Published in final edited form as:

Sci Signal. ; 8(358): ra1. doi:10.1126/scisignal.2005379.

The kinase activity of the Ser/Thr kinase BUB1 promotes TGF- β signaling

Shyam Nyati^{1,2}, Katrina Schinske-Sebolt², Sethuramasundaram Pitchiaya^{3,4}, Katerina Chekhovskiy², Areeb Chator², Nauman Chaudhry², Joseph Dosch⁵, Marcian E. Van Dort⁵, Sooryanarayana Varambally⁶, Chandan Kumar-Sinha^{6,7}, Mukesh Kumar Nyati², Dipankar Ray², Nils G. Walter^{3,4}, Hongtao Yu^{8,9}, Brian Dale Ross^{1,5}, and Alnawaz Rehemtulla^{1,2,*}

¹Center for Molecular Imaging, University of Michigan, Ann Arbor, MI-48109, USA

²Department of Radiation Oncology, University of Michigan, Ann Arbor, MI-48109, USA

³Single Molecule Analysis in Real-Time (SMART) Center, University of Michigan, Ann Arbor, MI-48109, USA

⁴Department of Chemistry, University of Michigan, Ann Arbor, MI-48109, USA

⁵Department of Radiology, University of Michigan, Ann Arbor, MI-48109, USA

⁶Department of Pathology, University of Michigan, Ann Arbor, MI-48109, USA

⁷Michigan Center for Translational Pathology, University of Michigan, Ann Arbor, MI-48109, USA

⁸Howard Hughes Medical Institute (HHMI), University of Texas Southwestern Medical Center, Dallas-75390, Texas

⁹Department of Pharmacology, University of Texas Southwestern Medical Center, Dallas-75390, Texas

Abstract

Transforming growth factor- β (TGF β) signaling regulates cell proliferation, differentiation, and development. The binding of TGF β to TGF β receptor 2 (TGFBR2) induces the interaction between TGF β receptor 1 (TGFBR1) and TGFBR2, leading to the phosphorylation and activation of transcriptional regulators SMAD2 and SMAD3. Using an siRNA screen of the human kinome and a live-cell reporter for TGFBR activity, we identified BUB1 (budding uninhibited by benzimidazoles-1), a Ser/Thr kinase, as an essential mediator of TGF β signaling. BUB1 interacted with TGFBR1 in response to stimulation with TGF β and promoted the heterodimerization of TGFBR1 and TGFBR2. Additionally, BUB1 interacted with TGFBR2, suggesting the formation

*Corresponding author. alnawaz@med.umich.edu.

Author Contributions: SN, BDR and ALR conceived and designed the experimental approach, SN performed the majority of experiments. SN, BDR and ALR wrote the manuscript with input from all authors. KS helped with high-throughput screen, SP performed TIRF microscopy analysis. KC, AC, NC and JD contributed to experiments. MEVD synthesized the 2OH-BNPP1 inhibitor. SV, CKS contributed in analyzing the HTS data, NGW, MKN and DR analyzed the data. HY provided reagents, suggested experiments and assisted in the interpretation of results.

Competing interests: The authors declare that they have no competing interests.

Data and materials availability: Data for the kinome siRNA screen are deposited at PubChem (<https://pubchem.ncbi.nlm.nih.gov/assay/assay.cgi?aid=1117269>), accession ID 1117269. Materials may be requested from the corresponding author. There is a patent pending for the use of targeting BUB1 therapeutically.

of a ternary complex. Knocking down BUB1 prevented the recruitment of SMAD3 to the receptor complex, the phosphorylation of SMAD2/3 and their interaction with SMAD4, SMAD-dependent transcription, and TGF β -mediated changes in cellular phenotype including epithelial-mesenchymal transition (EMT), migration, and invasion. Non-canonical signaling cascades of the TGF β pathway mediated by the kinases AKT and p38 MAPK also mediated by BUB1, suggesting an upstream positioning for BUB1 in the TGF β pathway. Although the substrate for BUB1 was elusive, its function in promoting TGF β signaling was dependent on its kinase function: A small-molecule inhibitor of BUB1 kinase (2OH-BNPP1) and a kinase-deficient mutant of BUB1 abrogated TGF β signaling and formation of the ternary complex in various normal and cancer cell lines. 2OH-BNPP1 administration to mice bearing lung carcinoma xenografts reduced the amount of phosphorylated SMAD2 in tumor tissue. These findings provide evidence for a role of BUB1 as a kinase in mediating TGF β -dependent signaling beyond its established function in cell-cycle regulation and chromosome cohesion.

Introduction

The transforming growth factor- β (TGF β) family of cytokines regulates many processes such as immune suppression, angiogenesis, wound healing and epithelial-to-mesenchymal transition (EMT) (1, 2). Abnormal TGF β signaling is linked to autoimmune and autoinflammatory diseases, fibrosis, tumor formation and metastasis, and various other disorders. Early in tumorigenesis, the proliferation of epithelial cells retains exquisite sensitivity to TGF β , wherein TGF β elicits a tumor suppressive response. However, transformed cells become refractory to TGF β -mediated growth inhibition and acquire a phenotype wherein the intracellular signaling circuitry is altered, leading to tumorigenic and metastatic effects in response to TGF β (3). These responses are diverse, depending on signaling from growth factor receptors activated in parallel (4), cell density and cell cycle phase (5), and the abundance and activity of transcriptional regulators (6).

BUB1 is a serine/threonine kinase that is encoded by the *BUB1* gene (40 kbp, 25 exons) in humans. During mitosis, BUB1 binds kinetochores and plays a key role in establishing the mitotic spindle checkpoint and aligning chromosomes (7) in addition to its central role in ensuring fidelity during chromosomal segregation into daughter cells (8). Three main regions have been identified in BUB1: a conserved 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 (9). Mutations in *BUB1* are associated with aneuploidy and several types of cancer.

Ligand-dependent activation of TGF β receptors and regulation of their subsequent kinase activity is a complex process that can involve several posttranslational modifications of the receptors [including autophosphorylation (10), cross-phosphorylation (11), *trans*-phosphorylation (12), ubiquitylation (13), sumoylation (14), and dephosphorylation (15)] as well as internalization of the receptor-ligand complex (16-19). With so much complexity in one pathway, we set out to find potentially new regulators of TGF β receptor activity.

Results

Kinase targeted high-throughput siRNA screen identifies regulators of TGF β signaling

To identify proteins that regulate signaling by the type I TGF β receptor (TGFBR1), we transfected small interfering RNAs (siRNAs) against each of the 720 known and predicted kinases into A549 lung adenocarcinoma and MDA-231-1833 breast cancer cells that stably expressed a reporter for TGFBR1 kinase activity [(BTR (4))] and screened for an increase in bioluminescence after the addition of TGF β ligand to the culture medium (fig. S1A). We used a quartile-based method to identify proteins that, when knocked down, induced over 7.14-fold activation of the reporter in A549 cells and over 13.91-fold in MDA-231-1833 cells (high stringency hits, targeted error rate $\alpha = 0.0027$), followed by experiment- and plate-wise analysis of the normalized fold induction (Fig. 1, A to C, and table S1) as previously described (20). Eight hits were identified in A549-BTR cells (Fig. 1A), whereas fifteen were recovered in MDA-231-1833-BTR cells (Fig. 1B). Two of these hits were the kinases BMPR2 (in A549-BTR cells) and RPS6KB1 (in MDA-231-1833-BTR cells), which are already associated with the TGF β pathway (table S2). Analysis at a lower stringency (5.4-fold in A549 cells, 10.29-fold in MDA-231-1833 cells, targeted error rate $\alpha = 0.046$) revealed additional hits, several of which also have known roles in regulating TGF β signaling (table S2), thereby further validating the screen.

A logical relations analysis of the A549-BTR and MDA-231-1833-BTR screens revealed that a large majority of the significant hits were distinct for each cell line (Fig. 1C); of these, seven were common (Fig. 1C). Signaling pathway impact analysis demonstrated that signaling networks previously implicated in promoting the cellular response to TGF β [including those mediated by receptor tyrosine kinases (RTKs), mitogen-activated protein kinase (MAPK), and phosphoinositide 3-kinase (PI3K)–AKT] were identified as hits in the screen (table S3 and fig. S1, B and C) (4). Targets within the progesterone-mediated oocyte maturation and the oocyte meiosis pathways, in which BUB1 is a key component, consistently exhibited high perturbation accumulation in A549-BTR and MDA-231-1833-BTR cells (fig. S1, B and C).

BUB1 promotes canonical and non-canonical TGF β signaling

In an effort to biochemically validate these findings, we knocked down select hits in MRC5 (human fetal lung fibroblast), Het1A (human esophageal epithelial), A549-BTR (lung adenocarcinoma), MDA-231-1833-BTR (breast cancer), MCF7 (breast cancer) and MCF10A (human mammary epithelial) cells. BUB1 knockdown induced the most robust decrease in TGF β -dependent SMAD2 phosphorylation (fig. S2) and was selected for further study. Similar to a positive control, in which TGFBR1 was silenced, knockdown of BUB1 in lung cancer (A549 and NCI-H358) and breast cancer (MDA-231-1833) cell lines using small interfering RNAs (siRNAs) abrogated the activation of SMAD2 and SMAD3 as well as components of the non-canonical signaling cascade (c-JUN, p38 MAPK, and AKT) in each of the cell lines (Fig. 1D), key downstream effectors of TGF β signaling (21, 22). Transfection with BUB1 siRNA caused a similar decrease in TGFBR1 abundance as did TGFBR1 siRNA, but only in A549 cells. Even still, in this cell line, the effects of BUB1 siRNA did not mimic the effects of TGFBR1 siRNA. Thus the effects of BUB1 knockdown

on downstream markers of TGF β signaling was not an artifact of off-target or indirect knockdown of TGFBR1.

BUB1 mediates TGF β -dependent recruitment of R-SMADs to the activated receptor

Because depletion of BUB1 reduced TGF β -mediated SMAD2/3 phosphorylation, we hypothesized that BUB1 may mediate the efficient recruitment of SMAD2/3 to the activated receptor in the presence of ligand. To test this hypothesis, A549 and HEK293T (human embryonic kidney 293T) cells were transfected with Flag-tagged SMAD3 and His-tagged TGFBR1 in the presence of control or TGFBR1- or BUB1-targeted siRNA and subsequently treated with TGF β for 1 hour. Co-immunoprecipitation analysis revealed that either TGFBR1 or BUB1 knockdown impaired SMAD3 binding to TGFBR1 in the presence of ligand (Fig. 2, A and B, fig. S3, A and B) as well as binding induced by ligand (fig. S3C).

Upon phosphorylation induces receptor-regulated SMADs (R-SMADs, including SMADs 1, 2, 3, 5, and 8/9) translocate to the nucleus wherein they regulate transcription of specific target genes. We evaluated the effect of BUB1 knockdown on the nuclear translocation of SMAD2 as a surrogate for the activation of the TGF β pathway by immunofluorescence microscopy. A549 and H358 cells were transfected with control or TGFBR1- or BUB1-targeted siRNA stimulated with TGF β for 1 hour. In control cells, SMAD2 was predominantly localized to the nucleus upon TGF β stimulation; in contrast, in cells depleted of either TGFBR1 or BUB1, SMAD2 translocation to the nucleus was similarly impaired (fig. S3D). Together, these results suggest that BUB1 plays an important role in promoting canonical and non-canonical TGF β signaling.

BUB1 promotes R-SMAD/SMAD4 complex formation and target gene transcription

To extend our finding that depletion of BUB1 leads to reduced R-SMAD recruitment to the receptor complex and hence reduced phosphorylation and nuclear translocation, we investigated whether BUB1 also promotes SMAD2/3-SMAD4 complex formation using coimmunoprecipitation assays. As expected, depletion of TGFBR1 in A549 cells impaired TGF β -induced formation of a SMAD2/3-SMAD4 complex (Fig. 2C, fig. S4). To evaluate a role for BUB1 in TGF β -induced gene expression, we used the SBE4-Luc reporter (6). In A549 cells transfected with control siRNA, TGF β induced a time-dependent increase in reporter activity, but this induction was abrogated by depletion of BUB1 (Fig. 2, D and E). Similar observations were seen in lung (NCI-H358), breast (MDA-231-1833), and cervical (HeLa) cancer cell lines (Fig. 2, F-H).

One of the expression signatures induced by TGF β signaling is that which promotes EMT, a process in epithelial cells that occurs during development and disease progression in response to specific stimuli (23). Addition of TGF β to epithelial cells in culture induces features of EMT, including the adoption of a spindle-like mesenchymal morphology and invasive behavior (24-26). Therefore, we investigated the role of BUB1 in TGF β -mediated EMT. As expected, cultures of A549 or NCI-H358 cells exhibited a transition to a mesenchymal phenotype in response to TGF β ; however, TGFBR1 or BUB1 knockdown prevented this transition (fig. S5, A to E). Similar to the effect of silencing TGFBR1, knocking down BUB1 significantly inhibited TGF β -mediated induction of cell migration

and invasion in culture (fig. S6, A to D). These results further support a role for BUB1 in mediating TGF β signaling.

Catalytic activity is required for BUB1 to mediate TGF β signaling

To confirm that the kinase activity of BUB1 mediated TGF β signaling, we conducted rescue experiments in A549 cells using siRNA-resistant constructs of BUB1 (27). In cells depleted of endogenous BUB1, restoration with wild-type BUB1 but not a kinase-deficient BUB1 mutant rescued SMAD2/3 phosphorylation in a ligand-dependent manner (Fig. 3A, fig. S7A). In agreement with this observation, reporter activity in BUB1-depleted A549 cells expressing the SBE4-Luc promoter was restored upon expression of wild-type but not a kinase-deficient BUB1 (Fig. 3B), demonstrating a requirement for BUB1's kinase activity in its role in TGF β signaling. The concentration of 2OH-BNPP, a catalytic BUB1 inhibitor (9), negatively correlated with the phosphorylation of SMAD2 and SMAD3 (fig. S7B-D). Blocking the activity of BUB1 with 2OH-BNPP1 dose-dependently impaired the TGF β -induced phosphorylation of proteins that mediate canonical and non-canonical TGF β signaling in multiple cancer cell lines (Fig. 3, C to E) and normal cell lines (fig. S7, E and F). Similar to the effects of a pharmacological TGFBR1 inhibitor [SB431542 (28)], 2OH-BNPP impaired the nuclear translocation of SMAD2 (fig. S7, G and H), SMAD2/3-SMAD4 complex formation (Fig. 3, F and G), and SBE4-Luc reporter activity (Fig. 3, H and I; fig. S7, I and J) in response to TGF β in various cell lines.

BUB1 co-localizes and interacts with TGFBR1 in response to TGF β stimulation

On the basis of the observed role of BUB1 in promoting the recruitment of SMAD3 to TGFBR1 (Fig. 2 A and B, fig. S3A and B) and previous evidence of a scaffolding function for BUB1 (7-9, 29), we investigated whether BUB1 physically interacts with TGFBR1 to mediate its activity using total internal reflection fluorescence (TIRF) microscopy (30). TIRF images revealed the presence of BUB1 and TGFBR1 in punctate structures within the plasma membrane plane (Fig. 4A). Spectral analysis detected negligible colocalization of TGFBR1 and BUB1 at 1 and 24 hours after ligand stimulation (fig. S8A), possibly because of the signal from the relatively high abundance of cytoplasmic BUB1. In contrast, by 72 hours with TGF β , the proportion of colocalized TGFBR1 and BUB1 signals doubled (Fig. 4, A to C).

We next examined whether forced overexpression of BUB1 using a myc-tagged construct was sufficient for its interaction with TGFBR1 and if this would affect the phosphorylation of SMAD2/3. Heterologous expression of BUB1 in A549 cells resulted in the coimmunoprecipitation of BUB1 and TGFBR1 regardless of TGF β ligand (Fig. 4D, fig. S8B), but this was not sufficient to initiate SMAD2/3 phosphorylation in the absence of ligand, only modestly increased phosphorylation of SMAD3 (but not SMAD2) in the presence of TGF β (Fig. 4D, fig. S8C). Overexpression of both BUB1 and TGFBR1 induced a markedly greater TGF β -stimulated interaction between BUB1 and TGFBR1 and further increased the phosphorylation of SMAD3 (Fig. 4D, fig S8B and C). These results demonstrate that over-expression of TGFBR1 and/or BUB1 enhances the activation of the signaling pathway only in the presence of the TGF β ligand. Myc-tagged BUB1

coimmunoprecipitated with a His-tagged cytoplasmic tail of TGFBR1 from A549 cell lysates (Fig. 4E), demonstrated that BUB1 may interact with the cytoplasmic tail of TGFBR1.

BUB1 mediate TGF β dependent TGFBR1-TGFBRII heteromeric complex formation

In the presence of ligand, TGFBR1 is recruited to the type II receptor (TGFBRII) and forms a stable receptor complex through trans-phosphorylation, (31). To examine the role for BUB1 in the formation of this heteromeric receptor complex, A549 cells were transfected with control or BUB1-targeted siRNA and treated with TGF β , and TGFBR1 immunoprecipitates from the resulting lysates were probed for TGFBRII. BUB1 depletion abrogated the formation of the TGFBR1-TGFBRII complex (Fig. 4F). The abundance of SARA [SMAD anchor for receptor activation (32)] was detected in the immunoprecipitated fraction irrespective of BUB1 expression (Fig. 4F). TGF β signaling is known to depend on the cell cycle (33) and BUB1 is a known regulator of the cell cycle (8); however, we did not observe cell cycle arrest in BUB1 depleted cells (fig. S8D).

To test whether BUB1 kinase activity played a role in formation of the heteromeric receptor complex, we transfected A549 cells with His-tagged TGFBR1 followed by treatment with 2OH-BNPP1 for 1 hour and stimulation with TGF β for an additional hour. The TGFBR1 inhibitor SB431542 was used in parallel experiments as a control. Inhibition of BUB1 kinase activity significantly reduced the formation of the TGFBR1-TGFBRII complex (Fig. 4G, fig. S9). Inhibition of BUB1 kinase activity also decreased the coimmunoprecipitation of myc-tagged BUB1 with His-tagged TGFBR1 (Fig. 4H). Because the kinase activity of BUB1 promoted the TGFBR1-II complex formation and its own interaction with TGFBR1, we investigated whether BUB1 could directly phosphorylate TGFBR1. In vitro kinase assays revealed that BUB1 was not a direct kinase for TGFBR1 (fig. S10), and although Myc-BUB1 and Flag-tagged SMAD2 coimmunoprecipitated when expressed in HEK293T cells (fig. S11), Myc-BUB1 did not phosphorylate purified SMAD3 in vitro (fig. S12). Thus, albeit important, the target of BUB1's kinase activity in the TGFBR1 signaling complex was elusive.

BUB1 interacts with TGFBRII in response to TGF β stimulation

We next evaluated whether BUB1 also interacted with TGFBRII, which would be indicative of the formation of a ternary complex between BUB1, TGFBR1, and TGFBRII. HEK293T cells were transfected with His-TGFBR1 and Myc-BUB1-WT and treated with 2OH-BNPP1 for 1 hour, followed by stimulation with TGF β for an additional hour.

Coimmunoprecipitation assays revealed an increase in TGF β -stimulated interaction between TGFBRII and BUB1 in HEK293T cells in a kinase-dependent manner (Fig. 4I). In parallel studies, treatment of cells with the TGFBR1-specific inhibitor SB431542 did not impact this interaction (Fig. 4I).

BUB1 inhibitor 2OH-BNPP1 abrogates SMAD2 phosphorylation in vivo

We followed up on our cellular studies by evaluating the abundance of phosphorylated SMAD2 in subcutaneous A549 tumor xenografts harvested 4 hours after mice were treated with 2OH-BNPP1. Similar to tumors from mice treated with the TGFBR1 inhibitor, tumors from 2OH-BNPP1-treated mice had a significantly decreased abundance of phosphorylated

SMAD2 compared with tumors from vehicle-treated mice (Fig. 5, A and B), supporting a role for BUB1 in SMAD activation.

Together, our data indicate the formation of a ternary complex between TGFBR1, TGFBR2, and BUB1, and that the kinase activity of BUB1 may promote canonical and non-canonical TGF β signaling (Fig. 6).

Discussion

Reverse genetic screens in mammalian cells using small interfering RNA (siRNA) have been utilized for assessing gene function, target validation, pathway analysis, gene redundancy, and therapeutic targeting of several disorders. In an effort to elucidate novel regulators of the TGF β pathway, siRNA library screens have identified key regulators of TGF β signaling (34-40), although screens using TGFBR1 kinase activity as a readout have not been conducted. The BTR reporter is unique in that loss of TGFBR1 kinase activity results in a gain of function (increase in bioluminescence signal), and thus is less likely to result in non-specific or off-target biological effects than commonly observed in traditional luciferase based screens (4). Recent studies using a GFP fused SMAD2 reporter in a large-scale RNAi screen observed that about 1% of hits exhibited off-target effects (41). In contrast, depletion of target genes that lead to cellular toxicity or off-target effects would be scored as false negative but not false positive when using the BTR reporter. This provided us with confidence that siRNAs which resulted in an increase in reporter activity represented true hits. A lack of overlap between hits derived from the two cell lines is not surprising considering that the physiologic response of cells to TGF β stimulation is often cell type and context dependent (42, 43). We focused on six hits that were common between the two cell lines, assuming that these may represent important biological regulators. Of these, BMPR2, RPS6KB1, NEK6 and JAK2 have been described to be associated with the TGF β pathway (44-48). However, the identification of BUB1 in our siRNA screen using the TGFBR1 kinase assay was surprising and unexpected due to the preponderance of literature describing BUB1 as a component of the mitotic machinery wherein it ensures bipolar attachment to spindle microtubules so that chromatid segregation can occur with high fidelity (7, 8). Confirmatory studies conducted in normal cells (MCF10A, MRC5 and Het1A) as well as tumor cell lines of various origins (A549, NCI-H358, MCF7 and MDA-231-1833) demonstrated a requirement for BUB1 expression in the cellular response to TGF β stimulation, and thus was followed up for mechanistic studies. Additional confidence for BUB1 as a true hit was derived from studies demonstrating that depletion of BUB1 in three independent cell lines resulted in abrogation of canonical (phosphorylated SMAD2 and phosphorylated SMAD3) as well as non-canonical (phosphorylated P38MAPK, AKT and c-JUN) downstream effector molecules. Since TGF β signaling has been shown to be cell cycle dependent (49,50), we addressed the possibility that BUB1 (a regulator of mitosis) knockdown results in a decrease in TGF β mediated signaling due to the arrest of cells in mitosis. Existing evidence indicates that siRNA mediating complete depletion of BUB1 does not result in a cell cycle arrest (51), a finding confirmed in our studies (fig. S9). In addition, cell cycle dependence of TGF β mediated cellular responses is understood to be a downstream event wherein changes in R-SMAD phosphorylation are not altered in a cell cycle dependent manner but are rather regulated through interaction with

SKI and SNON (5). Lastly, the ability of a BUB1 kinase inhibitor to abrogate TGF β dependent signaling within an hour of treatment, a period during which a cell cycle arrest would not have occurred further, supports a direct role for BUB1 in receptor mediated signaling.

The functional insights obtained in the current study suggest that BUB1 is recruited to TGFBR1 in a ligand dependent manner. Co-immunoprecipitation studies demonstrated recruitment of BUB1 to TGFBR1 within an hour of TGF β treatment, while TIRF microscopy demonstrated co-localization only at the 72 hour but not earlier time points. Superior sensitivity and signal to noise of the co-immunoprecipitation experiments may explain this discordant observation. Alternatively, BUB1-TGFBR1 interactions potentially occur at membrane proximal regions, which are optically inaccessible by TIRF microscopy, at earlier time points and these complexes are enriched at the plasma membrane only 72 h post ligand treatment – a hypothesis that is still consistent with co-immunoprecipitation experiments that sample whole cell lysates. Additionally, a requirement for BUB1 in the stabilization of the Type II and I receptor complex, R-SMAD recruitment to the receptor as well as co-SMAD/R-SMAD complex formation, and therefore in the downstream transcriptional response, was also demonstrated. Furthermore, our findings using a kinase dead mutant of BUB1 as well as a small molecule inhibitor demonstrated that the kinase activity of BUB1 promotes the recruitment of BUB1 to TGFBR1 and TGFBR2 as well as for TGFBR1-TGFBR2 complex formation upon ligand stimulation, leading to the propagation of the cellular response to TGF β . The biological significance of BUB1's role in TGF β signaling was emphasized by the finding that treatment of tumor bearing animals with 2OH-BNPP1 resulted in a loss of TGF β mediated SMAD2-phosphorylation to an extent similar to SB431542, an inhibitor of TGFBR1 kinase activity. Our in-vitro kinase assays failed to demonstrate TGFBR1 or SMAD3 as direct substrates of BUB1, although we observed an interaction between BUB1 and SMAD2 in HEK293T cells. Future studies will focus on identification of substrates of BUB1 kinase activity within the TGF β signaling pathway. Although we show that BUB1 forms a ternary complex by interacting with TGFBR1 as well as TGFBR2, the requirement for TGFBR2 in the recruitment of BUB1 to TGFBR1 was not directly investigated. Our studies in H358 cells, which lack TGFBR2 (52-54), demonstrated a requirement of BUB1 expression and kinase activity in propagation of the TGF β signaling pathway, suggesting that TGFBR2 may be dispensable for the interaction of BUB1 with TGFBR1.

In summary, we provide compelling evidence that BUB1 and its kinase activity are an integral component of TGF β signaling and promote TGFBR1/II receptor complex formation, thus regulating downstream signaling cascades, including the SMAD, MAPK, and PI3K/AKT pathways. We further show that BUB1 interacts with both, TGFBR1 and TGFBR2 and mediate TGF β dependent EMT, cell migration and invasion. We anticipate that our results will lay the foundations for future studies that provide new avenues for therapeutic targeting of TGF β signaling in disease.

Materials and Methods

Plasmid DNA

Wild-type (WT) BTR reporter has been described earlier (4). SBE4-Luc (60) reporter plasmid was provided by Dr. Bert Vogelstein (Addgene plasmid #16527). pCMV5-TGFBRI-His (#19161), pCS2-Flag-SMAD2 (#14042), and pCS2-Flag-SMAD3 (#14052) were provided by Dr. Joan Massague (61), pCMV5B-TGFBRI-K232R (#11763) was kindly gifted by Dr. Jeff Wrana (32). siRNA resistant Myc-BUB1-WT, and Myc-BUB1-kinase dead (KD) have been previously described (27).

Cell Culture and Transfection

The human lung carcinoma cell line A549 (American Type Culture collection, ATCC) was maintained in RPMI-1620 media supplemented with 10% heat-inactivated fetal bovine serum, 1% glutamine, and 0.1% penicillin/streptomycin/gentamycin (GIBCO-Invitrogen). Cell cultures were grown in a humidified incubator at 37°C and 5% CO₂. Breast cancer cell line 1833(62) 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. NCI-H358 lung cancer cell-line was provided by Dr. David Beer (University of Michigan, Ann Arbor). Normal lung fibroblast cell line MRC5, esophageal cell line Het-1A, breast epithelial line MCF7, cervical cancer cell line HeLa and normal kidney cell line HEK293T were maintained in DMEM while normal breast epithelial cell line MCF-10A was maintained in special media. All cell lines were obtained from ATCC. Cell cultures were grown in a humidified incubator at 37°C and 5% CO₂. A549 and MDA-231-1833 cell lines stably expressing BTR WT and mutant reporter were generated and maintained as described (4).

Antibodies, Reagents and siRNA library

Antibodies to phosphorylated (pSer^{465/467}) or total SMAD2, TGFBRI, TGFBRII, pSer⁶³ c-JUN, pSer⁴⁷³ or total AKT, pThr¹⁸⁰/Tyr¹⁸² or total p38MAPK, and GAPDH were all from Cell Signaling Technology. Antibody against SMAD3 was from Invitrogen; antibodies to TGFBRI (H100) and TGFBRII (L21) were from Santa Cruz; antibodies to Myc-tag and firefly luciferase were from Millipore; antibodies to N-terminal of BUB1 were from Abcam (rabbit) or Santa Cruz (goat); antibody to Flag-HRP was from Sigma; and antibodies to His-HRP were from Invitrogen (clones H3 and C-term) or Millipore (H8 clone). HRP-conjugated and fluorophore-conjugated secondary antibodies were from Jackson ImmunoResearch. Recombinant human TGFβ1 was obtained from HumanZyme. TGFBRI inhibitor SB431542 was obtained from Cayman Chemical. D-Luciferin was from Xenogen Corp. The siGENOME Smart Pool siRNA library targeted against all human kinases, non-silencing siRNA (NSS), and individual siRNA from the siGENOME Smart Pool, and custom siRNA against BUB1 were obtained from Dharmacon. A specific, small molecule inhibitor of BUB1 kinase activity (2-[(4-amino-1-(*tert*-butyl)-1*H*-pyrazolo[3,4-*d*]pyrimidin-3-yl)methyl]phenol; (2OH-BNPP1) (9) was synthesized in house.

High-throughput siRNA screening against human kinases

For the high throughput screen, A549 (5000 cells/well) and MDA-231-1833 cells (6000 cells/well) stably expressing the BTR-WT reporter were plated in clear-bottom white walled 96 well plates (Corning Inc.), one day before transfection. 20 μ l of 2 μ M of each siRNA from the siRNA kinase library was added to a V bottom intermediate plate. Similar amount of control siRNA were also added in column 1 and 12 in the intermediate plate (Suppl. figure S1a). The siRNA were diluted by adding 40 μ l Optimem and incubated for 5 min at room temperature. 80 μ l of Dharmafect-1 and Optimem mix was added to intermediate plate and incubated for additional 30 min. All liquid handling was done using Beckman Biomek NX^P Laboratory Automation Workstation (Beckman Coulter Inc.). The final assay concentration of each siRNA was 50 nM while 0.2 μ l DF-1 was used for each well. Cells were incubated for 72h in LiCONiC StoreX STX44 IC precision incubator (Liconic Instruments), which was connected to Plate Handler II robot (Perkin Elmer) and Envision 2104 Plate Reader (Perkin Elmer) with luciferin injector. 10 ng/mL TGF β was added to cells one hour prior to adding 50 μ g/mL D-luciferin and measuring bioluminescence. All the processes were automated. Fold change in reporter activity was calculated over change in activity in non-silencing control siRNA (NSS) transfected cells.

To validate hits, 100 nM siRNA to selected genes along with TGFBR1 siRNA and NSS were transfected in A549, NCI-H358 and MDA-231-1833 cells using Dharmafect1. The transfected cells were incubated for 60 hours, serum starved overnight, and 10 ng/mL TGF β added 1 hour prior to harvesting cells for Western blotting for phosphorylated SMAD2/3, P38MAPK, c-JUN and AKT. Additionally MRC-5, Het1A, MCF7, and MCF-10A cells lines were transfected in parallel experiments and western blotting was done to probe for phosphorylated SMAD2.

Analysis of the siRNA screen

Data from the siRNA screen was initially analyzed on MS Excel. A quartile-based method was used for HTS hit selection (20). Normalization was done with a control non-targeting siRNA (NSS) placed on each individual assay plate, median (Q2), first (Q1), and third (Q3) quartile values were calculated for all normalized values and subjected to plate-by-plate analysis. To facilitate experiment-wide analysis, Q1, Q2, and Q3 values were calculated as described above. Averages and SE calculations were done for triplicates of $\log(x/\text{median})$ values. Upper and lower boundaries were calculated as $Q3 + 2c(Q3 - Q2)$ and $Q1 - 2c(Q2 - Q1)$, respectively, for $c = 1.7239$, corresponding to a high stringency targeted error rate ($\alpha = 0.0027$; over 7.14 fold activation in A549 cells and over 13.91 fold in MDA-231-1833 cells), and $c = 0.9826$, corresponding to a lower stringency targeted error rate ($\alpha = 0.046$; over 5.4 fold in A549, over 10.29 fold in MDA-231-1833 cells). High stringency hits were chosen as those targets that scored $\alpha = 0.0027$ in both plate-by-plate and experiment-wide analyses. Selected targets distributed equally among all the plates which indicates that our data did not show any “alphabetical clustering” rather agreeing on the sparse-hit hypothesis, which assumes that hits are randomly spaced throughout the dataset. Selected top hits were subjected to Pathway Analysis using Pathway-Guide (Advaita Corp, MI) to identify the pathways that are significantly impacted in the BTR assay. This software uses an impact analysis that includes the classical statistics and also considers the magnitude of each gene's

expression change, their type and position in the given pathways, their interaction etc.(63, 64) Two-way evidence plot, total perturbation accumulation (TA) plot, and pathway-maps were generated by Pathway-Guide. Functional protein association network analysis of the hits was carried out using String (<http://string-db.org/>). Heat-maps of low and high-stringency hits were generated using tMEV(65) to show data-reproducibility.

Western blot analysis

Western analysis was carried out using standard protocols. Cells were grown in culture dishes, transfected with specific siRNA or treated with select compounds for designated time periods, and cell lysates were resolved on SDS-PAGE gels and transferred to PVDF membranes. Membranes were probed against specific primary antibodies followed by HRP conjugated secondary antibodies then visualized using the Enhanced Chemiluminescence (ECL) Western Blotting System (GE Healthcare). Signal intensity was measured using an image processing and analysis program (ImageJ,v1.45) (66).

Coimmunoprecipitation

For co-immunoprecipitation (CO-IP) studies, HEK293T, or A549 cells were transfected with various plasmids. Lysates were made 48 hours post-transfection in native lysis buffer (50mM Tris PH 7.4, 1% NP40, 0.25% Deoxycholate sodium salt, 150mM NaCl, 10% Glycerol, and 1mM EDTA) supplemented with 1X PhosStop (Roche), 1X Protease inhibitor cocktail (Roche), Sodium Ortho Vanadate, Sodium fluoride, PMSF, and β -Glycerol phosphate (2 μ M each). Cell pellet was solubilized in 600 μ l lysis buffer, rocked for 60 min at 4°C and centrifuged at 14000 rpm for 15 min. Protein estimation was performed using detergent compatible Dc assay kit (Pierce). Lysates were pre-cleared by incubating with 50 μ l Agarose beads for 1 hour at 4°C and centrifuged. Co-immunoprecipitation was carried out by incubating pre cleared cell lysate (400 μ g protein) with 1-1.5 μ g specific antibody overnight at 4 °C. The immune complex was captured using 30 μ l slurry of protein A/G-coupled Sepharose beads (GE Healthcare) for 2-3 hours, washed four times with lysis buffer. The resulting pellet was resolved by SDS-PAGE and transferred to PVDF membrane for western-analysis.

SBE4-Luc reporter Assay

For SBE4-Luc reporter studies, 1.25×10^5 cells (A549, NCI-H358, MDA-231-1833, and HeLa) were plated one day prior to transfection. Cells were transfected next day with 100 ng SBE4-Luc plasmid, 100 nM siRNA, 500 ng various DNA and 20 ng Gaussia-Luciferase for each well with Lipofectamine 2000. Cells were serum starved 24 hours post transfection for 6 hours and treated with 10 ng/mL TGF β for 24 hours (or up to 72 hours for A549) before measuring SBE4-Luc bioluminescence activity. Alternately, cells were treated with 10 μ M SB431542 or 2OH-BNPP1 in presence of TGF β . Cells were washed twice with PBS and kept in fresh media for 8 to 24 hours before measuring Gaussia-Luc activity. Relative firefly luciferase activity was normalized to Gaussia luciferase activity.

Cell migration and invasion assay

Multiwell cell culture inserts (8.0 μm pore size, BD Bioscience) were used for in vitro migration assays while Matrigel pre-coated inserts (8.0 μm pore size, BD Bioscience) were used for invasion assays. A549 cells were transfected with 100 nM siRNA, serum starved for 6 hours with 0.5% FBS containing media, treated with 10 ng/mL TGF β or mock for 72 hours, trypsinized, washed and plated in serum free-media in the inserts (25K for migration, 50K for invasion). Media with 5% serum was added in the bottom chamber providing a chemotactic gradient. Plates were incubated for 8 hours (migration assay) or for 24 hours (invasion assay). At the end of the assay, cells were removed from the top side of the insert using a cotton swab. Cells that penetrated to the underside surface were fixed using absolute methanol and stained with 0.5% crystal violet (Sigma) and counted under the microscope. The assay was repeated three times. The mean of three repeats run in triplicates is plotted.

Immunofluorescence

A549 cells were plated on glass cover-slips in 6 well plates and transfected with 100 nM siRNA, serum starved over-night, treated with 10 ng/mL TGF β for 1 hour and processed for immunofluorescence. For the inhibitor studies, cells were plated on glass-cover slips, serum starved for 24 hours and treated with 10 ng/mL TGF β in presence of 10 μM inhibitors (SB431542 or 2OH-BNPP1) for 1 hour. Cells on cover-slips were washed with PBS and fixed in 10% (v/v) buffered-formalin (5 min at room temperature, RT) followed by methanol fixation at -20°C for 20 min. Cover-slips were rehydrated by incubating 3 times in PBS for 5 min and permeabilized in 0.2% (v/v) Triton X100- PBS containing 1% (w/v) BSA on ice for 5 min. Cover slips were blocked by 5% (w/v) BSA, 5% (v/v) goat serum, 10% (v/v) donkey serum in PBS for 1 hour. Anti-SMAD2 antibody at 1:200 dilution was added in PBS containing 0.5% (w/v) BSA and incubated for 1 hour at 37°C in humidified chamber. Cover glasses were washed 3 times in PBS containing 0.025% (v/v) Triton X-100 and incubated with anti-Rabbit-488 secondary antibody for 1 hour. After washing, cover slips were mounted in ProLongGold (Invitrogen) containing DAPI, dried overnight at room temperature and stored at -20°C until microscopy. Micrographs were taken using Olympus BX-51 upright fluorescent microscope fitted with an Olympus DP-70 high resolution digital color camera.

Tumor xenograft experiments and immunohistochemistry

Cell line-derived xenografts were established by implanting 2.5×10^6 A549 cells subcutaneously into each flank of 4-6 week old male SCID mice. When tumors reached a volume between 40 to 60 mm^3 , mice were injected with a single intraperitoneal dose of SB431542 (10 mg/kg body weight), a dose previously reported to inhibit TGF β signaling in mouse models (67), or 2OH-BNPP1 (50 mg/kg), or vehicle (DMSO). Tumors were excised 4 hours after treatment and fixed in formalin. Paraffin-embedded sections were stained using an antibody for phosphorylated SMAD2, and micrographs were taken with an Olympus microscope fitted with an Olympus DP-70 high resolution digital camera. The proportion of nuclei that stained positive for phosphorylated SMAD2 were counted in three random fields per tumor per treatment condition [vehicle (n=4), SB431542 (n=2), and 2OH-BNPP1 (n=5)]. A two-sided Student's *t*-test was performed to assess statistical significance. Slides were

adjusted for brightness and contrast with Adobe Photoshop CS2 (Adobe Inc.), but the micrographs underwent no further manipulations. All mouse experiments were approved by the University Committee on the Use and Care of Animals (UCUCA) of the University of Michigan.

Cell cycle analysis

A549 cells were plated in 6 well plates and transfected with 100 nM siRNA. Cells were trypsinized 72 hours after transfection, counted, resuspended in PBS (50,000 cells/mL), fixed using 50% ethanol, stained with 50 µg/mL propidium iodide supplemented with RNase A (100 µg/mL), and read on a flow cytometer. The mean of at least three experiments is plotted.

TIRF Microscopy

A549 cells were plated on glass cover-slip bottom 35 mm dishes (MatTek) and serum starved next day for 24 hours. Cells were treated with 10 ng/mL TGFβ for 1, 24 or 72 hours and washed with PBS. Cells were fixed in 10% (v/v) buffered-formalin for 5 min at room temperature, followed by methanol fixation at -20°C for 20 min. Cells were rehydrated by incubating 3 times in PBS for 5 min and permeabilized in 0.2% (v/v) Triton X100-PBS containing 1% BSA on ice for 5 min. Cells were blocked using 5% (w/v) BSA, 5% (v/v) goat serum, 10% (v/v) donkey serum in PBS for 1 hour. Primary antibodies at appropriate dilution (anti-TGFBRI 1:500, anti-BUB1 (Goat) 1:35) were added in PBS containing 0.5% (w/v) BSA and incubated for 1 hour at room temperature in a humidified chamber. Plates were washed 3 times in PBS containing 0.025% (v/v) Triton X-100 and incubated with fluorophore labeled secondary antibodies for 1 hour in the dark. After washing, fresh PBS was added into the plates and TIRF microscopy was performed at the SMART Center using a cell-TIRF system based on an Olympus IX81 microscope, equipped with a 60x 1.49 NA oil-immersion objective (Olympus). Samples were placed on a nanometer-precision motorized stage (Princeton Instruments) with xyz-position piezo control. Images were acquired on an Ixon-Ultra EM-CCD camera (512x512 pixels, Andor). Alexa-488 and Alexa-647 dyes conjugated to the secondary antibodies were excited, one after another, by the 488 nm and 640 nm lasers, respectively. Their fluorescence was filtered through a customized quad-band filter cube consisting of a ZT405/488/561/640rpc dichroic filter (Chroma) and a ZET405/488/561/640m emission filter (Chroma). Images of BUB1 (Alexa 488) and TGFBRI (Alexa-647) were processed using a custom compiled ImageJ macro to identify spatially distinct particles and calculate extent of co-localization.

In vitro kinase assay

In vitro kinase assays were performed using 200 ng of protein (BUB1-WT, or a kinase inactive mutant BUB1-KD), and 2 µg of substrate (TGFBRI-WT, TGFBRI-KD, SMAD3 or H2A) in 1X kinase buffer (50 mM Tris-HCl, 150 mM NaCl, 10 mM MgCl₂, 1% (v/v) Glycerol, 0.1% (v/v) Triton X 100, DTT, PMSF, Na₃VO₄ (1 mM each), 2 mM NaF and β-glycerol phosphate) in 20 µL volume containing 10 µCi ³²p-ATP and 300 µM cold ATP. Reactions were run at 30 °C for 0.5-1 hour and quenched using Laemmli buffer and resolved using a 4-12% Bis-Tris gel. Quantitative autoradiography was performed using a Typhoon FLA 9000 scanner (GE Healthcare).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

We thank Steven Kronenberg for help in figure graphics and Tania Cunningham with preparation of the manuscript. We also thank the Microscope & Image analysis Laboratory and DNA sequencing core at the University of Michigan Medical School.

Funding: This work was supported by grants from the National Institute of Health P01CA85878, P50CA093990 and R01CA136892 (BDR and AR), P50CA093990-11 (Career Development Award, SN), as well as NSF MRI-R2-ID award DBI-0959823 (NGW).

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Figure 1. BUB1 mediates TGFβ ligand-dependent SMAD2/3 phosphorylation as well as MAPK and AKT activation
(**A and B**) Plot of the induction of a TGFBR1 reporter (BTR) in A549 cells (**A**) and MDA-231-1833-BTR (**B**) cells transfected with siRNAs targeting human kinases. Red or blue triangles and circles indicate high-stringency (triangles) or low-stringency (circles) hits in either plate-wise and experiment-wise analyses (red) or in experiment-wise analysis only (blue). Data are representative of 3 independent experiments. (**C**) Heat map of low-stringency hits in A549-BTR and MDA-231-1833-BTR cells from triplicate experiments. Gene name in *italics* indicates common hits. (**D**) Western blot analysis of TGFβ effector molecules in A549, NCI-H358, and MDA-231-1833 cells transfected with siRNA against either BUB1 (siBUB1) or TGFBR1 (siTGFBR1) or a scrambled control siRNA (NSS) in the presence of TGFβ ligand for 1 hour. Blots are representative of 3 independent experiments.

Figure 2. BUB1 promotes the recruitment of SMAD3 to TGFBR1, SMAD2/3-SMAD4 complex formation and transcriptional response

(**A and B**) Immunoprecipitation (IP) for the TGFBR1 antibody followed by immunoblotting (IB) for the Flag or His tags in HEK293T (**A**) and A549 (**B**) cells transfected with control siRNA (NSS), TGFBR1 siRNA, or BUB1 siRNA along with Flag-tagged SMAD3 (FL-SMAD3) and 6XHis-tagged TGFBR1 (His-TGFBR1) and treated with TGFβ (1 hour). (**C**) IP for SMAD2/3 followed by blotting for SMAD4 in lysates from A549 cells transfected with control siRNA, TGFBR1 siRNA, or BUB1 siRNA and treated with TGFβ (1 hour). (**D**) Relative firefly luciferase activity (normalized to Gaussia luciferase) after addition of TGFβ (10 ng/mL) and transfection with mock (NSS) or BUB1 siRNA for 24 to 72 hours in A549 cells transiently transfected with a SBE4-Luc reporter and GLuc plasmids. (**E**) Immunoblotting for luciferase and BUB1 in lysates from A549 cells transfected and treated as in (**D**) and harvested 24 hours after TGFβ treatment. (**F to H**) Relative luciferase activity in NCI-H358 (**F**), MDA-231-1833 (**G**), and HeLa (**H**) transfected as in (**D**) and treated with TGFβ (10 ng/mL) for 24 hours. Blots are representative of 3 independent experiments. Data are means ± S.E.M. of 3 independent experiments. **P<0.001, two-sided Student's *t*-test.

tagged BUB1 followed by blotting for TGFBR2 in lysates from HEK293T cells transfected with Myc-BUB1 WT and His-TGFBRI, serum starved, treated with 2OH-BNPP1 or SB431542 (10 μ M, 1 hour) followed by TGF β for an additional hour. (n = 2 independent experiments).

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Figure 5. BUB1 inhibitor blocks TGF β signaling in vivo

A, SCID mice harboring A549 xenografts treated with 50 mg/kg 2OH-BNPP1, 10 mg/kg SB431542 or vehicle control (DMSO), tumor harvested 4h post-treatment, and stained with phosphoSMAD2 antibody. Scale bar 200 μ m. **B**, Quantification of number of cells staining positive for nuclear phosphorylated SMAD2 for control (n=4), SB431542 treated (n=2), and 2OH-BNPP1 treated (n=5) tumors in three random fields for each tumor. Statistical analysis: **p< 0.001 calculated by a two-sided Student's t-test.

Figure 6. A model for a role of BUB1 in TGF β signaling

Based on our findings we propose that BUB1 forms a ternary complex with TGF β RI and TGF β RII. Our data suggest that the interaction of BUB1 with TGF β receptors is enhanced upon TGF β stimulation, requires the kinase activity of BUB1, and promotes stabilization of the heteromeric complex between TGF β RI-TGF β RII, R-SMAD recruitment, and subsequent canonical and non-canonical TGF β signaling cascades.