XB130/Tks5 scaffold protein interaction regulates Src-mediated cell proliferation and survival

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ABSTRACT **The scaffold protein XB130 regulates cell growth, survival, and migration. Yeast two-hybrid screening suggests that XB130 interacts with another scaffold protein, Tks5. We hypothesized that XB130 and Tks5 form a macromolecular complex to mediate signal transduction cascades for the regulation of cell growth and survival. Coimmunoprecipitation demonstrated that XB130 and Tks5 interact endogenously and form a complex with Src tyrosine kinase. Structure–function studies showed that the fifth SH3 domain of Tks5 binds to the N-terminus of XB130, which contains polyproline-rich motifs. Cell growth and survival studies revealed that down-regulation of XB130 and/or Tks5 reduced cell proliferation, resulting in cell cycle inhibition at the G1 phase and increased caspase 3 activity and apoptosis. Moreover, cell proliferation and survival were increased by overexpression of XB130 or Tks5 but decreased when XB130/Tks5 binding was disrupted by overexpression of XB130 N-terminal deleted mutant and/or Tks5 fifth SH3 domain W1108A mutant. Furthermore, down-regulation of XB130 and/or Tks5 inhibited serum- and growth factor–induced Src activation and downstream phosphorylation of PI3K and Akt. Our results suggest that Tks5, similar to XB130, plays a role in cell proliferation and cell survival and that the interaction between XB130 and Tks5 appears to be critical for regulation of Src-mediated cellular homeostasis.**

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INTRODUCTION

Scaffold and adaptor proteins are critical components of signal transduction pathways by coordinating association, assembly, and transport of molecules (Pawson and Scott, 1997; Flynn 2001; Pawson, 2007). Several studies have shown the importance of scaffold and adaptor proteins in normal cell proliferation, differentiation, and motility (Flynn, 2001; Yablonski and Weiss, 2001; Xu *et al.*, 2007). Moreover, dysregulation of these proteins is linked to cell dysfunction, resulting in disease development and progression (Pawson and Scott, 1997; Flynn, 2001; Shiozaki and Liu, 2011; Bai *et al.*, 2014). For example, X11α, Fe65, and Jun kinase–interacting protein are associated with Alzheimer's disease, and Nck, Grb2, tyrosine kinase substrate with five SH3 domains (Tks5), and XB130 are involved in tumorigenesis and metastasis (King and Turner, 2004; Blouw *et al.*, 2008; Oikawa *et al.*, 2008; Stylli *et al.*, 2009; Shiozaki *et al.*, 2011, 2012).

XB130 appears to be a classical example of the scaffold protein, containing various molecular-binding domains and acting as a signal transduction molecule to influence cell growth, survival, and migration (Xu *et al.*, 2007; Lodyga *et al.*, 2009, 2010; Snyder *et al.*, 2011). DNA sequence homology data illustrate that XB130 belongs to the actin filament–associated protein (AFAP) family and is also known as actin filament–associated protein 1–like 2 (AFAP1L2; Xu *et al.*, 2007; Snyder *et al.*, 2011). Similar to AFAP, XB130 contains Src homology 2 (SH2) and Src homology 3 (SH3) binding motifs in its N-terminus (Figure 1A). XB130 also contains two pleckstrin homology (PH) domains for phosphatidylinositol lipid binding at cellular membranes (Figure 1A; Xu *et al.*, 2007; Snyder *et al.*, 2011). Unlike AFAP, XB130 lacks an actin-binding domain and is not associated with actin under basal conditions. However, epidermal growth factor (EGF), NNK (a nicotine-derived metabolite) and constitutively activated Rac1 (CA-Rac) induce XB130 translocation from the

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Abbreviations used: AFAP1L2, actin filament–associated protein 1–like 2; coIP, coimmunoprecipitation; GST, glutathione-*S* transferase; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium; PI3K, phosphoinositide-3 kinase; Tks5, tyrosine kinase substrate with five SH3 domains.

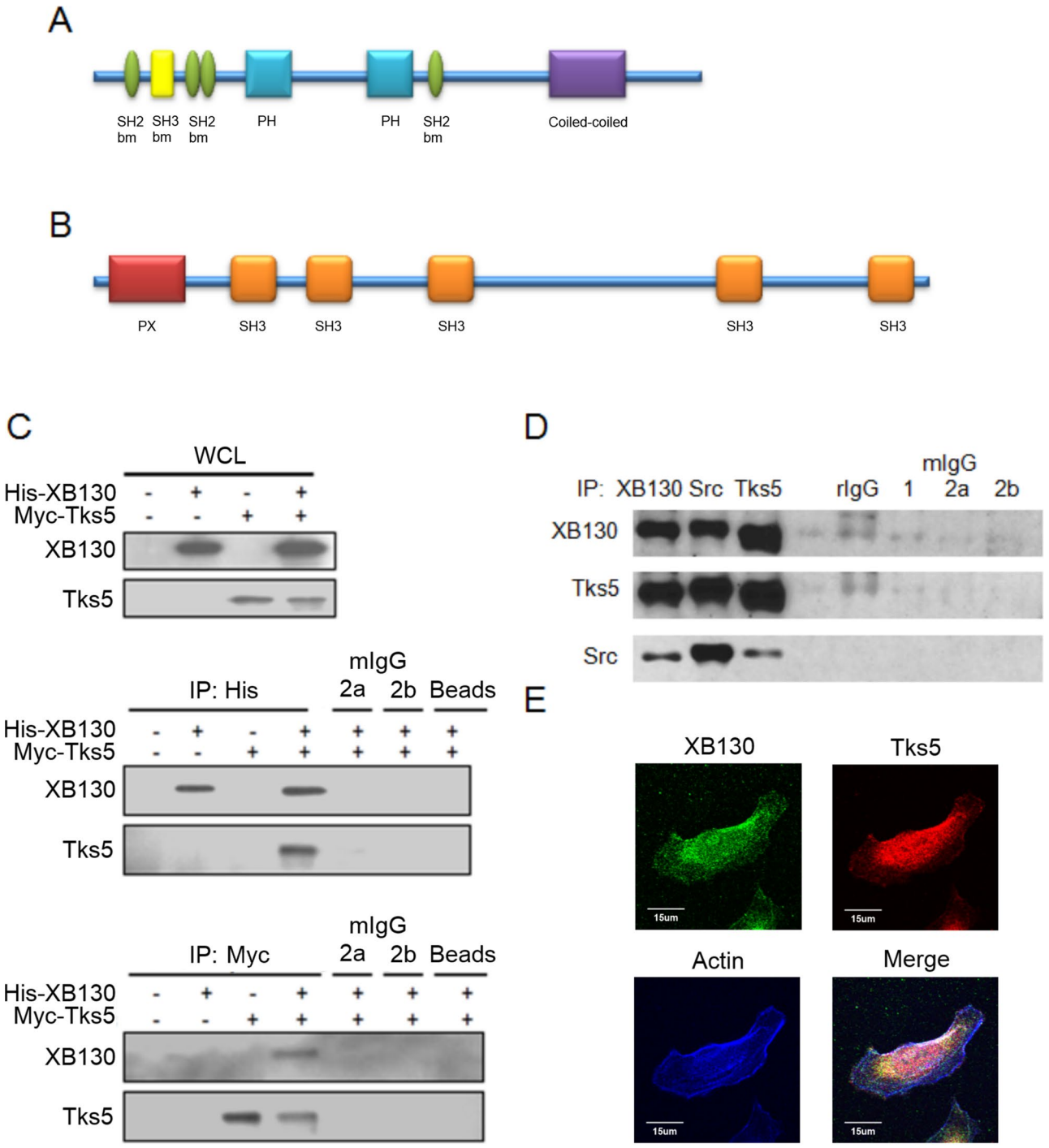

FIGURE 1: XB130 interacts with Tks5 and Src tyrosine kinase. (A) Schematic diagram of XB130 structure showing the Src homology 2 domain–binding motifs (SH2bm, green oval) and an SH3 domain–binding motif (SH3bm, yellow rectangle), two pleckstrin homology domains (PH, blue square), and a coiled-coiled region (purple rectangle). (B) Schematic diagram of Tks5 structure showing a phox homology domain (PX, red rectangle) and five SH3 domains (SH3, orange square). (C) CoIP of His-XB130 and Myc-Tks5 and immunoblot of XB130 and Tks5 in COS-7 cells. (D) CoIP of endogenous XB130, Tks5, and Src in BEAS-2B cells. Lane 4 was loaded with molecular weight markers. (E) Coimmunofluorescence staining shows that under unstimulated conditions, endogenous XB130 and Tks5 are both located in the cytoplasm and in the perinuclear region of BEAS-2B cells.

perinuclear cytoplasm to lamellipodia (Lodyga *et al.*, 2010; Wu *et al.*, 2015). Altered XB130 function has been shown to modulate several characteristics of cell migration, such as wound healing, cytoskeletal dynamics, cell spreading, and cell polarity (Lodyga *et al.*, 2010).

XB130 is a tyrosine kinase substrate that interacts with Src, Lck, Rearranged during transfection/papillary thyroid carcinoma (RET/PTC), and phosphoinositide 3-kinase (PI3K) to modulate cell proliferation and survival (Xu *et al.*, 2007; Lodyga *et al.*, 2009; Bai *et al.*, 2014). Src-mediated tyrosine phosphorylation of XB130 leads to the

transactivation of transcription factors through activator protein-1 (AP-1) and serum response element (SRE) binding sites (Xu *et al.*, 2007). Constitutively active RET/PTC promotes XB130 association with the p85α subunit of PI3K, resulting in phosphorylation of Akt and regulation of cell cycle progression and survival (Lodyga *et al.*, 2009). A subsequent study showed that XB130 modulates phosphorylation of the PI3K/Akt downstream targets p21Cip1/WAF1, p27Kip1, FOXO3a, and GSK3β and activation of caspase 8 and 9, thereby regulating cell proliferation and survival in WRO and TPC-1 human thyroid cancer cells and A549 human lung cancer cells (Shiozaki *et al.*, 2012; Bai *et al.*, 2014). XB130 also regulates expression of tumor- suppressive microRNAs (Takeshita *et al.*, 2013).

Using yeast two-hybrid screening, we found multiple putative XB130 binding partners, of which Tks5 had the most positive hits. The Tks5 protein shares close sequence and structural homology to its sister protein, Tks4, and to the p47phox family of NADPH oxidase (NOX) organizers (Diaz *et al.*, 2009). Tks5 contains an N-terminal phox homology (PX) domain for phosphatidylinositol lipid binding and five SH3 domains for protein–protein interactions (Figure 1B; Crimaldi *et al.*, 2009; Diaz *et al.*, 2009). Under unstimulated conditions, Tks5 is found in the cytoplasm, but upon stimulation, it translocates to the cell periphery, where it localizes to actin-rich, cytoskeletal outgrowths of the ventral cell membrane known as podosomes, which are instrumental in cell invasion (Courtneidge *et al.*, 2005; Seals *et al.*, 2005; Crimaldi *et al.*, 2009; Stylli *et al.*, 2009).

As a novel, multidomain scaffold protein, XB130 interacts with lipids and proteins to regulate several cellular processes, such as cell growth, proliferation, survival, and motility (Bai *et al.*, 2014). Whether Tks5 also shares a similar role for the regulation of cell proliferation and survival has yet to be examined. Thus, in this study, we sought to determine whether XB130 and Tks5 physically interact and whether such an interaction is necessary to mediate or affect downstream signaling responses and cellular functions.

RESULTS

XB130 interacts with Tks5 and protein tyrosine kinase, Src

A yeast two-hybrid screen was performed using plasmids encoding XB130 cDNA fused to the binding-domain fragment of the GAL4 transcriptional activator and a library of human brain cDNA fragments fused to the activating domain of the GAL4 transcriptional activator (Young, 1998). The yeast two-hybrid screen identified 35 putative interacting partners (unpublished data). Tks5 (also known as SH3 and PX domains 2A, SH3PXD2A; Figure 1B) was identified as the most highly captured binding partner, with five positive clones.

To confirm the interaction of XB130 with Tks5, we transfected COS-7 cells with Myc-Tks5 and histidine (His)-XB130 plasmids and performed coimmunoprecipitation (coIP) with cell lysates. Anti-His antibody pulled down both XB130 and Tks5, and anti-Myc antibody precipitated both Tks5 and XB 130 (Figure 1C). Isotype-specific immunoglobulin Gs (IgGs) had no similar effects. The interaction between endogenous XB130 and Tks5 was confirmed in human bronchial epithelial BEAS-2B cells using coIP (Figure 1D). Both XB130 and Tks5 are known Src tyrosine kinase substrates (Courtneidge *et al.*, 2005; Xu *et al.*, 2007), and endogenous Src in BEAS-2B cells also coimmunoprecipitated with XB130 and Tks5 (Figure 1D). Similar results were obtained in the thyroid cancer cell lines WRO and TPC-1 (unpublished data). Coimmunofluorescence microscopy showed that endogenous XB130 and Tks5 are colocalized in fine punctate structures in the cytoplasm and in perinuclear regions (Figure 1E).

The XB130 N-terminus is required for binding to the fifth SH3 domain of Tks5

Protein–protein interaction requires specific physical contact and orientation between proteins as a result of electrostatic forces and biochemical processes (Jones and Thornton, 1996). Protein sequences often contain well-known or canonical structural domains and motifs, which are required for specific binding to other proteins (Pawson and Nash, 2000). We probed the protein-binding regions and structural domains of XB130 and Tks5 to determine the specific physical binding sites between these partners. SH3 domains, although similar in overall structures, do not share identical amino acid sequences and thus may have very specific binding partners (Weng *et al.*, 1995). To determine whether XB130 binds specifically to one or more of the SH3 domains of Tks5, we performed a glutathione *S*-transferase (GST)–fusion protein pull-down assay in which the DNA sequence of each of the five SH3 domains of Tks5 was fused with the GST DNA sequence to express GST-tagged Tks5 SH3-domain recombinant proteins (Figure 2A). The individual GST-tagged Tks5 SH3-domain recombinant proteins were incubated with cell lysates from COS-7 cells, which were transfected with

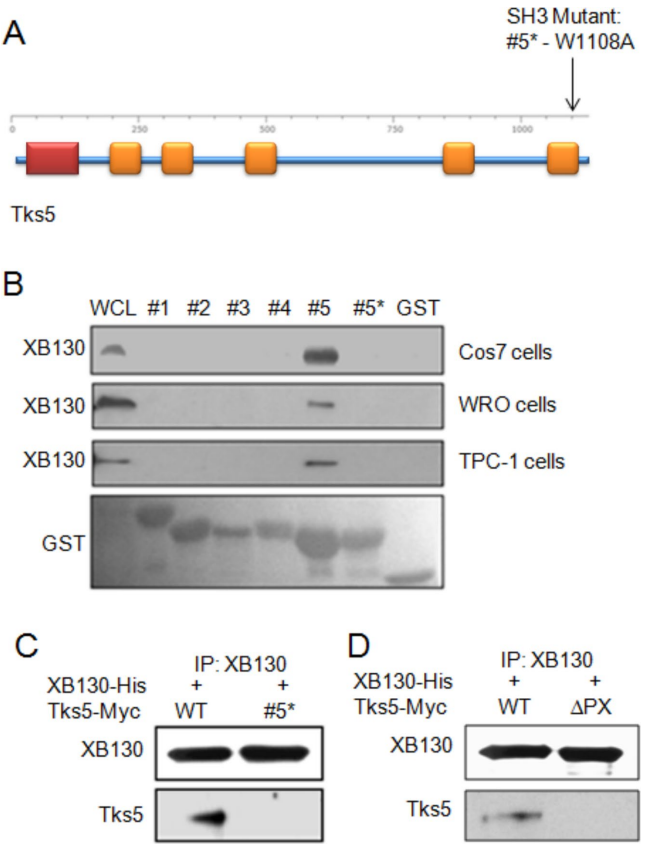

FIGURE 2: XB130 binds to the fifth SH3 domain of Tks5. (A) Schematic diagram of Tks5, in particular showing the location of the SH3 #5 single–amino acid substitution mutant, tryptophan 1108 to alanine (W1108A). (B) GST-fusion protein pull-down assay and immunoblot of XB130 shows that XB130 binds to only the fifth SH3 domain of Tks5 in COS-7 (transfected with His-XB130), WRO, and TPC-1 cells. (C) Immunoprecipitation of XB130 shows that single amino acid substitution of tryptophan (W) 1108 to alanine (A) in the fifth SH3 domain of Tks5 inhibited coIP between Tks5 and XB130 in COS-7 cells. (D) Immunoprecipitation of XB130 shows that deletion of PX domain of Tks5 (Myc-Tks5 ΔPX) also inhibited Tks5 binding to XB130 in COS-7 cells.

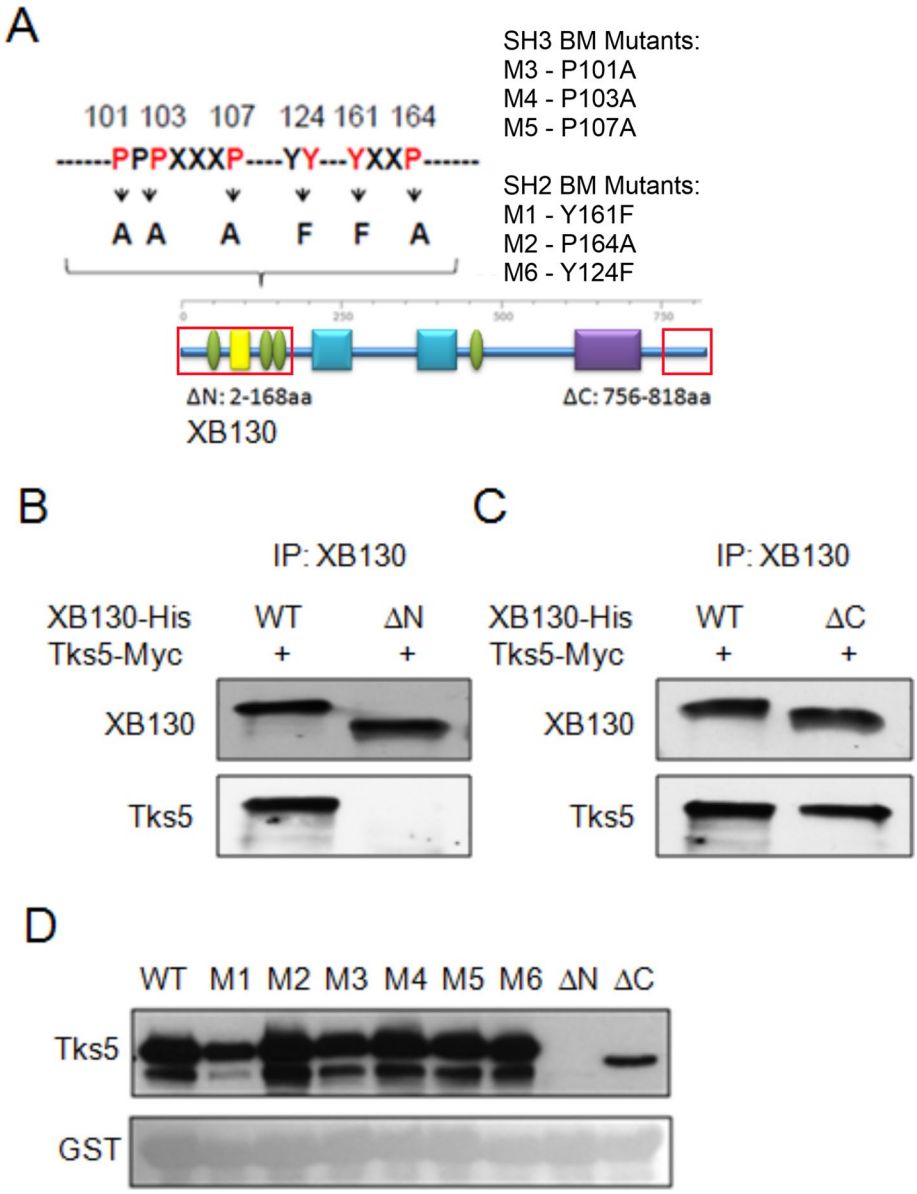

FIGURE 3: Tks5 binds to the SH2 and SH3 domain–binding motifs in the N-terminus of XB130. (A) Schematic diagram of XB130 showing the locations of the N-terminal deletion (ΔN), C-terminal deletion (ΔC), and SH3 domain–binding motif (SH3 BM) mutants and SH2 domain–binding motif (SH2 BM) mutants. (B) Deletion of the XB130 N-terminus–inhibited coIP of XB130 with Tks5 in COS-7 cells. (C) Deletion of the XB130 C-terminus slightly decreases coIP of XB130 with Tks5 in COS-7 cells. (D) GST-fusion protein pull down in COS-7 cells transfected with XB130 SH3 or SH2 BM mutants shows that Tks5 detection was decreased by SH2 BM mutant Y161F (M1) or SH3 BM mutant P101A (M3) in COS-7 cells. Moreover, N-terminal deletion of XB130 completely and C-terminal deletion of XB130 partially blocked binding with Tks5.

His-XB130–tagged vectors. XB130 was found to bind specifically to the fifth SH3 domain of Tks5 (Figure 2B). Similarly, endogenous XB130 in WRO and TPC-1 cells also bound specifically to the fifth SH3 domain of Tks5 (Figure 2B). The Tks5 protein sequence in the fifth SH3 domain contains an SH3 domain–binding pocket with a protein-binding tripeptide of two sequential tryptophans (W) and a tyrosine (Y) at positions 1108–1110. It has been shown that single–amino acid mutation of W1108 to alanine (W1108A, previously annotated as W1056A; Diaz *et al.*, 2009; see *Discussion*) abolishes the association of the fifth SH3 domain of Tks5 with ADAM19 (Abram *et al.*, 2003). GST-fusion protein pull-down of GST-tagged Tks5 SH3 #5 containing the single amino acid mutation of W1108A prevented XB130 binding to Tks5 (Figure 2B). Overexpression of His-XB130 and mutant Myc-Tks5 W1108A in COS-7 cells showed that the coIP between XB130 and Tks5 was abolished when the W1108A mutant was used (Figure 2C). It has been demonstrated that the PX domain of Tks5 is critical for its intermolecular interaction with phosphatidylinositol lipids, as well as the translocation of Tks5 and Tks5-bound actin-associated proteins to the cell periphery (Abram *et al.*, 2003). In COS-7 cells, anti-His antibody, used to detect XB130, did not pull down PX domain deleted Myc-Tks5 mutant (Figure 2D).

It has been well documented that SH3 domains bind to polyproline-rich (PPR) sequences within SH3-binding motifs (SH3bm). XB130 contains an SH3bm and several SH2bms within the N-terminus, as well as a PPR region from position 768–783 in the C-terminus (Figure 3A). Expression of the XB130 mutant with deletion of the N-terminal region (XB130ΔN) from position 2–168 abolished its binding with Tks5 (Figure 3B), whereas deletion of the C-terminal region (XB130ΔC) from position 756–818 did not significantly affect XB130/Tks5 binding (Figure 3C). The N-terminus of XB130 contains the polyproline-rich sequence PPPxxxP at position 101–107 and several putative tyrosine phosphorylation sites YxxA/E at position 124–127 and YxxP at position 161–164 (Figure 3A). To verify the potential significance of these residues in binding to Tks5, single–amino acid residues within the SH3bm at P101, P103, and P107 and within the SH2bms at Y124, Y161, and P164 were mutated (Figure 3A). GST-fusion protein pull down was performed using GST-tagged XB130, GST-tagged, XB130 single–amino acid mutants, GST-tagged, N-terminal–deleted XB130, and GST-tagged, C-terminal–deleted XB130 and incubated with COS-7 cell lysate containing overexpressed Myc-Tks5. Deletion of the N-terminus completely abolished binding with Tks5, and deletion of the C-terminus reduced binding of XB130 to Tks5. Moreover, Y161F (M1) and P101A (M3) mutants of XB130 showed reduced Tks5 binding (Figure 3D). These results suggest that the N-terminal of XB130 is critical for binding to the fifth SH3 domain of Tks5.

Down-regulation of XB130 and/or Tks5 leads to inhibition of cell proliferation and survival

Unlike XB130, the role of Tks5 in cell growth, proliferation, and survival is unknown. Because we showed that XB130 and Tks5 directly interact, we hypothesized that, depending on the extracellular stimulus, XB130 and Tks5 may act in conjunction to regulate cell growth and proliferation and cell survival. A 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium viability

screen showed that down-regulation of XB130 or Tks5 in BEAS-2B cells with specific small interfering RNA (siRNA) resulted in inhibition of cell viability in a dose-dependent manner (unpublished data). A growth curve shows that at 72 and 96 h after siRNA transfection, down-regulation of XB130 and/or Tks5 significantly reduced the numbers of cells compared with the growth of nontransfected control BEAS-2B cells and BEAS-2B cells transfected with a nontargeting siRNA (Figure 4A). To tease out the role of XB130 and Tks5 in DNA synthesis, we performed a bromodeoxyuridine (BrdU) incorporation assay. Down-regulation of XB130 or Tks5 resulted in significantly lower levels of BrdU incorporation as compared with cells receiving no siRNA or cells transfected with nontargeting siRNA (Figure 4B). To analyze cell cycle progression, we performed propidium iodide (PI) staining followed by flow cytometry analysis. Down-regulation of XB130 and/or Tks5 results in the accumulation of cells in the G1 (resting) phase and a decrease of cells in the DNA synthesis and G2 cell growth phase as compared with the control or nontargeting siRNA–transfected cells (Figure 4C). Moreover, expression of the cell-proliferative marker Ki67 was decreased, whereas a cell cycle regulation marker, p21, was increased in cell lysates from BEAS-2B cells transfected with XB130 and/or Tks5 siRNA (Figure 4D). The evidence of down-regulation of XB130 and Tks5 by respective siRNAs is shown in Figure 4E. Of interest, down-regulation of both XB130 and Tks5 resulted in similar inhibition on cell growth, BrdU incorporation, cell cycle progression, and individual siRNA of XB130 or Tks5 with no additive effect (Figure 4).

Using the same siRNA knockdown strategy, we assessed cell survival and apoptosis. Down-regulation of XB130 alone, Tks5 alone, or both XB130 and Tks5 led to a significant increase in caspase 3 activity over control cells or cells transfected with nontargeting siRNA. A caspase 3 inhibitor (AC-DEVD-CHO) and activator (staurosporine) were used to validate the response of the caspase 3 activity in BEAS-2B cells (Figure 5A). To further understand the role of XB130 and Tks5 in apoptosis, we performed PI/annexin V costaining with flow cytometry. A plot of annexin V as a function of PI was gated to determine living, early apoptotic, late apoptotic, and necrotic cells (Figure 5B). Down-regulation of XB130, Tks5, or both XB130 and Tks5 significantly reduced live cells and increased both early and late apoptosis populations, with a more significant effect in early apoptotic cell populations than in the control cells (Figure 5B). Again, no additive effects were seen when both XB130 and Tks5 siRNAs were used.

XB130/Tks5 interaction is important for regulation of cell proliferation and survival

Because we showed that the N-terminus of XB130 and the ligand-binding sequence in the fifth SH3 domain of Tks5 were critical for their interaction (Figure 6A), we were curious about whether physical binding of XB130 and Tks5 was required in the regulation of cell proliferation and survival. The green fluorescent protein (GFP)–tagged XB130 N-terminal deletion (XB130ΔN) mutant or Myc-tagged Tks5 SH3#5 W1108A (Tks5 W1108A) mutant was overexpressed in BEAS2B cells either alone or together with its wild-type (WT) or mutant binding partner. Cells were analyzed for BrdU incorporation, as an indicator of cell proliferation, and analyzed for caspase 3 activity, as an indicator of cell survival. Overexpression of XB130 WT and/or Tks5 WT increased BrdU incorporation (sign of DNA synthesis), whereas overexpression of XB130ΔN and/or Tks5 W1108A decreased BrdU incorporation relative to control cells (Figure 6B). Overexpression of the XB130 WT or Tks5 WT protein in conjunction with its mutant binding partner, Tks5 W1108A or XB130ΔN, respectively, showed similar BrdU incorporation results as seen in the mutant-alone groups (Figure 6B).

Furthermore, overexpression of XB130 WT, Tks5 WT, or both XB130 WT and Tks5 WT appears to have a protective effect on cells by decreasing caspase 3 activity compared with control cells (Figure 6C). In contrast, overexpression of XB130ΔN or together with Tks5 W1108A significantly increased caspase 3 activity (Figure 6C). Overexpression of the XB130 WT or Tks5 WT protein in conjunction with its mutant binding partner, Tks5 W1108A or XB130ΔN, respectively, also showed increased caspase 3 activity (Figure 6C).

XB130 and Tks5 interaction regulate Src activation and signaling

As scaffold proteins and Src-tyrosine kinase substrates, we hypothesized that XB130 and Tks5 may modulate cell proliferation and survival through Src-mediated signaling pathways. BEAS-2B cells were treated with normal DMEM containing either 10% fetal bovine serum FBS) or 50 ng/ml EGF for up to 60 min. Western blot analysis showed that tyrosine phosphorylation of BEAS-2B cellular proteins was rapidly enhanced by 10% FBS or 50 ng/ml EGF treatment after a 10-min interval (Figure 7A). Cells were then transfected with XB130 siRNA or Tks5 siRNA or both XB130 and Tks5 siRNA and treated with either 10% FBS or 50 ng/ml EGF for 10 min. Of importance, total cell lysate tyrosine phosphorylation and phosphorylation of Src Y416 (a sign of Src activation) was clearly enhanced in the control and control siRNA cells treated with 10% FBS or 50 ng/ml EGF (Figure 7B). Down-regulation of XB130, Tks5, or both XB130 and Tks5 inhibited the FBS- or EGF-induced tyrosine phosphorylation of cellular proteins and inhibited the phosphorylation of Src Y416 (Figure 7B).

Src is a well-known regulator of several kinase-mediated signaling pathways involved in the regulation of cell proliferation and survival, such as the PI3K signal transduction pathway. Like Src, XB130 is also a binding partner of PI3K (Lodyga *et al.*, 2009). Moreover, Tks5 has been implicated in PI3K activity and PI3K-mediated cell membrane dynamics (Fekete *et al.*, 2013). XB130 and/or Tks5 siRNA also blocked FBS- or EGF-induced phosphorylation of PI3K p85α subunit and Akt (Figure 7B).

DISCUSSION

This study elucidated the formation of a unique protein complex. Our results indicate that XB130 and Tks5 are endogenous binding partners that form a putative protein complex with Src tyrosine kinase. As scaffold proteins, both XB130 and Tks5 are highly involved in the potential binding, recruitment and translocation of proteins, lipids, and nucleic acids (Courtneidge *et al.*, 2005; Xu *et al.*, 2007; Crimaldi *et al.*, 2009; Lodyga *et al.*, 2009; Murphy and Courtneidge, 2011; Shiozaki and Liu, 2011). Our results show that the fifth SH3 domain of Tks5 binds to the polyproline-rich region in the N-terminus of XB130. Previous Tks5 studies showed that tryptophan residue 1056 (W1056) is located within a ligand-binding surface of the fifth SH3 domain of Tks5 and is important for protein–protein interactions (Diaz *et al.*, 2009). However, the human Tks5 gene sequence has been annotated, and database analysis shows that the fifth SH3 domain of Tks5 spans amino acid residues 1071–1133, and W1056 is now counted as amino acid residue 1108 (www.ncbi.nlm.nih.gov/protein/Q5TCZ1.1). The canonical SH3 binding pocket consists of two hydrophobic grooves that are lined by the aromatic residues tryptophan (W), phenylalanine (F), histidine (H), or tyrosine (Y) and may also contain an acidic amino acid residue similar to the aspartic acid residue in the Src kinase SH3 domain (Weng *et al.*, 1995). In the fifth SH3 domain of Tks5, the aromatic amino acid sequence WWY from residues 1108–1110 is located in close proximity to D1115 and is also flanked by hydrophobic regions (Seals *et al.*, 2005). Of

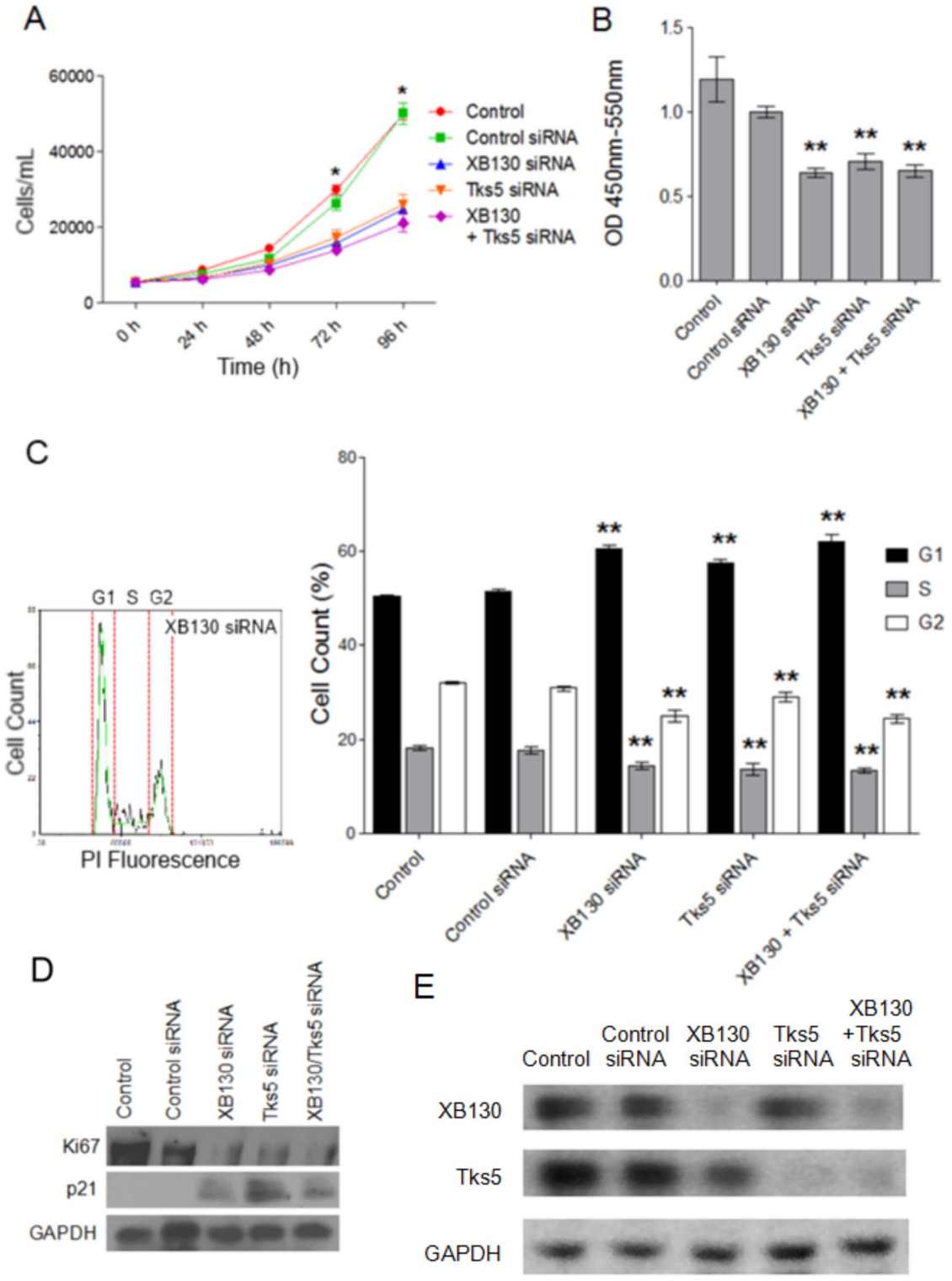

FIGURE 4: Down-regulation of XB130 and/or Tks5 leads to inhibition of cell proliferation and accumulation of cells in G1 phase. (A) Cell counting of BEAS-2B cells at 24 , 48 , 72, and 96 h shows that siRNA down-regulation of XB130, Tks5, or both XB130 and Tks5 reduced the number of cells/milliliter after 72 and 96 h. (B) BrdU colorimetric incorporation assay shows that siRNA of XB130 and/or Tks5 significantly decreases DNA synthesis. (C) PI staining and flow cytometry analysis shows that siRNA of XB130 and Tks5 results in accumulation of cells in the G1 phase and a decrease of cells in the S and G2 phases. Data from A–C are summarized from three independent experiments and presented as mean ± SD. *$p < 0.05$, **$p < 0.01$ compared with controls (nontransfected BEAS-2B cells and nontargeting siRNA–transfected cells). (D) Western blot analysis shows that the proliferative marker Ki67 is reduced, whereas p21 is up-regulated, in XB130 and/or Tks5 siRNA cell lysates. (E) Expression of XB130 and Tks5 in BEAS-2B cells was reduced by specific siRNA. Note: no transfection (Control), transfection of nontargeting siRNA (Control siRNA), or transfection of XB130 and/or Tks5 siRNA. GAPDH is shown as a loading control for D and E.

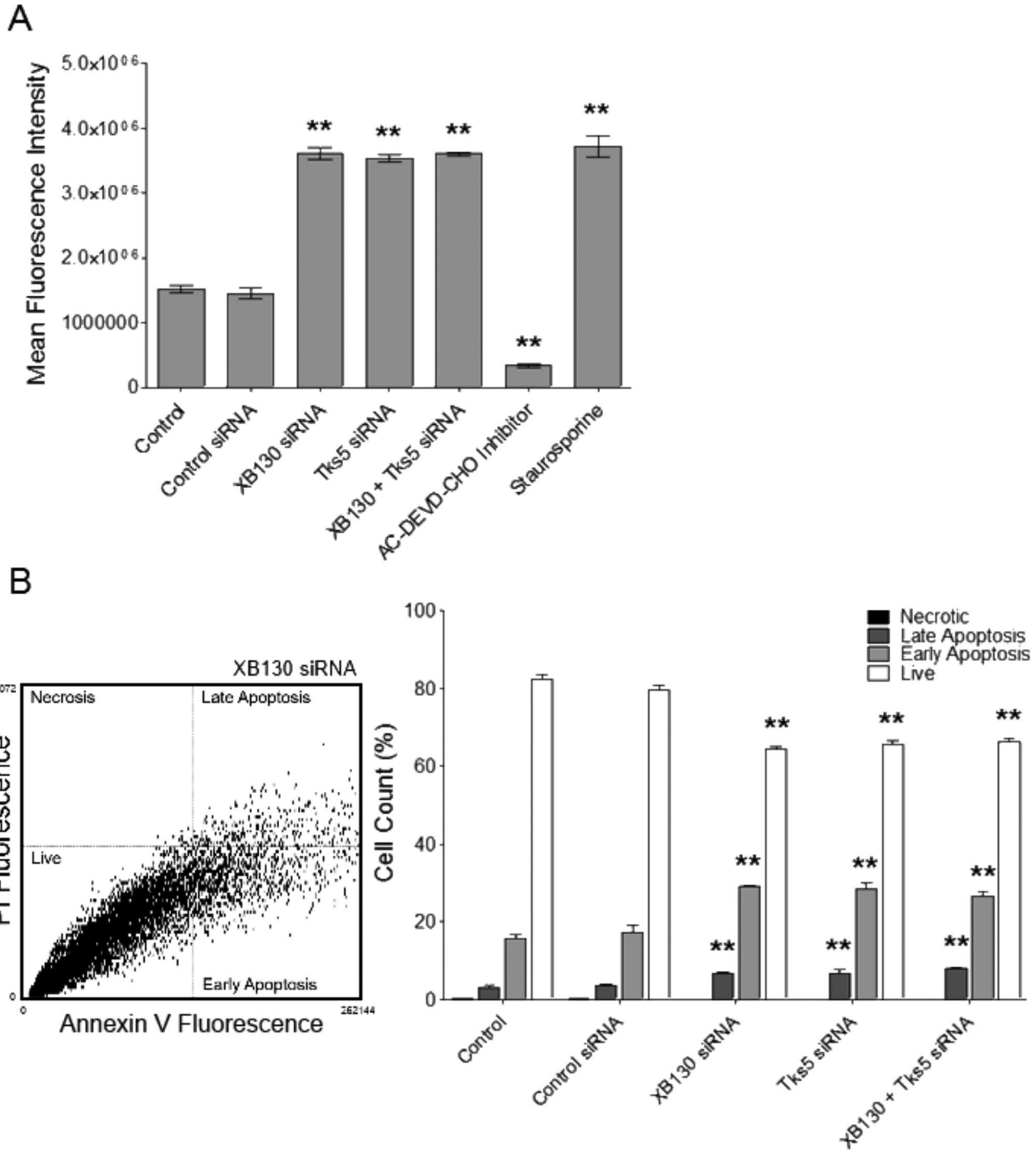

FIGURE 5: Down-regulation of XB130 and/or Tks5 leads to caspase 3 activation and increase in apoptotic cell populations. (A) EnzChek Caspase 3 activity fluorometric assay shows that siRNA of XB130 and/or Tks5 significantly increased caspase 3 activity. Caspase 3 inhibitor AC-DEVD-CHO (10 mM for 30 min) was used as a specificity control, and staurosporine (240 nM for 30 min) was used as a positive control to validate the assay. (B) PI/annexin V staining and flow cytometry analysis shows that siRNA of XB130 and/or Tks5 results in an increase of cells in early and late apoptosis with a decrease of viable cells. Data are summarized from three independent experiments and presented as mean ± SD. $^{**}p < 0.01$ compared with controls (nontransfected BEAS-2B cells and nontargeting siRNA–transfected BEAS-2B cells).

interest, we showed that the single amino acid mutation of tryptophan 1108 to alanine (W1108A) in the fifth SH3 domain of Tks5 appears to inhibit XB130 binding to Tks5. We also showed that deletion of the N-terminal PX domain of Tks5 abolishes XB130 binding to Tks5. Some studies suggest that the PX domain plays a role in conformational stability of Tks5 (Oikawa *et al.*, 2012) and may explain the lack of binding between PX domain–deleted Tks5 and XB130. We also demonstrated that compared with the GST-fusion protein pull-down of wild-type XB130, only SH2bm mutant Y161F and SH3bm mutant P101A showed decreased Tks5 protein

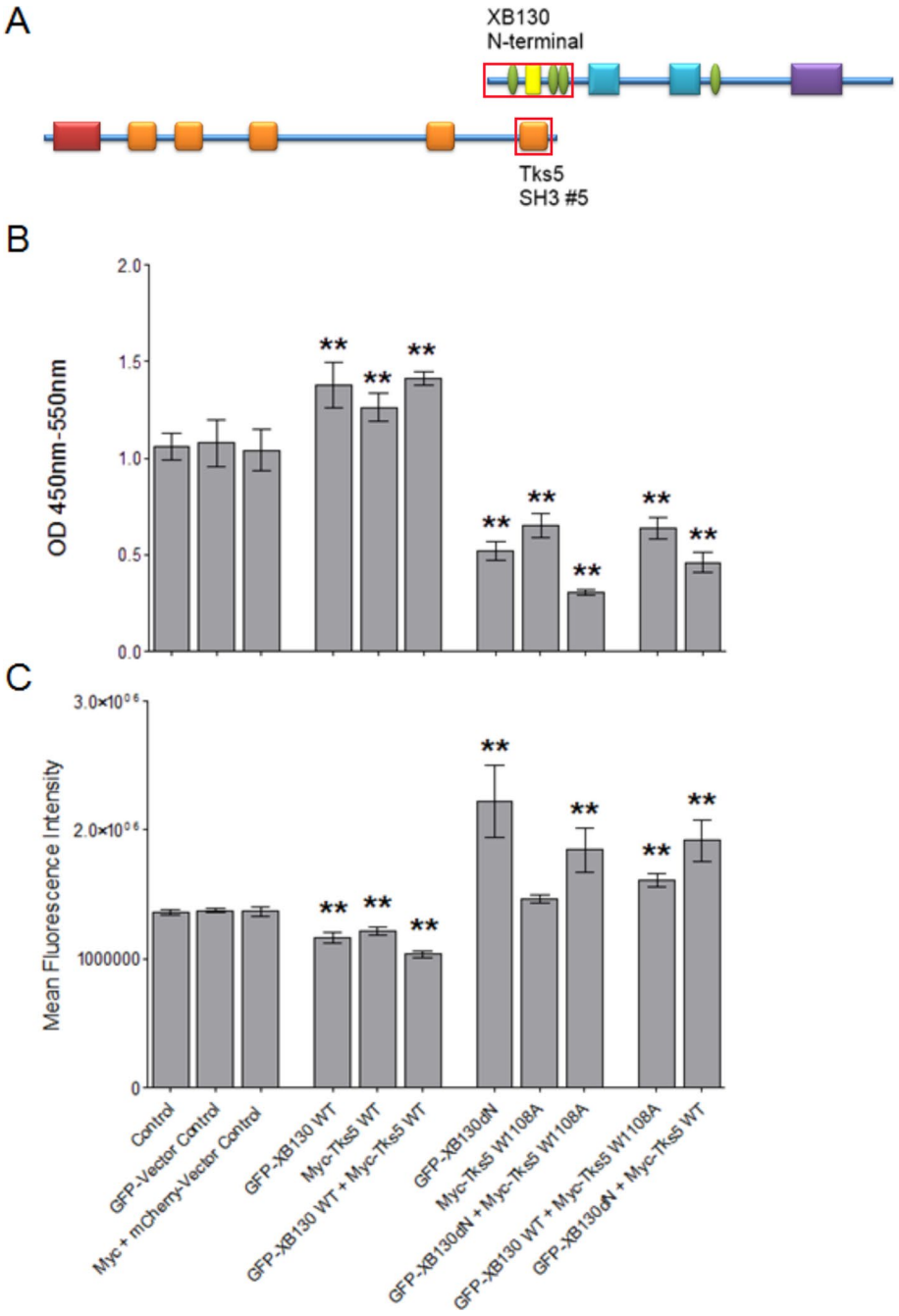

FIGURE 6: Deletion of the XB130 N-terminal and mutation of the fifth SH3 domain of Tks5 negatively affects cell proliferation and survival. (A) Diagram identifying the XB130 N-terminus, which binds to the fifth SH3 domain of Tks5. (B) BrdU colorimetric incorporation assay shows that overexpression of GFP-XB130 WT and/or Myc-Tks5 WT increased cell proliferation, whereas overexpression of the binding mutants GFP-XB130ΔN and/or Myc-Tks5 W1108A decreased cell proliferation. Overexpression of the WT protein with their mutant binding partner does not rescue the decreased cell proliferation. (C) EnzChek Caspase 3 activity fluorometric assay shows that overexpression of XB130 WT and/or Tks5 WT decreases caspase 3 activity, whereas overexpression of XB130ΔN or it together with Myc-Tks5 W1108A significantly increased caspase 3 activity. Overexpression of the WT protein with its mutant binding partner still shows increased caspase 3 activity. Data from B and C are summarized from three independent experiments and presented as mean ± SD. $^{**}p < 0.01$ compared with controls (nontransfected BEAS-2B cells and nontargeting siRNA–transfected BEAS-2B cells).

detection. These XB130 mutations are located within the polyproline-rich motif KxxPxxP$_n$ at amino acid residues 95–103 in the SH3bm and a second polyproline-rich motif PYxxPxP at amino acid residues 160–166 in the SH2bm (Xu *et al.*, 2007). These polyproline-rich motifs are essential for SH3 domain binding and promote increased affinity for SH3 domain–containing proteins such as Tks5 (Kay *et al.*, 2000).

XB130 has been demonstrated to play a key role in cell proliferation and survival. For example, XB130 associates with the p85α subunit of PI3K, resulting in downstream activation of Akt and regulation of cell proliferation and cell cycle progression (Lodyga *et al.*, 2009; Shiozaki *et al.*, 2011, 2012). Unlike XB130, Tks5's role in cell growth, proliferation, and survival has not previously been investigated. In our study, expression of Ki67, a nuclear protein that is up-regulated in actively replicating cells (Scholzen and Gerdes, 2000), was decreased in XB130 and/or Tks5 siRNA–down-regulated cell lysates. BrdU incorporation into DNA during the synthesis phase of the cell cycle was then used to quantify cell proliferation (Shi *et al.*, 2014). BrdU incorporation showed that cell proliferation was equally inhibited by down-regulation of XB130, Tks5, or both XB130 and Tks5, suggesting that both XB130 and Tks5 regulate cell cycle progression at or before the DNA synthesis phase. PI staining showed that down-regulation of XB130, Tks5, or both XB130 and Tks5 results in the accumulation of cells in the G1 (resting) phase and a decrease of cells in the DNA synthesis and replication phase and G2 (premitosis, rapid cell growth, and protein synthesis) phase of the cell cycle. Moreover, we observed that p21, a cyclin-dependent inhibitor that regulates the G1- and S-phase cell cycle progression (Gartel and Radhakrishnan, 2005), was up-regulated in XB130 and/or Tks5 down-regulated cells, suggesting that cells were under growth arrest at the G1 and/or S phase of the cell cycle. It is possible that the absence of XB130 and/or Tks5 results in the p53/p21-dependent G1-phase cell cycle arrest. These results are consistent with previously published cell proliferation data on XB130 shRNA stably transfected WRO cells (Shiozaki *et al.*, 2011). Our results indicate that XB130 and Tks5 may be involved in the regulation of the G1 checkpoint, which is important for determination of proper cell size and nutrient levels, as well as the occurrence of possible DNA damage (Bertoli *et al.*, 2013). Our results also demonstrate that both XB130 and Tks5 are involved in cell survival. It is possible that the role of XB130 and Tks5 in the G1 checkpoint may also relate to the proteins' roles in early apoptosis by which cells deemed unsuitable for DNA synthesis and cell cycle progression are signaled toward programmed cell death. Consequently, the depletion or degradation of XB130 and Tks5 may

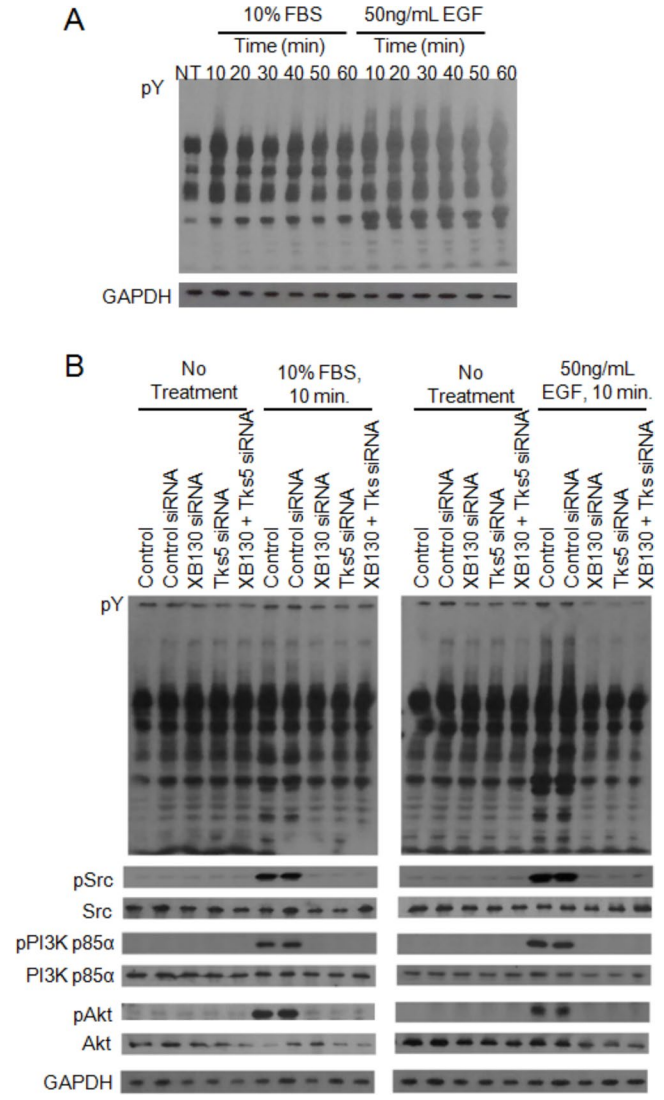

FIGURE 7: XB130 and Tks5 regulate Src activation and signaling. (A) Serum-starved BEAS-2B cells treated with either 10% FBS or 50 ng/ml EGF for up to 60 min show increased total protein tyrosine phosphorylation after 10 min. Nontreated (NT) BEAS-2B cells were not stimulated with FBS or EGF after 18-h serum starvation. (B) Down-regulation of XB130 and/or Tks5 inhibited total protein tyrosine phosphorylation, Src Y416, PI3K p85α Y458, and Akt S473 phosphorylation, compared with control BEAS-2B cells treated with 10% FBS or 50 ng/ ml EGF for 10 min. GAPDH is shown as a loading control.

prove to be an indicator of early apoptosis in cells. Moreover, down-regulation of both XB130 and Tks5 did not additively affect cell proliferation or survival, suggesting that these proteins may act on the same pathway.

Our results also demonstrate that the XB130/Tks5 interaction is important for cell proliferation and survival. Deletion of XB130's N-terminus or mutation of Tks5's ligand-binding site inhibited both cell proliferation and survival, even when expressed with their wild-type binding partner. Ultimately, the overexpression of wild-type XB130 and Tks5 did not alleviate the inhibition or help to regulate cell proliferation and survival if in the presence of its mutant binding partner. Thus, the specific interaction between these two proteins is required for the downstream cellular functions in which they are involved. In the case of XB130, removal of the N-terminus may result in protein instability or conformational change. It is also possible that the XB130 mutant may prevent the binding of Tks5 with other proteins. Moreover, without its N-terminus, XB130 is unable to bind to certain protein partners that are required for regulation of cell proliferation and survival. Further studies are necessary to test these possible mechanisms.

As scaffold proteins, XB130 and Tks5 are involved in various signal transduction pathways. However, both of these scaffold proteins are also binding partners with Src tyrosine kinase, which, of interest, also interacts with several other scaffold proteins to influence different signaling pathways. For example, the scaffold proteins growth factor receptor–bound protein 2 (Grb2), SHC-transforming protein 1, and proto-oncogene c-Crk are all known to interact with Src tyrosine kinase and participate in signaling cascades to regulate cell growth and motility (Lundgren *et al.*, 2006; Oikawa *et al.*, 2008; Watanabe *et al.*, 2009; Burger *et al.*, 2014). In this study, we first showed that under basal conditions, both XB130 and Tks5 bind to Src and form a putative protein complex. Furthermore, down-regulation of XB130 and Tks5 appear to inhibit Src activation by preventing phosphorylation of Src tyrosine residue 416. XB130 and Tks5 expression also influences whole-cell protein tyrosine phosphorylation and, more specifically, PI3K p85α and Akt phosphorylation. These results suggest that XB130 and Tks5 are upstream regulators of growth factor–induced Src activation and Src-mediated tyrosine kinase–dependent activation of cellular proteins that promote cell growth, proliferation, and survival. Src activation may require the binding of Src to XB130 and Tks5, as well as the binding of the two scaffolds, in order to stably change to an open, active conformation for efficient activation of kinase activity. This XB130/Tks5/Src interaction may also support the formation of a larger molecular complex for the signal transduction to downstream molecules that promote cell proliferation and survival (Figure 8). These results may also provide the foundation for an understanding of the important role of scaffold proteins in physiological processes such as development or disease, as in tissue injury and repair and tumorigenesis.

MATERIALS AND METHODS

Cell culture

TPC-1 cells were cultured in DMEM supplemented with 5% FBS and 2 mmol/l L-glutamine (all from Gibco, Carlsbad, CA). WRO cells were maintained in RPMI 1640 medium (Gibco) supplemented with 10% FBS, 2 mmol/l L-glutamine, 1 mmol/l sodium pyruvate (Gibco), and 1 mM nonessential amino acid (Gibco). COS-7 cells and human bronchial epithelial BEAS2B cells (ATCC, Manassas, VA) were cultured in low-glucose DMEM (Life Technologies, Rockville, MD) with 10% FBS (Gibco). All cells were cultured at 37°C with 5% CO2 in a humidified cell incubator.

Reagents, plasmids, and antibodies

siRNA targeting human Tks5, control siRNA, GST, and Myc (clone 9E10) antibodies, goat anti-Tks5 polyclonal antibody (M-20), and p21 antibody were from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit anti-Tks5 polyclonal antibody was a gift of S. Courtneidge (Sanford/Burnham Medical Research Institute, La Jolla, CA). XB130 monoclonal antibody was generated as previously described (Xu *et al.*, 2007). XB130 siRNA and control siRNA were from GE Healthcare Dharmacon (Lafayette, CO). RGS-His antibody to detect 6xHis-tagged proteins was from Qiagen (Valencia, CA). Src (clone GD11) monoclonal antibody, phosphotyrosine (clone 4G10) monoclonal antibody, and GADPH antibody were from Upstate Biotechnology

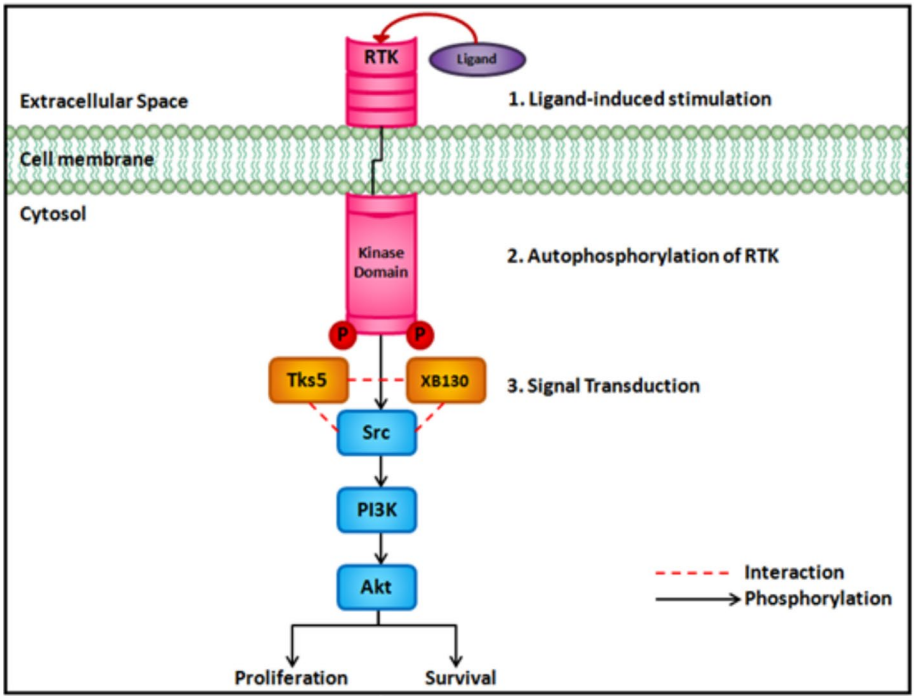

FIGURE 8: Proposed role of XB130/Tks5 interaction in mediating cell proliferation and survival. Diagram depicting extracellular ligand binding resulting in receptor tyrosine kinase (RTK) autophosphorylation and initiation of signal transduction that is relayed via the scaffold proteins XB130 and Tks5, which regulate phosphorylation of Src, as well a phosphorylation of downstream effectors PI3K and Akt, thereby leading to regulation of cell proliferation and survival.

(Lake Placid, NY). Horseradish peroxidase–conjugated goat anti-mouse or anti-rabbit secondary antibodies were from Amersham Pharmacia Biotech (Piscataway, NJ). PI3K p85α, phosphotyrosine 458 PI3K, Akt, and phosphoserine 473 Akt antibodies were from Cell Signaling Technology (Beverly, MA). Anti-GAPDH antibodies were from Abcam (Cambridge, United Kingdom). Human Ki67 antibody and rabbit anti-Tks5 (SH3#1) were from EMD Millipore (Merck, Darmstadt, Germany).

Cell transfection

Myc-Tks5, Myc-Tks5 W1108A, and mCherry vector plasmids were provided by S. Courtneidge, and His-XB130, pEGFP-XB130, and pEGFP-XB130ΔN plasmids were previously constructed (Xu *et al.*, 2007). His-, Myc-, GFP-, and mCherry-tagged constructs were transfected using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). siRNA was transfected into BEAS2B cells by Oligofectamine (Invitrogen) following the manufacturer's protocol. COS-7 cells were seeded in six-well plates, and BEAS-2B cells were seeded in 100-mm cell culture plates. All cells were transfected at ~70% confluence and incubated over 48 h before analysis.

Biochemical/immunoblot protein expression studies

Cells were lysed with RIPA buffer for Western blot analysis or Triton X-100 lysis buffer for immunoprecipitation procedures. Protein samples were analyzed using the Pierce 660-nm protein assay (Thermo Scientific, Rockford, IL), and an initial protein concentration of 250 μg/ml of the whole-cell lysate was used as the input for immunoprecipitation reactions. Equal volumes were used for the reactions. The whole-cell lysate was incubated with primary antibody against each protein (XB130 monoclonal antibody was generated as previously described; Xu *et al.*, 2007), rabbit anti-Tks5 (SH3#1) from EMD Millipore, and Src (clone GD11) monoclonal antibody from Upstate Biotechnology, washed with Triton X-100 lysis buffer (1.0% Triton X-100, 150 mM NaCl, 50 mM Tris-HCl, pH 8.0), and eluted by boiling for 10 min at 50°C with 50 μl of 2× SDS Laemmli buffer (no dithiothreitol), and 15 μl of sample was loaded on each lane. Coimmunoprecipitation used protein G–Sepharose beads precleared with normal mouse IgG. Rabbit IgG and mouse IgG2a and IgG2b were used as controls for Tks5 and XB130 immunoprecipitation. SuperSignal Dura Chemiluminescent substrate (Thermo Scientific, Rockford, IL) was used to detect protein signal after transfer.

GST-fusion protein expression, purification, and pull-down assay

Escherichia coli expressing GST-fusion proteins containing individual SH3 domain of Tks5 were cultured in lysogeny broth (LB) medium and induced by 100 μM isopropyl-1-thio-β-D-galactopyranoside. GST-fusion proteins were purified from the soluble fraction of the sonicated *E. coli* lysates with glutathione–Sepharose 4B beads. Thyroid cancer cell lysates or COS-7 cell lysates expressing His-tagged XB130 wild type or mutants were incubated with GST-fusion protein beads overnight at 4°C. The precipitates were washed with immunoprecipitation buffer and then mixed with SDS protein loading dye and analyzed by SDS–PAGE. After transfer to nitrocellulose membranes, the pull-down proteins were detected by immunoblotting for XB130 or GST.

Immunofluorescence microscopy

BEAS-2B cells were seeded on coverslips and incubated overnight in DMEM plus 10% FBS at 37°C with 5% CO2 in a humidified cell incubator. Cells were fixed using 4% paraformaldehyde and permeabilized with 0.1% Triton X-100 in phosphate-buffered saline (PBS). Cells were incubated with anti-XB13 (mouse IgG, homemade) and anti–Tks5 SH3#1 (rabbit IgG; EMD Millipore), primary antibody and secondary antibody conjugated with Alexa Fluor 594 (Molecular Probes, Eugene, OR), or Oregon green 488 (Molecular Probes). Actin was stained using CytoPainter phalloidin-iFluor 405 reagent (Abcam). Coverslips were mounted onto glass slides with Dako fluorescence mounting medium (Dako, Mississauga, Canada). Images were obtained using an Olympus FluoView Confocal FV1000-ASW and analyzed by Olympus FluoView FV10-ASW (Olympus, Tokyo, Japan) and ImageJ (National Institutes of Health, Bethesda, MD).

Flow cytometry cell sorting

BEAS-2B cells were transfected with pEGFP vector, pEGFP-XB130, pEGFP-XB130ΔN mutant, m-Cherry vector with myc-Tks5, or myc-Tks5 W1108A. Cells were collected after 48 h of transfection, washed with PBS, resuspended in DMEM plus 2% FBS, and subjected to flow cytometry (FC500; Beckman Coulter, Fullerton, CA) cell sorting. The GFP- and/or mCherry-positive cells were acquired by double gating and collected in 100% FBS on ice and then reseeded on plates in DMEM plus 10% FBS for subsequent studies. Protein expression was validated by Western blot.

PI and annexin V staining and flow cytometry analysis

Cells were collected and washed with PBS. Cells were diluted to a density of 1×10^6 cells/ml and then stained with 100 µg/ml PI and fluorescein isothiocyanate–conjugated-annexin V in 500 µl of annexin V binding buffer for 15 min at room temperature. Samples were analyzed at 530 and 575 nm on a BD FACSCanto II (BD Biosciences, San Jose, CA). Data analysis was performed using FlowJo Data Analysis Software (FlowJo, Ashland, OR).

BrdU incorporation assay

BEAS-2B cells were seeded and transfected before BrdU incorporation using EMD Millipore BrdU Cell Proliferation Assay. BrdU reagent was added with the reintroduction of 10% FBS and incubated for 4 h at 37°C. Mouse monoclonal antibody was used to detect BrdU. Samples were incubated with goat anti-mouse IgG peroxidase secondary antibody and TMB peroxidase substrate. The samples were analyzed by spectrophotometer at 450 and 550 nm.

Caspase 3 activity assay

BEAS-2B cells were seeded and transfected before performing EnzChek caspase-3 activity assay kit #1 (Molecular Probes). Cells were lysed and centrifuged. The supernatant was aliquoted in wells, and 10 mM Z-DEVD-AMC substrate was added to each sample. The samples were analyzed by fluorimeter at 342-nm excitation and 441-nm emission after 30 min.

Statistical analyses

Data are expressed as mean ± SD from at least three experiments and analyzed by two-way analysis of variance with $\alpha \leq 0.05$, and Bonferroni correction was used to establish a significance of $p \leq 0.01$.

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