The type II TGF-β receptor phosphorylates Tyr^{182} in the type I receptor to activate downstream Src signaling

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Abstract

Transforming growth factor–β (TGF-β) signaling has important roles during embryonic development and in tissue homeostasis. TGF-β ligands exert cellular effects by binding to type I (TβRI) and type II (TβRII) receptors and induce both SMAD-dependent as well as SMAD-independent intracellular signaling pathways. Activation of the tyrosine kinase Src is one such SMAD-independent consequence of TGF-β signaling. We investigated the mechanism by which TGF-β stimulation activates Src in human and mouse cells. Before TGF-β stimulation, inactive Src was present in a complex with TβRII. Upon TGF-β1 stimulation, which induces the formation of a complex of TβRI and TβRII, TβRII phosphorylated TβRI on serine and threonine residues, which promotes TβRI kinase activity, and on Tyr^{182}. The SH2 domain of Src bound to phosphporylated Tyr^{182}, leading to activation of Src kinase activity. Interaction of the Src SH3 domain with a proline-rich region in TβRI also contributed to binding. TGF-β1–induced Src activation depended on the kinase activity of TβRII but not on that of TβRI, indicating that binding to TβRI activated Src through a non-enzymatic mechanism. Activated Src then phosphorylated TβRI on several tyrosine residues, which may stabilize Src binding to the receptor. In functional assays, Src activation was required for the TGF-β–induced production of fibronectin and for migration in human breast carcinoma cells and for the induction of α-smooth muscle actin (α-SMA) and actin reorganization in mouse fibroblasts. Thus, TGF-β induces Src activation by stimulating a direct interaction with TβRI that depends on tyrosine phosphorylation of TβRI by TβRII.

Introduction

The transforming growth factor–β (TGF-β) superfamily of cytokines has 33 human genes, encoding members of the TGF-β, bone morphogenetic protein (BMP), and growth and differentiation factor (GDF) families, as well as activins, inhibins, nodal, and anti-Müllerian hormone (*1*). All have important roles during embryonic development and in tissue homeostasis through their abilities to regulate cell proliferation, migration, and differentiation. Perturbation of signaling by TGF-β family members is often seen in different diseases, including malignancies, as well as inflammatory and fibrotic conditions (*2*).

Activation of the TGF-β signaling pathway is initiated by the binding of a TGF-β ligand to a heterotetrameric complex of type I (TβRI) and type II (TβRII) receptors (*3*, *4*). Although the receptors are most often referred to as serine-threonine kinase receptors, their kinase domains have structural characteristics similar to both serine-threonine and tyrosine kinases (*4*). Several tyrosine residues in TβRII has been identified as autophosphorylation sites (*5*). TβRI also is autophosphorylated on tyrosine residues in addition to serine/threonine residues, but their location is not known (*6*). After ligand binding, the constitutively active TβRII phosphorylates TβRI in a region of the juxtamembrane domain rich in glycine and serine residues (the GS domain), leading to a conformational change in TβRI and activation of its kinase activity. Important substrates for TβRI are SMAD2 and SMAD3, which, after phosphorylation, assemble into heteromeric complexes with SMAD4 and translocate to the nucleus where they regulate the expression of specific genes in a contextual manner (*3*). In addition, certain non-SMAD signaling pathways are also activated downstream of TGF-β receptors, including the extracellular signal–regulated kinases 1 and 2 (Erk1/2), Jun N-terminal kinase (JNK), and p38 mitogen-activated protein

(MAP) kinase pathways, phosphatidylinositol 3´-kinase (PI3K), the kinase Src, and Rho GTPases (*7*–*10*).

The gene encoding the nonreceptor tyrosine kinase Src was one of the first identified proto-oncogenes (*11*, *12*). Src is a signaling hub that phosphorylates a broad spectrum of cytoplasmic targets, thereby linking extracellular signals to cell proliferation, migration, and apoptosis (*13*). In human cancers the proto-oncogene *SRC* is rarely mutated; however, increased amounts of Src protein and activity have been observed in numerous types of cancers (*14*–*16*). Src can be activated by several types of receptors, including receptor tyrosine kinases, G protein–coupled receptors, and integrins. In these cases, Src is activated by binding of its SH2 and SH3 domains to the activating receptors (*11*, *12*, *17*), which opens up the active site of the Src kinase domain, followed by autophosphorylation at a conserved tyrosine (Tyr^{416} in chicken or Tyr^{419} human) in the kinase activation loop, thereby locking the kinase into its catalytically competent conformation (*11*, *17*, *18*). Antibodies recognizing this phosphorylated tyrosine (referred to as Src-$pTyr^{416}$ regardless of species-specific amino acid numbering) have been used to evaluate the kinase activity of Src by immunoblotting analysis or by immunohistochemistry (*19*, *20*). Src activation is critically involved in tissue fibrosis and is important for promoting epithelial-mesenchymal transition (EMT) (*21*). Several papers have reported that TGF-β stimulation activates Src (*22*–*28*).

In the present study, we provide evidence for the molecular mechanism whereby TGF-β stimulation activates Src. We showed that the kinase activity of TβRII and the direct interaction between TβRI and Src was crucial for the activation of Src by TGF-β1 in human and mouse cells. We also demonstrated that TGF-β–induced actin reorganization and cell

migration in human breast carcinoma MCF10A cells and mouse embryonic fibroblasts (MEFs) depended on the activation of Src.

Results

TGF-β1 activates Src and induces the formation of a complex containing TβRI and Src

Stimulation with TGF-β1 activated the tyrosine kinase Src in human embryonic kidney (HEK) 293T cells, human prostate cancer (PC3U) cells, and mouse embryonic fibroblasts (MEFs), as determined by immunoblotting using an antibody specific for Src-pTyr^{416} (Fig. 1, A to C, and fig. S1, A and B). In MEFs, the onset of TGF-β1–induced Src activation coincided in time with SMAD2 and SMAD3 C-terminal phosphorylation and reached a maximum after about 30 minutes (fig. S1C). Activation of Src by TGF-β1 was not affected by SB505124, a pharmacological inhibitor of TβRI kinase activity of (Fig. 1A, fig. S1A), whereas it was completely blocked by SU6656, which inhibits the kinase activity of Src (Fig. 1B, fig. S1B). Transfection of HEK293T cells with increasing amounts of a plasmid encoding HA-tagged TβRI (HA-TβRI) resulted in Src activation proportional to the amount of the expression construct that was introduced into cells (fig. S1D). Ligand-dependency of Src activation was observed mainly in cells expressing a low amount of HA-TβRI and was less obvious when more receptor was expressed (fig. S1D). TGF-β1 induced Src activation to a similar extent in HEK293T cells overexpressing wild-type TβRI, the constitutively active mutant TβRI(T204D), or the kinase-dead mutant TβRI(K232R) (Fig. 1D). These results suggest that TGF-β1–induced Src activation does not depend on the kinase activity of the type I receptor but does depend on the kinase activity of Src itself.

Analyzing lysates of HEK293T and PC3U cells by co-immunoprecipitation, we found that Src formed a complex with HA-TβRI in a TGF-β1–dependent manner (Fig. 1, E and F, fig. S2, A and B). Endogenous Src also co-immunoprecipitated with endogenous TβRI from lysates of MEFs, and the interaction was stronger when the cells were treated with TGF-β1 (Fig. 1G). An interaction between TβRI and Src was also detected in reverse experiments in which HA-TβRI co-immunoprecipitated with endogenous Src (fig. S2C). TGF-β1–induced interaction between TβRI and Src did not depend on the kinase activity of the receptor or Src, because treatment of the cells with SB505124 or SU6656 did not affect their co-immunoprecipitation (Fig. 1, E and F, fig. S2, A and B). Moreover, Src co-immunoprecipitated not only with wild-type TβRI, but also with constitutively active and kinase-dead receptor mutants, from lysates of TGF-β1–treated HEK293T cells (fig. S2D), and a kinase-deficient mutant Src (K295R, Y527F) co-immunoprecipitated with the receptor to a similar extent as did wild-type Src (fig. S2E). These results indicate that activation of Src by TGF-β1 is induced by ligand binding, is independent of TβRI kinase activity, and involves the formation of a complex between TβRI and Src.

The SH2 and SH3 domains of Src interact with TβRI

To identify the epitopes in Src that are involved in the interaction with TβRI, we tested deletion mutants of Src (Fig. 2A) in immunoprecipitation assays. The C-terminal part of Src, encompassing its kinase domain, did not co-precipitate with TβRI, whereas the N-terminal part, including the SH2 and SH3 domains and the unique region of Src, co-precipitated with the receptor (Fig. 2B). Further analyses of N-terminal deletion mutants of Src revealed that deletion mutants lacking the Src unique region (Src(91-536)) or the unique region and the SH3 domain (Src(151-536)) co-precipitated with the receptor (Fig. 2C). This implies that the SH2 domain of Src is sufficient for its interaction with HA-TβRI.

However, the results of glutathione S-transferase (GST) pulldown experiments suggested that, in addition to the SH2 domain, the SH3 domain of Src also interacted with TβRI (Fig. 2D).

We analyzed further the possible contribution of the SH3 domain of Src for the binding to TβRI. Analysis of the TβRI structure revealed that it contains a proline-rich sequence, KLRPNIPNR (fig. S3A), that is similar to binding motifs for class I (R/KxxPxxP) and class II (PxxPxR/K) SH3-domains (*28*). Point mutations in this region of TβRI (R451A, P455A, or R457A) led to reduced Src co-immunoprecipitation (Fig. 2E and fig. S3B) and a reduction in binding to a GST-Src SH3 domain fusion protein (fig. S3C). These results suggest that the SH3 domain of Src contributes to Src binding to TβRI by interacting with the proline-rich motif in the receptor.

The kinase activity of TβRII is necessary for Src activation by TGF-β1

The binding of TGF-β1 to TβRII and TβRI is known to stabilize the receptor complex (*3*, *4*). Therefore, we studied the role of TβRII in Src activation by TGF-β1 (Fig. 3, A to D). Overexpression of TβRII in HEK293T cells led to an increase of TGF-β1–induced activation of Src, as determined by immunoblotting for Src-pTyr^{416} (Fig. 3A). As expected, an increased amount of TβRII also led to an enhanced phosphorylation of SMAD2 in response to TGF-β1 stimulation. No ligand-induced increase in Src activation was observed in cells transfected with kinase-defective TβRII (Fig. 3A). These results indicate that, unlike the case for TβRI, the kinase activity of TβRII is necessary for the activation of Src by TGF-β1.

TβRII promotes the interaction between TβRI and Src by phosphorylating tyrosine residue(s) in TβRI

We investigated whether TβRII also binds Src and found that endogenous Src co-immunoprecipitated with endogenous TβRII from the lysates of MEFs regardless of whether or not the cells were treated with TGF-β1 (Fig. 3B). TβRII kinase activity or co-expression with TβRI did not affect its interaction with Src (Fig. 3, C and D). However, as determined by immunoblotting using the antibody specific for Src-pTyr^{416}, the interaction with wild-type or kinase-deficient TβRII did not activate Src (Fig. 3C). In contrast, activated Src was detected in TβRI immunoprecipitates (Fig. 3D).

Having found that TβRI interacted with both the SH2 and SH3 domains of Src (Fig. 2D), we analyzed the role of TβRII in these interactions. Co-expression of wild-type TβRII, but not the kinase-deficient mutant, increased the amount of TβRI that precipitated with GST-Src(SH2) in pull-down experiments, whereas the low amount of TβRI that precipitated with GST-Src(SH3) did not depend on the kinase activity of co-expressed TβRII (Fig. 4A). These findings suggest that phosphorylation of TβRI by TβRII increases its affinity for GST-Src(SH2), suggesting that TβRII phosphorylates TβRI on tyrosine residue(s), thus providing bindings site(s) for the SH2 domain of Src.

Immunoblotting TβRI immunoprecipitates with the pY99 antibody, which recognizes all phosphorylated tyrosine residues, showed that TβRI was phosphorylated on tyrosine residues when it was co-expressed with TβRII (Fig. 4B). A TβRI mutant lacking all tyrosine residues in the cytosolic portion of the receptor (HA-TβRI(11Y>F)) was not recognized by the pY99 antibody, indicating specificity of the assay (Fig. 4B). Subsequent analysis showed that mutation of Tyr^{182} in TβRI considerably suppressed the phosphorylation of the

receptor by TβRII, suggesting that this residue is a major target in TβRI for tyrosine phosphorylation by TβRII (Fig. 4C). The involvement of tyrosine residues of TβRI in its interaction with Src was supported by a GST pulldown assay demonstrating that the HA-TβRI(11Y>F) mutant was not pulled down with GST-Src(SH2) (Fig. 4D). In accordance, co-immunoprecipitation of Src was much weaker with the HA-TβRI(11Y>F) mutant compared with the wild-type receptor (Fig. 4E).

TβRI activates Src in vitro

To further investigate the activation of Src by TβRI, we performed in vitro kinase assays using purified, tagged proteins (Fig. 5, A to D). Immunoblotting with antibodies to Src-pTyr^{416} demonstrated that GST-TβRI activated GST-Src in vitro (Fig. 5A). Similarly, GST-TβRI strongly enhanced the phosphorylation of endogenous Src immunoprecipitated from lysates of PC3U cells, as shown by immunoblotting with the pY99 antibody (Fig. 5B).

TβRI has been reported to be a dual-specificity kinase that possesses an intrinsic kinase activity directed to serine, threonine, and tyrosine residues (*6*). To investigate the possibility that tyrosine residues in Src were phosphorylated by GST-TβRI, we performed in vitro kinase reactions in the presence of radiolabelled ATP (γ-^{33}P-ATP). The SH2 and SH3 domains of Src were not phosphorylated by GST-TβRI, whereas, as expected, GST-SMAD2 was phosphorylated by the receptor under the same conditions (Fig. 5C).
Using in vitro kinase reactions with immunoprecipitated Src overexpressed in HEK293T cells, we found that its activation in the presence of GST-TβRI was abolished by AZM475271, an inhibitor of Src kinase activity, but not by SB505124, an inhibitor of TβRI kinase activity (Fig. 5D). As expected, the antibody to Src-pTyr^{416} did not bind to a kinase-

deficient Src mutant (Fig. 5D). These results suggest that TβRI does not phosphorylate Src directly, but activates its kinase, thereby inducing Src autophosphorylation.

Src phosphorylates TβRI on tyrosine residues

It has been reported that TβRII is phosphorylated on tyrosine residues by Src (*29*). We investigated the possibility that TβRI is also phosphorylated by Src. Only low amounts of tyrosine phosphorylation were observed in TβRI immunoprecipitated from HEK293T cells, with or without TGF-β1 stimulation, as determined by immunoblotting with the pY99 antibody (Fig. 6A, lane 1). However, co-expression with Src enhanced tyrosine phosphorylation of TβRI, and this was slightly increased upon treatment of the cells with TGF-β1 (Fig. 6A, lanes 2 and 3). The phosphorylation of TβRI on tyrosine residues was dependent on Src activity, because it was not detected when the receptor was co-transfected with a kinase-deficient Src mutant (Fig. 6B).

We further explored the phosphorylation of endogenous TβRI using MEFs. Phosphorylation of TβRI on tyrosine residues, was observed in wild-type MEFs and enhanced by TGF-β1 stimulation (Fig. 6C). However, in MEFs deficient three Src family members (Src, Fyn, and Yes; MEF(SYF$^{-/-}$) cells), very little tyrosine phosphorylation of TβRI was seen with or without stimulation with TGF-β1, as determined by immunoblotting with pY99 antibodies (Fig. 6C). In addition, TGF-β1–enhanced phosphorylation of TβRI on tyrosine residues was abolished in MEFs treated with the Src inhibitor AZM475271, but not in cells treated with the TβRI inhibitor SB505124 (Fig. 6D). Thus, TβRI was phosphorylated on tyrosine residues in cells mainly by Src kinase(s).

Because endogenous TβRI migrates during electrophoresis as a wide band and may overlap with the area in which Src migrates, we analyzed TβRI immunoprecipitates from TGF-β1–treated MEFs for the presence of Src. Immunoblotting for Src did not detect Src in immunoprecipitates of endogenous TβRI from the lysates of MEF cells (fig. S4A). Thus, endogenous Src did not co-precipitate with endogenous TβRI when RIPA lysis buffer was used for protein extraction. These results confirmed that the band shown in the immunoblot with the pY99 antibody (Fig. 6, C and D) represents endogenous TβRI phosphorylated on tyrosines and not phosphorylated Src.

The ability of Src to phosphorylate TβRI was analyzed by in vitro kinase assays with purified GST-Src and GST-TβRI (fig. S4, B and C). GST-Src underwent autophosphorylation, as did GST-TβRI. The TβRI inhibitor SB505124 strongly reduced autophosphorylation of the receptor but did not affect the phosphorylation of GST-TβRI by GST-Src (fig. S4B). Immunoblotting with pY99 antibodies revealed no autophosphorylation of TβRI on tyrosine residues, suggesting that the observed autophosphorylation (fig. S4B) occurred on serine and threonine residues; however, it confirmed the phosphorylation of GST-TβRI on tyrosine residues by Src (fig. S4D).

The intracellular domain of TβRI contains 11 tyrosine residues, six of which are potential sites for phosphorylation by Src (PhosphoMotif Finder (*30*)) (fig. S4D). According to the TβRI crystal structure, three of these residues – Tyr^{182}, Tyr^{429}, and Tyr^{476} – are located close to the surface of the folded molecule (PDB Entry: 1B6C, DOI: 10.2210/pdb1b6c/pdb) and may therefore be primary targets for phosphorylation. To identify the tyrosine residues in TβRI that are phosphorylated by Src, we performed in vitro kinase reactions using GST-tagged Src and GST-tagged kinase-deficient mutants of TβRI (K232R) with certain

tyrosine residues replaced by phenylalanine residues as substrates (Fig. 6E). The pY99 antibody recognized kinase-deficient TβRI with intact tyrosine residues, but not the mutant in which all 11 tyrosine residues were mutated (11Y>F), after phosphorylation by GST-Src, confirming the specificity of the assay (Fig. 6E). Mutation of Tyr^{182} or Tyr^{476} in TβRI did not decrease TβRI(K232R) phosphorylation by Src in vitro, whereas mutation of Tyr^{428} or Tyr^{429} decreased TβRI(K232R) phosphorylation (Fig. 6E). Analysis of TβRI phosphorylation in cells revealed that wild-type HA-TβRI, but not the mutant in which the tyrosines at positions 182, 249, 282, 291, 295, 428, 429, and 476 were mutated to phenylalanines (HA-TβRI(8Y>F)), was phosphorylated on tyrosine residues when it was coexpressed with Src in HEK293T cells (Fig. 6F). TβRI mutants in which different subsets of these tyrosines were substituted [TβRI(Y182,428,429,476F) or TβRI(Y182,295,428,429F)] showed diminished, but not completely suppressed, phosphorylation on tyrosine residues (Fig. 6F).

To ensure that the band observed in the pY99 blot (Fig. 6, A, B and F) represented TβRI and not co-immunoprecipitated Src, which has a similar size, we carefully compared the migration of HA-TβRI and Src during electrophoresis (fig. S4, E and F). We found that the pY99 band migrated exactly as TβRI, whereas Src migrated slightly slower (fig. S4E). To confirm that the pY99 antibody interacted with phosphorylated TβRI, we did consecutive immunoprecipitation of the receptor (fig. S4F). We did not detect any co-precipitated Src after the second round of immunoprecipitation with HA antibody (fig. S4F). The pY99 antibody interacted with wild-type and constitutively active TβRI, but not with the TβRI(8Y>F) mutant, confirming phosphorylation of the receptor on tyrosine residues (fig. S4F). These observations suggest that TβRI can be phosphorylated by Src on multiple

sites, however, Tyr^{182}, identified in the current study as a major site phosphorylated by TβRII, is not a major residue for Src phosphorylation.

Src is important for cellular responses to TGF-β

In order to investigate the importance of Src activation in TGF-β–induced signaling, we determined the effect of Src inhibitors on cellular responses known to be regulated by TGF-β. The TGF-β1–induced production of fibronectin in human breast carcinoma MCF10A cells was blocked by the TβRI inhibitor SB505124 and also strongly reduced by the Src inhibitor AZM75271 (Fig. 7, A and B). At the same time, phosphorylation of SMAD2 and the abundance of plasminogen activator inhibitor-1 (PAI-1) were not appreciably affected by Src inhibition but were decreased in cells treated with SB505124 (Fig. 7, A and B).

The induction of α-smooth muscle actin (α-SMA) by TGF-β1 in MEFs was not inhibited by the Src inhibitor SU6656 and did not depend on Src family kinases (Fig. 7C). However, immunofluorescence staining revealed that TGF-β1–induced actin filament formation was inhibited in MEFs treated with SU6656 (Fig. 7D). Moreover, TGF-β1 did not induce actin filament formation in MEFs deficient for Src family kinases [MEF($SYF^{-/-}$) cells], but transfection with Src partially rescued filament formation (Fig. 7D).

We also found that the kinase activity of Src was important for TGF-β1–induced cell migration. In a cell culture wound healing assay, SU6656 inhibited the TGF-β1–induced migration of MCF10A and PC3U cells (Fig. 7E, fig. S5A). The TβRI inhibitor SB505124 also inhibited TGF-β1–induced migration of MCF10A cells, but it had only a minor effect on the migration of PC3U cells (Fig. 7E, fig. S5A). The importance of Src for TGF-β1–induced

cell migration was confirmed using MEF(SYF$^{-/-}$) cells. Whereas migration of these cells in cell culture wound healing assays was stimulated moderately by TGF-β1, restoration of Src expression enhanced migration in response to TGF-β1 stimulation (Fig. 7F).

Src is known to cooperate with the tyrosine kinase focal adhesion kinase (FAK) to promote cell migration. We found that TGF-β1 promoted phosphorylation of FAK at Tyr^{397}, Tyr$^{576/577}$, and Tyr^{925} in wild-type MEFs (fig. S5B). In MEF(SYF$^{-/-}$) cells, we detected TGF-β1–induced phosphorylation of Tyr^{397} and Tyr^{925} but no phosphorylation of Tyr$^{576/577}$ (fig. S5B). These observations suggest that TGF-β1 stimulation of FAK phosphorylation at Tyr$^{576/577}$, known to be important in cell migration (*31*), is dependent on Src. It remains to be explored whether and to what extent these effects of Src on TGF-β1-induced cellular responses are mediated by phosphorylation of TβRI on tyrosine residues.

Discussion

Here, we have shown that Tyr^{182}, located in proximity to the GS domain of TβRI, was a major tyrosine residue phosphorylated by TβRII in response to TGF-β stimulation. This promoted the association of Src with TβRI, leading to Src autophosphorylation. Based on these results, we propose a model for the mechanism by which TGF-β stimulation activates Src (Fig. 8, A to C). Before TGF-β stimulation, inactive Src occurs in a complex with TβRII (Fig. 8A). After TGF-β–induced formation of the heterotetrameric receptor complex of TβRI and TβRII, TβRII phosphorylates TβRI on serine and threonine residues to activate its kinase activity, and on Tyr^{182}, and possibly other tyrosine residue(s), to which the SH2-domain of Src can bind, leading to activation of the kinase activity of Src and Src autophosphorylation (Fig. 8B). An interaction of the Src SH3-domain with a proline-rich motif in TβRI contributes to the strength of Src binding to TβRI. Src thereafter

phosphorylates TβRI on several tyrosine residues, which may enforce Src binding and possibly also have other effects on TGF-β signaling (Fig. 8C). Such phosphorylation of the receptor was enhanced in MEFs treated with TGF-β1 but was not detected in fibroblasts deficient in Src family kinases (Fig. 6C). Notably, the mechanism we describe has similarities to the well-established manner in which tyrosine kinase receptors activate Src, specifically through the binding of the Src SH2 domain to phosphorylated tyrosine residues in the receptors, whereafter the catalytic site of Src is opened (*11*, *17*).

Whereas TGF-β–induced phosphorylation of SMAD2 and SMAD3, leading to activation of SMAD signaling, has major roles in TGF-β signaling (*3*), there are also SMAD-independent signaling pathways initiated by the activated TGF-β receptors, including the Erk1/2, JNK, and p38 MAP kinase pathways, PI3-kinase, Rho GTPases, and Src, that are implicated in specific downstream cellular responses (*7*, *32*). Activation of these pathways often involves the recruitment of adaptor proteins and is dependent on the cell type and the subcellular localization of the receptors (*8*–*10*). For example, TGF-β has been reported to activate Src indirectly through increased production of protein tyrosine phosphatase receptor type kappa (PTPRK) and αvβ3 integrins (*25*, *26*), activation of epidermal growth factor (EGF) receptor by tumor necrosis factor α converting enzyme (TACE, also called ADAM17) (*22*, *33*), and by the production of reactive oxygen species (*34*). In addition, in macrophages, TGF-β also enhances *Src* expression in a manner that depends on SMAD3 (*21*). Whether these mechanisms can occur in parallel to the mechanism involving a direct binding of Src to TβRI, as described here, remains to be determined.

Because the interaction of Src with TβRI and its activation were enhanced by TGF-β1 stimulation and required the kinase activity of TβRII, we investigated whether TβRII

phosphorylated TβRI on tyrosine residues, in addition to the serine and threonine residues that are involved in its activation. Immunoblotting with antibodies specific to phosphorylated tyrosine revealed that this was the case (Fig. 4B). It is known that TβRII is autophosphorylated on Tyr^{259}, Tyr^{336}, and Tyr^{424} (*5*); however, no information has been available on whether TβRII also can phosphorylate TβRI or other proteins on tyrosine residues. Here we provide evidence that Tyr^{182}, located close to the GS domain of TβRI, is a major tyrosine residue phosphorylated by TβRII (Fig. 4C).

Mutation of tyrosine residues in TβRI reduced but did not completely block Src binding to the receptor (Fig. 4E). As shown by GST pulldown experiments, the SH3 domain of Src could also bind to TβRI (Fig. 2D); in contrast to the binding by the SH2 domain, this interaction did not depend on the kinase activity of TβRII or treatment with TGF-β1 (Fig. 4A). Thus, it is possible that the interaction between TβRI and Src involves different epitopes that bind the SH2 and SH3 domains of Src, respectively. However, given the need for the kinase activity of TβRII for efficient Src activation, it is likely that interaction of the SH2 domain of Src with phosphorylated residue(s) in TβRI is of major importance for Src activation.

Src activation by, for example, the EGF receptor (*35*), the platelet-derived growth factor (PDGF) receptor (*36*, *37*), and FAK (*38*), involves binding of the SH2 domain of Src to phosphorylated tyrosine residues in the activating proteins, which promotes Src activation by disrupting intramolecular inhibitory interactions within the Src molecule (*11*, *12*). In many cases, Src in turn phosphorylates the bound protein on additional tyrosine residues that modulate their activity and creates new sites for interaction with other signaling molecules. Thus, Src phosphorylates the EGF receptor on multiple sites and integrates the

EGF receptor with other non-related membrane receptors and intracellular signaling molecules (*14*). Src also phosphorylates the PDGFβ receptor, which modulates chemotaxis and mitogenesis (*39*). Similarly, autophosphorylation of FAK on Tyr^{397}, induced by ligand-bound receptor tyrosine kinases or clustered integrins, creates a high-affinity binding site for Src, leading to phosphorylation of FAK at additional residues and promoting downstream signaling (*38*).

We found that Src phosphorylates TβRI at several tyrosine residues. Replacement of Tyr^{182}, which was phosphorylated by TβRII, with a phenylalanine residue had no appreciable effect on Src phosphorylation of TβRI, whereas mutation of Tyr^{428} and Tyr^{429} decreased TβRI phosphorylation. However, mutations of Tyr^{182}, Tyr^{428}, Tyr^{429}, and Tyr^{476}, or mutations of Tyr^{182}, Tyr^{295}, Tyr^{428}, and Tyr^{429}, did not completely inhibit tyrosine phosphorylation of TβRI (Fig. 6F). These observations indicate that TβRI is phosphorylated by TβRII and Src at different tyrosine residues and that Src phosphorylates several residues. It has been shown that Src phosphorylates TβRII at Tyr^{284} (*26*, *29*) and Tyr^{470} (*40*). Phosphorylation of Tyr^{284} promotes the binding of the adaptors Shc and Grb2, which leads to activation of the Erk1/2 MAP kinase pathway (*41*). The possible role of TβRI phosphorylation by Src for TGF-β signaling requires further studies.

Upon TGF-β stimulation, activated TβRI can directly phosphorylate ShcA on tyrosine and serine residues (*6*). We investigated whether TβRI also phosphorylates Src. The results of our experiments suggest that this is not the case and that the increased phosphorylation of Src observed in the presence of TβRI is the result of Src activation and its

autophosphorylation (Fig. 5, A to D). This observation supports the notion that TβRI activates Src by binding to its SH2 and SH3 domains.

Src transmits external signals to the interior of the cell to regulate a variety of normal and oncogenic processes, including proliferation, differentiation, survival, motility, and angiogenesis (*12*, *14*, *42*). To accomplish these activities, Src interacts with numerous cellular molecules, including receptor tyrosine kinases, integrins, FAK, and adaptor proteins such as p130Cas and Shc (*12*, *14*). It has been reported that Src family tyrosine kinases modulate cellular responses to TGF-β, such as growth arrest (*28*, *43*), cytoskeletal reorganization (*23*), cell migration (*28*, *44*), and invasion (*12*, *45*). The kinase activity of Src is also important for TGF-β–induced myofibroblast differentiation (*46*–*48*) and macrophage-to-myofibroblast transition (*49*). In our experiments, inhibition of Src strongly decreased TGF-β1–induced fibronectin production, as well as actin reorganization and migration, but had little effect on TGF-β1–induced PAI-1 accumulation or SMAD2 phosphorylation (Fig. 7, A to F, fig. S5A).

Increased abundance and/or catalytic activity of Src have been detected in a number of human cancers, including lung, skin, colon, prostate, breast, ovarian, endometrial, pancreatic, and head and neck malignancies (*14*–*16*, *50*). Similarly, many cancers are characterized by enhanced TGF-β signaling (*51*–*53*). It is possible that inhibition of TGF-β–activated Src could be beneficial for treatment of cancer patients. As follows from our results, activation of Src by TGF-β does not depend on the kinase activity of TβRI, suggesting that TβRI kinase inhibitors would not effectively block the protumorigenic consequences of Src activation by TGF-β.

In summary, we have identified a detailed molecular mechanism whereby ligand-induced activation of TGF-β receptors results in the activation of the tyrosine kinase Src. Because such a mechanism does not require the kinase activity of TβRI, our work provides important information for the development of anticancer drugs targeting the protumorigenic signaling of TGF-β.

Materials and Methods

Cell culture and transfection

293T (ATCC CRL3216) and MCF10A (ATCC CRL10317) cells were obtained from ATCC. Mouse embryonic fibroblasts (MEFs) or MEFs deficient in Src family kinases (MEF(SYF$^{-/-}$)) were kind gifts from Dr. Johan Lennartsson (Uppsala University). MEFs, MEF(SYF$^{-/-}$), and 293T cells, were grown in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal bovine serum (FBS) and 2 mM L-glutamine, 100 units/ml penicillin and 100 µg/mL streptomycin. MCF10A cells were grown in DMEM/F12 (Gibco), supplemented with 5% FBS (Biowest), 20 ng/ml EGF (PeproTech), 100 ng/ml cholera toxin (Sigma-Aldrich), 0.5 µg/ml hydrocortisone (Sigma-Aldrich) and 10 µg/ml insulin (Sigma-Aldrich) (complete medium). The human prostate (PC-3U) cancer cell line (*54*) were cultured in RPMI-1640 medium supplemented with 10% FBS and 2 mM L-glutamine, 100 units/ml penicillin and 100 µg/ml streptomycin. All cell cultures were incubated at 37°C in the presence of 5% CO_2. For TGF-β stimulation experiments, PC-3U, MEF, and MEF(SYF$^{-/-}$) cells were starved for 16 h in media supplemented with 0.1% FBS; 293T and MCF10A cells were starved for 16 h in media supplemented with 1% FBS. Cells were then stimulated with 5 ng/ml TGF-β1.

For transient transfection, cells were seeded at 60% density in the corresponding culture medium. Cells were transfected with plasmid DNAs using FuGENE HD (E2312, Roche) according to the manufacturer's protocols. Transfection media were replaced with fresh media after 24 h.

Antibodies and reagents

Antibodies or antisera against the following proteins were used in immunoblotting (IB), immunoprecipitation (IP) or immunofluorescence (IF), at the indicated dilutions: HA (Y-11, 1:500), TβRI (V22, 1:250 and 1:100 for IP; the specificity of this antibody has previously been reported (*55*)), TβRII (E6, 1:500), GST (B-14, 1:500), and p-Tyr (PY99, 1:500) were from Santa Cruz Biotechnology; a phosho-SMAD2 (1:500) antisera were generated in rabbits in-house; antibodies against Src (36D10 or L4A1, 1:1000) and p-Y419-Src (2101, 1:1000) were from Cell Signaling Technology; antibodies against β-actin (1:10000), β-tubulin (1:1000), Flag (M2, 1:1000), HA (HA-7, 1:250 for IP) and α-SMA (1:1000 for IB and 1:50 for IF) were from Sigma; TβRI (ab31013, 1:1000 for IB and 1 µg/mg of lysate for IP) and TβRII (EPR24349-124, 1:1000 for IB and 1:250 for IP) from Abcam; Horseradish peroxidase-coupled secondary antibodies (1:10000) were from Jackson ImmunoResearch Laboratories; Alexa Fluor labeled antibodies (1:1000) for IF were from Invitrogen.

4,6-Diamidino-2-phenylindole dihydrochloride (DAPI) fluorescent dye to visualize cell nuclei by microscopy was from Merck; Protein-G Sepharose was from Invitrogen; Clarity Western ECL Blotting Substrate was from BioRad; PageRuler prestained protein ladder was from ThermoFisher; Protease inhibitors cocktail was from Roche; kinase inhibitors SB505124 and SU6656, were from Sigma; AZM475271 was from Tocris. All inhibitors were added 1 h before TGF-β1 stimulation.

Plasmids and small interfering RNAs

The plasmids pCDNA3-TβRI/HA for expression of full length TβRI (UniProt ID P36897) with an HA tag fused to the C-terminus, pCDNA3-TβRI(T204D)/HA, and pCDNA3-TβRI(K232R)/HA were provided by Dr Peter ten Dijke (University of Leiden, Netherlands), plasmids pCMV5-TβRII(K227R)/His for expression of full length TβRII (UniProt ID P37173) with 6XHis tag at the C-terminus and pCMV5-TβRII/His were from Dr Joan Massagué (Memorial Sloan Kettering Cancer Center, New York, USA). FLAG-tagged TβRI was from Dr Kohei Miyazono (Department of Molecular Pathology, Graduate School of Medicine, University of Tokyo, Tokyo, Japan) and Dr Takeshi Imamura (Division of Biochemistry, Cancer Institute of the Japanese Foundation for Cancer Research, Tokyo, Japan). The GST-TβRI construct, containing a Flag-tag in the C-terminus, was described earlier (*56*). All mutations in human TβRI were made by the QuikChange site-directed mutagenesis kit (Stratagene) using pCDNA3-ALK5/HA and pGEX4T-1-TβRI/Flag as templates. Primers used for site-directed mutagenesis are listed in table S1.

Expression vectors encoding Src (#13663 and #42202), Src(K295R,Y527F) (#13657), Src (91-536) (#42206), Src (151-536) (#42207), Src/HA (1-249) (#42204), Src/HA (250-536) (#42209), and GST-Src (SH2) (#46510) were purchased from Addgene. The plasmid pGEX6P3-Src for expression of GST-Src was a gift from Dr. Jun Zhou (Shandong University, China) (*57*). The plasmid for GST-Src(SH3) expression was constructed by cloning the SH3 domain of Src (amino acids 84-144; UniProt ID P12931) into a pGEX4T-1 vector using primers described in table S1.

Site-directed mutagenesis

Site-directed mutagenesis to generate TβRI mutants was performed using the QuikChange XL Site-Directed Mutagenesis Kit (Agilent) according to the manufacturer's instructions.

Immunoblotting, immunoprecipitation, and GST binding assays

Cell lysates were prepared in a lysis buffer containing 0.5% Triton X-100, 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 2 mM NaF, 2 mM $Na_4P_2O_7$, 1 mM β-glycerophosphate, 2 mM Na_3VO_4, and protease inhibitors. The lysates were cleared by centrifugation at 13,000 rpm for 15 min at 4°C and protein concentrations were measured by Bradford assay (500-0006, Bio-Rad Laboratories). For protein expression analysis, total cell lysates with adjusted protein concentration were separated by sodium dodecylsulfat polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose or PVDF membranes, which were incubated with the indicated antibodies. Bound antibodies were visualized by enhanced chemiluminescence.

For co-immunoprecipitation analysis, cell lysates were first cleared by incubation with Protein G Sepharose for 1 h, and then incubated with indicated antibody for 18 h at 4°C before incubation with Protein G Sepharose for 1 h at 4°C. The beads were washed four times with an ice-cold lysis buffer and twice with Tris-buffered saline (TBS), and immuno-complexes were eluted from the beads by adding 2× Laemmli SDS-sample buffer and boiling for 5 min. Immunoblot analysis of the precipitated proteins was performed using indicated primary antibodies and corresponding anti-rabbit or anti-mouse IgG light chain specific antibodies. Immunoblots were analyzed by Image Lab software (Bio-Rad Laboratories).

For GST binding assays, cells were lysed in RIPA buffer (1.0% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris, pH 8.0, 150 mM NaCl, 2 mM NaF, 2 mM $Na_4P_2O_7$, 1 mM β-glycerophosphate, 2 mM Na_3VO_4, and protease inhibitors). The lysates were cleared by incubation with 50 µg GST and Glutathione Superflow Agarose (Pierce) for 2 h at 4°C, and then incubated with GST fusion proteins for 18 h at 4°C before incubation with Glutathione Agarose for 1 h at 4°C. Bound proteins were eluted and analyzed by immunoblotting, as above.

In vitro kinase assays

In vitro kinase reactions were performed using purified GST fusion proteins expressed in bacteria or proteins immunoprecipitated from the cell lysates as indicated in the figure legends. The proteins were mixed in the Kinase Assay Buffer (25 mM MOPS, pH 7.2, 12.5 mM β-glycerophosphate, 20 mM $MgCl_2$, 12.5 mM $MnCl_2$, 5 mM EGTA, 2 mM EDTA, and 0.25 mM dithiothreitol. Small molecular weight kinase inhibitors were added when indicated. The reactions were initiated with the addition of 50 µM ATP. In some cases, 5 µCi γ-^{33}P-ATP (PerkinElmer) was added to the mixtures. After the 30 minutes of incubation at 30 °C, the reactions were terminated by addition of an SDS-containing sample buffer and boiling for 5 min. The reaction products were separated by SDS-PAGE and analyzed by immunoblotting with PY99 antibody. Radioactive samples were exposed on x-ray film or in Pharos FX Plus Molecular Imager.

Immunofluorescence assays

Immunofluorescence assays were performed as previously described (*58*). The α-SMA antibody was diluted 1:50 when used for immunofluorescence. Standard fluorescence

microscopy digital images were acquired at room temperature using "Plan-Neofluar" 10x/0.3 objective on Axio Imager.M2 fluorescence microscope (Carl Zeiss Microscopy, Germany) equipped with a Retiga EXi camera (QImaging, Canada) and controlled by ZEN software (Carl Zeiss MicroImaging). Acquired images were processed and analyzed using ZEN 3.1 (blue edition) software.

Cell migration assay

Cells were grown to confluence in 6-well plates (Sarstedt AG & Co.), incubated for 16 h in starvation medium, and wounds were made using a pipette tip. TGF-β1 (5 ng/mL) and inhibitors were added to the culture media immediately after wounding. Phase contrast images were captured at indicated times and the imaging data were analyzed by the TScratch software (*59*). Motility of the cells was expressed as a percentage of the original open wound area.

Statistical analysis

All quantitative data were presented as the mean ± SEM (standard error of the mean) or the mean ± SD (standard deviation) as indicated of at least three independent experiments by Student’s t test for between-group differences. $P < 0.05$ was considered statistically significant.

Supplementary Materials

Figs. S1–S5.

Table S1.

References and Notes

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Figure Legends

Fig. 1. TGF-β1 activates Src and induces interaction between TβRI and Src.

(**A to C**) HEK293T cells were treated with TGF-β1 for the indicated time periods in the presence of the TβRI kinase inhibitor SB505124 (A) or the Src inhibitor SU6656 (B). Mouse embryonic fibroblasts (MEFs) were treated with TGF-β1 alone (C). Cell extracts were subjected to immunoblotting (IB) using antibodies against Src phosphorylated at Tyr^{416} (Src-pY416), Src, and β-tubulin. Quantification of Src phosphorylation (means ± SEM) reflects the normalized signal for Src $pTyr^{416}$ relative to total Src. n = 3 independent experiments. *, $p < 0.05$ by Student's t test. (**D**) HEK293T cells were transfected with HA-tagged wild-type TβRI (HA-TβRI), constitutively active TβRI (HA-TβRI(T204D)), or kinase-deficient TβRI (HA-TβRI(K232R)) and treated or not with TGF-β1 for 15 min. Cell extracts were subjected to IB using the indicated antibodies. Src $pTyr^{416}$ was quantified and normalized to total Src (means ± SEM). n = 3 independent experiments. (**E and F**) HEK293T cells were transfected with HA-TβRI and treated or not with TGF-β1 for the indicated time periods in the presence of SB505124 (E) or SU6656 (F). Cell extracts were subjected to immunoprecipitation (IP) using HA antibodies, followed by IB for Src and HA. #, HA antibodies and lysate of non-transfected cells; ¤, non-specific IgG and lysate of cells transfected with HA-TβRI. Immunoprecipitated Src was quantified and normalized to the amount of total Src in whole-cell extracts (WCE). Blots are representative of n = 3 independent experiments. *, $p < 0.05$ by Student's t test. (**G**) MEFs were treated or not with TGF-β1 for 15 min. Endogenous TβRI was immunoprecipitated from the cell extracts using the V22 antibody against TβRI or a non-specific IgG (NS), followed by IB using the indicated antibodies. As a control, V22 antibodies without cell lysates were added to the first lane. Arrowhead designates heavy chain IgG. Immunoprecipitated Src was quantified

and normalized to the amount of total Src in WCE. Blots are representative of n = 3 independent experiments. *, $p < 0.05$ by Student's t test.

Fig. 2. The SH2 and SH3 domains of Src bind to TβRI.

(**A**) Schematic diagram showing the structure of the Src molecule and of the fragments of Src used in this study. Numbers in parentheses indicate the amino acids present in each fragment. (**B and C**) HEK293T cells were transfected with Flag-tagged TβRI and the indicated HA-tagged fragments of Src (A) or Flag-tagged TβRI and either untagged full length Src (1-536) or the indicated fragments of Src (B), then subjected to immunoprecipitation (IP) with Flag antibodies, followed by immunoblotting (IB) for HA, Src, and Flag. In (B), endogenous Src appears as a 60 kDa band in all lanes in which TβRI was transfected. (**D**) HEK293T cells were transfected with HA-tagged TβRI. Cell extracts were subjected to pulldown using GST-Src(SH2) or GST-Src(SH3), followed by SDS gel electrophoresis and staining with Ponceau red and IB for HA. (**E**) HEK293T cells were transfected with HA-tagged wild-type (WT) TβRI or TβRI(R451A), TβRI(P455A) or TβRI(R457A) mutants and treated or not with TGF-β1 for 15 min. Cell extracts were subjected to IB for Src, HA, and β-tubulin. Immunoprecipitated Src was quantified and normalized to the amount of total Src in whole-cell extracts (WCE). Blots are representative of n = 3 independent experiments. *, $p < 0.05$ by Student's t test.

Fig. 3. The kinase activity of TβRII is necessary for Src activation by TGF-β1.

(**A**) Extracts from HEK293T cells transfected with His-tagged wild-type TβRII or kinase-deficient TβRII(K227R) were treated or not with TGF-β1 for 30 min, and then subjected to immunoblotting (IB) for Src-pY416, Src, phosphorylated SMAD2 (p-SMAD2), SMAD2, His, and β-tubulin. Src phosphorylation ($pTyr^{416}$) was quantified and normalized to total Src

(means ± SEM). n = 3 independent experiments. *, $p < 0.05$ by Student's t test. (**B**) MEFs were treated or not with TGF-β1 for 15 min. Endogenous TβRII was immunoprecipitated (IP) from the cell extracts, followed by IB with the indicated antibodies. LB, lysis buffer; NS, non-specific IgG. Immunoprecipitated Src was quantified and normalized to the amount of total Src in whole-cell extracts (WCE). Blots are representative of n = 3 independent experiments. *, $p < 0.05$ by Student's t test. (**C**) Extracts from HEK293T cells transfected with His-tagged wild-type TβRII or kinase-deficient TβRII(K227R) were stimulated or not with TGF-β1 for 30 min, and then subjected to IP with His antibodies, followed by IB for Src-pY416, Src, p-SMAD2, His, and β-tubulin. Blots are representative of n = 3 independent experiments. (**D**) HEK293T cells transfected with HA-tagged TβRI and His-tagged wild-type TβRII or kinase-deficient TβRII(K227R) were treated or not with TGF-β1 for 30 min. Cell lysates were then subjected to IB for Src-pY416, Src, HA, TβRII, and β-tubulin. Blots are representative of n = 3 independent experiments.

Fig. 4. TβRII promotes the interaction between TβRI and Src.

(**A**) HEK293T cells were transfected with HA-tagged TβRI and His-tagged wild-type TβRII or kinase-deficient TβRII(K227R) and treated or not with TGF-β1 for 15 min. Cell extracts were subjected to pulldown using GST-Src(SH2) or GST-Src(SH3) and subjected to SDS gel electrophoresis followed by staining with Ponceau red and immunoblotting (IB) for HA. Quantification of precipitated HA-tagged TβRI reflects the normalized signal for HA in pulldown relative to signal for HA in the input (whole-cell extracts, WCE). Blots are representative of n = 3 independent experiments. (**B and C**) HEK293T cells were transfected with His-tagged TβRII plus HA-tagged wild-type TβRI or the TβRI(11Y>F) mutant (B), His-tagged TβRII plus HA-tagged wild-type TβRI, TβRI(Y182F), or TβRI(Y476F) mutants (C) and treated or not with TGF-β1 for 30 min. Cell extracts were

subjected to immunoprecipitation (IP) with HA antibodies and IB for phosphotyrosine (pY99), HA, TβRII, and β-tubulin. Total proteins on the membrane were visualized by labeling with No-Stain Reagent. Blots are representative of n = 3 independent experiments. (**D**) HEK293T cells were transfected with HA-tagged wild-type TβRI or a receptor mutant lacking all tyrosine residues in the cytosolic portion (TβRI(11Y>F)) and treated with TGF-β1 for 15 min. Cell extracts were subjected to pulldown using GST-Src(SH2) or GST alone and subjected to SDS gel electrophoresis, followed by staining with No-Stain Reagent, and IB for HA. The arrowhead indicates nonspecific background bands. Blots are representative of n = 3 independent experiments. (**E**) HEK293T cells were transfected with His-tagged TβRII and HA-tagged wild-type TβRI or TβRI(11Y>F) and treated or not with TGF-β1 for 30 min. Cell extracts were subjected to IP with HA antibodies and IB for Src, HA, phosphorylated SMAD2 (p-SMAD2), and TβRII. Blots are representative of n = 3 independent experiments.

Fig. 5. TβRI activates Src in vitro.

(**A and B**) Purified GST-Src (A) or endogenous Src purified by immunoprecipitation (IP) from PC3U cells (B) was incubated with GST-TβRI in an in vitro kinase assay, and then subjected to immunoblotting (IB) for phosphotyrosine (pY99), pSrc-Y416, Src, and Flag. Quantification of Src phosphorylation reflects the normalized signal for Src-pTyr^{416} relative to total Src. Blots are representative of n = 3 independent experiments. *, $p < 0.05$ by Student's t test. (**C**) Purified GST-tagged TβRI was incubated with purified GST-tagged SMAD2, or the SH2 or SH3 domains of Src in a kinase buffer containing [γ-^{33}P]-ATP and then subjected to SDS-gel electrophoresis and autoradiography. Total protein amounts in the reactions are shown by Coomassie staining. Images are representative of n = 3 independent experiments. (**D**) Wild-type Src and kinase-deficient Src(K295R,Y527F) were

purified by IP from transfected HEK293T cells, incubated with or without purified GST-TβRI in kinase buffer, and subjected to IB with for phosphotyrosine, Src, and Flag. Quantification of Src phosphorylation reflects the normalized signal for phosphotyrosine relative to total Src. Blots are representative of n = 3 independent experiments. *, $p < 0.05$ by Student's t test.

Fig. 6. Src phosphorylates TβRI on tyrosine residues.

(**A**) HEK293T cells transfected with HA-tagged TβRI and Src were treated or not with TGF-β1 for 30 min. Cell extracts were subjected to immunprecipitation (IP) for HA and immunoblotting (IB) for phosphotyrosine (pY99), HA, and Src. WCE, whole-cell extracts. Blots are representative of n = 3 independent experiments. **, $p < 0.01$ by Student's t test. (**B**) HEK293T cells were transfected with HA-tagged TβRI and wild-type Src or kinase-deficient Src(K295R,Y527F) and treated or not with TGF-β1 for 15 min. Cell extracts were subjected to IP with HA antibodies, followed by IB with for phosphotyrosine, HA and Src antibodies. Blots are representative of n = 3 independent experiments. **, $p < 0.01$ by Student's t test. (**C**) MEFs or MEF($SYF^{-/-}$) cells were treated or not with TGF-β1 for 30 min. Cell extracts were subjected to IP with TβRI antibodies (V22), followed by IB for phosphotyrosine, TβRI, Src, and β-tubulin. LB, lysis buffer; NS, non-specific IgG. The amount of phosphorylated TβRI (IP: HA, IB: pY99) was normalized to the immunoprecipitated TβRI (IP: HA, IB: HA). Blots are representative of n = 3 three independent experiments. *, $p < 0.05$ by Student's t test. (**D**) MEFs were treated or not with TGF-β1 for 30 min in the presence of the Src inhibitor AZM475271 or the TβRI inhibitor SB505124. Cell extracts were subjected to IP with TβRI antibodies, followed by IB for phosphotyrosine, TβRI, phosphorylated Smad2 (p-Smad2), Smad2, Src -$pTyr^{416}$, Src, and GAPDH. NS, non-specific IgG. Blots are representative of n = 3 three independent

experiments. (**E**) GST-tagged TβRI mutants were incubated with GST-Src or GST and then subjected to IB with for phosphotyrosine, Flag and Src. Blots are representative of n = 3 three independent experiments. (**F**) Extracts from HEK293T cells transfected with Src and HA-tagged wild-type TβRI or the indicated mutants were subjected to IP with HA antibodies, followed by IB for phosphotyrosine, HA, Src, and β-tubulin. Blots are representative of n = 3 three independent experiments.

Fig. 7. The kinase activity of Src is necessary for some cellular responses to TGF-β1.

(**A and B**) MCF10A cells were treated or not with TGF-β1 for 48 h (A) or 72 h (B), with or without SB505124 or AZM475271. Cell extracts were subjected to immunoblotting (IB) for fibronectin, phosphorylated SMAD2 (p-SMAD2), and β-tubulin (A), or fibronectin, PAI-1, and β-tubulin (B). Blots are representative of n = 3 independent experiments. *, $p < 0.05$ by Student's t test. (**C**) MEFs and MEF(SYF$^{-/-}$) cells were treated or not with TGF-β1 in the absence or presence of SU6656 for 48 h. Cell extracts were subjected to IB for α-smooth muscle actin (α-SMA), Src-pY416, and β-tubulin. Blots are representative of n = 3 independent experiments. *, $p < 0.05$ by Student's t test. (**D**) Wild-type MEFs, MEF(SYF$^{-/-}$), and MEF(SYF$^{-/-}$) transfected with Src, were treated with TGF-β1 in the absence or presence of SU6656 for 48 h. α-SMA (red) was detected by immunofluorescence. Arrowheads indicate α-SMA fibers in TGF-β1–treated MEF(SYF$^{-/-}$) cells transfected with Src. Scale bar, 100 μm. (**E and F**) MCF10A cells (E), and MEF(SYF$^{-/-}$) cells and MEF(SYF$^{-/-}$) cells transfected with Src (F) were subjected to a cell culture wound healing assay in the absence or presence of TGF-β1 and kinase inhibitors SB505124 or SU6656. Phase contrast images were captured at the indicated time points. Quantification was performed on 4 (E) or 12 (F) measurements in each experimental condition and expressed as a

percentage of the open wound area. Data represent means ± standard error of the mean. Brackets indicate the comparisons that showed significant differences of cell migration under treatments. *, $p < 0.05$. Representative images of cell migration after treatments for different time periods are shown. Scale bars, 200 μm.

Fig. 8. Schematic illustration of the molecular mechanism by which TGF-β stimulation activates Src.

(**A**) In the absence of TGF-β stimulation, inactive Src occurs in a complex with TβRII. (**B**) After TGF-β–induced formation of the heterotetrameric receptor complex of TβRI and TβRII, TβRII phosphorylates TβRI on Thr^{186}, Ser^{187}, Ser^{189}, and Ser^{191} to activate its kinase (*4*), and on Tyr^{182} and possibly other tyrosine residue(s). (**C**) Binding of the SH2 domain of Src to phosphorylated tyrosine residues in TβRI activates Src. An interaction of the SH3 domain of Src with a proline-rich motif in TβRI contributes to the strength of Src binding to TβRI. Thereafter Src phosphorylates TβRI on several tyrosine residues, including $Tyr^{428/429}$, which may enforce Src binding and possibly also have other effects on TGF-β signaling.

Figure 1

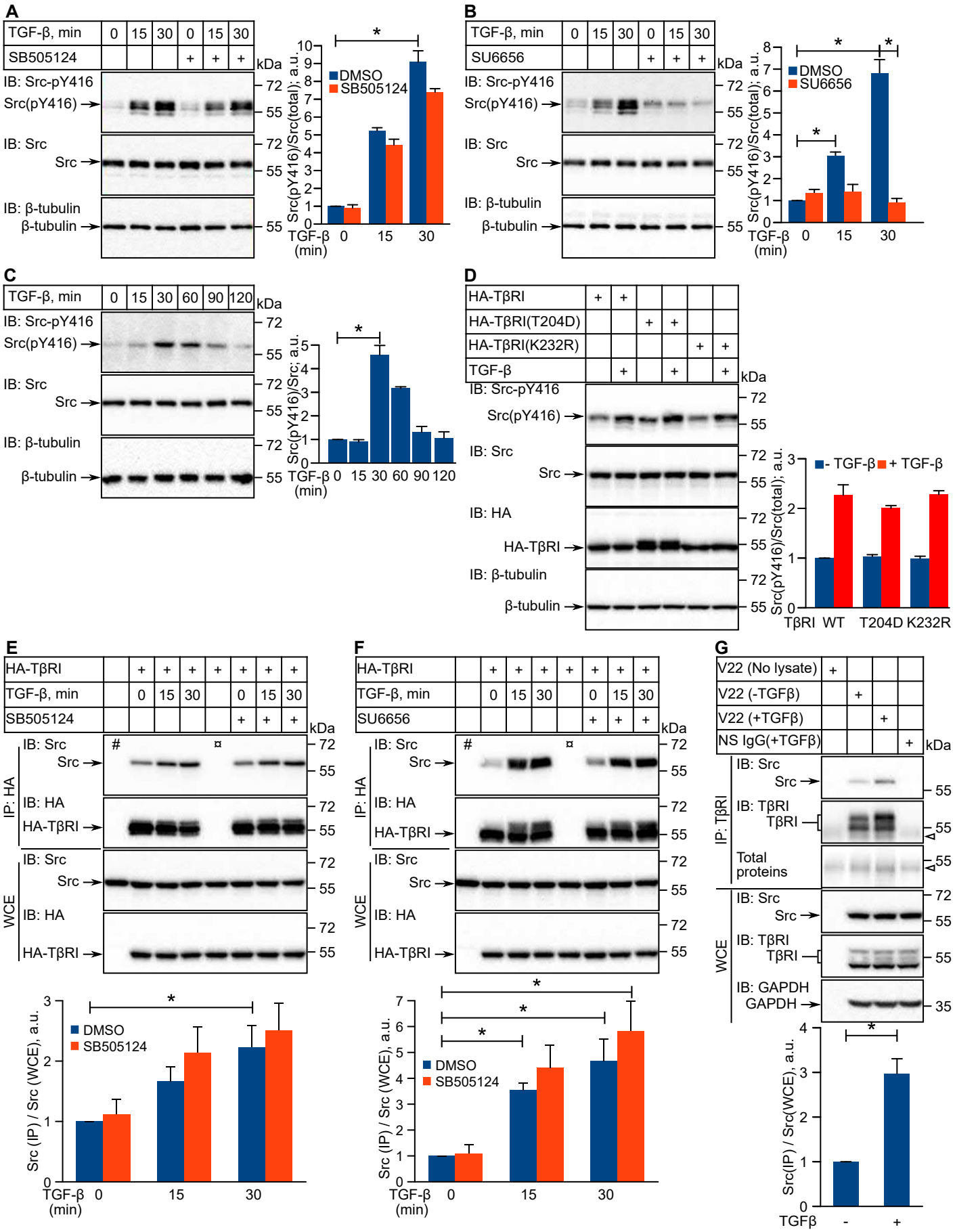

A
TGF-β, min
SB505124
IB: Src-pY416
Src(pY416)
IB: Src
Src
IB: β-tubulin
β-tubulin
kDa
DMSO
SB505124
Src(pY416)/Src(total); a.u.
TGF-β (min)
B
SU6656
DMSO
SU6656
C
D
HA-TβRI
HA-TβRI(T204D)
HA-TβRI(K232R)
TGF-β
IB: HA
HA-TβRI
- TGF-β
+ TGF-β
TβRI WT T204D K232R
E
IP: HA
WCE
Src (IP) / Src (WCE), a.u.
F
G
V22 (No lysate)
V22 (-TGFβ)
V22 (+TGFβ)
NS IgG(+TGFβ)
IP: TβRI
Total proteins
IB: GAPDH
GAPDH
Src(IP) / Src(WCE), a.u.
TGFβ

Figure 2

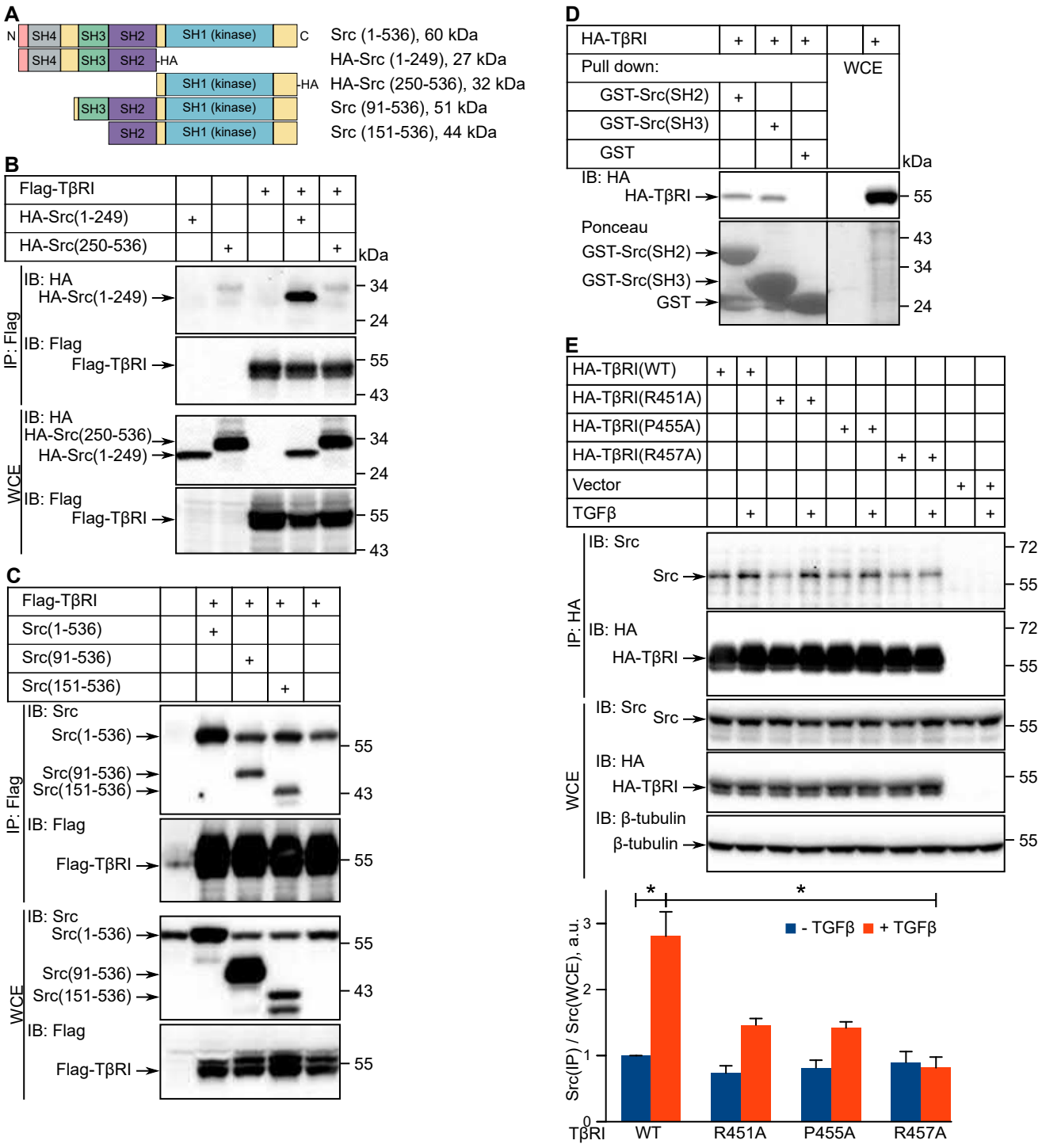

A
N
SH4
SH3
SH2
SH1 (kinase)
C
HA
Src (1-536), 60 kDa
HA-Src (1-249), 27 kDa
HA-Src (250-536), 32 kDa
Src (91-536), 51 kDa
Src (151-536), 44 kDa
B
Flag-TβRI
HA-Src(1-249)
HA-Src(250-536)
kDa
IP: Flag
IB: HA
IB: Flag
WCE
34
24
55
43
C
Src(1-536)
Src(91-536)
Src(151-536)
IB: Src
D
HA-TβRI
Pull down:
WCE
GST-Src(SH2)
GST-Src(SH3)
GST
Ponceau
E
HA-TβRI(WT)
HA-TβRI(R451A)
HA-TβRI(P455A)
HA-TβRI(R457A)
Vector
TGFβ
72
β-tubulin
Src(IP) / Src(WCE), a.u.
- TGFβ
+ TGFβ
TβRI
WT
R451A
P455A
R457A

Figure 3

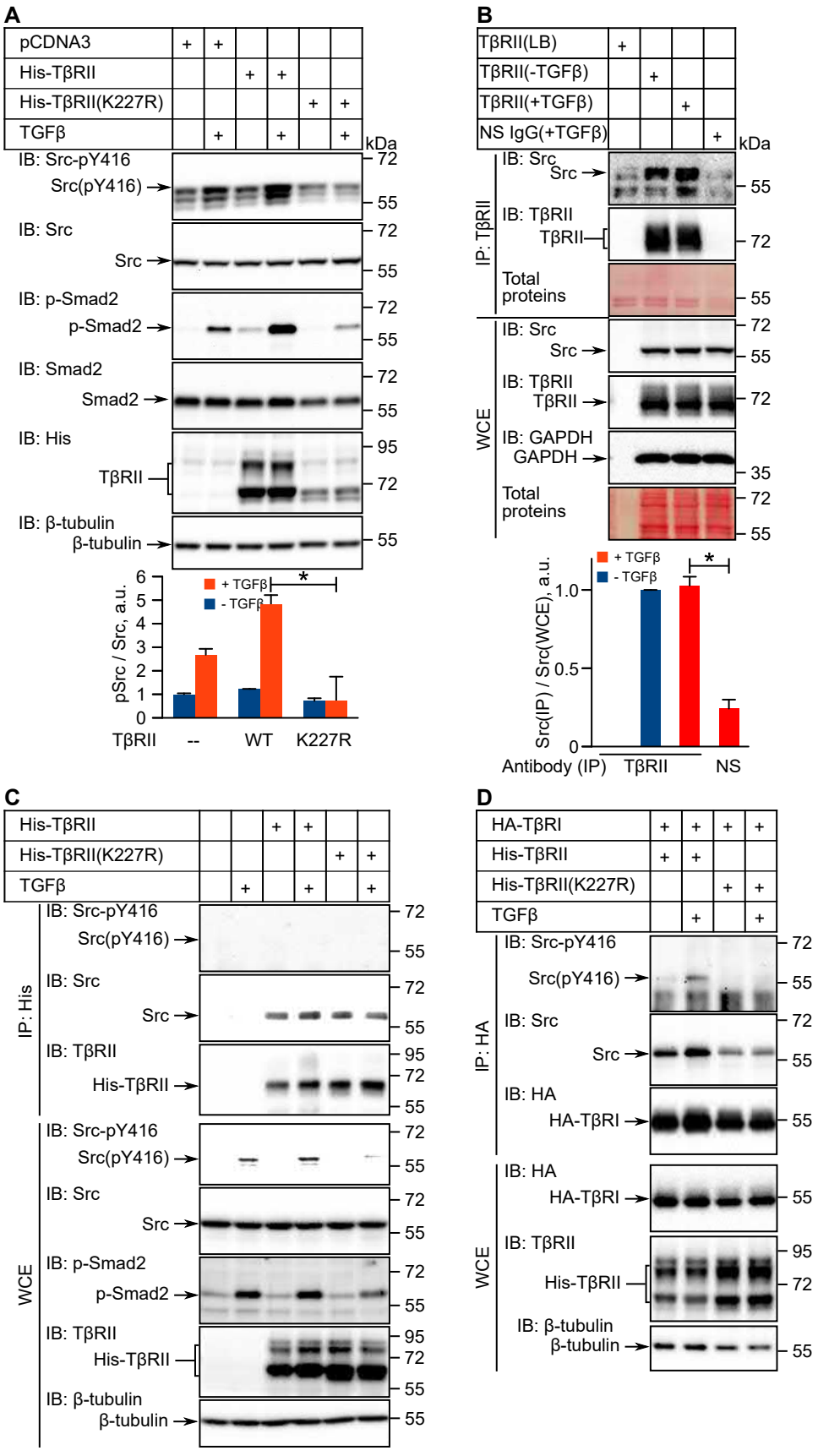

A
pCDNA3
His-TβRII
His-TβRII(K227R)
TGFβ
kDa
IB: Src-pY416
Src(pY416)
IB: Src
Src
IB: p-Smad2
p-Smad2
IB: Smad2
Smad2
IB: His
TβRII
IB: β-tubulin
β-tubulin
72
55
95
pSrc / Src, a.u.
+ TGFβ
- TGFβ
TβRII
--
WT
K227R
B
TβRII(LB)
TβRII(-TGFβ)
TβRII(+TGFβ)
NS IgG(+TGFβ)
IP: TβRII
IB: Src
IB: TβRII
Total proteins
WCE
IB: GAPDH
GAPDH
35
Src(IP) / Src(WCE), a.u.
Antibody (IP)
NS
C
His-TβRII
His-TβRII(K227R)
TGFβ
IP: His
WCE
IB: p-Smad2
p-Smad2
D
HA-TβRI
IP: HA
IB: HA
HA-TβRI

Figure 4

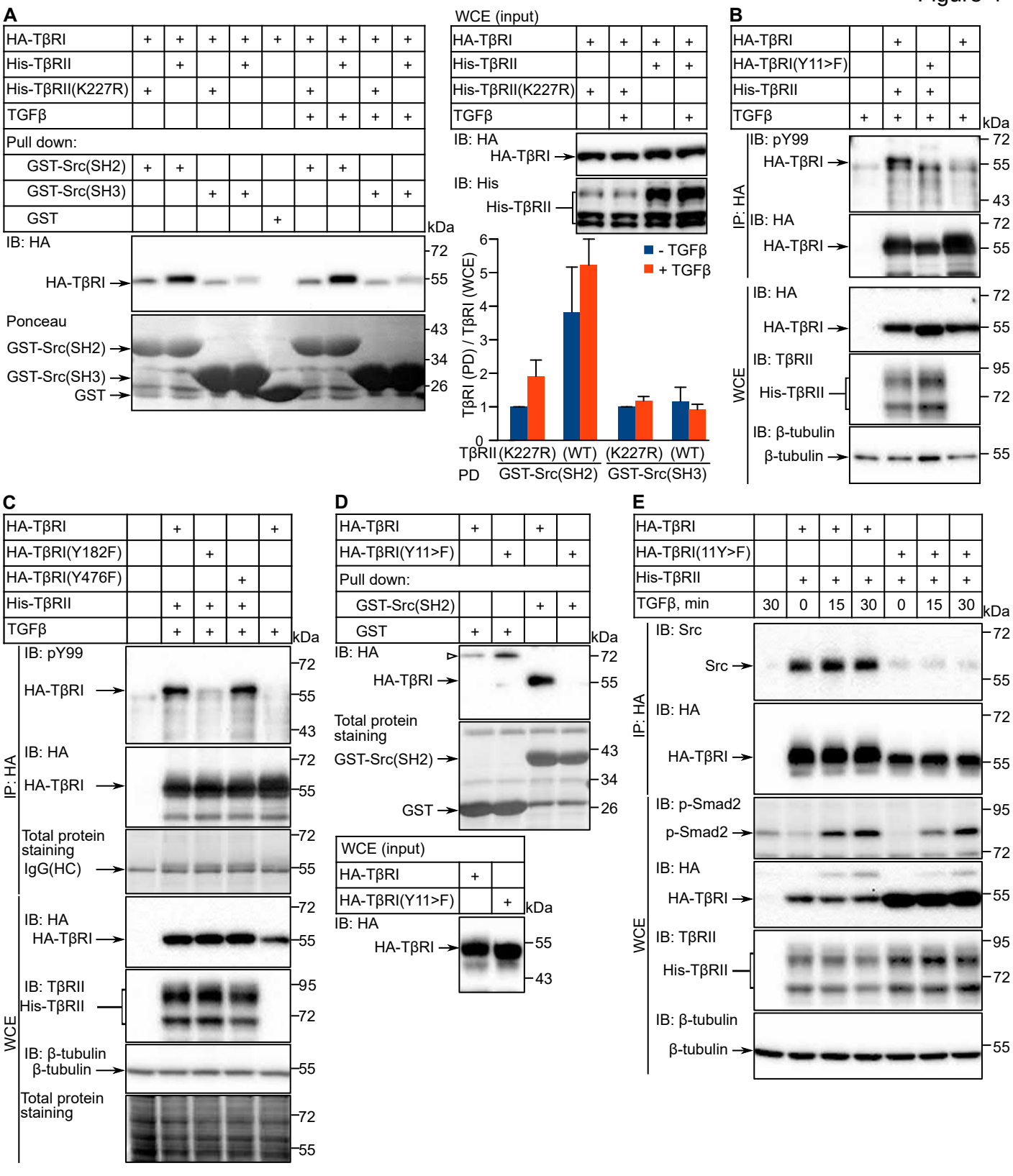
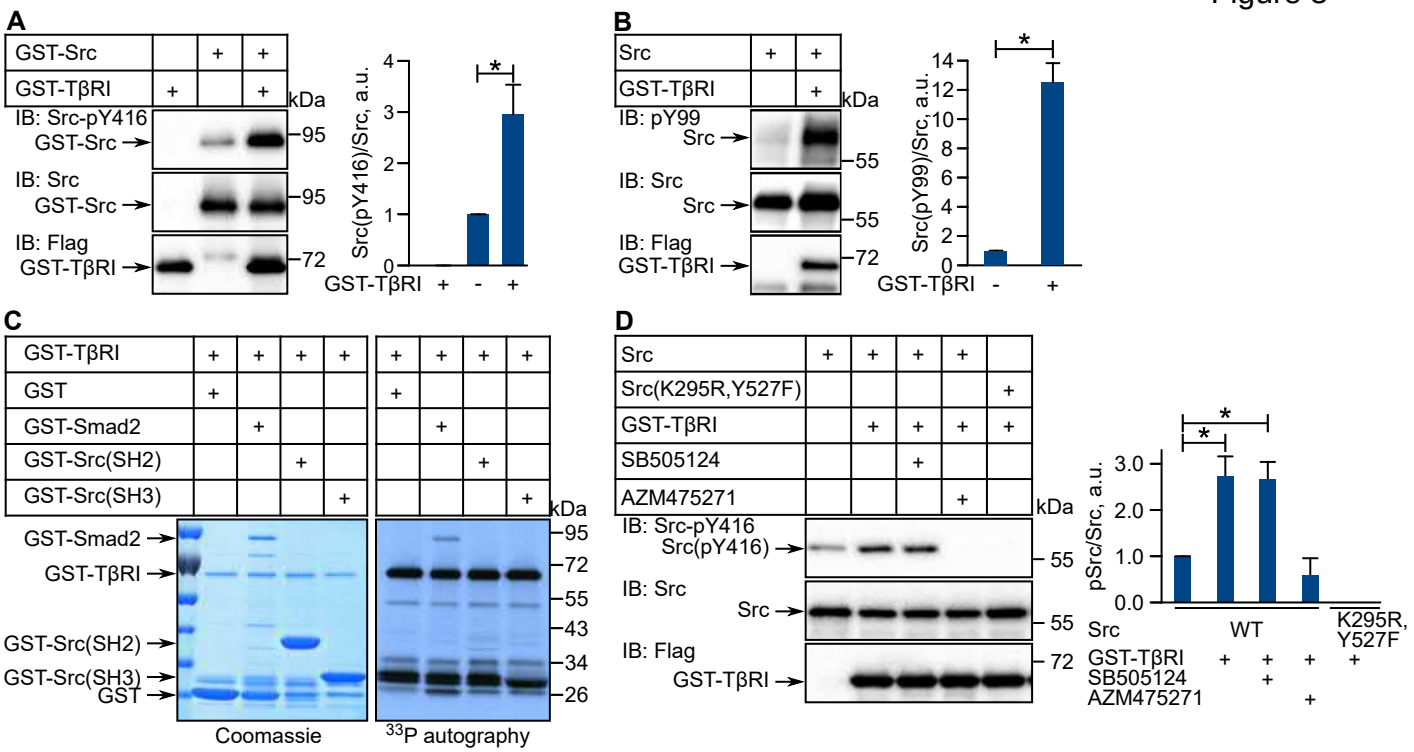

A
HA-TβRI
His-TβRII
His-TβRII(K227R)
TGFβ
Pull down:
GST-Src(SH2)
GST-Src(SH3)
GST
IB: HA
HA-TβRI
Ponceau
GST-Src(SH2)
GST-Src(SH3)
GST
kDa
WCE (input)
IB: His
His-TβRII
- TGFβ
+ TGFβ
TβRI (PD) / TβRI (WCE)
TβRII (K227R) (WT) (K227R) (WT)
PD GST-Src(SH2) GST-Src(SH3)
B
HA-TβRI(Y11>F)
IB: pY99
IP: HA
IB: TβRII
WCE
IB: β-tubulin
β-tubulin
C
HA-TβRI(Y182F)
HA-TβRI(Y476F)
Total protein staining
IgG(HC)
D
HA-TβRI(Y11>F)
E
HA-TβRI(11Y>F)
TGFβ, min
IB: Src
Src
IB: p-Smad2
p-Smad2

Figure 5


A
GST-Src
GST-TβRI
kDa
IB: Src-pY416
GST-Src
95
IB: Src
GST-Src
95
IB: Flag
GST-TβRI
72
Src(pY416)/Src, a.u.
GST-TβRI + - +
B
Src
GST-TβRI
kDa
IB: pY99
Src
55
IB: Src
Src
55
IB: Flag
GST-TβRI
72
Src(pY99)/Src, a.u.
GST-TβRI - +
C
GST-TβRI
GST
GST-Smad2
GST-Src(SH2)
GST-Src(SH3)
kDa
GST-Smad2
GST-TβRI
GST-Src(SH2)
GST-Src(SH3)
GST
95
72
55
43
34
26
Coomassie
33P autography
D
Src
Src(K295R,Y527F)
GST-TβRI
SB505124
AZM475271
kDa
IB: Src-pY416
Src(pY416)
55
IB: Src
Src
55
IB: Flag
GST-TβRI
72
pSrc/Src, a.u.
Src
WT
K295R, Y527F
GST-TβRI
SB505124
AZM475271

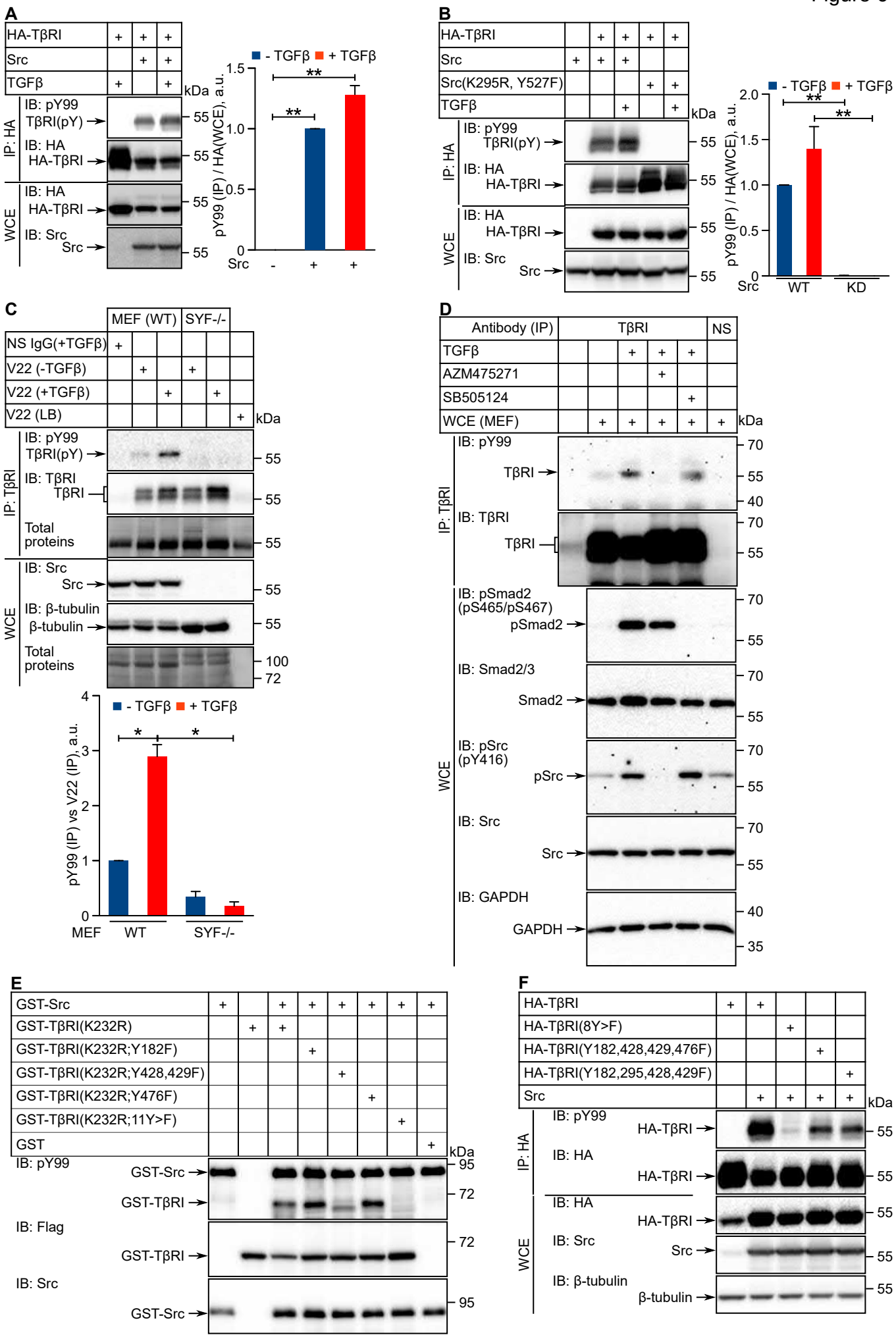

A
HA-TβRI
Src
TGFβ
IP: HA
IB: pY99
TβRI(pY)
IB: HA
HA-TβRI
WCE
IB: HA
HA-TβRI
IB: Src
Src
kDa
55
- TGFβ + TGFβ
pY99 (IP) / HA(WCE), a.u.
B
HA-TβRI
Src
Src(K295R, Y527F)
TGFβ
IP: HA
IB: pY99
TβRI(pY)
IB: HA
HA-TβRI
WCE
IB: HA
HA-TβRI
IB: Src
Src
WT
KD
C
MEF (WT)
SYF-/-
NS IgG(+TGFβ)
V22 (-TGFβ)
V22 (+TGFβ)
V22 (LB)
IP: TβRI
IB: pY99
TβRI(pY)
IB: TβRI
TβRI
Total proteins
WCE
IB: Src
Src
IB: β-tubulin
β-tubulin
Total proteins
100
72
pY99 (IP) vs V22 (IP), a.u.
MEF
WT
SYF-/-
D
Antibody (IP)
TβRI
NS
TGFβ
AZM475271
SB505124
WCE (MEF)
IP: TβRI
IB: pY99
TβRI
IB: TβRI
TβRI
70
55
40
WCE
IB: pSmad2 (pS465/pS467)
pSmad2
IB: Smad2/3
Smad2
IB: pSrc (pY416)
pSrc
IB: Src
Src
IB: GAPDH
GAPDH
35
E
GST-Src
GST-TβRI(K232R)
GST-TβRI(K232R;Y182F)
GST-TβRI(K232R;Y428,429F)
GST-TβRI(K232R;Y476F)
GST-TβRI(K232R;11Y>F)
GST
IB: pY99
GST-Src
GST-TβRI
IB: Flag
GST-TβRI
IB: Src
GST-Src
95
72
F
HA-TβRI
HA-TβRI(8Y>F)
HA-TβRI(Y182,428,429,476F)
HA-TβRI(Y182,295,428,429F)
Src
IP: HA
IB: pY99
HA-TβRI
IB: HA
HA-TβRI
WCE
IB: HA
HA-TβRI
IB: Src
Src
IB: β-tubulin
β-tubulin

Figure 7

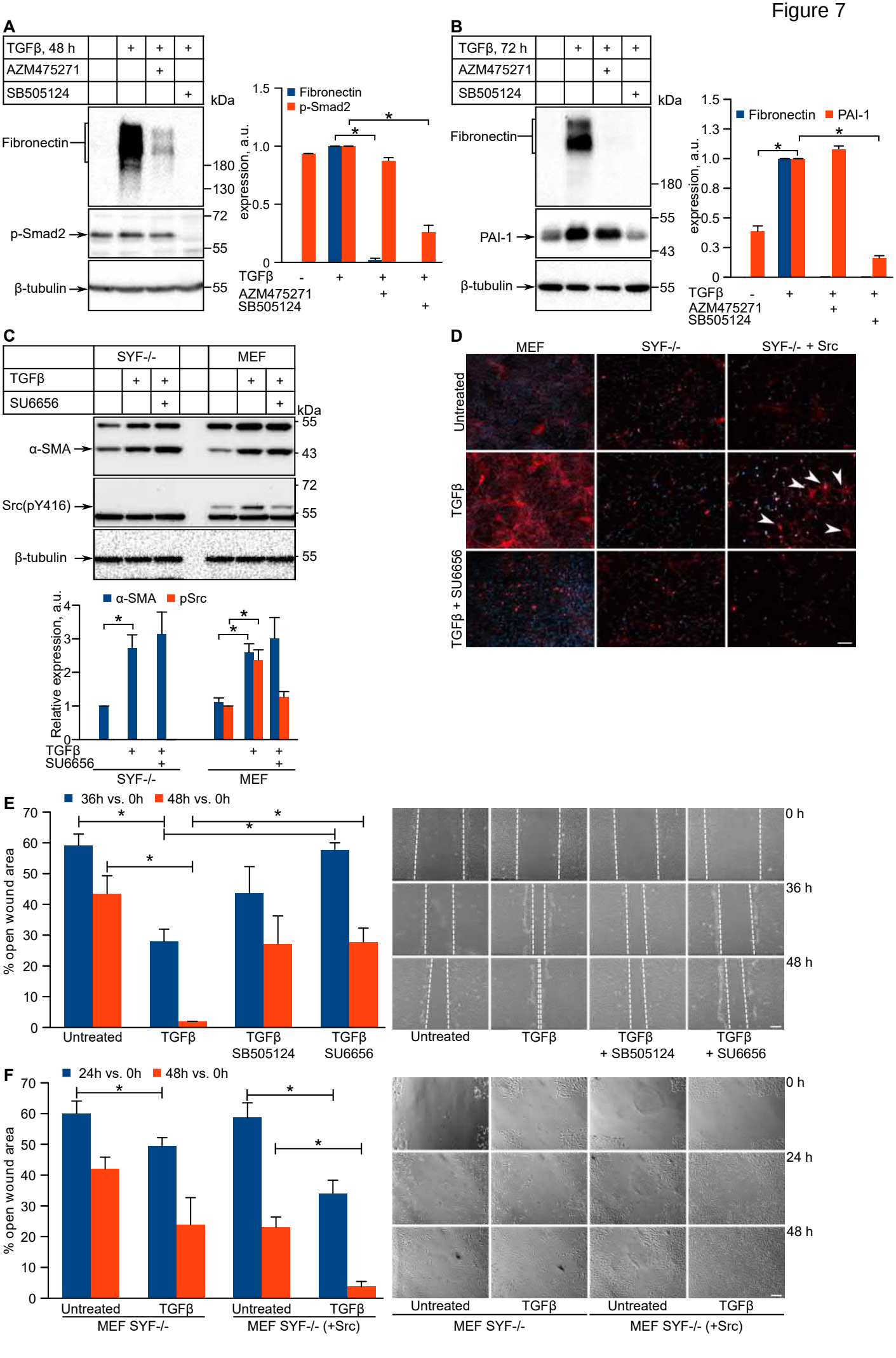
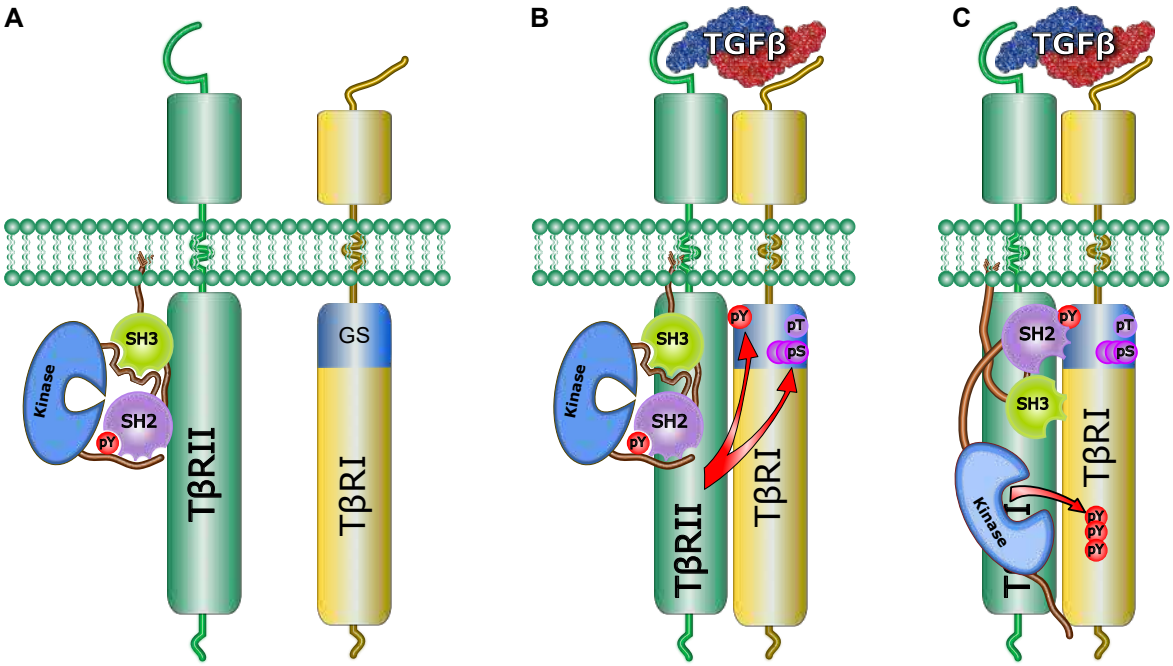

A
TGFβ, 48 h
AZM475271
SB505124
kDa
Fibronectin
p-Smad2
β-tubulin
180
130
72
55
55
Fibronectin
p-Smad2
expression, a.u.
1.5
1.0
0.5
0
TGFβ
AZM475271
SB505124
B
TGFβ, 72 h
AZM475271
SB505124
kDa
Fibronectin
PAI-1
β-tubulin
180
55
43
55
Fibronectin
PAI-1
expression, a.u.
1.5
1.3
1.0
0.8
0.5
0.3
0
TGFβ
AZM475271
SB505124
C
SYF-/-
MEF
TGFβ
SU6656
kDa
α-SMA
Src(pY416)
β-tubulin
55
43
72
55
55
α-SMA
pSrc
Relative expression, a.u.
4
3
2
1
0
TGFβ
SU6656
SYF-/-
MEF
D
MEF
SYF-/-
SYF-/- + Src
Untreated
TGFβ
TGFβ + SU6656
E
36h vs. 0h
48h vs. 0h
% open wound area
70
60
50
40
30
20
10
0
Untreated
TGFβ
TGFβ SB505124
TGFβ SU6656
0 h
36 h
48 h
Untreated
TGFβ
TGFβ + SB505124
TGFβ + SU6656
F
24h vs. 0h
48h vs. 0h
% open wound area
70
60
50
40
30
20
10
0
Untreated
TGFβ
Untreated
TGFβ
MEF SYF-/-
MEF SYF-/- (+Src)
0 h
24 h
48 h
Untreated
TGFβ
Untreated
TGFβ
MEF SYF-/-
MEF SYF-/- (+Src)

Figure 8



Supplementary Materials for
The type II TGF-β receptor phosphorylates Tyr^{182} in the type I receptor to activate downstream Src signaling

Ihor Yakymovych *et al.*

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DOI: 10.1126/scisignal.abp9521

The PDF file includes:
Figs. S1 to S5
Table S1

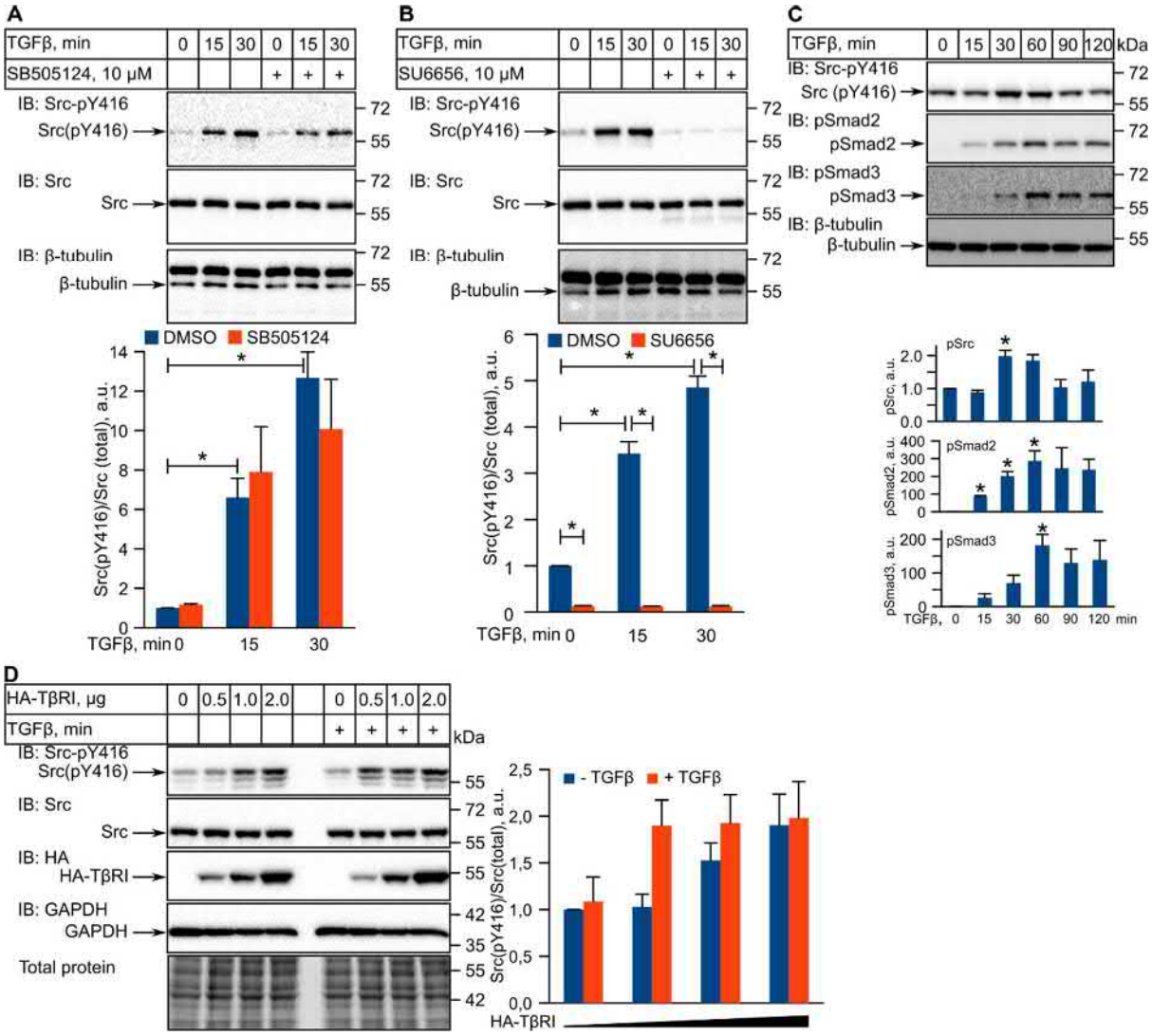

Fig. S1. TGF-β1 activates Src. (A and B) PC3U cells were treated with TGF-β1 for the indicated time periods in the presence of the TβRI inhibitor SB505124 (A) or Src inhibitor SU6656 (B). Cell extracts were subjected to immunoblotting (IB) using the indicated antibodies. Quantification of Src phosphorylation (means ± SEM) reflects the normalized pY416 signal relative to total Src. Blots are representative of n = 3 independent experiments. *, $p < 0.05$ by Student's t test. (**C**) MEFs were treated with TGF-β1 for the indicated time periods. Cell extracts were subjected to IB using antibodies against Src-pY416, phospho-SMAD2, phospho-SMAD3, and β-tubulin. Quantification shows the amount of phosphorylated protein normalized to β-tubulin. Blots are representative of n = 3 independent experiments. (**D**) HEK293T cells were transfected with indicated amounts of HA-TβRI plasmid and were treated or not with TGF-β1 for 15 min. Cell extracts were subjected to IB using antibodies against Src-pY416, Src, HA and GAPDH. Total proteins on the membrane were visualized by labeling with No-Stain Reagent. Blots are representative of n = 3 independent experiments.

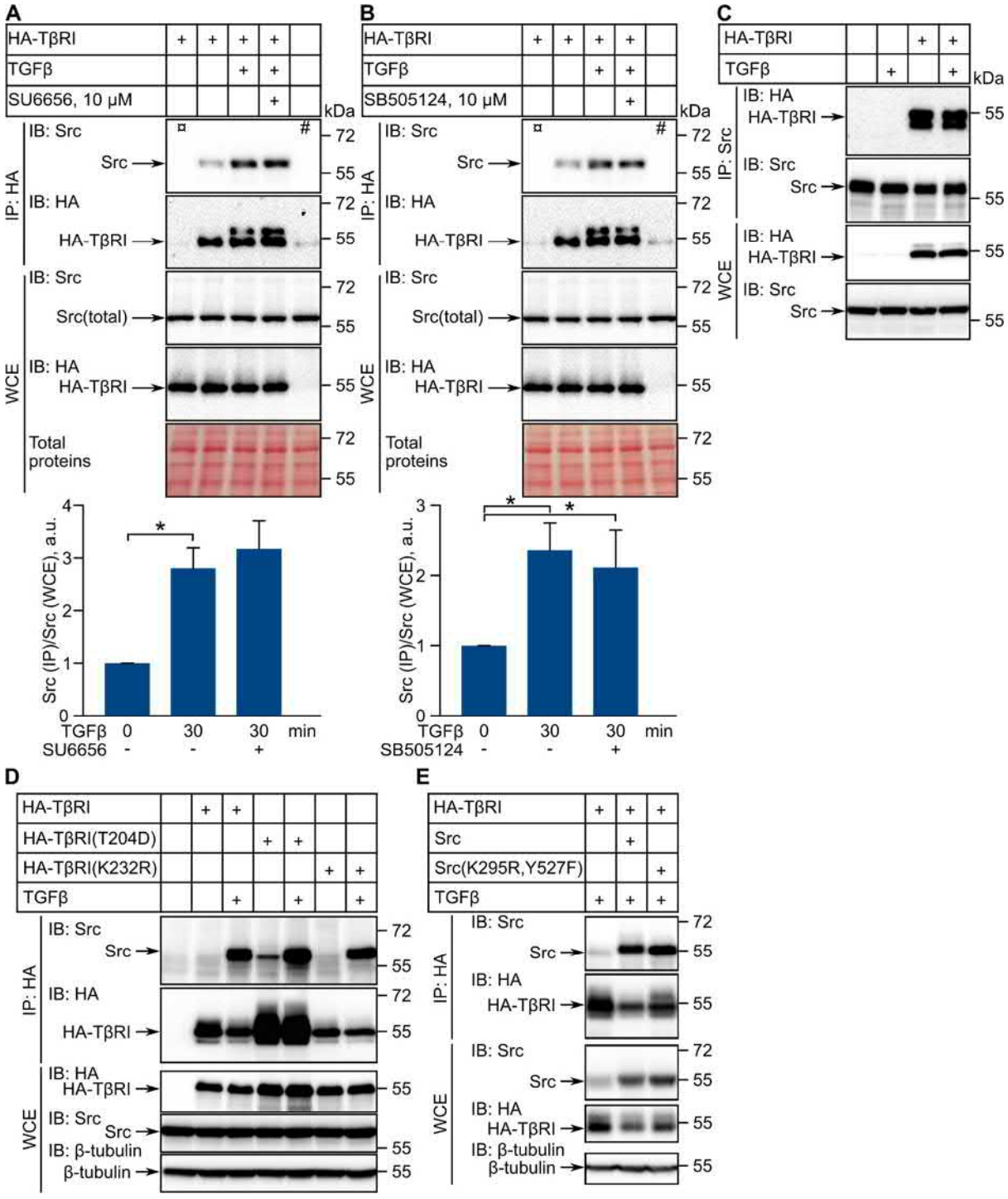
A
HA-TβRI
TGFβ
SU6656, 10 μM
kDa
IP: HA
IB: Src
Src
IB: HA
HA-TβRI
WCE
IB: Src
Src(total)
IB: HA
HA-TβRI
Total proteins
72
55
¤
#
Src (IP)/Src (WCE), a.u.
TGFβ 0 30 30 min
SU6656 - - +
B
HA-TβRI
TGFβ
SB505124, 10 μM
SB505124 - - +
C
IP: Src
D
HA-TβRI
HA-TβRI(T204D)
HA-TβRI(K232R)
IB: β-tubulin
β-tubulin
E
Src
Src(K295R,Y527F)

Fig. S2. TGF-β1 induces a complex between TβRI and Src. (**A and B**) PC3U cells were transfected with HA-tagged TβRI and treated or not with TGF-β1 for the indicated time periods in the presence of SU6656 (A) or SB505124 (B). Cell extracts were subjected to immunoprecipitation (IP) using HA antibodies, followed by immunoblotting (IB) using Src and HA antibodies. Total proteins on the membrane were visualized by staining with Ponceau red. #, HA antibodies and lysate of non-transfected cells; ¤, non-specific IgG and lysate of cells transfected with HA-TβRI. Immunoprecipitated Src was quantified and normalized to the amount of total Src in whole-cell extracts (WCE). Blots are representative of n = 3 independent experiments. *, $p < 0.05$ by Student's t test. (**C**) HEK293T cells were transfected with HA-TβRI and treated or not with TGF-β1 for 30 min. Cell extracts were subjected to IP with Src antibodies and IB using HA and Src antibodies. Blots are representative of n = 3 independent experiments. (**D and E**) HEK293T cells were transfected with wild-type TβRI (HA-TβRI), constitutively kinase active (HA-TβRI(T204D)) or kinase-deficient (HA-TβRI(K232R)) receptor mutants (D), or wild-type TβRI (HA-TβRI), wild-type Src or kinase-deficient Src(K295R,Y527F) mutant (E) and treated or not with TGF-β1 for 15 min. Cell extracts were subjected to IP with HA antibodies, followed by IB with Src, HA, and β-tubulin antibodies. Blots are representative of n = 3 independent experiments.

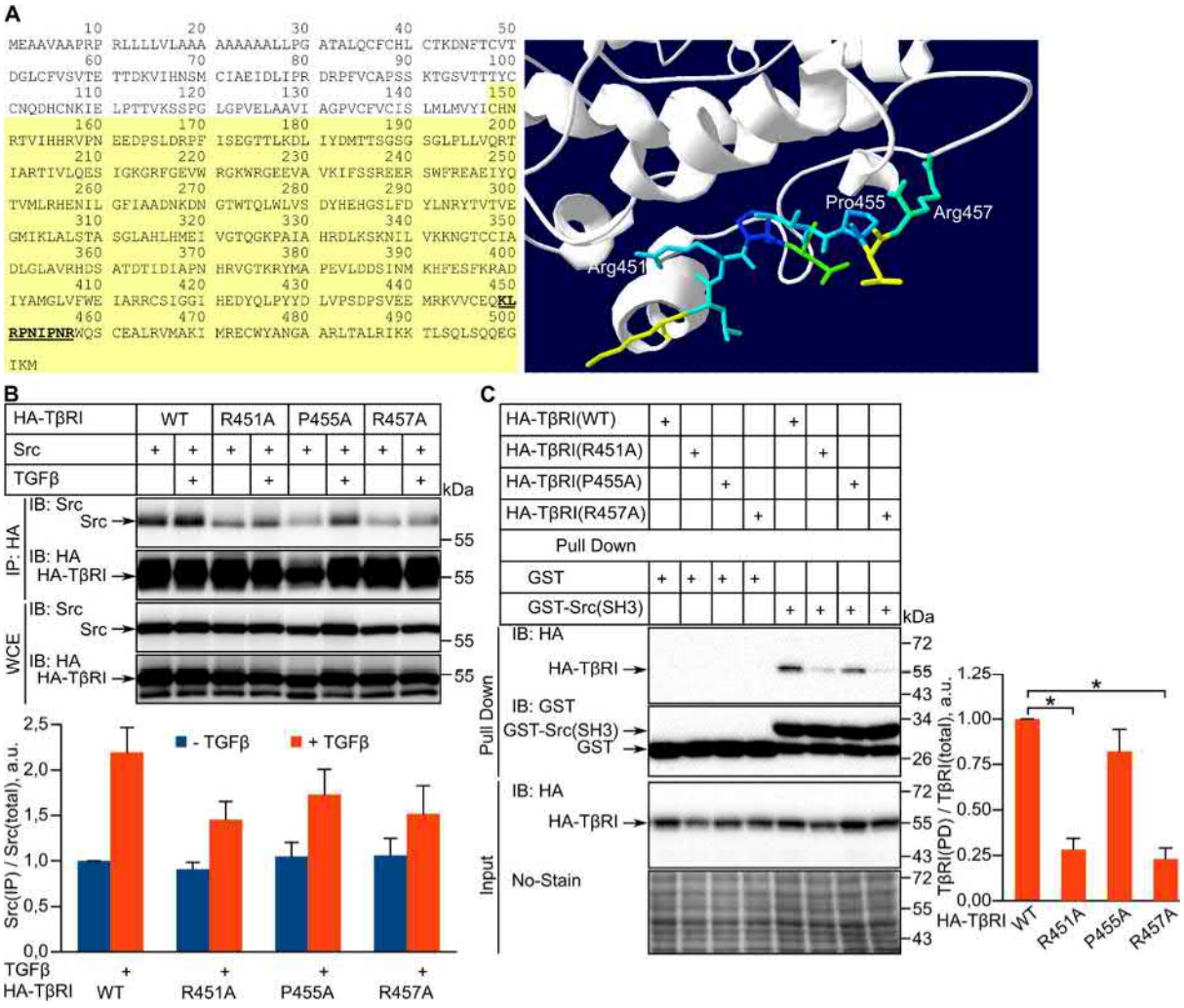

Fig. S3. Mutations in the proline-rich sequence K^{449}LRPNIPNR^{457} of TβRI decrease its affinity to the SH3 domain of Src. (**A**) Right: sequence of TβRI (UniProtKB P36897-1) with the proline-rich sequence K^{449}LRPNIPNR^{457} indicated in bold and underlined; yellow, intracellular domain. Left: magnified view of the intracellular domain of TβRI (PDB Entry: 1B6C, DOI: 10.2210/pdb1b6c/pdb) with residues of the proline-rich sequence K^{449}LRPNIPNR^{457} depicted. Residues selected for mutagenesis are indicated. (**B**) HEK293T cells were transfected with HA-tagged TβRI or TβRI R451A, P455A or R457A mutants, and Src, and treated or not with TGF-β1 for 15 min. Whole-cell extracts (WCE) and HA immunoprecipitates (IP) were subjected to immunoblotting (IB) with Src and HA antibodies. Quantification of immunoprecipitated Src normalized to the input (WCE) Src. Blots are representative of n = 3 independent experiments. (**C**) HEK293T cells were transfected with HA-tagged TβRI or TβRI(R451A), TβRI(P455A) or TβRI(R457A) mutants. Cell extracts were subjected to pulldown using GST-Src(SH3) or GST, followed by SDS-gel electrophoresis and IB with HA and GST antibodies. Loading was controlled by IB of whole cell extracts with HA antibodies. Quantification of precipitated HA-TβRI normalized to the input HA-TβRI. Blots are representative of n =3 independent experiments. *, $p < 0.05$ by Student's t test.

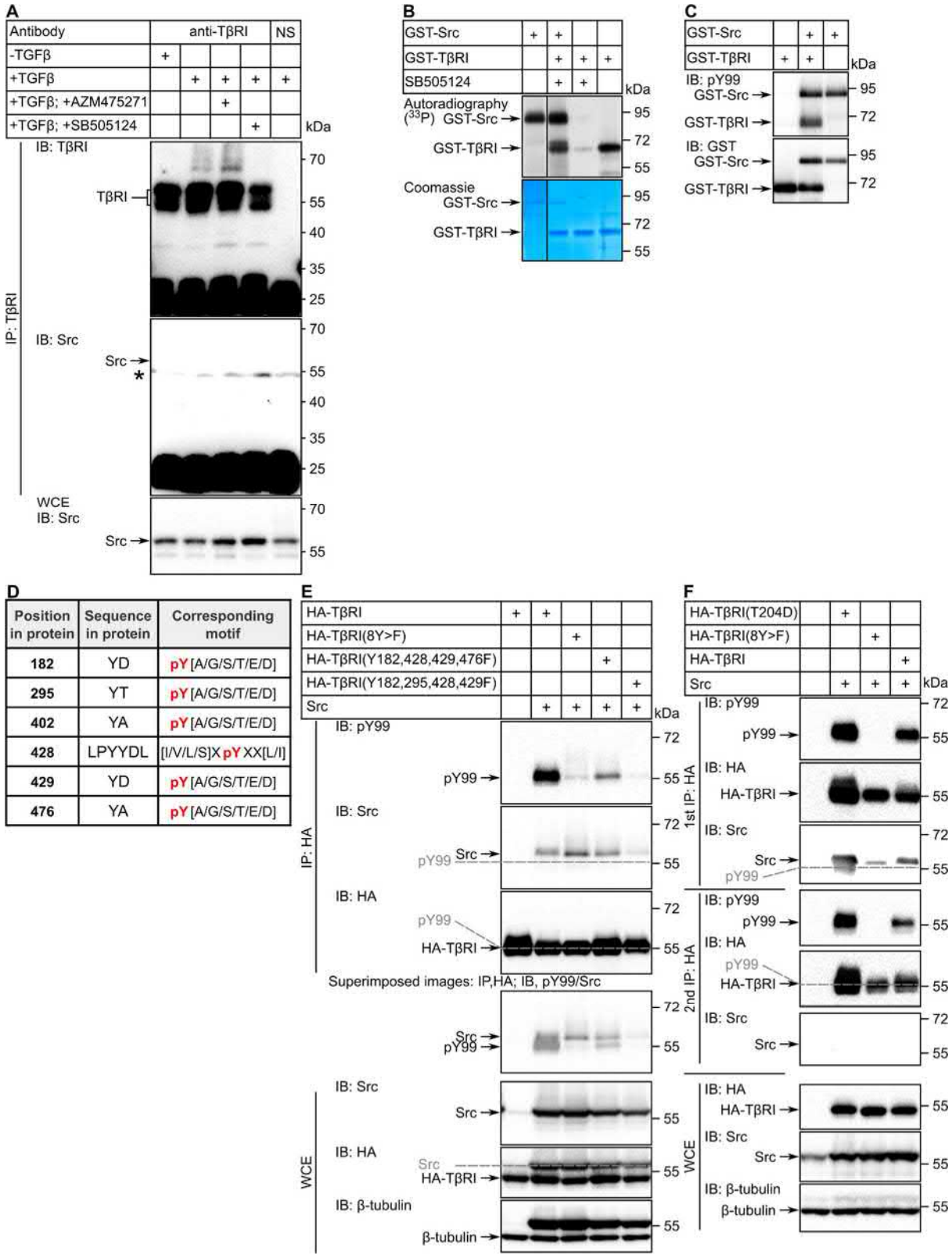
A
Antibody
anti-TβRI
NS
-TGFβ
+TGFβ
+TGFβ; +AZM475271
+TGFβ; +SB505124
kDa
IB: TβRI
TβRI
IP: TβRI
IB: Src
Src
WCE
IB: Src
B
GST-Src
GST-TβRI
SB505124
Autoradiography
(33P) GST-Src
GST-TβRI
Coomassie
GST-Src
GST-TβRI
C
GST-Src
GST-TβRI
IB: pY99
GST-Src
GST-TβRI
IB: GST
GST-Src
GST-TβRI
D
Position in protein
Sequence in protein
Corresponding motif
182 YD pY [A/G/S/T/E/D]
295 YT pY [A/G/S/T/E/D]
402 YA pY [A/G/S/T/E/D]
428 LPYYDL [I/V/L/S]X pY XX[L/I]
429 YD pY [A/G/S/T/E/D]
476 YA pY [A/G/S/T/E/D]
E
HA-TβRI
HA-TβRI(8Y>F)
HA-TβRI(Y182,428,429,476F)
HA-TβRI(Y182,295,428,429F)
Src
IP: HA
IB: pY99
pY99
IB: Src
pY99 Src
IB: HA
pY99
HA-TβRI
Superimposed images: IP,HA; IB, pY99/Src
Src
pY99
WCE
IB: Src
Src
IB: HA
Src
HA-TβRI
IB: β-tubulin
β-tubulin
F
HA-TβRI(T204D)
HA-TβRI(8Y>F)
HA-TβRI
Src
1st IP: HA
IB: pY99
pY99
IB: HA
HA-TβRI
IB: Src
Src
pY99
2nd IP: HA
IB: pY99
pY99
IB: HA
pY99
HA-TβRI
IB: Src
Src
WCE
IB: HA
HA-TβRI
IB: Src
Src
IB: β-tubulin
β-tubulin

Fig. S4. Src phosphorylates TβRI. (**A**) MEFs were treated or not with TGF-β1 for 30 min in the presence of the Src inhibitor AZM475271 or the TβRI inhibitor SB505124. Cell extracts were subjected to immunoprecipitation (IP) with TβRI antibodies, followed by immunoblotting (IB) with TβRI and Src antibodies. NS, non-specific IgG. *, IgG heavy chain. Blots are representative of n = 3 three independent experiments. (**B**) GST-tagged TβRI was incubated with or without GST-Src and SB505124 in kinase buffer containing [γ-^{33}P]-ATP and subjected to SDS-gel electrophoresis and autoradiography. Total protein levels in the reactions are shown by Coomassie staining. Images are representative of n = 3 independent experiments; the line indicates where the images were cut; all samples were run on one gel. (**C**) GST-tagged TβRI was incubated with or without GST-Src, and then subjected to IB with pY99 and GST antibodies. Blots are representative of n = 3 independent experiments. (**D**) Src kinase substrate motives in the TβRI intracellular domain (according to PhosphoMotif Finder (*31*)). (**E**) Extracts from HEK293T cells transfected with Src and HA-tagged wild-type TβRI or its mutants, were subjected to IP with HA antibodies, followed by IB with pY99, Src, and HA antibodies. HA-TβRI(8Y>F), HA-TβRI(Y182,249,282,291,295,428,429,476F) mutant. Relative position of pY99 band is shown in grey. Superimposed images of IB with pY99 and Src antibody are presented to show relative positioning of corresponding bands. Blots are representative of n = 3 independent experiments. (**F**) HEK293T cells were transfected with Src and HA-TβRI or mutant HA-TβRI(8Y>F). First, HA-tagged receptors were immunoprecipitated with HA antibody. Immunoprecipitates were then boiled in buffer containing 0.1 M Tris pH 7.4, 1% SDS and 10 mM DTT for 3 min. After cooling, iodoacetamide was added to a final concentration of 25 mM and samples were diluted 10 times with 1% Triton X-100, 0.1 M Tris pH 7.4. HA-tagged receptors were then immunoprecipitated with HA antibody a second time. Immunoprecipitates were analyzed by IB with pY99, Src, and HA. Relative position of pY99 band is shown in grey. Blots are representative of n = 2 independent experiments.

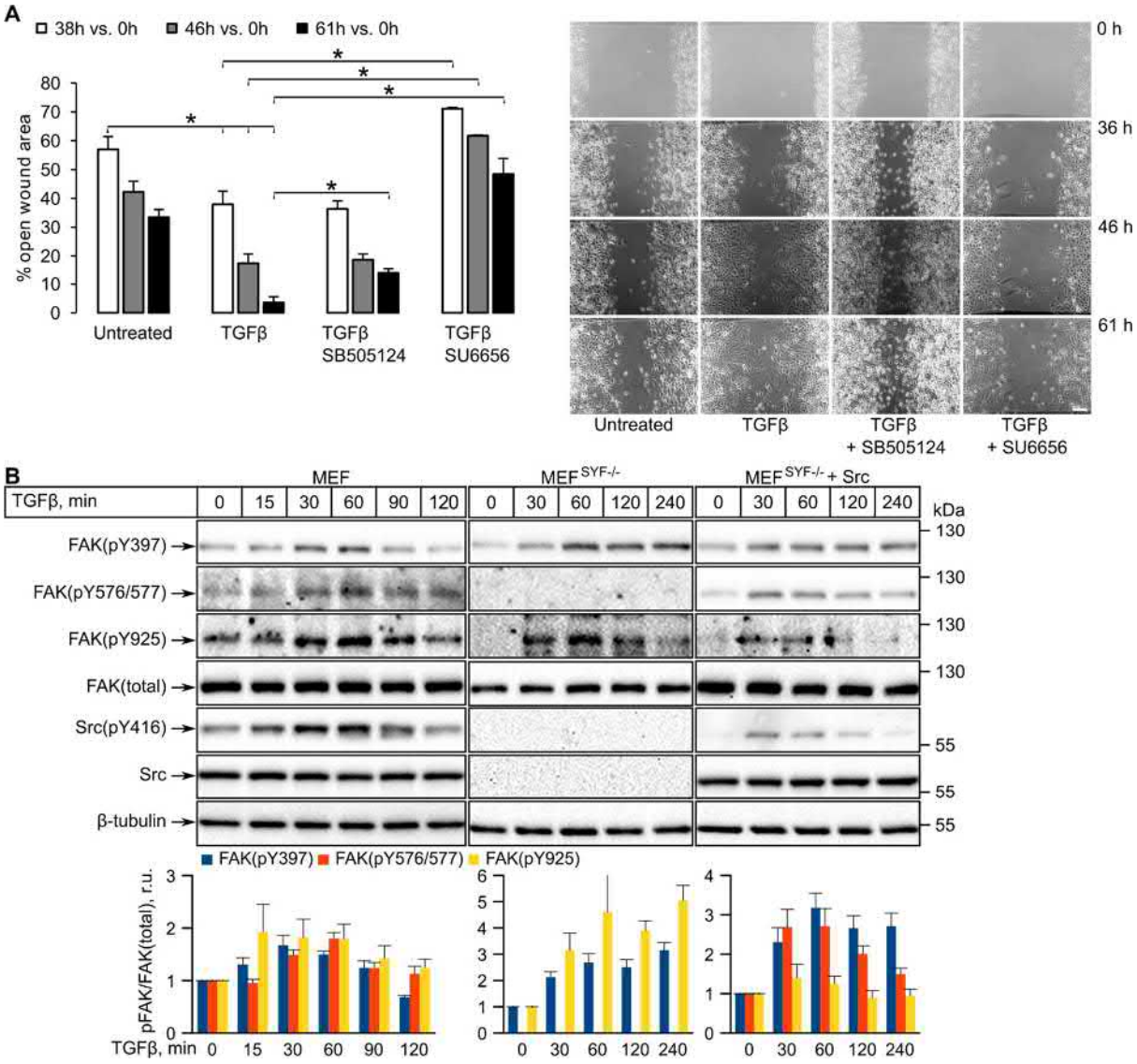

Fig. S5. Src mediates cellular responses to TGF-β1. (**A**) The TGF-β1-induced motility of PC3U cells was analyzed by an in vitro wound healing assay. TGF-β1 and SB505124 or SU6656 were added to culture medium supplemented with 0.1% FBS immediately after wounding. Phase contrast images were captured at the indicated time points. Quantification was performed of 4 measurements in each experimental condition and expressed as a percentage of the open wound area. Data represent means ± standard error of the mean. Brackets indicate the comparisons that showed significant differences of cell migration under treatments. *, $p < 0.05$. Representative images of the migration of PC3U cells in response to different treatments at different time points are shown (n = 3 independent experiments). Bar, 200 µm. (**B**) MEFs, MEFs that do not express Src, Fyn, and Yes ($SYF^{-/-}$), and MEF($SYF^{-/-}$) transfected with Src, were treated or not with TGF-β1 for the indicated time periods. Cell extracts were subjected to immunoblotting with pY397-FAK, pY576/Y577-FAK, pY925-FAK, FAK, Src-pY416, and Src antibodies. Quantifications of FAK phosphorylation (means ± SEM, of n = 3 independent experiments) reflect the normalized phospho-signal for the indicated tyrosines relative to total FAK.

Table S1. Primers used in this study.

Primer name	Primer sequence (5'-3')
Src-SH3 EcoRI_fwd	GCATGAGAATTCGCCGGTGGAGTGACCACCTTTGTG
Src-SH3 XhoI_rev	GCATGACTCGAGTCAGATGGAGTCGGAGGGCGCCAC
TbR1_Y182F_fwd	CGTTGAAAGACTTAATTTTTGATATGACAACGTCAGGTTCTGG
TbR1_Y182F_rev	CCAGAACCTGACGTTGTCATATCAAAAATTAAGTCTTTCAACG
TbR1_Y249F_fwd	GGTTCCGTGAGGCAGAGATTTTTCAAACTGTAATGTTACG
TbR1_Y249F_rev	CGTAACATTACAGTTTGAAAAATCTCTGCCTCACGGAACC
TbR1_Y282F_fwd	CTGGTTGGTGTCAGATTTTCATGAGCATGGATCCC
TbR1_Y282F_rev	GGGATCCATGCTCATGAAAATCTGACACCAACCAG
TbR1_Y291/5F_fwd	GGATCCCTTTTTGATTTCTTAAACAGATTCACAGTTACTGTGG
TbR1_Y291/5F_rev	CCACAGTAACTGTGAATCTGTTTAAGAAATCAAAAAGGGATCC
TbR1_Y378F_fwd	GTGGGAACAAAAAGGTTCATGGCCCCTGAAGTTC
TbR1_Y378F_rev	GAACTTCAGGGGCCATGAACCTTTTTGTTCCCAC
TbR1_Y402F_fwd	CTTCAAACGTGCTGACATCTTTGCAATGGGCTTAG
TbR1_Y402F_rev	CTAAGCCCATTGCAAAGATGTCAGCACGTTTGAAG
TbR1_Y424F_fwd	GTTCCATTGGTGGTATTCATGAAGATTTCCAACTGCC
TbR1_Y424F_rev	GGCAGTTGGAAATCTTCATGAATACCACCAATGGAAC
TbR1_Y428F_fwd	CATGAAGATTACCAACTGCCTTTTTATGATCTTGTACCTTCTGAC
TbR1_Y428F_rev	GTCAGAAGGTACAAGATCATAAAAAGGCAGTTGGTAATCTTCATG
TbR1_Y429F_fwd	GATTACCAACTGCCTTATTTTGATCTTGTACCTTCTGACC
TbR1_Y429F_rev	GGTCAGAAGGTACAAGATCAAAATAAGGCAGTTGGTAATC
TbR1_Y476F_fwd	GAGAGAATGTTGGTTTGCCAATGGAGCAGCTAGG
TbR1_Y476F_rev	CCTAGCTGCTCCATTGGCAAACCAACATTCTCTC
TbR1_R451A_fwd	GTTGTTTGTGAACAGAAGTTAGCGCCAAATATCCCAAACAG
TbR1_R451A_rev	CTGTTTGGGATATTTGGCGCTAACTTCTGTTCACAAACAAC
TbR1_P455A_fwd	GTTAAGGCCAAATATCGCAAACAGATGGCAGAGC
TbR1_P455A_rev	GCTCTGCCATCTGTTTGCGATATTTGGCCTTAAC
TbR1_R457A_fwd	CCAAATATCCCAAACGCATGGCAGAGCTGTGAAGC
TbR1_R457A_rev	GCTTCACAGCTCTGCCATGCGTTTGGGATATTTGG