NEOPLASIA
www.neoplasia.com
Volume 22 Number 4 April 2020 pp. 163–178

Original Research

TGFBR2 mediated phosphorylation of BUB1 at Ser-318 is required for transforming growth factor-β signaling

Shyam Nyati *; Brandon S. Gregg; Jiaqi Xu; Grant Young; Lauren Kimmel; Mukesh K. Nyati; Dipankar Ray; Corey Speers; Alnawaz Rehemtulla *

Department of Radiation Oncology, University of Michigan, Ann Arbor, MI, USA

Abstract

BUB1 (budding uninhibited by benzimidazoles-1) is required for efficient TGF-β signaling, through its role in stabilizing the TGFBR1 and TGFBR2 complex. Here we demonstrate that TGFBR2 phosphorylates BUB1 at Serine-318, which is conserved in primates. S318 phosphorylation abrogates the interaction of BUB1 with TGFBR1 and SMAD2. Using BUB1 truncation domains (1–241, 241–482 and 482–723), we demonstrate that multiple contact points exist between BUB1 and TGF-β signaling components and that these interactions are independent of the BUB1 tetratricopeptide repeat (TPR) domain. Moreover, substitutions in the middle domain (241–482) encompassing S318 reveals that efficient interaction with TGFBR2 occurs only in its dephosphorylated state (241–482 S318A). In contrast, the phospho-mimicking mutant (241–482 S318D) exhibits efficient binding with SMAD2 and its over-expression results in a decrease in TGFBR1-TGFBR2 and TGFBR1-SMAD2 interactions. These findings suggest that TGFBR2 mediated BUB1 phosphorylation at S318 may serve as a switch for the dissociation of the SMAD2-TGFBR complex, and therefore represents a regulatory event for TGF-β signaling. Finally, we provide evidence that the BUB1-TGF-β signaling axis may mediate aggressive phenotypes in a variety of cancers.

Neoplasia (2020) 22 163–178

Keywords: BUB1 (budding uninhibited by benzimidazoles-1), kinase, TGFb (transforming growth factor-beta), SMAD2, SMAD3, TGFBR1, TGFBR2, signaling, phosphorylation, regulation

Introduction

The transforming growth factor beta (TGF-β) family of cytokines regulates diverse yet critical physiological processes including angiogenesis, wound healing, immune suppression, and epithelial-to-mesenchymal transition (EMT) [1]. Abnormal TGF-β signaling is linked to autoimmune and auto-inflammatory diseases, chronic inflammatory lung diseases including asthma and chronic obstructive pulmonary disease (COPD), fibrosis, and the development and maintenance of cancer and metastasis. Ligand-dependent activation of TGF-β receptors and initiation of its signaling cascades is a complex process that can involve several posttranslational modifications of the receptors, internalization of the receptor-ligand complex as well as cross-talk with other cell surface receptors [2]. Although activation and regulation of TGF-β receptors has been extensively investigated, many important questions still remain, including contribution of novel adaptors, role of co-receptors and the regulation of receptor internalization upon activation [2,3].

The serine/threonine kinase BUB1 (budding uninhibited by benzimidazoles-1) was identified as an essential mediator of TGF-β signaling in an siRNA screen against a reporter for TGFBR-kinase activity [4–7]. BUB1's role in the mitotic spindle checkpoint assembly and chromosome congression is well appreciated, however, a molecular basis for its role in TGF-β signaling is ill defined [8,9], despite mounting evidence for non-cell-cycle related functions of BUB1 in receptor endocytosis [10] and viral entry [11]. BUB1 has multiple functional domains within its N-terminus which mediate protein–protein interactions. These include the tetratricopeptide repeats (TPR) domain (amino acid 99–132; [12]) and a Gli2-binding sequence (GLEBS)/BUB3 binding motif (residues 209–270; [13,14]). BUB1's function within the spindle assembly checkpoint is mediated by two KEN-box motifs as well as a D-Box for interac-

* Corresponding authors. Department of Radiation Oncology, University of Michigan, 1301 Catherine Road, Med Sci I, #4433D, Ann Arbor, MI-48109-5637, USA (S. Nyati); Department of Radiation Oncology, University of Michigan, 1600 Huron Parkway, NCRC, #520, Ann Arbor, MI-48109. USA (A. Rehemtulla).
e-mail addresses: shyamnya@med.umich.edu (S. Nyati), alnawaz@umich.edu (A. Rehemtulla).

Received 9 January 2020; accepted 10 February 2020

tion with CDH1 and CDC20 [15,16], co-activators of the anaphase promoting complex/cyclosome (APC/C).

Recruitment of BUB1 to the type 1 TGFB-receptor (TGFBR1) upon ligand induced activation and subsequent heterodimerization of TGFBR1 with TGFBR2 has been demonstrated [7], however, domains within BUB1 responsible, presumable with the cytosolic domains of the transmembrane receptors, are yet to be defined. The TPR domains and the GLEBS motif of BUB1 are surrounded by multiple hydrophobic residues which mediate protein–protein interactions [13,17,18]. We hypothesized that BUB1 may interact with TGFBR1 and/or TGFBR2 through these regions. Our finding that BUB1 interacted with TGFBR2 suggested that this possibly facilitates the formation of a ternary complex. In addition, siRNA mediated knock down of BUB1 prevents the recruitment of SMAD3 to the receptor complex, the phosphorylation of SMAD2/3 and their interaction with SMAD4, SMAD-dependent transcription, as well as TGF-β-mediated cellular phenotypes including epithelial-mesenchymal transition (EMT), migration, and invasion. Non-canonical TGF-β signaling cascades (AKT and p38 MAPK) also require the presence of BUB1. Lastly, a small-molecule inhibitor of BUB1 kinase activity (2OH-BNPP1) as well as a kinase-deficient mutant of BUB1 revealed that BUB1 may phosphorylate a component of the TGF-β receptor signaling complex in response to ligand [7]. In an effort to provide mechanistic insight into these observations, we investigated if BUB1 may serve as a substrate for TGFBR kinase activity and if this phosphorylation event may provide functional insights into the role for BUB1 in the regulation of TGF-β signaling.

Materials and methods

Plasmid DNA

pCS2-Flag-SMAD2 (Addgene plasmid #14042), and pCMV5-TGFBRI-His (#19161) were provided by Dr. Joan Massague [19]. pCMV5B-TGFBRII-WT (#11766) was kindly gifted by Dr. Jeff Wrana [20]. siRNA resistant Myc-BUB1-WT, and Myc-BUB1-kinase dead (KD) and BUB1 truncation mutants (1–241, 242–482 and 482–723) were provide by Dr. Hongtao Yu [15]. Point mutations (S318A and S318D) were carried out using single primer site directed mutagenesis protocol with the primers listed in Table 1.

Antibodies and reagents

Antibodies to pSMAD2 S465/567 (#3108), SMAD2 (#3122), pSMAD3 S423/425 (#9520), TGF□R1 (#3712), TGFBR2 (K105 clone #3713), and Actin (all from Cell Signaling), SMAD3 (Invitrogen), TGFBR1 (V-22 #SC-398, H-100 #SC-9048), TGFBR2 (L-21 #SC-400), His-tag (clone H3; #8036), Myc-tag (clone 9E10; SC-40) and SARA (Santa Cruz biotechnology), Flag M2-HRP (Sigma Aldrich #8592), His-tag-HRP (clone C-terminal, Invitrogen), and Myc-tag (clone 9E10) and His-tag (clone H8) were from Millipore-Sigma. BUB1 (#Ab70372, #Ab9000), Actin-HRP (# ab20272), GAPDH-HRP (#Ab9485) were from Abcam. BUB1 (#TA306432) was from Origene. The HRP-conjugated secondary antibodies were from Jackson ImmunoResearch. Recombinant human TGF-β1 (#100-21B) was obtained from PeproTech, siRNA targeting human BUB1 (5'-GAGUGAUCAC GAUUUCUAAdTdT-), as well as non-silencing siRNA (NSS) were chemically synthesized by GE-Dharmacon. ^{32}p-ATP was obtained from Perkin Elmer, Recombinant GST-TGFBR2 (amino acid 190-end) was obtained from BPS Bioscience (#40707). Cloning and purification of BUB1-WT, BUB1-KD and BUB1-E has been described previously [15,21,22] and was provided by Dr. Hongtao Yu. BUB1-WT and mutants were cloned into pFAST-Bac plasmids and expressed along with Bub3. Recombinant human H2A (#M2502S) was obtained from New England Biolabs (NEB), 2OH-BNPP was synthesized in-house. SimplyBlue SafeStain was purchased from ThermoFisher Scientific. FuGENE 6 was from Roche while Lipofectamine 2000 was from Invitrogen.

Cell culture and transfection

The human lung carcinoma cell line A549 and normal kidney cell line HEK293T were obtained from American Type Culture collection (ATCC) maintained in RPMI-1620 or DMEM media respectively supplemented with 10% heat-inactivated fetal bovine serum, 1% glutamine, and 0.1% penicillin/streptomycin/gentamycin (GIBCO-Invitrogen). Cells were grown in a humidified incubator at 37□C and 5% CO2. Breast cancer cell line 1833 (15) derived from MDA-MB-231 was kindly provided by Dr. Joan Massague (Memorial Sloan Kettering Institute, NY) and maintained in DMEM media in the same conditions mentioned above. All cell lines utilized in the study were tested routinely for mycoplasma contamination. Acquired cultures were expanded immediately upon receipt and low passage cells were maintained in liquid nitrogen. Cell lines in culture were freshly replenished routinely (1–2 months) to ensure that a genetic drift was not occurring. HEK293T cells were used in these studies as these cells respond to TGF-β ligand and consistently demonstrate high transfection efficiency. Plasmids were transfected in HEK293T cells using FuGENE 6 while plasmids alone (in A549 or MDA-231-1833 cells) or in combination with siRNAs were transfected using Lipofectamine 2000. HEK293T cells were transfected with 500–1000 ng plasmid in 6-well plates or between 1–2 μg DNA in 10 cm dishes while A549 or MDA-231-1833 cells were transfected using 1 μg DNA/well in 6-well plate or 2–3 μg DNA in 10 cm dishes. siRNA was transfected at a concentration of 60–100 nM. Plasmids, siRNA and transfection reagents (4 μl transfection reagent for 1 μg plasmid) were diluted with pre-warmed OptiMEM media separately. These were mixed to form transfection complex and were overlaid on cells plated in growth media with serum only. Transfection mixes were allowed to sit on the cells until ready to treat or harvest (30–60 h). HEK293T cells demonstrated far superior transfection efficiency compared to A549 and MDA-231-1833 cells. There was no cytotoxicity observed with Fugene6 transfections while minimal cytotoxicity (<10%) was observed with Lipofectamine 2000. Cells were routinely

Table 1. A list of primers used for various mutagenesis of BUB1.

Primer	Sequence
S318D Fwd	CCCGCTTCCCAGGAAAGGgaCGAGGTTAATCCAGCACGT
S318A Fwd	CCCGCTTCCCAGGAAAGGgCCGAGGTTAATCCAGCACGT
dTPR1 Fwd	CCTCTTGGTGAATGGGAAAGAAAGAAATACCACAATGACCCAAG
dTPR2 Fwd	GAATTTTTAGATAAGAAGAAATACCACACCCTGTCATCCCCTCTGTACA
dTPR3 Fwd	CATGGGATTGGAACCCTGTCAAGAGAGTTCCTGCAACAACAA
L45G-L49G Fwd	TTCCTGAGAATAAAGAATACggGATAACTTTAggAGAACATTTAATGAAGGAATT
A106D Fwd	CCTCTGTACATTGCCTGGGatGGGCATCTGGAAGCCCAA
L122G Fwd	CAGCATGCCAGTGCTGTCggTCAGAGAGGAATTCAAAACCA

plated at higher densities (as suggested by the manufacturer) for Lipofectamine 2000 transfections to minimize toxicity.

Western blot analysis

Western analysis was carried out using standard protocols. Cells were grown in culture dishes, transfected with specific siRNA or plasmids, or treated with select compounds and 5 ng/mL TGF-β for 30–60 min. After treatments, cells were washed with ice-cold PBS and IP-lysis buffer (50 mM Tris PH 7.4, 1% NP40, 0.25% Deoxycholate sodium salt, 150 mM NaCl, 10% Glycerol, and 1 mM EDTA) in the presence of protease and phosphatase inhibitor cocktails (Roche) was used to prepare cell lysates. Samples were rocked for 30–60 min at 4 °C and centrifuged at 14,000 rpm for 20–30 min at 4 °C. Protein concentration of the cell lysates was determined and normalized using lysis buffer for further analysis (approx. 1 μg/μl). Cell lysates were run on 4–12% Novex Bis-Tris SDS-PAGE gels in MOPS buffer. SeeBlue Plus2 (ThermoFisher, MA) pre-stained molecular weight marker was run on all the gels. The gels were transferred to PVDF membranes in Tris-Glycine western transfer buffer containing 20% Methanol (v/v) as recommended by the manufacturer. The PVDF membranes were blocked with 5% milk or 5% BSA in TBST for 1hour at room-temperature and were probed against specific primary antibodies (generally 1:1000 dilution with over-night incubation at 4 °C). The membranes were washed three times (10 min each at room-temperature) and were incubated with HRP conjugated secondary antibodies (generally 1:10,000 dilution at room-temperature for 1 h) then visualized using the Enhanced Chemiluminescence (ECL) Western Blotting System (GE Healthcare). Primary antibodies against over-expressed proteins were used at higher dilutions (Myc-tag and His-tag 1:2000 to 1:20,000, FL-tag 1:5000 to 1:50,000) for inputs and CO-IPs.

Co-immunoprecipitation

For co-immunoprecipitation studies HEK293T cells were transfected with the indicated plasmids in 10-cm plates. 48 h post-transfection cells were treated with TGF-β (5–10 ng/mL) for 30–60 min. Cells were washed with ice cold PBS twice and lysates were made in 1 ml IP-lysis buffer supplemented with 1× PhosStop (Roche), 1× Protease inhibitor cocktail (Roche), Sodium Ortho Vanadate, Sodium fluoride, PMSF, and β-Glycerol phosphate (2 μM each). Protein estimation was performed using detergent compatible Dc assay kit (Pierce). 400–500 μg lysates were pre-cleared by incubating with normal Rabbit-IgG or normal Mouse-IgG for 1 h followed by 30 μl protein A/G-coupled Sepharose beads (GE Healthcare) for an additional hour at 4 °C and centrifuged. Co-immunoprecipitation was carried out by incubating pre-cleared cell lysate (400 μg protein) with 1–2 μg Myc-tag, TGFBR1 or TGFBR2 specific antibodies overnight at 4 °C on a rotating platform. The immune complex was captured using 30 μl slurry of protein A/G-PLUS Sepharose beads for 2–4 h, washed four times with lysis buffer. Alternatively, FL-beads were used for 2 h at room temperature for pull-downs. After washing, the beads were boiled in 60–80 μl 1.2× Laemmli buffer diluted with IP-lysis buffer containing protease and phosphatase inhibitors to elute bound protein complexes. The Laemmli buffer was supplemented with 1–2% β-mercaptoethanol and 0.01% SDS to denature the proteins. The resulting pellet was resolved by 4–12% Bis-Tris SDS-PAGE gels in MOPS buffer and transferred to PVDF membrane for western-analysis.

In vitro kinase assay

In vitro kinase assays were performed using 200 ng of catalyst (recombinant TGFBR2 protein), and 200 ng–2 μg of substrate (BUB1-WT, or a kinase inactive mutant BUB1-KD) in 1× kinase buffer (50 mM Tris-HCl PH 7.5, 150 mM NaCl, 10 mM $MgCl_2$, 1% (v/v) Glycerol, 0.1% (v/v) Triton X 100, DTT, PMSF, Na_3VO_4 (1 mM each), 2 mM NaF and β-glycerol phosphate) in 20 μl volume containing 10 μCi ^{32}p-ATP and 300 μM cold ATP. Reactions were run at 30 °C or 37 °C for 3 h at atmospheric pressure (760 mm Hg). The reactions were quenched using Laemmeli buffer and resolved using a 4–12% Bis-Tris gel. Autoradiography was performed using a Typhoon FLA 9000 scanner (GE Healthcare). Alternatively, His-TGFBR1 WT or TGFBR1-cytoplasmic fragment containing GS region (TGFBR1-cyto) were overexpressed in HEK293T cells and pulled down using Ni-beads. These beads were washed 3 times in lysis buffer and 3 times in 1× kinase buffer and used directly in the in vitro kinase reactions.

MS/MS analysis

In vitro kinase assays were performed without ^{32}p-ATP labeled ATP and were run on a SDS–PAGE in laminar flow hood. The gels were washed with milli-Q water and stained with SimplyBlue SafeStain (Invitrogen) and destained with milli-Q water. The stained bands were cut, digested in-gel with trypsin. The peptides were resolved on the C18 reverse phase column and high-resolution MS and MS/MS spectra were acquired on Orbitrap Fusion Tribrid mass spectrometer. The peptide/protein identification was performed by searching the MS/MS data against a human protein database using Proteome Discoverer (v1.4). Phosphorylation at Ser, Thr and Tyr were considered as potential modifications. Data were filtered at 1% false discovery rate.

Bioinformatics analyses

BUB1 protein sequences were downloaded from pubmed. Reference sequences with largest transcript (transcript 1 or transcript X1) were downloaded and used in analysis. Multiple sequence alignment was performed on Clustal X2 with default parameters. Gape opening penalty 10, gap extension 0.2, delay divergent sequences by 30%, and Gonnet series was selected for protein weight matrix. The aligned data matrix (sequences) were exported in postscript format.

A search for any known disease associated variation found none at Ser318 in Leiden Open Variation Database (LOVD; https://databases.lovd.nl/shared/variants/BUB1).

SNP analysis was performed on NCBI database, using SNP: gene view. A search for any cancer related mutations in BUB1 was performed on COSMIC (catalog of somatic mutations in cancer) database.

BUB1 expression and TGF-β pathway member expression was evaluated by downloading clinical datasets from GEO or by using Oncomine.org. Comparison of BUB1 expression between cancer vs. normal was performed using a Student's *t*-test. Correlation was performed comparing BUB1 expression versus all other genes, including TGF-β pathway genes, in the clinical cohorts as indicated. Genes retained with heatmap were those with a false discover rate (FDR) p-value <0.02 and log2 fold change þ1.10. The Spearman's correlation coefficient rho was generated by correlating BUB1 versus each other gene. Difference between multiple groups was assessed using analysis of variance (ANOVA) testing. Datasets were accessed from GEO as follows:

METABRIC- http://www.ebi.ac.uk/ega/studies/EGAS00000000083
TCGA- http://tcga-data.nci.nih.gov/tcga/
Richardson- http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE3744
Zhao- http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE3971
Bittner- http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE2109

Results

TGFBR2 phosphorylates BUB1 at Ser318

Based on the finding that the kinase activity of BUB1 was required for TGF-β signaling, we investigated if BUB1 may phosphorylate the TGFBR1. Expression of His-tagged full length TGFBR1 or its cytosolic domain in HEK293T cells [23], followed by immunoprecipitation of each using Ni-beads, we carried out a kinase reaction (Fig. 1A) in the presence of recombinant BUB1. A kinase dead mutant of BUB1 (BUB-KD, [15]) was used as a negative control. Although TGFBR1 or its cytoplasmic domain could not be demonstrably phosphorylated by BUB1 ([7]; Fig. 1A), we observed incorporation of radiolabel (^{32}P) in BUB1 as well as BUB1-KD. Unlike BUB1, which undergoes autophosphorylation (lane 8) [24], the kinase dead mutant of BUB1 was not expected to be phosphorylated, hence the observed incorporation of ^{32}P (lane 5 vs. lane 9) in the presence of the TGFBR1 cytoplasmic domain was surprising. We speculated that TGFBR1, or an associated kinase, co-purified upon immunoprecipitation, may be responsible for phosphorylating BUB1. The very faint signal observed for BUB1 in the lanes 1 and 7 could be endogenous BUB1 that efficiently pulled-down with over-expressed TGFBR1-cytoplasmic tail in HEK-293T cells. We have previously shown that BUB1 interacts with both, full-length as well as cytoplasmic tail of TGFBR1 [7].

Using baculovirus derived recombinant TGFBR2 as the kinase and BUB1-WT or BUB1-KD as substrate in in vitro kinase reactions (Fig. 1B), we observed that BUB1-KD was efficiently phosphorylated by TGFBR2 (lane 6 vs. lane 7; Fig. 1B). BUB1-WT appeared to be extensively autophosphorylated in in vitro kinase reactions (Fig. 1A and B). This massive autophosphorylation can be completely blocked as demonstrated using a BUB1 kinase inhibitor, 2OH-BNPP1 (Fig. 1D). To further confirm that BUB1-WT serves as a substrate for TGFBR2 kinase

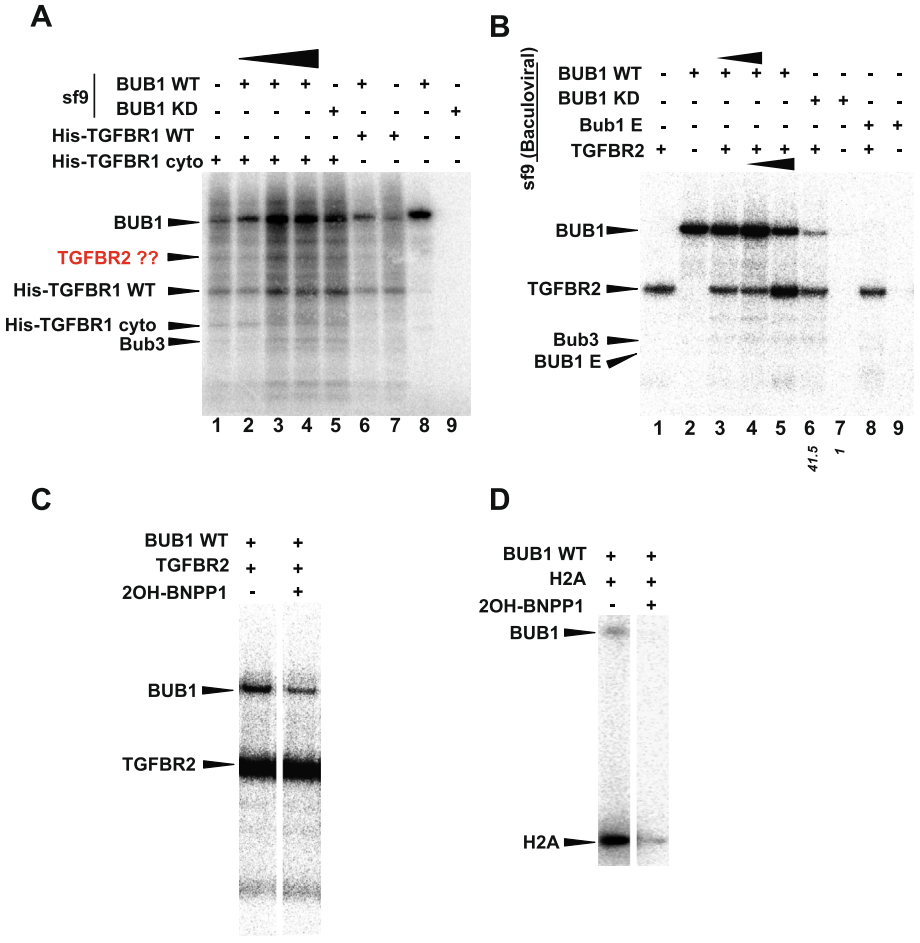
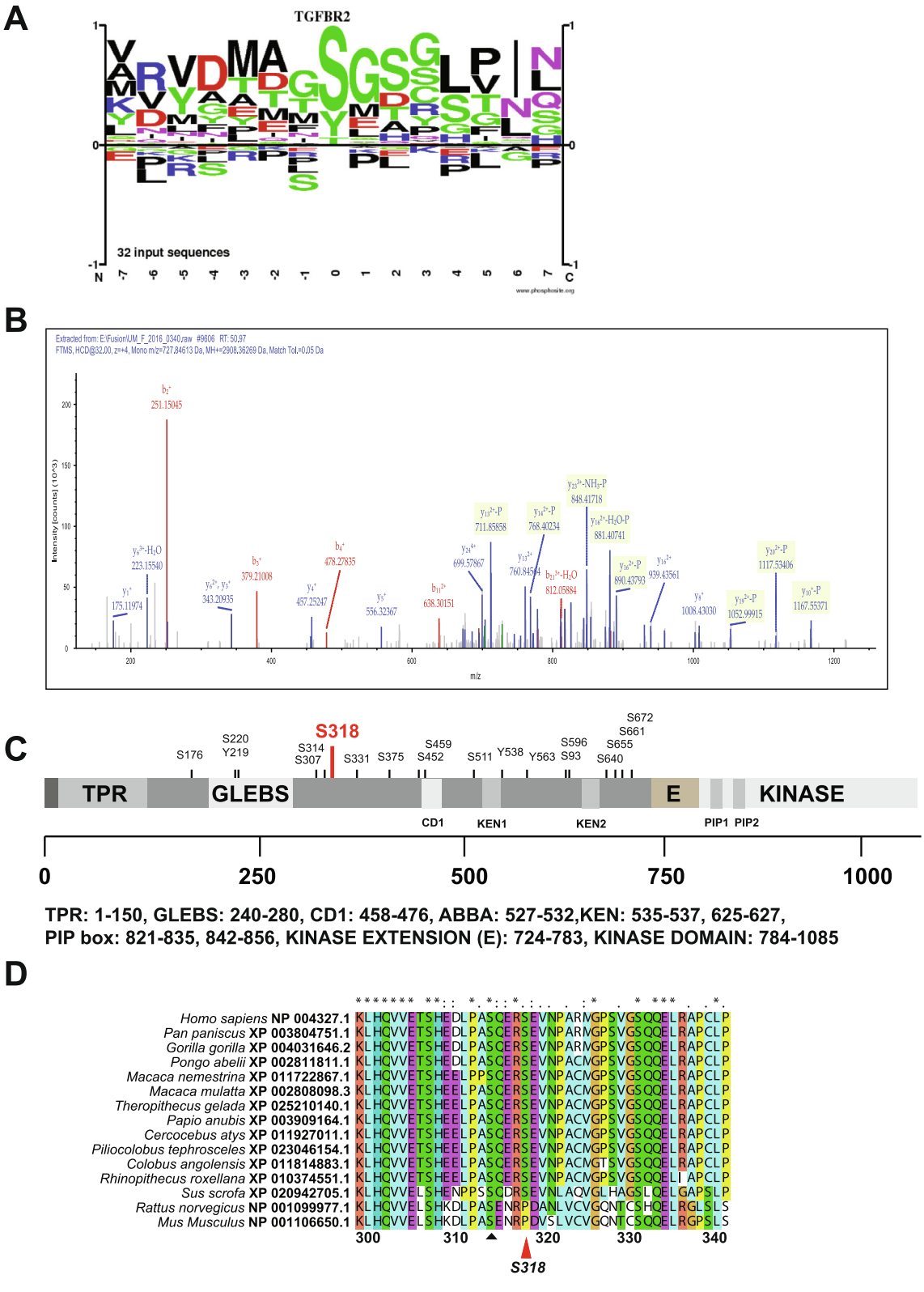


Fig. 1. TGFBR2 is a BUB1 kinase. (A) Radio-phosphoimages of in vitro kinase assay where His-tagged TGFBR1-WT and TGFBR1-cyto domain only (purified using Ni-NTA beads from HEK293T cells) were used as kinases. Varying concentrations of Sf9 baculovirally purified BUB1-WT (200 ng, 500 ng, 1 μg/lane) and BUB1-KD (1 μg/lane) were used as substrates. The reactions were run at 37 °C for 3 h with 5–10 μCi 32p-ATP. Laemmli quenched reactions were run on SDS-PAGE gels and imaged on phosphoimager. Arrows point to the proteins as detected by protein-specific antibodies on the western blots. (B), Sf9 purified BUB1 WT (1 μg in lanes 2 &3, 2 μg in lane 4, 500 ng lane 5), BUB1-KD (1 μg), or BUB1-E (1 μg) were used as substrates while baculovirally purified GST-TGFBR2 (200 ng/lane in all lanes except lane 5 where 500 ng) was used as a kinase in in vitro kinase reactions run at 37 °C for 3 h with 5–10 μCi hot-ATP. A representative radio-phosphoimage is shown. Numbers below the lanes show average fold enrichment (of BUB1-KD phosphorylation) from three different replicates. Arrows point to the proteins detected with specific antibodies in western-blots. Bub3 and BUB1-E are undetectable in radio-phosphoimages. (C) In vitro kinase assay where BUB1-WT (200 ng), GST-TGFBR2 (1 μg) along with BUB1 inhibitor 2OH-BNPP1 (10 μM) was used. The in vitro kinase reaction was performed in same conditions as in Fig. 1A and B. (D) In vitro kinase assay where purified BUB1-WT (100 ng) was used as a kinase and histone-H2A (500 ng) was used as a substrate in the presence of 10 μM 2OH-BNPP1. Radio-phosphoimage shows 2OH-BNPP1 inhibits BUB1 kinase activity in this assay.

activity, we performed in vitro kinase reaction with BUB1-WT and TGFBR2 in the presence or absence of 2OH-BNPP1 (Fig. 1C). The addition of 2OH-BNPP1 reduced BUB1-WT phosphorylation where TGFBR2 was added (Fig. 1C, lane 2), compared to where it completely blocked it (Fig. 1D, lane 2). This data demonstrates that the observed phosphorylation (in Fig. 1C, lane 2) could be attributed to TGFBR2 and not BUB1 mediated autophosphorylation. Additionally, these observations also suggest that autophosphorylation of BUB1-WT is blocked by 2OH-BNPP1 even in the presence of TGFBR2 but TGFBR2 mediated phosphorylation of BUB1 is not affected (by 2OH-BNPP1). In parallel experiments, addition of 2OH-BNPP1 in reactions wherein BUB1-WT was the kinase and H2A the substrate (histone 2A, a well characterized substrate for BUB1), confirmed that under the conditions described, 2OH-BNPP1 efficiently inhibited BUB1 kinase activity (Fig. 1D).



A computational analysis to define a consensus target motif for TGFBR2 substrates was performed on PhosphositePlus [25,26] (https://www.phosphosite.org/homeAction). This tool creates a consensus motif using all the known target phosphorylation sites/motifs including autophosphorylation sites, if any. A consensus motif based on 32 different substrate motif sequences available till now within the database indicated that BUB1 may indeed be a substrate for TGFBR2 (Fig. 2A). To confirm this, we performed an in vitro kinase reaction where BUB1-KD was used as a substrate and TGFBR2 was used as a kinase. BUB1-KD only reaction was used as a control. These samples were subjected to tandem mass spectrometry (MS/MS) analysis. These studies identified Ser318 of BUB1 (Fig. 2B, Table 2) as a target of the TGFBR2 kinase. Although in excess of 40 phosphorylation sites, including 28 autophosphorylation sites, have been described within BUB1 [Fig. 2C, [24,27,28]], phosphorylation at Ser318 has not been previously reported. Ser318 may therefore, play BUB1s' previously unappreciated role in TGF-β signaling. Interestingly, mutations within Ser318 have not been reported in The Cancer Genome Atlas (TCGA). A comparison of protein sequence alignment demonstrates that S318 residue is conserved between humans and non-human primates (including chimpanzee, gorilla, monkeys, and baboon) and other higher mammals (e.g. pigs) but is not conserved in lower mammals including mice and rats (Fig. 2D). Not surprisingly, the site S314, which is attributed to BUB1's cell-cycle functions [29] was found to be conserved among all the animal taxa tested. Moreover, an analysis of the available data on publically available databases found only one single nucleotide polymorphism (SNP) in BUB1 at Ser318 which did not cause any changes in the encoded amino acid (synonymous change). This SNP (rs763002902) has a mutant allelic frequency reaching zero (0.00002) and a similarly low heterozygosity score (0.000) suggesting that this SNP may not be functionally relevant. Moreover, no mutations were found at this residue in COSMIC database (out of >36,000 unique samples, as of 10-18-2019) again indicating that Ser318 of BUB1 is conserved in humans.

Ser318 phosphorylation status specific interaction of BUB1 with components of the TGF-β signaling complex

To elucidate the functional significance for Ser318 phosphorylation on the propagation of TGF-β signaling as well as interaction of BUB1 with TGFBR1, TGFBR2 and SMAD2, we generated phospho-mimic (Ser318-Asp; S318D) and phospho-deficient (Ser318Ala; S318A) mutants of full-length BUB1-WT. HA-tagged TGFBR2 and Myc-tagged BUB1 (WT, S318A or S318D mutants) were over-expressed in HEK293T cells, followed by TGF-β1 treatment for 1 hour prior to analysis. Co-immunoprecipitation revealed that mutation of Ser318 did not alter the interaction of full-length BUB1 to TGFBR2 (Fig. 3A, Table 3). In contrast, the BUB1 S318A mutant interacted more efficiently with His-TGFBR1 (Fig. 3B, Table 3) as well as FL-SMAD2 (Fig. 3C, Table 3).

BUB1 truncation mutant harboring Ser318 interacts minimally with TGF-β signaling components

In order to delineate polypeptide domains within BUB1 involved in interaction with TGF-β signaling components including TGFBR1, TGFBR2, and SMAD2, we utilized Myc-tagged BUB1 truncation mutants 1–241, 241–482, and 482–723 [8] for co-immunoprecipitation studies. BUB1 truncation mutants were over-expressed in HEK293T cells along with the His-TGFBR1 cytosolic fragment [23] or HA-TGFBR2 or FL-SMAD2. As BUB1 activity, protein levels, [15] and interaction with TGFBR1 are cell cycle dependent and highest during G2/M (Figs. S1), cells were treated with either TGF-β1 (Fig. 4A and C) or synchronized to G2/M using nocodazole (Fig. 4B). Full-length Myc-BUB1-WT was used as positive control in these experiments. The resulting lysates were immunoprecipitated using Myc-tag (Fig. 4A and B) or FL-tag (Fig. 4C) specific antibodies. Myc-BUB1-WT interacted with HA-TGFBR2 (Fig. 1A), irrespective of TGF-β1 ligand treatment status. Additionally, BUB1 truncation mutants 1–241 and 482–723 interacted with HA-TGFBR2 and the peptide 241–482 encompassing Ser318 interacted weakly with TGFBR2 (Fig. 2A and Table 3). This data suggested that BUB1 may interact with TGFBR2 through at least two, and potentially more, contact points. Additionally, these results suggest that this interaction with TGFBR2 may be minimally impacted by Ser318 phosphorylation status.

Next, BUB1-WT and all three truncation mutants (as above) were co-transfected with the His-TGFBR1 cytoplasmic domain in HEK293T cells followed by nocodazole synchronization. Immunoprecipitation using a Myc-tag specific antibody and western analysis using a His-tag antibody showed that the full-length Myc-BUB1-WT interacted with the cytoplasmic tail of TGFBR1 and that this interaction was enhanced in G2/M arrested cells (Fig. 4B). These findings were consistent with the results

Fig. 2. BUB1 is phosphorylated at Serine 318 by TGFBR2. (A) The phosphorylation target consensus motif in putative substrates of TGFBR2 kinase activity as predicted by PhosphoSite (https://www.phosphosite.org/homeAction.action) using default parameters. (B) In vitro kinase assay was performed on BUB1-KD protein (1–2 μg) with TGFBR2 (200 ng) for 3hrs at 37 °C. Control reactions were run where TGFBR2 protein was omitted. The reactions were run on 4–12% Bis-Tris SDS-PAGE gel and gel slices were cut and digested with trypsin, the peptides were introduced into a high-resolution mass spectrometer (Orbitrap Fusion Tribrid) and MS/MS data were acquired. The MS/MS spectrum indicates that the Serine 318 of BUB1 is phosphorylated by TGFBR2. Observed *b*- and *y*-ions are indicated. The MS/MS was performed on BUB1-KD with and without TGFBR2 and the data were compared to identify the TGFBR2 dependent site (see Table 2). (C) Schematics of BUB1 protein showing different functional and structural domains and the known phosphorylation sites including the newly identified TGFBR2 dependent phosphorylation target site serine 318 (S318) in red and bold. TPR: tetratricopeptide repeat motif, GLEBS: GLE2p-binding sequence; Gle2 and BUB3 binding sequence, CD1: conserved domain 1, ABBA: degron sequence present in Cyclin A, BUBR1, BUB1 and Acm1, KEN: motif containing Lys-Glu-Asn, PIP box: proliferating cell nuclear antigen (PCNA) interaction motif, KINASE EXTENSION domain: amino acids 724–783 and KINASE domain: 784–1085. 2D, Partial protein sequence alignment surrounding Ser318 of human BUB1 along with non-human primates, pig, mouse and rat. Genus and species name is indicated along with the accession number for the reference protein sequences. Complete sequence alignment is marked with an asterisk (*), while colon (:) indicates conservation between groups of strongly similar properties (score >0.5 in the Gonnet PAM250 matrix), partial alignments are marked with a period (.) indicating conservation between groups of weakly similar properties (score = <0.5 in the Gonnet PAM250 matrix). Sequences for only the longest isoform were used for the analysis. The small black arrowhead shows S314 of BUB1 which is required for its cell-cycle related functions and is conserved across all species tested. Ser318 is present in primates and pig and is absent in mouse and rat. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 2. Table showing the phosphorylation events of BUB1 detected by MS/MS in the current study. This includes the autophosphorylation sites previously described as well as the TGFBR2 dependent site newly identified. References for the previously identified sites are also provided.

BUB1 alone	BUB1 and TGFBR2	Target site in peptide	Target site	Kinase	Reference
	LHQVVETSHEDLPASQERsEVNPAR	S19(Phospho)	318	TGFBR2	
DGKFsPIQEKsPK	DGKFsPIQEKsPK	S5(Phospho); S11(Phospho)	655, 661	BUB1 (autophos.)	Asghar et al
LPsKPKEEVPHAEEFLDDSTVWGIR	LPsKPKEEVPHAEEFLDDSTVWGIR	S3(Phospho)	563	BUB1 (autophos.)	Asghar et al
DGKFsPIQEK	DGKFsPIQEK	S5(Phospho)	655	BUB1 (autophos.)	Asghar et al
FSPIQEKsPK	FSPIQEKsPK	S8(Phospho)	661	BUB1 (autophos.)	Asghar et al

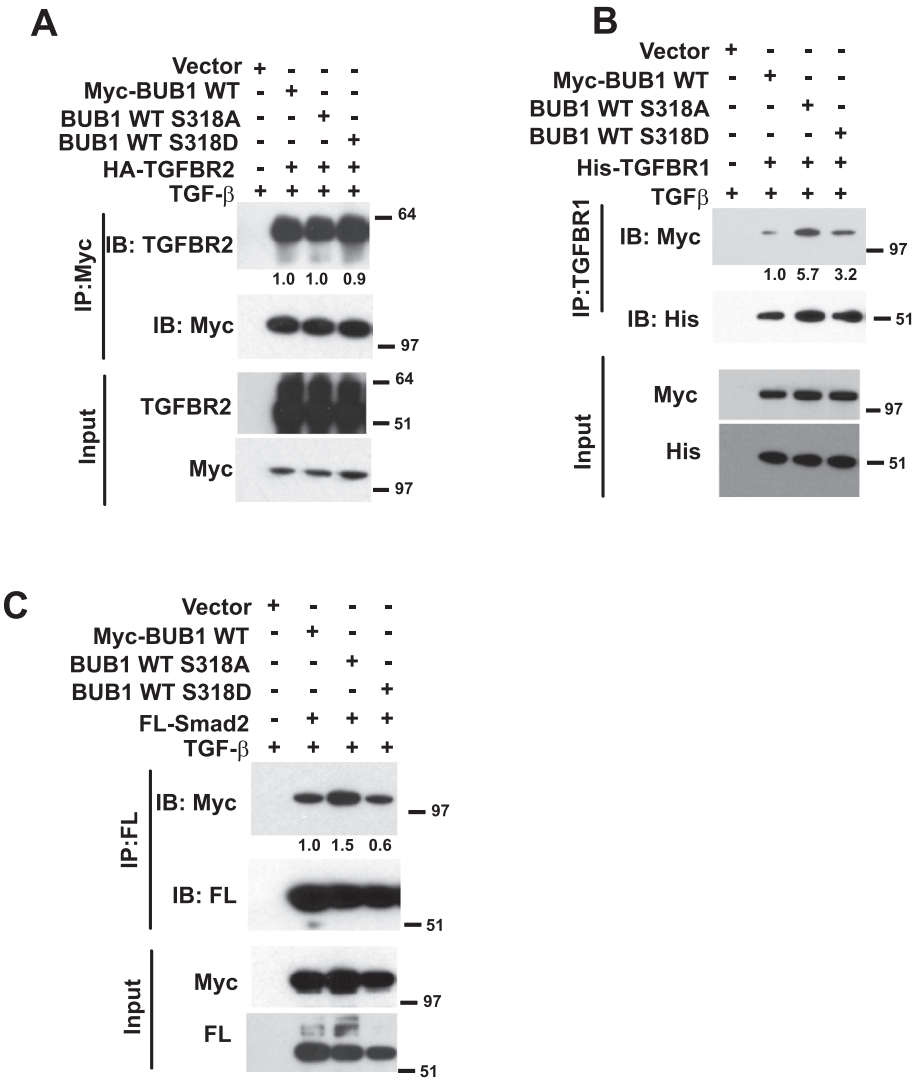


Fig. 3. Phosphorylation of BUB1 at Ser318 causes reduction in interaction with TGFBR1 and SMAD2. (A) HEK293T cells were transfected with Myc-BUB1-WT, S318A, S318D mutants and HA-tagged TGFBR2, serum starved and treated for an hour with TGF-β (5 ng/mL). Lysates were made 40–48 h post-transfections. Immunoprecipitation was performed using Myc-tag antibodies and blots were probed with TGFBR2 and Myc-tag antibodies. (B) IP for TGFBRI and then blotting for Myc in lysates from HEK293T cells transfected with Myc-BUB1-WT, S318A, S318D mutants and His-tagged TGFBR1, serum-starved, and treated with TGF-β (5 ng/mL) for 1 h. (C) IP for FLAG and then blotting for Myc in lysates from HEK293T cells transfected with BUB1-WT, S318A and S318D mutants and FL-SMAD2, serum starved and treated with TGF-β (5 ng/mL) for 1 h.

using the full-length TGFBR1 and BUB1-WT (S1 Fig). In contrast to its interaction with TGFBR2, the BUB1 1–241 polypeptide was found to interact with TGFBR1 while the 241–482 and 482–723 mutants had minimal interaction (Fig. 4B, Table 3).

To better define BUB1 residues or domains which may be responsible for BUB1 interaction with TGFBR1, we generated mutants which were either devoid of various tetratricopeptide repeats (TPR) and generated TPR domain point mutants (L45-49G, A106D and L122G)) in full-length as well as in the N-terminal fragment (1–241) of BUB1. The TPR domains of BUB1 span the N-terminal region of the protein and are involved in its interaction with multiple proteins, including Blinkin and Borealin [12,17,30]. When either TPR deleted or fragments spanning residue 1–241 of BUB1 (Figs. S2A and S2B) were overexpressed in HEK293T cells along with His-TGFBR1 and subjected to co-IP using

Table 3. A list of BUB1 mutants and their interaction efficiency with TGFBR1, TGFBR2 and SMAD2. Nt = not tested.

BUB1 mutants	TGFBR1	TGFBR2	SMAD2
WT	+	++++	+
WT S318A	+++	++++	+++
WT S318D	+	++++	+
1–241	+++	++++	++
241–482	-	+	+
482–723	-	+++	++
241–482 S318A	-	++++	-
241–482 S318D	-	-	++++
WT dTPR1	+	nt	nt
WT dTPR2	+	nt	nt
WT dTPR3	+	nt	nt
1–241 dTPR1	++++	nt	nt
1–241 dTPR2	++	nt	nt
1–241 dTPR3	++	nt	nt
WT L45-49G	++	nt	nt
WT A106D	++	nt	nt
WT L122G	++	nt	nt
1–241 L45-49G	++	nt	nt
1–241 A106D	++	nt	nt
1–241 L122G	++	nt	nt

a TGFBR1 antibody, we detected no loss in the interaction of TGFBR1 either to full-length or 1–241 BUB1 fragment devoid of either TPR1, TPR2, TPR3, or point-mutants of TPRs (Figs. S2A, S2B). However, we observed an increased interaction of TPR1-deleted BUB1 to TGFBR1. These data suggest that TPR2 and TPR3 domains of BUB1 may not be involved in direct interaction with TGFBR1 while TPR1 may play a role in BUB1-TGFBR1 interaction. We substituted hydrophobic amino acids (Leucine and Alanine) to electrically charged (Aspartic acid, A106D) or non-polar (Glycine, L44-49G, L122G) amino acids in BUB1 to test whether hydrophobic interactions played a major role in these interactions. We did not observe detectable changes in these point-mutated BUB1's interaction with TGFBR1 (Figs S2A and S2B). These results suggest that hydrophobic interactions alone may not be responsible for BUB1's ternary complex formation with TGFBR1/2 and R-SMADs.

Next, to identify domains of BUB1 responsible for interaction with R-SMADs, Flag-tagged SMAD2 was overexpressed along with Myc-tagged BUB1-WT and its truncation mutants. Cells were treated with TGF-β1 or untreated as control. Resulting lysates were immunoprecipitated with FL-tag antibody. In agreement with our previous observation [7], a strong BUB1-WT interaction with SMAD2 was detected. The 1–241 and 482–723 fragments interacted with SMAD2 while 241–482 did not show detectable interaction (Fig. 4C). This data suggests that BUB1 may have multiple contact points for TGF-β1 signaling components. These contact points primarily reside within the 1–241 and 482–723 polypeptide domains.

Ser318 phosphorylation status dictates BUB1(241–482) interaction with TGFBR2 and SMAD2

To better understand the contribution of TGFBR2 mediated BUB1 Ser318 phosphorylation on TGF-β signaling, we generated S381A and S318D mutant fragments (241–482) of BUB1. These mutants were

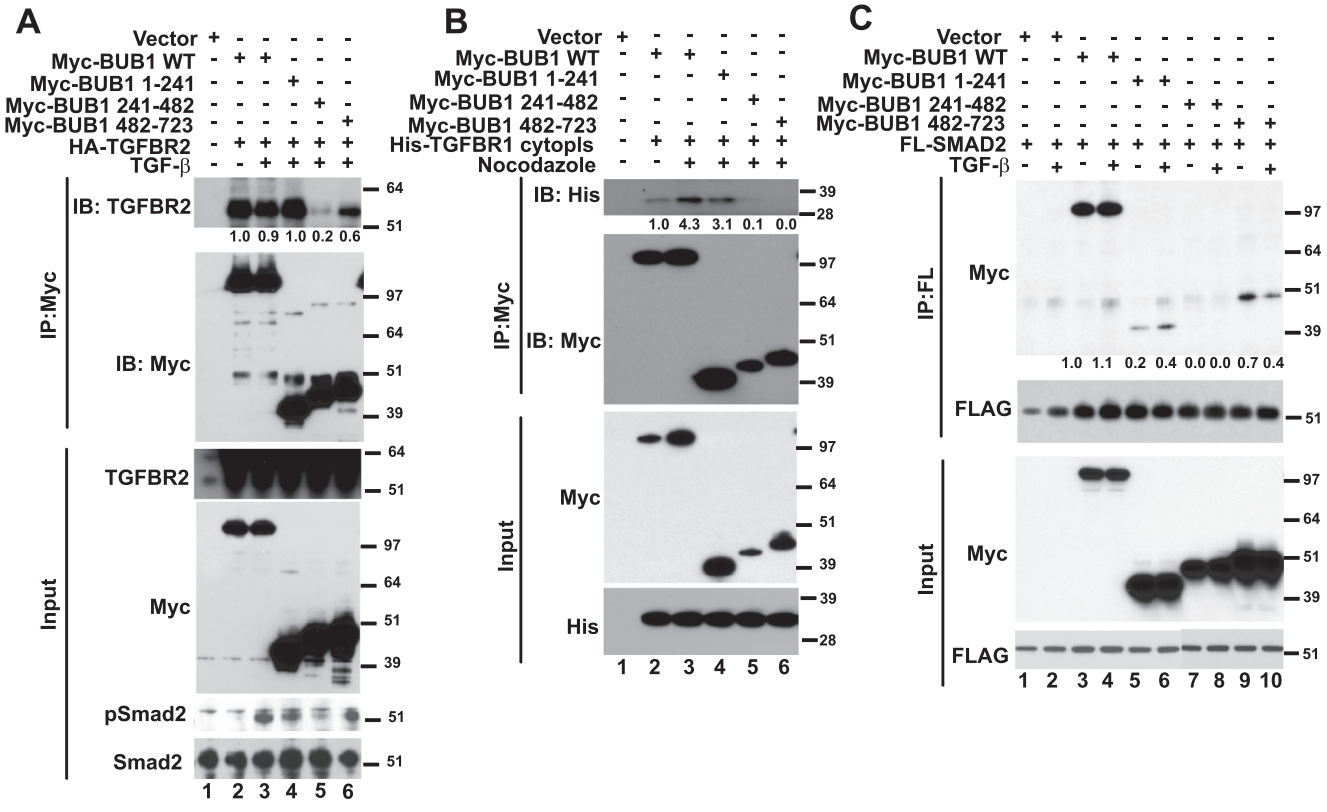


Fig. 4. BUB1 may have multiple contact points for TGF-β signaling components. (A) IP for Myc and then blotting four TGFBR2 in lysates from HEK293T cells transfected with Myc-BUB1 truncation mutants (1–241, 242–481 and 482–723) and HA-TGFBR2, serum starved and treated with TGF-β (5 ng/mL) for 1 h. (B) IP for Myc and then blotting four His in lysates from HEK293T cells transfected with Myc-BUB1 truncation mutants and His-TGFBR1 cytoplasmic tail, synchronized in G2/M by nocodazole. (C) IP for FLAG and then blotting for Myc in lysates from HEK293T cells transfected with BUB1 truncation mutants and FL-SMAD2, serum starved and left untreated or treated with TGF-β (5 ng/mL) for 1 h.

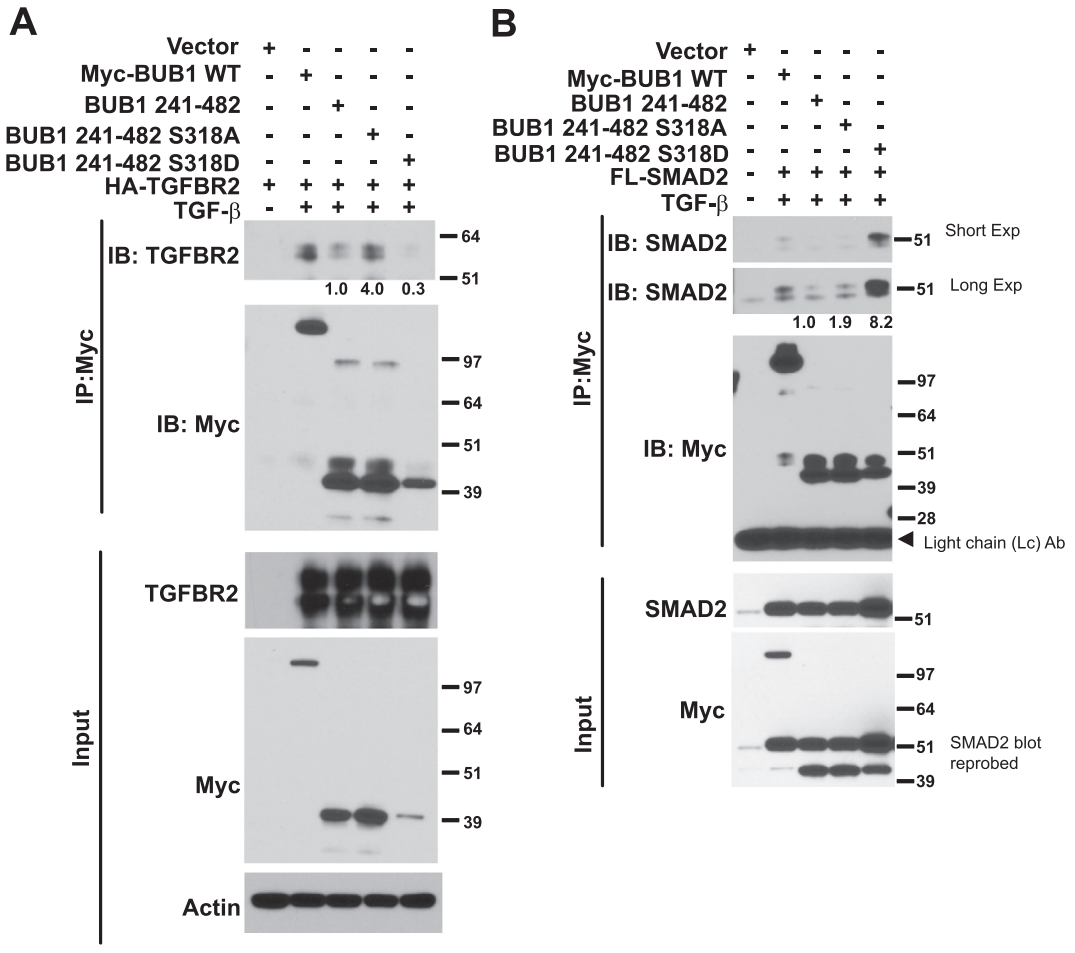


Fig. 5. TGFBR2 mediated phosphorylation of BUB1 reduces its interaction with TGFBR2 and SMAD2. (A) IP for Myc and then blotting for TGFBR2 in lysates from A549 cells transfected with Myc-BUB1 truncation mutant 241–282 and phospho-deficient (S318A) or phospho-mimicking (S318D) mutants along with HA-TGFBR2. Cells were serum starved and treated with TGF-β (5 ng/mL for 1 h) before harvesting. (B) IP for Myc and then blotting for SMAD2 in lysates from HEK293T cells transfected with Myc-BUB1 truncation mutant 241–282 and phospho-deficient (S318A) or phospho-mimicking (S318D) mutants along with FL-SMAD2. Cells were serum starved and treated with TGF-β (5 ng/mL for 1 hour) before harvesting.

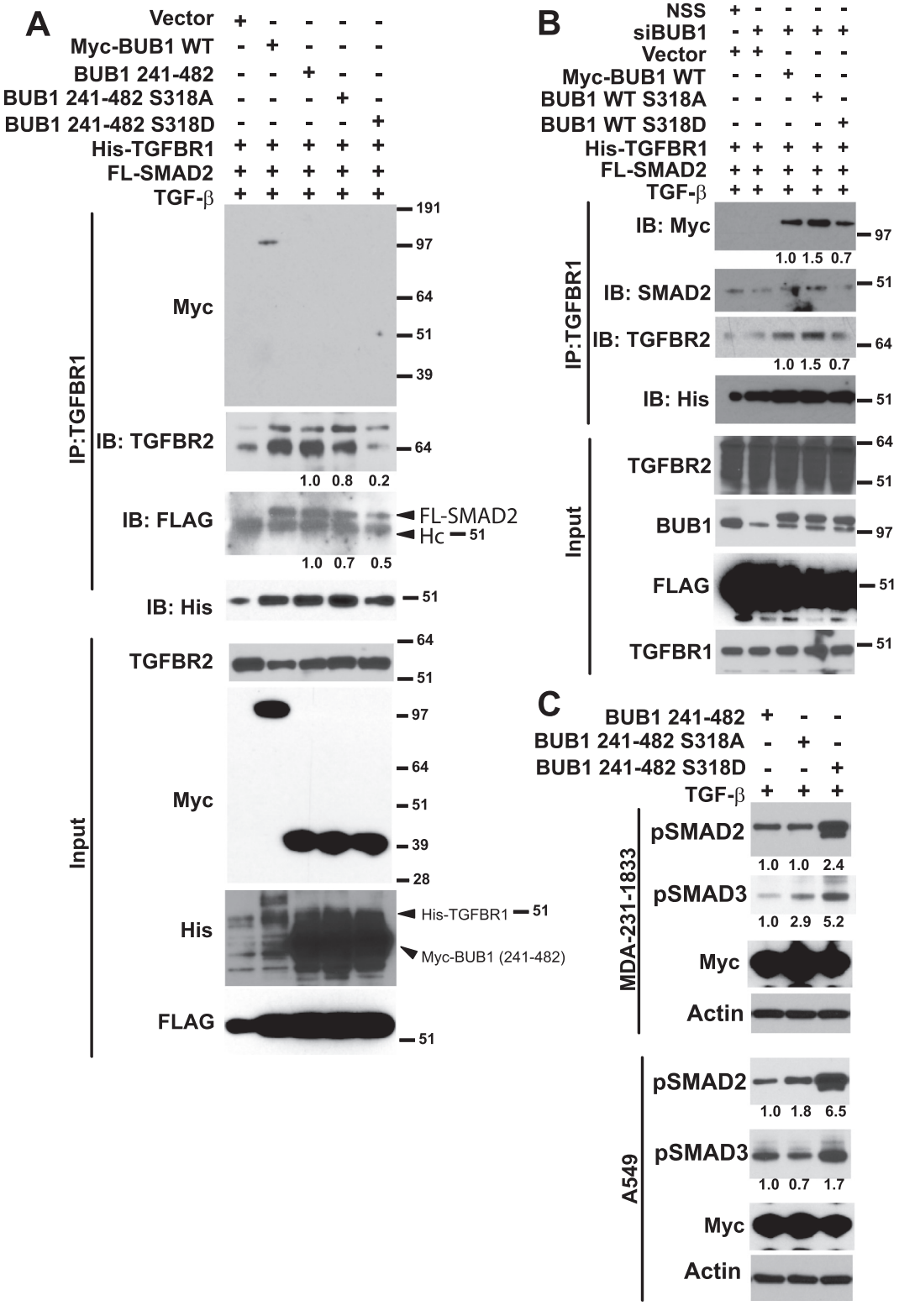
over-expressed in HEK293T cells along with HA-TGFBR2 or FL-SMAD2. Immunoprecipitation using a Myc-tag specific antibody confirmed that the BUB1 241–482 fragment interacts weakly with HA-TGFBR2 (Fig. 5A). Full length BUB1-WT was used as a positive control and showed a strong interaction with HA-TGFBR2 (Fig. 3A). In contrast to the wild-type BUB1 (241–482) fragment, the non-phosphorylatable S318A fragment showed an enhanced interaction with TGFBR2. In contrast, the phospho-mimic BUB1 (241–482)-S318D mutant had no appreciable interaction with TGFBR2 (Fig. 5A). This suggested to us that BUB1 may be recruited to TGFBR2 in its non-phosphorylatable form, and upon phosphorylation by TGFBR2 at Ser318, BUB1 may dissociate from the receptor. In parallel experiments, when FL-SMAD2 was co-expressed with BUB1 mutants (Fig. 5B), we noted an improved interaction of SMAD2 with the BUB1 (241–482)-S318D mutant compared to the phospho-deficient BUB1 (241–482)-S318A fragment (Fig. 5B). This demonstrates that unphosphorylated BUB1 may be recruited to the activated TGFBR2 in complex with SMAD2/3, which upon phosphorylation by the activated TGFBR complex at Ser318 within BUB1, may lead to the dissociation of the activated complex (see proposed model in Fig. 7). Our observation that SMAD2 interacted strongly with BUB1 241–482 S318D led us to postulate that phospho-SMAD2/3 may dissociate from the activated complex at the same time as phosphorylated BUB1. This hypothesis will be tested in future studies.

TGFBR2 mediated BUB1 Ser318 phosphorylation triggers TGFBR disassembly

To confirm if BUB1 Ser318 phosphorylation influences the stability of the activated complex, i.e. TGF-β dependent heteromerization of TGFBR1 and TGFBR2 and recruitment of R-SMADs, we overexpressed BUB1 241–482 truncation mutants along with His-TGFBR1 and FL-SMAD2 in HEK293T cells. BUB1-WT was used as a positive control for the assay. Transfected cells were serum-starved over-night and treated with TGF-β1 for 1 hour before harvesting. Cell extracts were immunoprecipitated using TGFBR1-specific antibody and probed with antibodies against TGFBR2, Flag-, Myc- and His-tags (Fig. 6A). BUB1-WT interacted well with TGFBR1 while the 241–482 fragment did not (similar to Fig. 4B). Importantly, we observed a substantial reduction in interaction of TGFBR2 and SMAD2 with TGFBR1 when the phospho-mimic BUB1(241–482)-S318D fragment was overexpressed (Fig. 6A). This data further supports our hypothesis that TGFBR2 mediated phosphorylation of BUB1 at S318 may be a signaling cue for the activated receptor complex to disassemble (Fig. 7).

To independently confirm that BUB1 Ser318 phosphorylation may lead to dissociation of the active TGFBR complex, we performed a rescue experiment wherein endogenous BUB1 was silenced using siRNA and full-length Myc-BUB1-WT or S318A or S318D mutants were

overexpressed along with His-TGFBR1 and FL-SMAD2 in HEK293T cells. Transfected cells were treated with TGF-β1 one hour before harvesting, resulting lysates were subjected to immunoprecipitation using TGFBR1 antibody. Immunoblotting of the immunoprecipitated pellets demonstrated an increased interaction of BUB1 S318A, SMAD2 and TGFBR2 to TGFBR1 (Fig. 6B) compared to BUB1-WT (lane 3 vs. 4). This interaction was diminished when the BUB1 S318D mutant was overexpressed (Fig. 6B). Next, we evaluated if BUB1 S318 phosphorylation had any effect on the phosphorylation of R-SMAD transcription factors. BUB1 241–482 along with S318A and S318D mutants were overexpressed in MDA-231-1833 or A549 cells. These cells were serum starved overnight and treated with 5 ng/mL TGFβ for 1 hour before harvesting. Western-blotting of the resulting lysates showed an increase in SMAD2 and SMAD3 phosphorylation in lanes where phospho-mimic



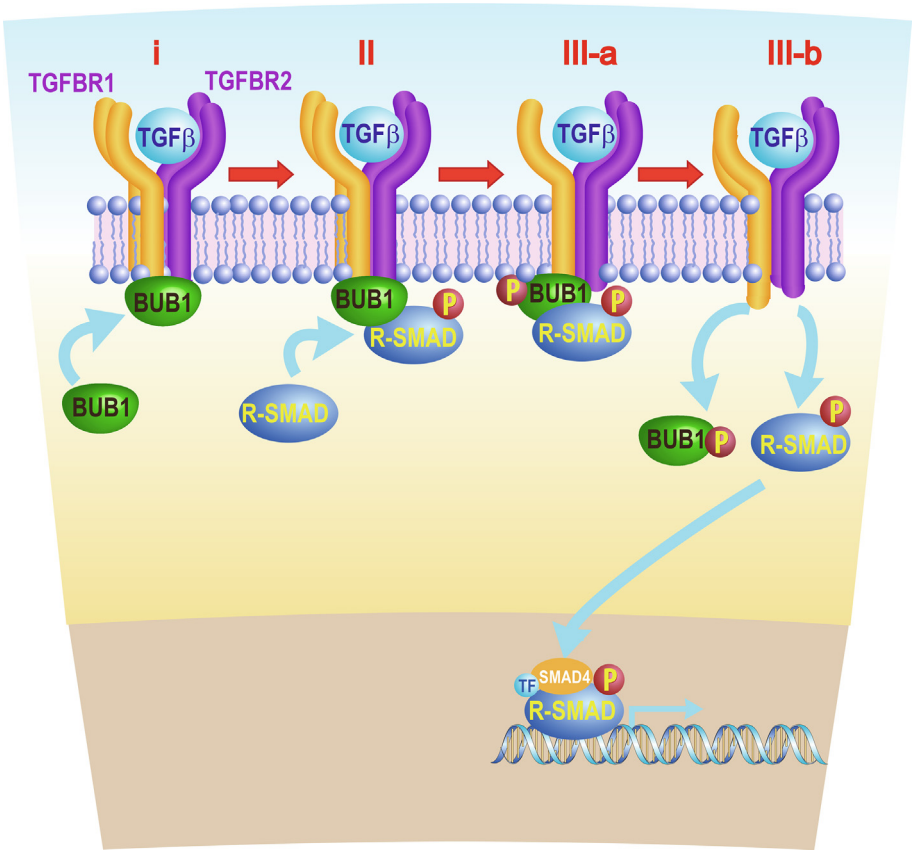


Fig. 7. A schematic representing the proposed model with steps involved in BUB1 mediated regulation of activated TGF-β signaling complex. (i) BUB1 is recruited to TGFBR1-TGFBR2 complex in response to ligand, (ii) BUB1 participates in the recruitment of SMAD2/3 to the receptor, and (iii) TGFBR2 phosphorylates BUB1 at S318, which triggers the disassembly of the activated complex.

mutant of BUB1 (S318D) was overexpressed (Fig. 6C). This data further supports the hypothesis that TGFBR2 mediated BUB1 phosphorylation at Ser318 may be a signaling cue for the dissociation of the active complex when optimal R-SMAD phosphorylation has been achieved after ligand mediated TGFβ signaling activation (Fig. 7).

BUB1 expression is significantly elevated in cancerous tissues and is correlated with TGF-β signaling in aggressive forms of breast cancer

Since the above findings provide compelling evidence for a key regulatory role for BUB1 in TGF-β signaling, we asked if BUB1 expression levels may have clinical significance by evaluating its expression in cancer versus normal tissues. Using various clinical datasets including The Cancer Genome Atlas (TCGA), we found that BUB1 expression was elevated in cancer compared to normal tissue in every tissue type examined (lung, breast, pancreas, colorectal, brain, ovary) and was more significantly elevated in the most aggressive tumor types, even within the same tissue (e.g. glioblastoma vs. oligodendroglioma for brain cancers) (Fig. 8A). We next hypothesized that the BUB1/TGF-β signaling axis described herein may confer a particularly poor clinical prognosis as our previous work identified BUB1 expression as being elevated in triple-negative breast cancers, the most aggressive subtype of breast cancer [31]. To test this hypothesis, we examined the correlation between BUB1 expression levels and TGF-β signaling pathway members using the TCGA and

Fig. 6. TGFBR2 mediated phosphorylation of BUB1 is a signaling cue for the activated TGFBR complex disassembly. (A) HEK293T cells were transfected with Myc-BUB1 WT (as positive control) and truncation mutant Myc-BUB1 241–482 along with S318A and S318D truncation mutants. His-TGFBR1 and FL-SMAD2 were co-transfected with BUB1 and cells were starved and treated with TGF-β (5 ng/mL for 1 h) before harvesting. Lysates were subjected to IP with TGFBR1 and blotted for Myc, TGFBR2 and FLAG. Arrow in the IB: SMAD2 blot denotes the FL-SMAD2 specific band. The band below is Heavy Chain of the antibody (Hc). The arrow in the input blot is His-TGFBR1. The His-tag blot in input is reprobed after probing with Myc-tag antibody. The thick bands below His-TGFBR1 (in inputs) are that of Myc-BUB1 truncation mutants. (B) HEK293T cells were transected with non-targeting control (NSS) or BUB1 siRNA along with Myc-BUB1 WT, S318A and S318D mutants, His-TGFBR1 and FL-SMAD2. Cells were starved and treated with TGF-β before harvesting. Lysates were subjected to IP with TGFBR1 and blotted for Myc, TGFBR2 and FLAG. (C) MDA-231-1833 and A549 cells were transfected with Myc-BUB1 241–482 and 241–482 S318A and 241–482 S318D mutants. 24 h post-transfection, cells were serum starved over-night. Cells were treated with 5 ng/mL for an hour before harvesting. The resulting lysates were resolved on SDS–PAGE gels and probed with antibodies against pSMAD2, pSMAD3, Myc-tag and Actin.

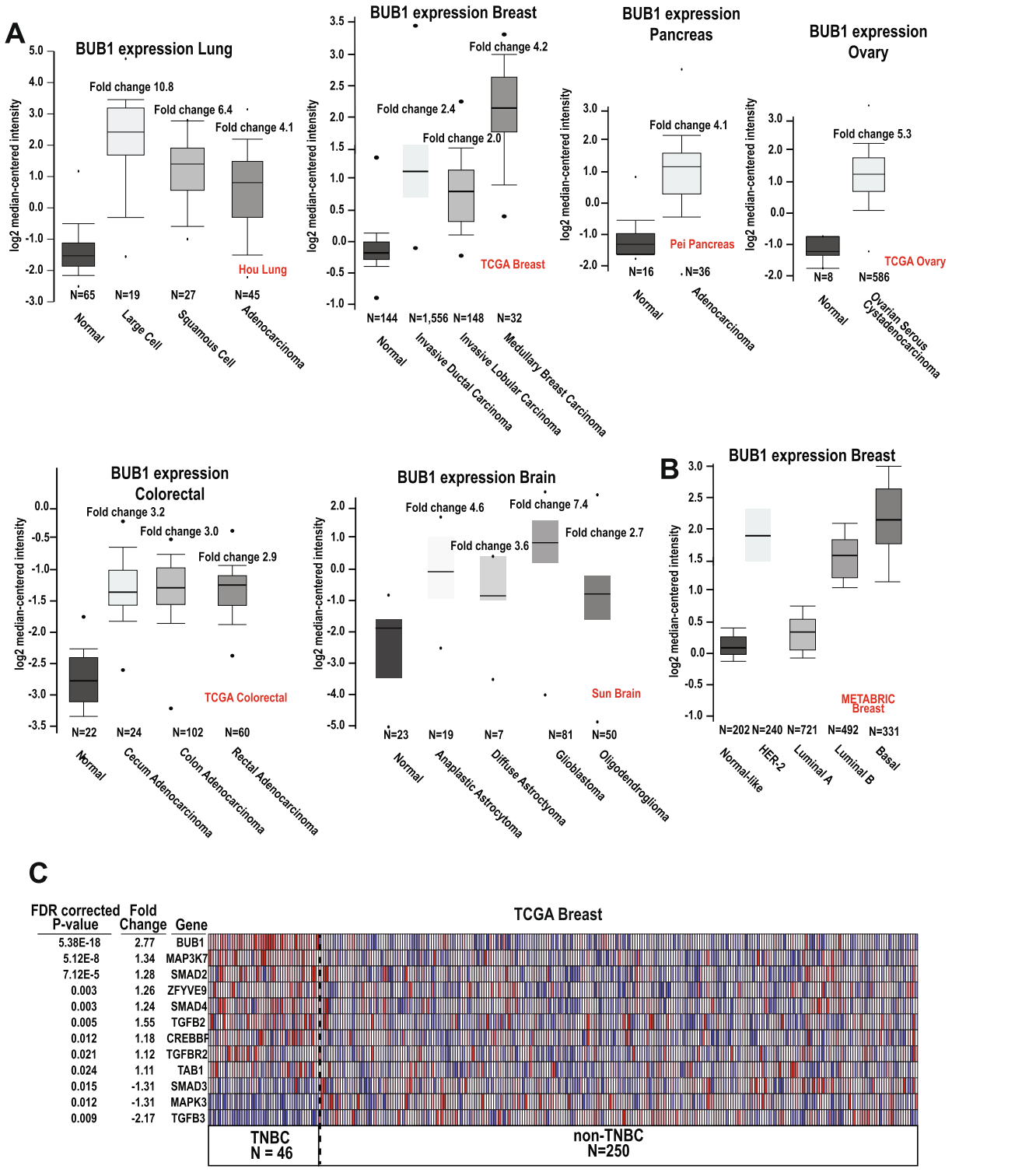


Fig. 8. BUB1 expression is significantly elevated in cancer tissue compared to normal and is significantly correlated with TGF-β signaling pathway proteins in patient tumor samples. (A) BUB1 expression is significantly elevated in cancer vs. normal tissue in numerous human tissues. (B) BUB1 expression is also elevated in the most aggressive subtype of breast cancer, the basal-like subtype in the METABRIC dataset. (C) BUB1 expression was correlated with TGF-β signaling pathway proteins and are coordinately expressed in basal-like, triple-negative breast cancer, especially MAPK3K7, SMAD2, and SMAD4. All expression data is log2 median centered. All groups significantly different on ANOVA testing with a fold change ≥2.0 with a *p*-value <0.05. TNBC = triple-negative breast cancer.

METABRIC datasets. Our analysis revealed that BUB1 was expressed most highly in basal-like breast cancer, the most aggressive form in both the TCGA and METABRIC datasets (Fig. 8B). We then used co-expression analysis to evaluate the expression levels of TGF-β signaling pathway members (as defined by KEGG and BIOCARTA) and BUB1 expression in TCGA. We found that BUB1 was one of the most highly

overexpressed genes in TNBC in TCGA (top 0.5%), and that the expression of TGF-β signaling pathway members was also significantly elevated in TNBC. In particular, there was significant correlation of BUB1 expression and MAP3K7 (FDR corrected *p*-value 0.00000005), SMAD2 (*p*-value 0.00007), SMAD4 (*p*-value 0.003), TGFB2 (*p*-value 0.005), and TGFBR2 (*p*-value 0.02; Fig. 8B). There was a significant inverse correlation in BUB1 expression and SMAD3 in the TCGA dataset (Fig. 8C). These findings were confirmed in three additional, independent breast cancer datasets that had gene expression data from TNBC patient tumors [32,33] These findings provide compelling evidence for the hypothesis that BUB1 expression levels and the TGF-β pathway members described herein are coordinately expressed and may regulate the processes that drive the aggressive phenotypes in cancer, including TNBC.

Discussion

Although a requirement for BUB1 and its kinase activity in TGF-β signaling [7] has been demonstrated, the underlying mechanistic understanding is ill defined. The findings described here demonstrate that BUB1 is a substrate for TGFBR2 in response to ligand-mediated activation of the TGF-β signaling pathway. Furthermore, phosphorylation status of the Ser318 residue within BUB1 appears to function as switch for ligand-induced assembly of the TGF-β signalosome. An immunoprecipitated fraction of the TGFBR1 cytoplasmic domain was shown to phosphorylate BUB1, and since over-expression of the TGFBR1 cytoplasmic domain revealed its interaction with several TGF-β signaling components, including endogenous TGFBR1 and TGFBR2 kinases [23,34], we hypothesized that an interacting partner of the TGFBR1 cytosolic domain may be responsible for BUB1 phosphorylation, since we have previously demonstrated that BUB1 was not a substrate for TGFBR1 kinase activity [7]. Using recombinant TGFBR2 in in vitro kinase assays, we demonstrate that TGFBR2 indeed phosphorylates BUB1 and that this phosphorylation occurred when full-length BUB1 was a substrate, but not the extended kinase-domain (BUB1-E). Furthermore, using 2OH-BNPP1 we confirmed that the observed phosphorylation of BUB1 was mediated by TGFBR2 and not due to autophosphorylation, commonly observed at additional residues within BUB1 [24,27,28] due to its constitutive kinase activity [27,35]. However, in the present studies we have not explored if autophosphorylated BUB1 (at a single or multiple sites) may serve as an optimal substrate for TGFBR2.

Post-translational autophosphorylation of BUB1 has been described at numerous residues, however, the present study identifies a previously undescribed and functionally distinct phosphorylation site at Ser318. Phosphorylation of the Ser318 site does not seem to alter BUB1 interaction with TGFBR2, but appears to modulate BUB1 interaction with TGFBR1 and SMAD2. An enhanced affinity of the S318A non-phosphorylatable mutant to TGFBR1 and SMAD2 was observed compared to the S318D phosphomimic. This suggests that the unphosphorylated form of BUB1 may participate in the recruitment of the SMADs to TGFBR1 upon ligand activation. Subsequently, phosphorylation of BUB1 by TGFBR2 (at Ser318), may be a signaling cue for the disassembly of the activated receptor complex. Additionally, we observed an increase in TGFβ mediated R-SMAD phosphorylation when the BUB1 241–482 S318D mutant was overexpressed (Fig. 6C) which further supports the above hypothesis. We have not systematically evaluated if R-SMAD phosphorylation precedes BUB1 phosphorylation due to unavailability of phospho-S318-BUB1 antibodies. However, our earlier finding that BUB1 protein is required for the TGFBR1-TGFBR2 heteromerization as well as efficient recruitment of R-SMADs to the activated TGFBR1 [7] supports this hypothesis. Furthermore, the current finding that over-expression of the BUB1 phospho-mimic mutants reduce TGFBR1-TGFBR2 and TGFBR1-SMAD2 interactions (Fig. 6) further supports this hypothesis. Although, based on our current results we cannot rule out an alternate hypothesis wherein TGFBR2 recruits BUB1 and phosphorylates it first (upon activation with TGFβ ligand) and that S318-phosphorylated BUB1 then helps forming/stabilizing TGFBR1-TGFBR2 heteromeric complex and recruiting R-SMADS to the activated receptor complex. After detecting optimal phosphorylation of R-SMADs, phospho-BUB1 then signals for the disassembly of the activated complex.

We have not tested the interaction of phosphorylated R-SMADs specifically with different BUB1 mutants. Also, we have not evaluated whether and how overexpression of BUB1 S318 WT, S318A, and S318D mutants affect the SBE4-Luc reporter activity.

An analysis of BUB1 protein sequences indicate that the Ser318 residue is likely present only in higher mammals (humans, non-human primates and pigs) and is absent in mouse and rats. It would be interesting to see whether TGFBR2 phosphorylates some other BUB1 residue in mouse and rats and what effect would that have on TGFβ signaling. Absence of cancer related mutations (COSMIC database) and presence of only a single silent (synonymous) SNP further suggests that this residue might have an important evolutionary role.

BUB1 is comprised of three main structural and functional regions; an N-terminal region that contains the kinetochore localization domain; an intermediate, non-conserved region that is required as a scaffold for the recruitment of proteins; and a C-terminal region that contains a catalytic serine/threonine kinase domain [21]. Our current data show that the N-terminal (1–241) and intermediate (482–723) regions of BUB1 may independently interact with SMAD2, TGFBR1, or TGFBR2 while the 242–481 domain encompassing S318 does not interact well to any of the TGF-β signaling components tested. These findings suggest that full length BUB1 may interact with TGF-β signaling components through distinct contact points (two or possibly more), and that S318 phosphorylation by TGFBR2 may represent a regulatory switch for disassembly of the activated receptor. Since the TPR domains are amphiphilic and provide a structural basis for mediation & stabilization of protein–protein interactions [12,17,30], these regions may be relevant for BUB1 interaction with TGFBR. BUB1 and BUBR1 have multiple TPR domains which indicate that these proteins may possess multiple contact points for protein–protein interactions. By utilizing TPR domain deletion as well as point mutations in BUB1 full-length and N-terminal fragments we confirmed that BUB1 interaction to TGFBR1 was independent of TPR2 and TPR3 and may utilize TPR1. These observations suggest that some additional domains/residues within BUB1 must be involved in interaction with TGF-β signaling components. Moreover, it is also possible that independent BUB1 molecules may interact with SMAD2, TGFBR1 and TGFBR2 as recently demonstrated for kinetochore proteins CENP-E and CENP-F [36].

Recent work by Overlack et al., [13] revealed a functional significance of residues surrounding S318 within BUB1 (BUB1 209–409), consisting of the Gli2-binding sequence (GLEBS)/BUB3 binding domain (B3BD) and an approximately140-residue C-terminal extension (CTE), although not required for kinetochore localization, was essential for kinetochore localization of BUBR1 and BUB1-BUBR1 interaction [13]. This observation in context of result presented here may provide a mechanistic basis for the often described association between TGF-β signaling and the cell-cycle [37]. Since BUB1 [38] and TGFBR1 [39] levels fluctuate during the cell cycle, the results presented here demonstrating that BUB1 and TGFBR1 interaction is cell cycle dependent and may provide a molecular underpinning for a cross-talk between the cell cycle machinery and TGF-β signaling.

Although, our findings suggest that TGFBR2 mediated phosphorylation of BUB1 may lead to dissociation of the activated TGFBR complex, the subcellular location where this takes place is unclear. The mechanisms that direct the internalization and compartmentalization of activated

receptors for turnover or signaling activation are not well defined [2]. Some results suggest a requirement for endocytosis into the EEA1-positive endosome for activation [40,41] while other studies demonstrate that TGFBR1 endocytosis is not required for SMAD activation [42,43]. We postulate that BUB1 participates in the formation & stabilization of heteromeric TGFBR1/2 complex at the membrane [7] and that Ser318 phosphorylated BUB1 may regulate endocytosis of the activated TGFBRs into the endosomal compartment, thus impacting the dissociation of the activated TGFBR1/2 complex and R-SMADs. In support of our findings on the role of BUB1 beyond chromatid segregation, recent studies using two hybrid screens [44] as well as siRNA screens [45] identified BUB1 as a component of receptor signaling micro-domains within membranes. Other studies identified BUB1 interaction with supervillin [44], Vps5 and β2-adaptins [46,47], as well as a role for BUB1 in virus particle entry into the cell through clathrin-dependent endocytosis of Drosophila C virus [11]. These studies emphasize distinct functions of BUB1 beyond mitosis, within membrane micro-domains wherein extracellular signals are communicated to the intracellular network. Interestingly, the BUB1 homologue and also a component of the mitotic spindle checkpoint, BUBR1, has been demonstrated to play an important role in insulin signaling by ensuring timely insulin receptor endocytosis [10].

Our initial finding that BUB1 complexes with SMADs [7] and since SMADs traverse along the kinesin and dynein protein trafficking machinery, (microtubules; [48]) it is possible that BUB1 could also traverse along microtubules, possibly together with SMADs and associated kinesins, to reach the TGF-β receptor [5]. As shown in Fig. 5B, there is an increased interaction of R-SMADs to the phospho-mimic mutant of BUB1, although this may also be a signaling cue for the activated TGFBR1/2 complex to dissociate. At this point we cannot rule out the possibility that BUB1 dissociates from the activated TGFBR complex in complex with R-SMADs. Although BUB1 deletion or TGF-β1 knockout are shown to rapidly lead to aneuploidy [49,50] as well as reduction in DNA damage recognition and increased sensitivity to ionizing radiation [51,52], we have not tested the combinatorial effects of these observations in the studies presented here.

Our observations that BUB1 is expressed highly in a variety of tumors (breast, lung, colorectal, ovarian, pancreas, brain) compared to normal tissues (Fig. 8A and B) highlight that BUB1 expression could potentially serve as a biomarker for diagnosis and prognosis in these cancers. Moreover, we observed high correlation between BUB1 expression and TGFβ pathway genes (Fig. 8C) in aggressive breast cancer (triple negative, TNBC). We observed negative correlation between BUB1 expression and SMAD3, MAPK3, and TGFB3 expression in TNBC (Fig. 8C). This negative correlation does not necessarily mean downregulation of these genes in TNBC. It just reflects that a small number of the pathway members RNA expression was not positively correlated with BUB1 expression. In contrast, we observed that the expression of 8 TGFβ pathway genes (including SMAD2, SMAD4, TGFB2, TGFBR2, ZFYVE9, among others) was significantly and positively correlated with BUB1 in TNBC (Fig. 8C). Pathway analysis suggests activation of this pathway (GSEA analysis) despite the 3 genes that were negatively correlated. Some of these positively correlated genes have been found to be associated with mutational hot-spots in a recent study encompassing 33 cancer types and >9000 patients [53]. These observations suggest that BUB1 and the TGF-β pathway members expression could be coordinated that may regulate the aggressive phenotypes in cancer. Since BUB1 is a kinase, this data theorizes targeting BUB1 to treat aggressive cancers which are driven by TGFβ.

A recent study has identified that USP2a, a de-ubiquitinase that directly interacts with the TGFBR1-TGFBR2 complex and removes the K33-linked polyubiquitin chains from TGFBR1, which promotes SMAD2 recruitment to the activated receptor complex and phosphorylation by TGFBR1 [54]. This data supports our above findings wherein BUB1 is phosphorylated by TGFBR2 which may act as a molecular switch for TGF-β signaling at the membrane. A number of outstanding mechanisms should be studied further: (i) whether BUB1 phosphorylates any additional components of TGF-β signaling cascade, (ii) whether BUB1 participate in endocytosis and sorting of the activated TGFBR complex to lipid-raft or non-lipid-raft vesicles, (iii) whether BUB1 shuttles with phosphorylated R-SMADs along microtubules and (iv) whether BUB1 also interacts with BMP-receptors and affects BMP signaling.

Funding

This study was supported by NIH-NCI grants CA216449 (AR), CA193690 (AR) and CA046592(AR).

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

We would like to thank Dr. Venkatesha Basrur, Ph.D., Katrina Ann Sebolt, and Steven Kronenberg from the University of Michigan on their scientific guidance and help in manuscript preparation. We also want to thank Dr. Hongtao Yu, Ph.D., University of Texas, SouthWestern Medical Center, Dallas, Texas for providing reagents and guidance. We thank University of Michigan Vector Core, Proteomics Core and Microscopy & Image Analysis Laboratory for their services.

Author contributions

SN and AL conceptualized the project and designed the experiments. SN, BSG, JX, GY and LK performed experiments. SN, MKN, DR, CS and AR analyzed the data. CS performed all the bioinformatics analyses. SN wrote the manuscript with feedback from other authors.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.neo.2020.02.001.

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