



Phosphoproteomics identifies potential downstream targets of the integrin $\alpha 2\beta 1$ inhibitor BTT-3033 in prostate stromal cells

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Background: Integrin $\alpha 2\beta 1$ inhibitor BTT-3033 (1-(4-fluorophenyl)-N-methyl-N-[4[[[(phenylamino)carbonyl]amino]phenyl]-1H-pyrazole-4-sulfonamide) was recently reported to inhibit neurogenic and thromboxane A₂-induced human prostate smooth muscle contraction, and thus represents a target with a different inhibition spectrum than that of $\alpha 1$ -blockers in benign prostate hyperplasia (BPH) treatments. Clarifying the underlying mechanisms of the inhibition effects will provide insights into the role of integrin $\alpha 2\beta 1$ in prostate contraction and enable new intracellular targets for smooth muscle contraction to be explored.

Methods: ProteomeHD was used to predict and enrich the top co-regulated proteins of integrin $\alpha 2$ (ITGA2). A phosphoproteomic analysis was conducted on human prostate stromal cells (WPMY-1) treated with 1 or 10 μ M of BTT-3033 or solvent for controls. A clustering analysis was conducted to identify the intracellular targets that were inhibited in a dose-dependent manner. Gene ontology (GO) and annotation enrichments were conducted to examine any functional alterations and identify possible downstream targets. A Kinase-substrate enrichment analysis (KSEA) was conducted to identify kinases-substrate relationships.

Results: Enrichments of the actin cytoskeleton and guanosine triphosphatases (GTPases) signaling were predicted from the co-regulated proteins with ITGA2. LIM domain kinases, including LIM domain and actin-binding 1 (LIMA1), zyxin (ZYG1), and thyroid receptor-interacting protein 6 (TRIP6), which are functionally associated with focal adhesions and the cytoskeleton, were present in the clusters with dose-dependent phosphorylation inhibition pattern. 15 substrates were dose-dependently inhibited according to the KSEA, including polo-like kinase 1 (PLK1), and GTPases signaling proteins, such as disheveled segment polarity protein 2 (DVL2).

Conclusions: In this study, we proposed that the mechanisms underlying the contractile and proliferative effects of integrin $\alpha 2\beta 1$ are the LIM domain kinases, including the ZYG1 family, and substrates, including PLK1 and DVL2.

Keywords: Integrin $\alpha 2\beta 1$; BTT-3033; benign prostatic hyperplasia (BPH); lower urinary tract symptoms (LUTS); phosphoproteomics

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Submitted Jun 09, 2021. Accepted for publication Aug 30, 2021.

doi: 10.21037/atm-21-3194

View this article at: <https://dx.doi.org/10.21037/atm-21-3194>

Introduction

Male lower urinary tract symptoms (LUTS) that are suggestive of benign prostate hyperplasia (BPH) are the result of a mechanical obstruction arising from an enlarged prostate and dynamic factors, such as prostate smooth muscle tone (1). The proliferation of prostate stromal cells has been reported to contribute significantly to BPH, especially symptomatic BPH (2). The tone of the prostate smooth muscle located in the prostatic stroma is also increased in BPH (3). BTT-3033 (1-(4-fluorophenyl)-N-methyl-N-[4[[[(phenylamino)carbonyl]amino]phenyl]-1H-pyrazole-4-sulfonamide) is an integrin $\alpha 2\beta 1$ inhibitor that has recently been shown to inhibit neurogenic and thromboxane A₂-induced prostate smooth muscle contraction in the human prostate at a concentration of 1 μ M, and thus represents a candidate target for BPH treatments with a different inhibition spectrum than those of $\alpha 1$ -blockers (4). It has been suggested that thromboxane A₂ may induce smooth muscle contraction by mechanisms other than endothelin-1 or adrenergic agonists; however, the mechanisms by which this occurs are still far from being understood. Thus, we sought to investigate the mechanisms underlying the contraction inhibition caused by BTT-3033, and determine whether BTT-3033 affects the proliferation of prostate stromal cells.

In mammals, a total of 24 different integrin heterodimers are formed by non-covalent bonds between 18 α -subunits and 8 β -subunits (5). Integrins mediate adhesion between cells and the extracellular matrix (ECM) (5). Distribution of the integrin subtype and corresponding functions is diverse in different organs due to various cell types and the distinct ECM components. This relatively specific feature may be able to provide high selectivity and therapeutic approaches with limited side effects, and thus has promising practical applications. Integrin $\alpha 2\beta 1$, a collagen-I-specific receptor that attaches stromal cells to the ECM, mediates multiple cell functions (6-8). The cytoplasmic part of integrin $\alpha 2\beta 1$ is the center for focal adhesion (FA) (9) assembly, with the assembling partners including the Src family kinase (SFK), focal adhesion kinase (FAK) and integrin-linked kinase (ILK) of which the modifications may affect biological processes (4,10,11). We conducted a preliminary test (data not shown)

that suggested that SFK, FAK, and ILK may not be the downstream targets of BTT-3033 at 1 μ M in the context of contraction inhibition. These preliminary results prompted the consecutive investigations reported in this article. In this study, we investigated the effects of integrin inhibitor BTT-3033 on the proliferation of the prostate stromal cell line WPMY-1, predicted the top co-regulated proteins of integrin $\alpha 2\beta 1$, and conducted a phosphoproteomics analysis to identify the potential mechanisms of effects of BTT-3033 on prostate stromal cells. We present the following article in accordance with the MDAR reporting checklist (available at <https://dx.doi.org/10.21037/atm-21-3194>).

Methods

Cell culture

We purchased WPMY-1 (catalog no. CRL-2854), an immortalized cell line from human prostate stroma, without any evidence of malignancy, from American Type Culture Collection (Manassas, VA, USA). WPMY-1 cells show characteristics of myofibroblasts and prostate smooth muscle cells, including the expression of vimentin, α -smooth muscle actin, calponin and α_{1A} -adrenoceptors, but lack the expression of cytokeratin and tyrosine hydroxylase (12). Consequently, WPMY-1 is commonly used to investigate mechanisms of smooth muscle contraction and cell proliferation (12-14). The WPMY-1 cells were grown in Dulbecco's Modified Eagle Medium (Biological Industries, USA) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin at 37 °C with 5% carbon dioxide. The WPMY-1 cells were randomly grouped and treated with solvent, and BTT-3033 at different concentrations, including 1 μ M in the context of smooth muscle contraction inhibition, and 10 μ M in the context of cell cycle arrest.

Proteins co-regulation prediction

ProteomeHD (15), an online proteome-wide co-regulation analysis tool, was used to identify the proteins that were co-regulated with the $\alpha 2$ integrin subunit (ITGA2) of the proteasome. It should be noted that unlike the $\beta 1$ integrin subunit (ITGB1), ITGA2 only assembles one kind

of integrin heterodimer, i.e. $\alpha 2\beta 1$. Enrichment analysis of KEGG pathways and gene ontology (GO) biological processes was undertaken via proteomeHD using the selected proteins.

AlamarBlue assay

Cell viability was assessed using an AlamarBlue assay. The WPMY-1 cells were seeded into 96-well plates (1,000 cells/100 μ L), and treated with 0.3, 1, 3, or 10 μ M BTT-3033 or dimethyl sulfoxide (DMSO) overnight. An AlamarBlue (Bio-Rad, UK) solution was then added to each well, and the cells were incubated for 3–4 hours at 37 °C. Subsequently, absorbance as an indicator of cell viability was measured with an excitation at 570 nm and an emission at 590 nm using Varioskan (Thermo) luminometer at 0, 24, 48, and 96 h. Readouts were saved as Excel files and transferred into Prism Graphpad (8.4.3) for analysis.

Protein sample preparation

Samples of WPMY-1 from the BTT-3033 low (1 μ M) and high (10 μ M) groups and the control group were lysed in lysis buffer (100 mM NH_4HCO_3 , 6 M urea, and 0.2% SDS, pH =8) with a phosphatase inhibitor (phosSTOP, Roche, 1 tablet per 10 mL). The enrichment of phosphopeptide was carried out using PHOS-SelectTM Iron Affinity Gel (Sigma, P9740). Lyophilized powder was dissolved and centrifuged after which the supernatant was loaded to the IMAC-Fe column, and the collection was centrifuged and eluted twice before being lyophilized.

Liquid chromatography with tandem mass spectrometry

The lyophilized powder was dissolved and centrifuged, after which 1 μ g supernatant was used for detection. Bottom-up proteomics analyses were performed in an EASY-nLCTM 1200 UHPLC system (Thermo Fisher) coupled with an Orbitrap Q Exactive HF-X mass spectrometer (Thermo Fisher) in the data-dependent acquisition mode. The separated peptides were analyzed by Q Exactive HF-X with an ion source of Nanospray FlexTM (ESI).

The identification and quantitation of protein

The resulting spectra from each sample were searched separately against the homo_sapiens_uniprot_2020.07.02.fasta (192283 sequences) database by Proteome Discoverer

2.4 (PD 2.4, Thermo) with false discovery rate (FDR) <1% at the level of proteins and peptides.

Analyses of motif and kinase-substrate relationships

The motif-x algorithm was used to identify the motifs enriched within a set of phosphate sites. All enrichments were carried out for 7 residues surrounding the central residue with occurrences >20 and $P < 10^{-6}$. All identified serine, threonine, and tyrosine phosphorylation sites were scored and calculated using a Kinase-substrate enrichment analysis (KSEA) (16).

Bioinformatic analysis

All the identified proteins were assigned their gene symbol via UniProtKB (<http://www.uniprot.org/>). A heatmap and clustering analysis based on the expression/modification of proteins or phosphorylation sites was conducted and visualized using R (the “pheatmap” package) (17) according to the (phospho-) proteomic assay. A protein-protein interaction (PPI) and enrichments of GO and annotation analyses were performed using STRING (<https://string-db.org/>) (18), and visualized using Cytoscape (Version 3.8.2).

Materials, drugs, and nomenclature

We purchased BTT-3033 (1-(4-fluorophenyl)-N-methyl-N-[4[[[(phenylamino)carbonyl]amino]phenyl]-1H-pyrazole-4-sulfonamide), a small molecule inhibitor with assumed selectivity for integrin $\alpha 2\beta 1$ heterodimers, from Tocris (Bristol, UK). Stock solutions (10 mM) were prepared in DMSO, and stored at –20 °C until used.

Statistical analysis

The quantitation of protein or phosphate sites between three groups were statistically analyzed by Kruskal-Wallis test and posthoc using R. $P < 0.05$ was defined as significance, were used to screen the differentially expressed proteins or differentially modified phosphate sites.

Results

Effects of BTT-3033 on the viability of WPMY-1 cells

To examine the effects of BTT-3033 on stromal proliferation, we assessed the effects of 0.3, 1, 3, 10 μ M

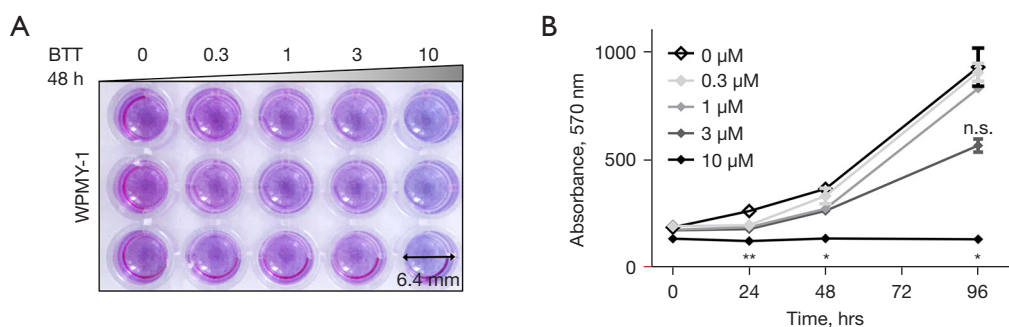


Figure 1 Viability of WPMY-1 cells after BTT-3033 treatment. (A) Representative picture of cell viability by the alamarBlue assay at 48 hrs. alamarBlue fluoresces and changes color in response to chemical reduction to reflect cell viability by the degree of the conversion. When cellular activity is high, alamarBlue is metabolically converted to a pink derivative. (B) WPMY-1 cells were analyzed by an alamarBlue assay at different time points after the application of BTT-3033 (0.3 or 10 μM) or DMSO. Lines with distinct node shapes represent different concentrations of BTT-3033. Ongoing cell proliferation is shown by absorbance at 570 nm. For the time-concentration viability curves, data are presented as mean \pm standard error of the mean (SEM) from $n=3$ experiments for each concentration and timepoint. ** represents significant ($0.001 \leq P$ value < 0.01), * represents significant ($0.01 \leq P$ value < 0.05), and n.s. represents not significant (P value ≥ 0.05).

of BTT-3033 on the viability of WPMY-1 cells using alamarBlue assays (see *Figure 1*). 0.3 and 1 μM of BTT-3033 did not alter the viability of the WPMY-1 cells within 96 h; while 3 μM of BTT-3033 compromised their viability at the 96-h timepoint (although without significance). The cells did not show any sign of proliferation with 10 μM of BTT-3033. Thus, while 1 μM of BTT-3033 inhibited tissue contraction, it did not inhibit proliferation. Conversely, 10 μM BTT-3033, stopped the cell growth. Thus, BTT-3033 was shown to have an inhibitory effect on contraction and proliferation at low (1 μM) and high (10 μM) concentrations, respectively.

Prediction of co-regulated proteins with integrin $\alpha 2\beta 1$

To better understand the mechanism underlying BTT-3033, the possible downstream targets or pathways with relationships to integrin $\alpha 2\beta 1$ were predicted using the bioinformatic tool ProteomeHD (15). The top 100 proteins co-regulated with ITGA2 were identified (see *Table S1*) and enriched in KEGG pathways, and the GO biological processes were analyzed thereafter (see *Figure 2A,2B*). Not surprisingly, integrin $\beta 1$ is among the top co-regulated proteins, along with myosin light chain kinase (MYLK), which is the key kinase to increase smooth muscle tone. The top enriched KEGG pathway and GO biological processes were the “regulation of actin cytoskeleton” and “small GTPase mediated signal transduction,” respectively. Consequently, BTT-3033 appeared to inhibit contraction by mediating alterations of the actin cytoskeleton and

GTPase signaling; however, it should be noted that these findings require further verification.

Effects of 1 and 10 μM of BTT-3033 on protein expression profiles

To verify the prediction and systematically identify real alterations of BTT-3033, a bottom-up proteomic analysis was conducted on WPMY-1 cells incubated with 1 or 10 μM BTT-3033 or DMSO for 6 hours (see *Figure 3A*). The sample quality of the cell lysates was confirmed to be “excellent” (see *Figure S1*). The proteomic clustering analysis recognized proteins with similar expression patterns among three groups and classified them into 6 clusters, which are presented as parallel coordinate plots (see *Figure 3B*). Proteins downregulated by BTT-3033 treatment were allocated into Clusters 2 and 5. The annotation groups in these two enriched clusters were mainly involved in mitosis, cell cycle, cell division, and centrosome (see *Figure 3C*). Notably, proteins associated with integrins or focal adhesion did not exhibit significant changes. Thus, there was only limited evidence that BTT-3033 reduces contraction by affecting protein expression levels.

Protein phosphorylation alterations by 1 and 10 μM of BTT-3033 using phosphoproteomic analysis

Other than protein expression level, phosphorylation is one of the most important posttranslational modifications of proteins that is profoundly involved in the regulation of cell

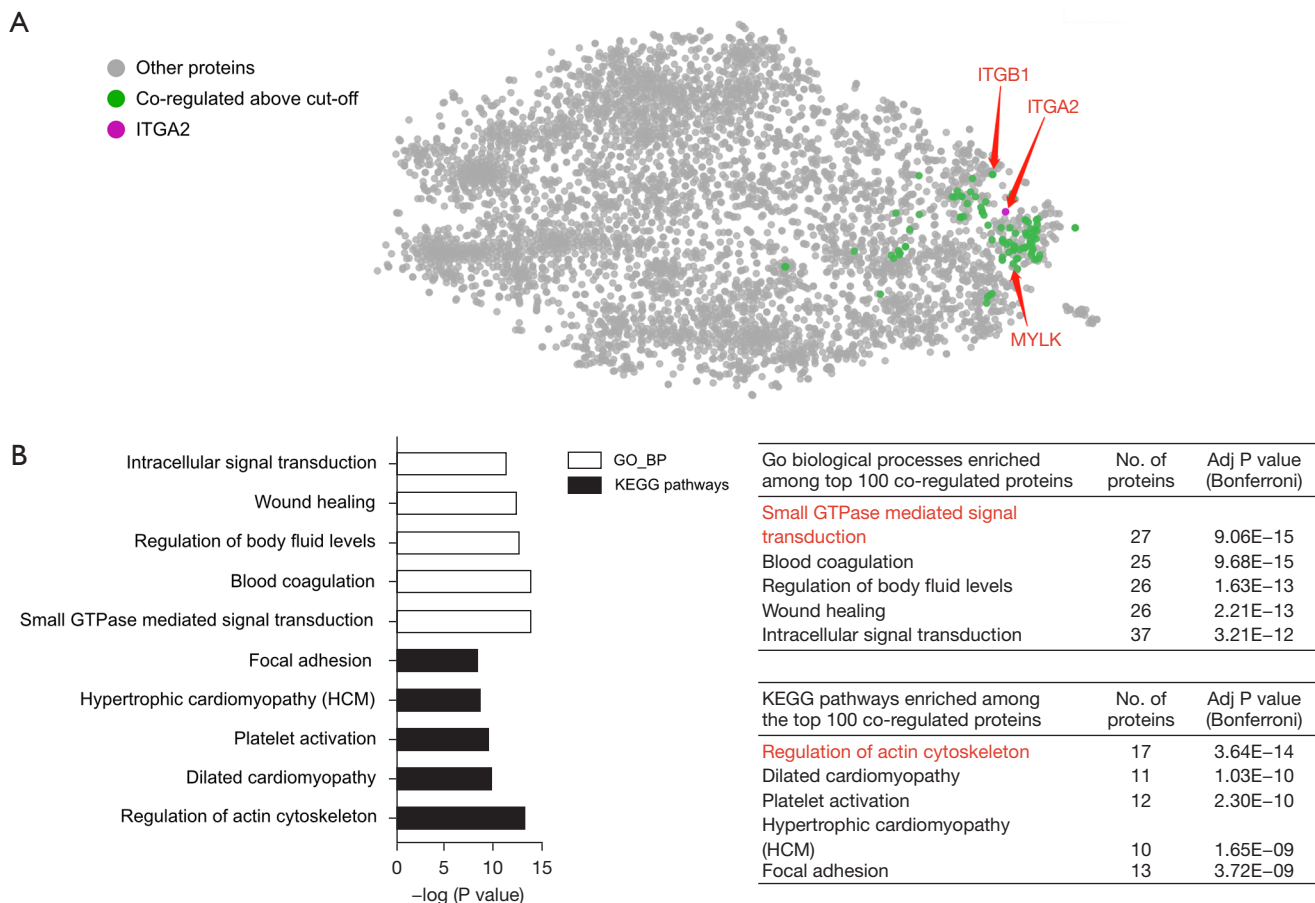


Figure 2 Proteins co-regulated with integrin alpha-2 (ITGA2). (A) Each dot represents a protein in the proteome-wide map. The purple-marked dot represents ITGA2 and the green-marked dots represent the top 100 proteins co-regulated with ITGA2 of the proteasome that were calculated using ProteomeHD (cutoff score: 0.803824). (B) Left: Bar diagram of the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways and gene ontology (GO) biological processes enriched among the top 100 co-regulated proteins. Right: KEGG pathways and GO biological processes enriched tables among the top 100 co-regulated proteins; ranked according to the adjusted P value (Bonferroni).

functions. Thus, the samples used in the proteomic analysis were simultaneously subjected to a phosphoproteomic analysis (see *Figure 4A*). The clustering analysis of the phosphorylated proteomics suggested that inhibited modified phosphate sites could be classified into two clusters (i.e., Clusters 5 and 6; see *Figure 4B*). Proteins with phosphate sites that were inhibited in a dose-dependent manner were classified into Cluster 6 that enriched in GO analysis including “actin binding”, and annotation including “LIM domain” (see *Figure 4C,4D*). Notably, we had previously reported the effects of LIM kinase inhibitors on contractions of human prostate strips and the actin organization of WPMY-1 cells, and LIM kinase inhibitors showed a similar inhibition pattern as that of BTT-3033 (see discussion). In relation to enrichment, LIM domain and actin-binding

protein 1 (LIMA1), thyroid receptor-interacting protein 6 (TRIP6), and zyxin (ZYG) were suggested to be able to functionally connect focal adhesion and cytoskeleton (see *Figure 4E*). Thus, among the downstream proteins of focal adhesion which regulate the cytoskeleton, 3 LIM domain proteins (i.e., LIMA1, TRIP6, and ZYG) were detected to have gradient-inhibited phosphate sites.

Kinase-substrate enrichment analysis

In addition to the individual phosphorylation level, the KSEA used the mean $\log_2(\text{FC})$ of known phosphosite substrates of a given kinase to systematically calculate kinase activity (16). A KSEA was conducted using the phosphoproteomic data, and it was suggested that the kinase

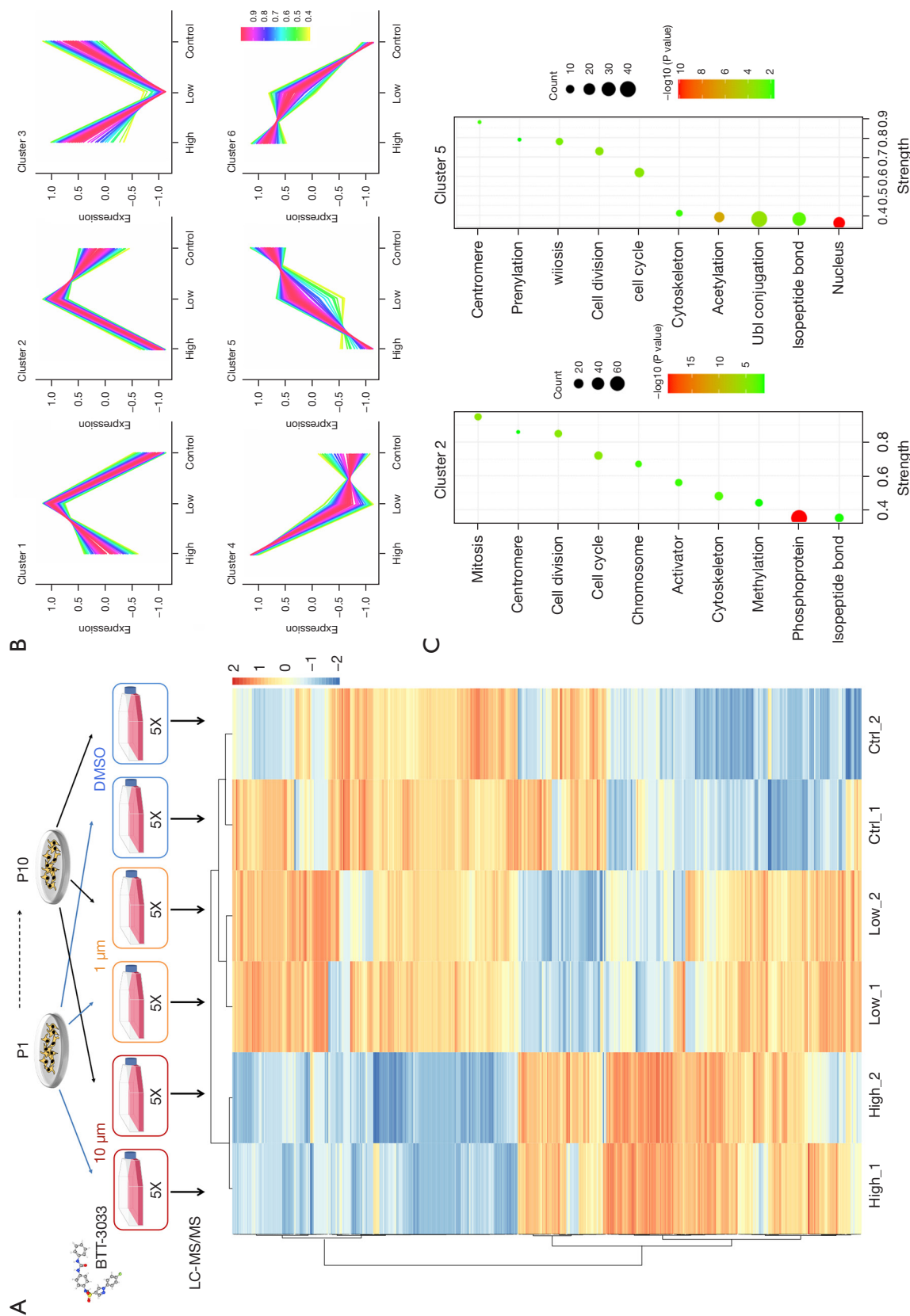


Figure 3 Overall pattern of DEPs of WPMY-1 cells after treatment of BTT-3033. (A) Upper: the workflow of the experimental design: WPMY-1 cells from passenger 1 and passenger 10 were respectively subjected to 10 or 1 μ M of BTT-3033 or dimethyl sulfoxide (DMSO), and a Liquid Chromatography with tandem mass spectrometer (LC-MS/MS) was conducted before the differentially expressed protein (DEP) analysis. Down: Heatmap of the DEPs for the 3 groups (High-1/2; 10 μ M BTT-3033; Low-1/2; 1 μ M BTT-3033, and Ctrl_1/2, DMSO). Upregulated or downregulated proteins are presented in orange and blue, respectively. The color scale representing the Z-score is shown on the right side. (B) Parallel coordinate plots of DEPs after the clustering of 3 groups. Each cluster of DEPs shows a similar expression change in the 3 groups. Clusters 2 and 5 show inhibition patterns. (C) Enrichment analysis of Clusters 2 and 5 according to annotation.

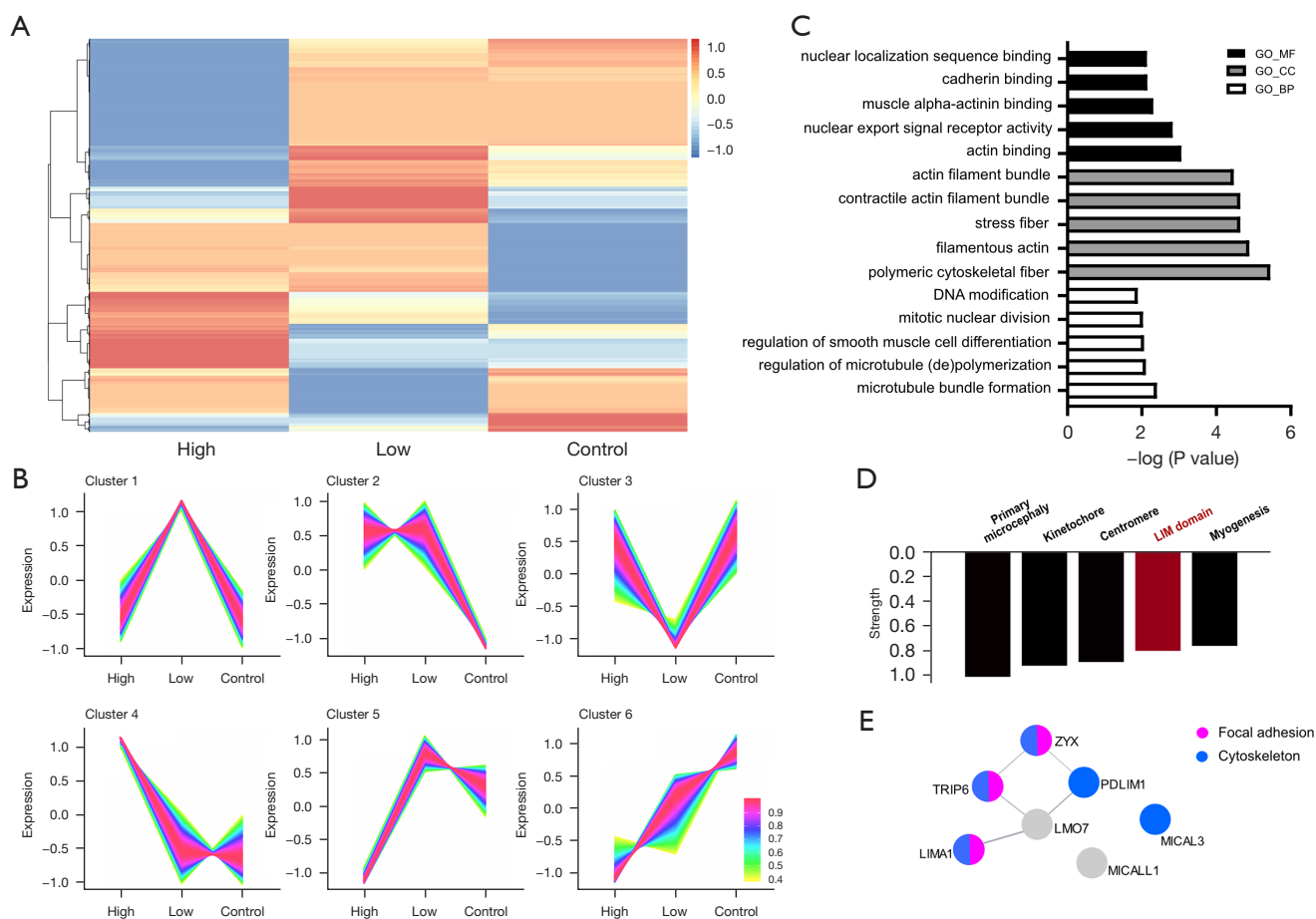


Figure 4 Phosphoproteomic analysis, and GO and annotation enrichment of WPMY-1 cells after BTT-3033 treatment. (A) Heatmap of differentially detected phosphorylated sites for the three groups (high: 10 μ M of BTT-3033; low: 1 μ M of BTT-3033, and Ctrl: DMSO). Upregulated or downregulated phospho-sites are presented in red and blue, respectively. The color scale representing the Z-score is shown on the right side. (B) Parallel coordinate plots of DEPs after the clustering of three groups. Each cluster of the DEPs shows a similar expression change in the three groups. Cluster 6 shows a gradient inhibition pattern. (C) Gene ontology (GO) enrichment of phosphoproteomic results. Black—GO enrichment of molecular function. Grey—GO enrichment of cellular component. White—GO enrichment of biological process. (D) Annotation enrichment of Cluster 6. (E) PPI analysis for the "LIM domain" enrichment. Violet dots and blue dots represent proteins for focal adhesion and the cytoskeleton, respectively.

substrates in the GSK3, ACTR2/ACTR2B/TGF β R2, TTK, AuroraA, and CDK2/CDK3/CDK1/CDK5 groups were deactivated by BTT-3033 (starting at 1 μ M) (see *Figure 5A*). Specifically, disks large homolog 1 (DLG1), cell division cycle 25B (CDC25B), disheveled segment polarity protein 2 (DVL2), myocyte-specific enhancer factor 2A (MEF2A), GLI family zinc finger 2 (GLI2), ABL proto-oncogene 1 (ABL1), centrosomal protein of 192 kDa (CEP192), nuclear factor of activated T-cells 5 (NFAT5), nucleophosmin (NPM1), polo-like kinase 1 (PLK1), E3 ubiquitin-protein ligase NEDD4-like (NEDD4L), nuclear mitotic apparatus

protein 1 (NUMA1), brain-specific angiogenesis inhibitor 1-associated protein 2 (BAIAP2), sperm-associated antigen 5 (SPAG5), and serine/threonine-protein kinase N2 (PKN2) are the substrates for which BTT-3033 gradually inhibits phosphorylation (see *Figure 5B*). A String network showed their correlations, and enrichment occurred in the Rho GTPase binding, cytoskeletal protein binding, and cell cycle (see *Figure 5C*). Notably, the PPI analysis showed that DVL2, a protein in the Rho GTPase signaling pathway, was able to establish a connection between cytoskeletal pathway proteins PLK1 and ABL1 (see discussion).

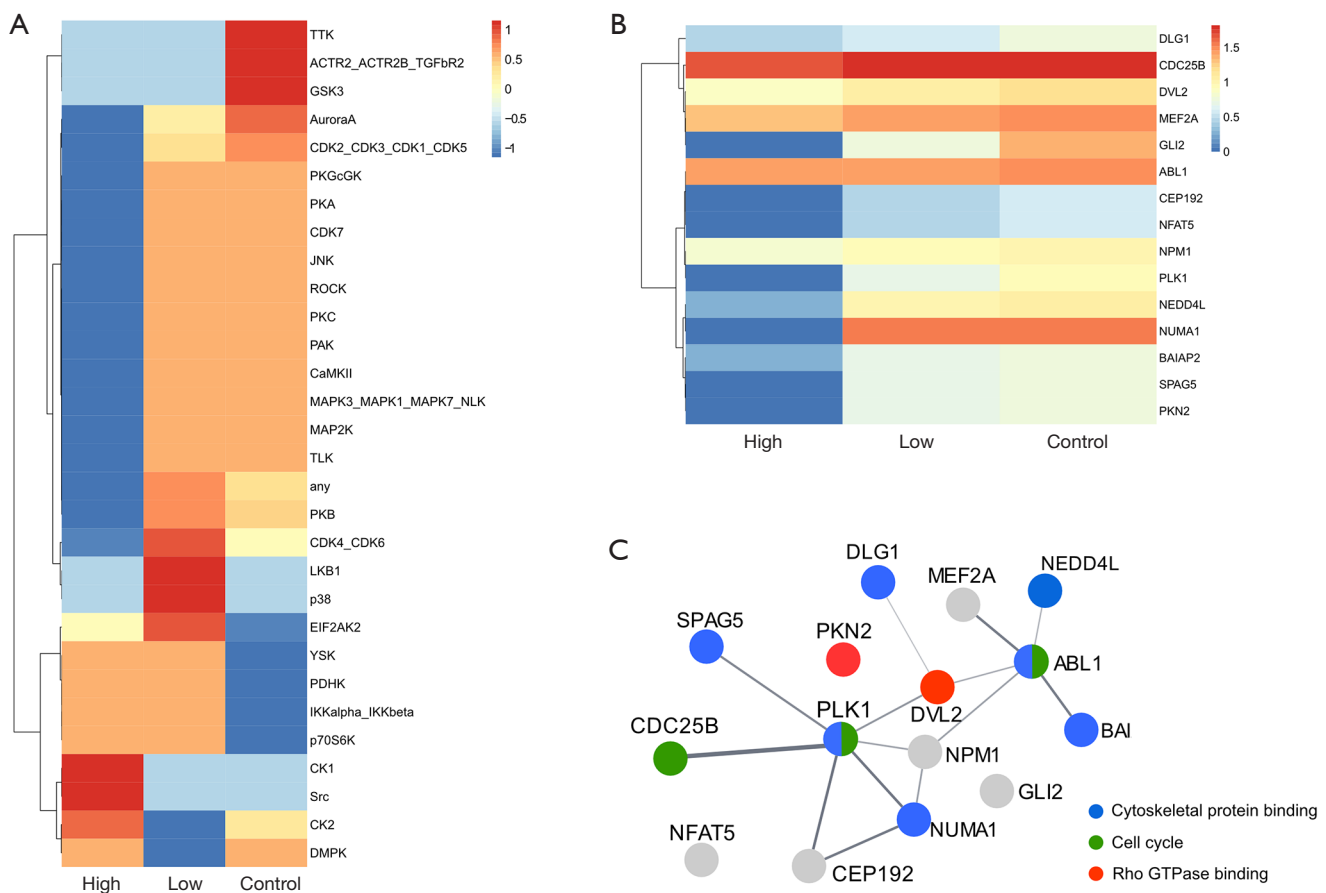


Figure 5 The KSEA and PPI analysis of substrates were predicted to be gradually inhibited by BTT-3033. (A) Alterations of kinase-substrate relationships in categories according to kinase-substrate enrichment analysis (KSEA). (B) The individual substrates predicted to be gradient-inhibited by BTT-3033. (C) PPI analysis of gradient-inhibited substrates. The red dots are substrates for Rho guanosine triphosphatase (GTPase) binding; the blue dots are substrates for cytoskeletal protein binding; the green dots are substrates for the cell cycle.

Discussion

In our previous study, we showed that 1 μ M of BTT-3033 (an integrin $\alpha 2\beta 1$ inhibitor) inhibited neurogenic and thromboxane A_2 -induced contractions of human prostate strips, but did not affect endothelin-1-induced contraction and limited the inhibition of contractions by adrenergic agonists (4). In that study, the cytotoxicity tests suggested that the effect of 1 μ M of BTT-3033 was reversible after its removal. This is in line with the cell viability assay in this study, which showed that 1 μ M of BTT-3033 did not compromise the viability of WPMY-1 cells. However, 10 μ M of BTT-3033 blocked the cell cycle. It is not surprising that an inhibitor in a higher concentration can inhibit proliferation given that it can suppress contraction,

and that functions related to cytoskeleton motility are fundamental to most cell processes, including mitosis and ribosome action (19–21). Proliferation inhibition is a molecular strategy for reducing stromal growth in the treatment of BPH, and if a potential medication component could be identified that affects contraction and proliferation at different concentrations, more accurate functional modulation could result. 10 μ M of BTT-3033, however, has been reported to have effects on platelet function, cardiac ventricular restriction, articular cartilage organization, and other biological processes; however, 1 μ M of BTT-3033 is less likely to have side effects on other cells (6,22–24). Consequently, the mechanisms by which BTT-3033 affects prostate contraction are the main focus of this discussion.

The cytoplasmic parts of integrins form FA complexes

with other FA components. FAs are dynamic attachment points for the cytoskeleton at the membrane, mediate cytoskeletal organization, and thus regulate contractility (4). SFK, FAK, and ILK are among the most investigated kinases, are structurally or functionally linked to the FA complex, and have all been shown to be involved in the contraction regulation of prostate tissues (4,25,26). However, our preliminary test (data not shown) indicated their expressions and phosphorylation were unaffected by 1 μ M of BTT-3033. As these results were inconsistent with our previous assumption, subsequent investigations were conducted. Specifically, we used proteomeHD to predict the top proteins co-regulated with ITGA2. Unsurprisingly, ITGB1 and MYLK were among the top co-regulated proteins, and the “regulation of actin cytoskeleton” was the leading KEGG pathway enrichment, which supported our previous findings on the effects of the integrin α 2 β 1 inhibitor BTT-3033. Conversely, “small GTPase mediated signal transduction” was the leading GO biological processes, which suggests that BTT-3033 inhibits contraction by mediating alterations of the actin cytoskeleton and GTPase signaling. Overall, proteomeHD reflects protein co-regulation patterns, but does not focus on specific cells or conditions. Thus, to better understand how BTT-3033 inhibits the contraction of prostate strips, a bottom-up (phospho-)proteomic analysis was conducted to explore possible downstream targets.

In relation to contraction inhibition, 1 μ M of BTT-3033 was of greater interest than 10 μ M of BTT-3033 in the context of growth impediment. However, we still conducted an analysis of three groups (i.e., the control group, the 1 μ M BTT-3033 group and the 10 μ M BTT-3033 group) for two reasons. First, it was thought that synchronously downregulated proteins/phosphate sites in both the 1 and 10 μ M BTT-3033 groups could corroborate each other and improve specificity. Second, it was thought proteins/sites gradually downregulated by gradient BTT-3033 could be the most direct downstream targets and could connect contractivity with the cell cycle.

According to the cluster analysis of the phosphoproteomics, several enrichments are in line with patterns of synchronous and gradual inhibition. Among these, the “LIM domain” caught our attention, as we had previously reported that the inhibitors for LIM kinases reduced human prostate smooth muscle contraction *ex vivo* (the human prostate strip) and *in vitro* (WPMY-1 cells) (14). Additionally, LIMA1, ZYX, and TRIP6 were shown to be related not only to the “cytoskeleton,” which is essential to contractivity, but

also to “focal adhesion,” which is more directly affected by BTT-3033. We assumed that LIMA1, ZYX, and TRIP6, as downstream molecules of integrin α 2 β 1 and FAs, regulate contractivity by modulating the cytoskeleton. The LIMA1 protein consists of two actin-binding domains separated by a central LIM domain. LIMA1 has been shown to be regulated by focal adhesion complex (9) and to modulate cell dynamics by spatiotemporally interacting with actin (27).

ZYX is a zinc-finger protein with a dual function. An N-terminal domain mediates its association to focal adhesions, and a C-terminal LIM domain mediates protein-protein and/or protein-deoxyribonucleic acid interactions. ZYX coordinates the organization of actin filaments at focal adhesions, and modulates gene expression in vascular smooth muscle cells by translocating to the nucleus (28). TRIP6, also known as ZYX-related protein-1 (ZRP-1), is an adaptor protein that belongs to the ZYX family of LIM proteins. TRIP6 is primarily localized in the cytosol or focal adhesion plaques, and may be associated with the actin cytoskeleton. Additionally, it is capable of shuttling to the nucleus to serve as a transcriptional coregulator (29).

LIMA1, ZYX, and TRIP6 have not been reported to mediate prostate contraction. LIM kinase 1 and 2 were not (technically) detected in the phosphoproteomic analysis. However, the inhibition pattern of the LIM kinase 1 and 2 inhibitors shares some common ground with that of BTT-3033 in that U46619-induced contractions are most susceptible to the inhibitors and endothelin-1-induced contractions are not affected at all. The divergent susceptibility of endothelin-1- and U46619-induced contractions to inhibitors such as BTT-3033 has not yet been well explained. As previously discussed, vascular smooth muscle contractions induced by different agonists may be mediated by different couplings of G protein-coupled receptors to different intracellular effectors. Thus, thromboxane receptors were supposed to predominantly couple with and activate $G_{\alpha_{12/13}}$ proteins, while endothelin receptors were supposed to predominantly activate G_{α_q} (or G_{α_q} in addition to $G_{\alpha_{12/13}}$). $G_{\alpha_{12/13}}$ activation leads to contraction (mainly via the small GTPase RhoA/Rho kinase pathway), and G_{α_q} activation mainly occurs in a Ca^{2+} -dependent manner. Further research needs to be conducted to show whether these processes are robust and also applies to prostate smooth muscles. Interestingly, the Rho GTPases pathway may partly support this process, as several types of Rho kinases have been shown to affect smooth muscle contraction in the prostate or in other organs, and these are mainly mediated by $G_{\alpha_{12/13}}$ and independent Ca^{2+} .

Notably, the KSEA showed that PLK1 was the substrate that gradually inhibited phosphate sites by BTT-3033. It has recently been reported that the PLK1 inhibitor onvansertib inhibits proliferation and adrenergic contraction in the prostate; however, a different spectrum to BTT-3033 was used (30). The relationships of PLK1 and Rho GTPases raised our interest for the following reasons: (I) A recent systematic study revealed that focal adhesion is the crucial place for complicated GTPases regulation (31), and RhoA and Rac have been shown to mediate prostate contraction, while various other Rho GTPases have been shown to be related to smooth muscle contractions in different organs (32); and (II) PLK1 can recruit RhoA guanine nucleotide-exchange factor (GEF) Ect2, and MyoGEF, and regulate RhoA activity (33,34), while the Rac/CDC42 downstream of integrin can activate PAK1, LIM kinases, and Aurora A/PLK1 (35,36). The upstream molecules of PLK1 are not yet known; however, the later also has a withdraw that the results of the (phospho-)proteomic analysis showed that BTT-3033 did not alter the expression or phosphorylation of PAK1.

KSEA suggested that DVL2 and PKN2 were also substrates which were gradually inhibited, and they were found to be related to GTPases in a more direct way. Previously reports showed that DVL2 enhanced the activation of the Rho family GTPases (37,38), and subsequently mediated various cell dynamic functions. DVL2, which is a downstream target of ABL1 (39), formed a complex with PLK1 (40), and consequently connected these two center nodes shown in the PPI analysis. PKN2 is a Rho/Rac effector protein that participates in the regulation of cell cycle progression, actin cytoskeleton assembly, cell migration, cell adhesion, tumor cell invasion and transcription (41-43).

In summary, the network of integrin and LIM domain kinases (especially the ZYX family, PLK1, and kinases, such as DVL2 that are involved in GTPases signaling) are interesting and promising in the context of smooth muscle contraction and proliferation, which are clinically highly relevant in the context of BPH and require further exploration.

The main limitation of this study is related to its sample numbers, as 6 samples may not be sufficient to avoid sampling bias or technique bias. However, any presented value was based on detection (0 means being detectable, but the total detection was very close to 0 instead of being not detected). Further, each sample was prepared by mixing 5 technical duplicates. However, three groups

(each comprising 2 samples) with gradient concentration changes may have better selectivity than two groups (each comprising 3 samples). If we had selected the proteins/sites from the intersection of significant inhibitions, and compared a BTT high group to a BTT low group, and a BTT low group to a control group, the results would have been more convincing even with the limited sample numbers. However, as we elected to sacrifice sensitivity to improve the selectivity of the suggested targets, this study may have failed to detect some of the downstream targets.

Conclusions

Based on the phosphoproteomic analysis, our findings identified some novel candidate effectors that are involved in the BTT3033-sensitive regulation of prostate smooth muscle contraction and stromal growth. Given the involvement of both processes in LUTS suggestive of BPH, these effectors have high clinical relevance. Future research should be conducted on the contraction effects of integrin $\alpha 2\beta 1$ and should focus on LIM domain kinases, including the ZYX family, and PLK1 or GTPase-related proteins, such as DVL2.

Acknowledgments

Funding: This study was supported by grants from the National Natural Science Foundation of China [grant number 81770693], the Deutsche Forschungsgemeinschaft [grant number HE 5825/8-1], the Postdoctoral International Exchange Program of China Postdoc Council (OCPC), and the Chinese Scholarship Council [grant number 201706370083 (BL) and 201706270197 (PL)].

Footnote

Reporting Checklist: The authors have completed the MDAR reporting checklist. Available at <https://dx.doi.org/10.21037/atm-21-3194>

Data Sharing Statement: Available at <https://dx.doi.org/10.21037/atm-21-3194>

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at <https://dx.doi.org/10.21037/atm-21-3194>). BL, BZ, ZC, YH and XC report funding support from the National Natural Science Foundation of China (the payments were made to the

institution); BL, QY, RH, RW, YL, and MH report funding support from the Deutsche Forschungsgemeinschaft (the payments were made to the institution); BL and PL report personal funding support from the Chinese Scholarship Council. The other authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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- (English Language Editor: L. Huleatt)

Cite this article as: Li B, Li P, Xia W, You B, Yu Q, Zhang B, Huang R, Wang R, Liu Y, Chen Z, Gan Y, He Y, Hennenberg M, Stief CG, Chen X. Phosphoproteomics identifies potential downstream targets of the integrin $\alpha 2\beta 1$ inhibitor BTT-3033 in prostate stromal cells. *Ann Transl Med* 2021;9(17):1380. doi: 10.21037/atm-21-3194