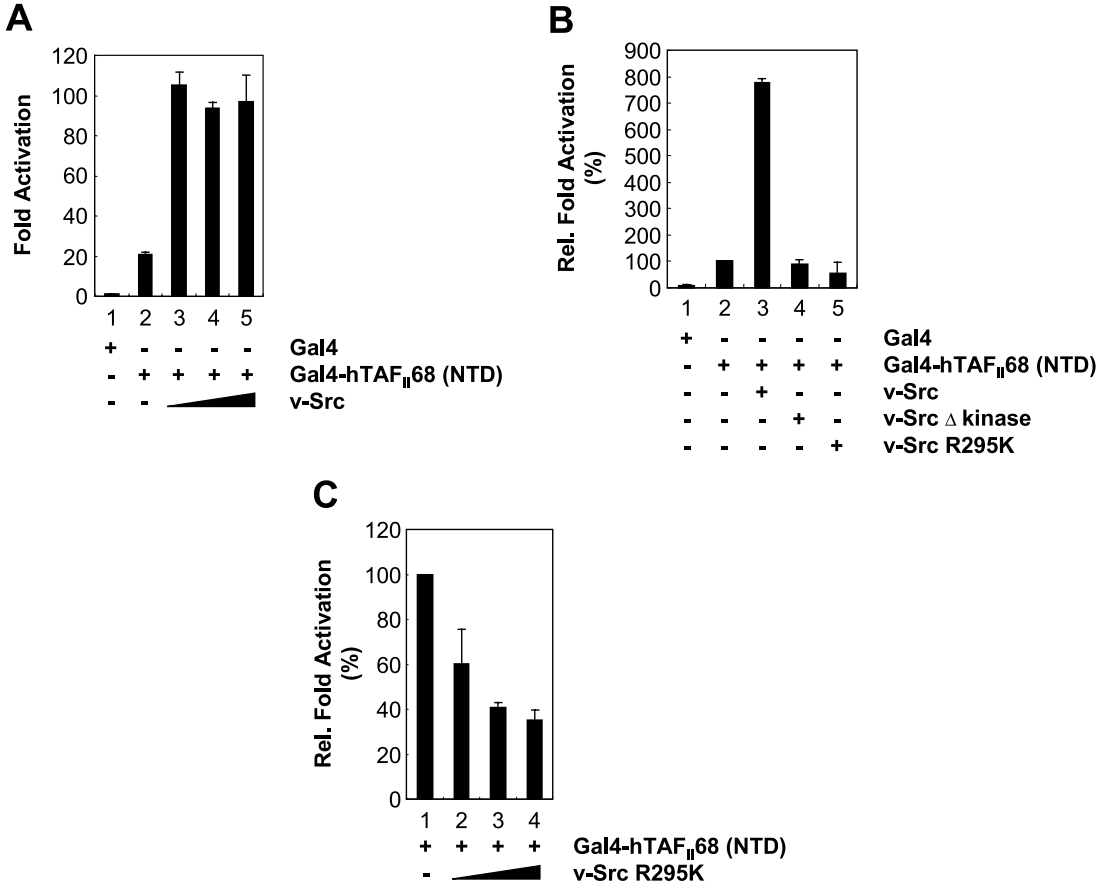

Fig. 7. Modulation of hTAF$_{II}$68 (NTD)-mediated transactivation by v-Src. A: Stimulation of transactivation activity of hTAF$_{II}$68 (NTD) by v-Src. C33A cells were transfected with 2 μg of Gal4 or Gal4-hTAF$_{II}$68 (NTD) plasmids alone or together with increased amounts of expression plasmids for v-Src (1, 3, and 5 μg) (bars 3–5). Cells were harvested 48 h post transfection in passive lysis buffer, and 10 μl was used in the luciferase assay. Values are expressed as mean increases ± S.D. relative to a value of 1 which was set from the reporter plasmid transfection alone. Results shown are means from two independent experiments performed in duplicate. B: The stimulation of hTAF$_{II}$68 (NTD)-mediated transactivation by v-Src is kinase-dependent. Two micrograms of Gal4 or Gal4-hTAF$_{II}$68 (NTD) expression plasmids were cotransfected with 1 μg of v-Src or v-Src kinase mutants into C33A cells, respectively. Cells were harvested 48 h post transfection in passive lysis buffer, and 10 μl was used in the luciferase assay. Luciferase activity was measured and normalized to *Renilla* luciferase activity. The relative transactivation values obtained with Gal4-hTAF$_{II}$68 (NTD) was taken as 100%. Results shown are means from two independent experiments performed in duplicate. The standard deviation for each experiment is shown by an error bar. C: Inhibition of hTAF$_{II}$68 (NTD)-mediated transactivation by dominant-negative v-Src. C33A cells were cotransfected with 2 μg of Gal4 or Gal4-hTAF$_{II}$68 (NTD) expression plasmids and increasing amounts (1, 3, and 5 μg) of dominant-negative v-Src mutant, v-Src R295K. Luciferase activity was measured and normalized to *Renilla* luciferase activity. The relative transactivation values obtained with Gal4-hTAF$_{II}$68 (NTD) was taken as 100%. Results shown are the mean from two independent experiments performed in duplicate. The standard deviation for each experiment is shown by an error bar.

assay (Fig. 7A). v-Src Δkinase and v-Src R295K are a kinase domain deletion mutant and a kinase-negative mutant, the latter harboring a lysine to arginine missense mutation in the kinase domain. As shown in Fig. 7B, both mutants failed to stimulate transcription mediated by hTAF$_{II}$68 (NTD), suggesting a requirement for the kinase activity of v-Src for this observed effect (compare bars 4 and 5 to bar 3).

To assess the possibility that a dominant-negative form of v-Src can block hTAF$_{II}$68 (NTD)-mediated transcription, we determined the effect of v-Src R295K on hTAF$_{II}$68 activity. When cells were cotransfected with Gal4-hTAF$_{II}$68 (NTD) and v-Src R295K, hTAF$_{II}$68 function was inhibited up to 65% in a dose-dependent manner (Fig. 7C). Taken together, these results suggest that the transcriptional activation function of hTAF$_{II}$68 (NTD) can be significantly increased by activation of Src, that this effect is dependent on Src kinase activity, and cellular Src activity is likely to be important for hTAF$_{II}$68 (NTD)-mediated transcription activation.

4. Discussion

In this report, we demonstrate that the transcriptional properties of hTAF$_{II}$68 can be modulated by the Src protein tyrosine kinase. The hTAF$_{II}$68 polypeptide shows extensive similarities to the EWS and TLS proteins [17,49]. The two latter genes, which were originally identified as the amino-terminal portions of translocation-generated fusion genes in Ewing's sarcomas and myxoid liposarcomas, encode putative RNA binding proteins containing a central RNA binding domain and three glycine-, arginine-, and proline-rich motifs [50,51]. We have previously documented that EWS/WT1 is phosphorylated by protein tyrosine kinases and this modification regulates DNA binding, self-association, and transactivation of the fusion protein [29,30,51], demonstrating that EWS (NTD)-mediated transcription could be regulated by signaling pathways under control of cytoplasmic kinases. In contrast to our understanding of EWS function, the regulation of hTAF$_{II}$68 remains poorly characterized.

Our results demonstrate that the hTAF$_{II}$68 (NTD) contains several potential tyrosine phosphorylation sites (Fig. 1A,C) and is a substrate for v-Src in vitro and in vivo (Fig. 1B,D). Src kinases have been reported to regulate a variety of cell functions, including cell cycle progression, growth, survival, and migration [24,52]. Studies using human tumor tissues and tumor-derived cell lines have demonstrated that enhanced tumorigenicity in vivo is correlated with elevated c-Src expression and tyrosine phosphorylation [53]. Our results suggest that v-Src resides upstream of hTAF$_{II}$68 and modulates hTAF$_{II}$68 function.

The finding that a dominant-negative form of v-Src blocks hTAF$_{II}$68 (NTD)-mediated transactivation (Fig. 7C) suggests that endogenous Src activity is required for hTAF$_{II}$68 activation in vivo. It is interesting to note that signaling through v-Src is known to perturb the transcription of cellular proteins. For instance, studies using the activated viral form of the Src protein have demonstrated that the ectopic introduction of v-Src can stimulate or repress the amounts of transcripts encoding a wide variety of cellular proteins [54–63]. In addition, other data have shown that transformation of cells by non-nuclear oncoproteins leads to deregulated transcription in the nucleus. For example, Ha-Ras, a cytoplasmic signaling protein, is critical for the activation of the *Egr-1* promoter and *Cox-2* gene (also known as *TIS10* and prostaglandin synthase) in v-Src-transformed cells [64,65]. Likewise, the serine/threonine kinase c-Raf is critical for the expression of *Egr-1* but plays no role in the activation of *Cox-2* [66,67]. Our studies provide a molecular mechanism for a specific case – that is, of Src signaling to hTAF$_{II}$68.

We have recently shown that the NTD of EWS contains six PxxP regions, which have been shown to interact with the SH3 domains [29,30]. It has been well documented that SH3 domains mediate protein–protein interactions involved in the signal transduction, cytoskeletal organization, and subcellular localization of proteins [68,69]. In fact, these domains consists of two anti-parallel β-sheets packed at right angles to one other [70,71]. Two SH3 variable loops, the RT and n-Src loops, flank a ligand binding region formed by the contribution of residues that are well conserved in the SH3 family and this region binds proteins with the consensus sequence PxxP [70,72–77]. We have previously reported the presence of two SH3 domain-interacting motifs within the proline-rich region of EWS (NTD) that correspond to the consensus motif (PxxxxPxxP) identified in a number of c-Abl targets [30]. In addition, three proline-rich motifs of EWS (NTD) could provide binding sites for the v-Src SH3 domain [29].

The interaction between hTAF$_{II}$68 (NTD) and v-Src SH3 domain was unexpected as hTAF$_{II}$68 (NTD) does not contain any proline-rich region corresponding to the consensus motif (PxxP) identified in a number of SH3 domain-interacting proteins. However, although SH3 domains are well established protein modules that recognize extended proline-rich sequences, a modification of this theme has recently been reported [78,79]. For example, Mongiovi et al. reported that sequences with the consensus PxxDY, derived from the e3b1/Abi-1 and RN-tre proteins, interact with the Eps8 SH3 domain [78]. In addition, Kato et al. [80] and Lewitzky et al. [81] reported that sequences with the consensus Px[VI][DN]RxxKP, from the UBPY, Gab1, AMSH, and SLP-76 proteins, bind to the SH3 domains in the Hbp, STAM, and Grb2 proteins. Similarly, the SH3 domain of p53BP2 interacts with a peptide sequence in p53 that is not proline-rich [82]. It is therefore likely that the hTAFII68 (NTD)/Src interaction falls into the class of SH3 interactions in which the partner lacks the prototypical PxxP motif.

Using recombinant proteins in a GST pull-down assay, we showed that hTAF$_{II}$68 harbors at least two independent binding sites for the v-Src protein (Fig. 4B): a high affinity binding site located in hTAF$_{II}$68 (75–159) and a low affinity binding site in hTAF$_{II}$68 (1–72). The binding observed with hTAF$_{II}$68 (29–105) may be due to a third site, or to the presence of the low affinity site detected with hTAF$_{II}$68 (1–72). Interestingly, v-Src also contains two independent hTAF$_{II}$68 (NTD)-interacting motifs – the SH3 and SH2 domains (Fig. 5A). These results suggest that the association of v-Src with hTAF$_{II}$68 (NTD) could be mediated by two different domains on each molecule or that the stoichiometry of the interacting proteins is greater than 1:1. Further experiments will be required to distinguish among these possibilities.

It has been shown that several SH3-containing adapter proteins, such as Crk, Grb2, and Nck, bind to the proline-rich region immediately C-terminal of the tyrosine kinase domain [83–85]. These adapter proteins play critical roles in the assembly of signaling complexes [84]. In general, such an adapter binds to a tyrosine-phosphorylated protein through its SH2

domain and a proline-rich protein through its SH3 domain and these simultaneous bindings allow the relay of a signal from one partner to the other [69]. Because the SH3 domain of Crk binds to hTAF$_{II}$68 (Fig. 6A), this adapter protein may recruit hTAF$_{II}$68 to specific signaling pathways.

Our finding that v-Src can phosphorylate and associate with hTAF$_{II}$68 (NTD) in vitro and in vivo indicates that v-Src resides upstream of hTAF$_{II}$68. However, it is not clear how the cytoplasmic membrane-anchored v-Src regulates activity of nuclear-localized hTAF$_{II}$68. One possibility that could explain these v-Src effects on hTAF$_{II}$68 (NTD) function is that the hTAF$_{II}$68 (NTD)-mediated transactivation is modulated by non-membrane-anchored v-Src. It is well documented that the N-terminal glycine residue of the Src kinase is cotranslationally myristoylated during protein synthesis [44,86–89] and this fatty acid modification is required for its plasma membrane association [44–46]. However, although myristoylation of c- or v-Src is required for association with the cytoplasmic membrane, not all myristoylated Src proteins are associated with the plasma membrane [90,91]. According to a previous report, significant amounts of myristoylated c- and v-Src kinases are also associated with the perinuclear membrane [47]. Furthermore, it has been estimated that as much as 16% of v-Src may not be modified in vivo [87]. Interestingly, myristoylation-deficient Src proteins do not simply lose the ability to bind with a high affinity to plasma membrane-associated receptor [88,92–94], but they also require the ability to translocate inside the nucleus [48]. Consistent with these reports, we also found that some v-Src protein is associated with the perinuclear membrane and present in the nucleus (Fig. 3B).

The other possibility that could explain these v-Src effects on hTAF$_{II}$68 (NTD)-mediated transactivation is that Src protein first affects induction or modification of nuclear transcriptional regulatory proteins which subsequently modulate transactivation properties of hTAF$_{II}$68. In fact, activation of gene expression is a common consequence following stimulation of receptor or non-receptor tyrosine kinases by growth factors such as epidermal growth factor, platelet-derived growth factor, or colony-stimulating factor 1 [24,95]. We also have shown that hTAF$_{II}$68 is able to bind to the SH2 or SH3 domains of several signaling molecules. Although the functional significance of these interactions still remains undefined, it is conceivable that other signaling proteins can also modulate hTAF$_{II}$68 functions.

hTAF$_{II}$68 was originally identified on the basis of its association with a distinct TFIID subpopulation [17]. TFIID is the main sequence-specific DNA binding components of the RNA polymerase II transcription machinery [96,97]. It is a multiprotein complex composed of the TBP and TBP-associated factors and is the factor that nucleates preinitiation complex formation on RNA polymerase II-mediated transcription [98]. Additionally, hTAF$_{II}$68 is also associated with another multiprotein complex, the human RNA polymerase II complex [99]. Interestingly, hTAF$_{II}$68 is able to enter into the preinitiation complex together with RNA polymerase II, suggesting that hTAF$_{II}$68 has a role in transcription initiation and/or elongation. Thus, the hTAF$_{II}$68 fusion product in human cancers may stimulate transcription in a similar fashion to that proposed for hTAF$_{II}$68. In addition, it is conceivable that hTAF$_{II}$68 (NTD) functions as a bridging factor by mediating a stable interaction between DNA and the transcription apparatus. Furthermore, it would also be interesting to attempt to determine if ectopic expression of v-Src could modulate the transcription initiation and/or elongation through hTAF$_{II}$68 modification.

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