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The small molecule BC-2059 inhibits Wnt-dependent gene transcription in cancer through disruption of the Transducin Beta-Like 1 (TBL1)-β-catenin protein complex

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Running Title: Mechanism of Wnt pathway inhibition by BC-2059 in cancer

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Abbreviations: AML, Acute myelogenous leukemia; APC, adenomatous polyposis coli; AXIN2, axin-like protein; CETSA, In cell thermal shift; GPS2, G protein pathway suppressor 2; MYC, MYC Proto-Oncogene, NCoR1, Nuclear receptor co-repressor 1; NFκB, nuclear factor

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kappa B; SIAH1, Seven In Absentia Homolog 1. SIP, SIAH1-interacting protein; SMRT, Nuclear receptor co-repressor 2; TBL1, transducin β-like 1; TBLR1, TBL receptor 1; TBLR1, TBL1 Related Protein 1; TCF7L2/LEF, Transcription Factor 7-Like 2 /lymphoid enhancer factor; WRE, Wnt-responsive element

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Abstract

The central role of β-catenin in the Wnt pathway makes it an attractive therapeutic target for cancers driven by aberrant Wnt signaling. We recently developed a small molecule inhibitor, BC-2059, that promotes apoptosis by disrupting the β-catenin /transducin β-like 1 (TBL1) complex through an unknown mechanism of action. In this study, we show that BC-2059 directly interacts with high affinity for TBL1 when in complex with β-catenin. We identified two amino acids in a hydrophobic pocket of TBL1 that are required for binding with β-catenin, and computational modeling predicted that BC-2059 interacts at the same hydrophobic pocket. Although this pocket in TBL1 is involved in binding with NCoR/SMRT complex members GSP2 and SMRT and p65 NFkB subunit, BC-2059 failed to disrupt the interaction of TBL1 with either NCoR/SMRT or NFkB. Together, our results show that BC-2059 selectively targets TBL1/β-catenin protein complex, suggesting BC-2059 as a therapeutic for tumors with deregulated Wnt signaling pathway.

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Significance Statement

This study reports the mechanism of action of a novel Wnt pathway inhibitor, characterizing the selective disruption of the TBL1/β-catenin protein complex. As Wnt signaling is dysregulated across cancer types, this study suggests BC-2059 has the potential to benefit patients with tumors reliant on this pathway.

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Introduction

The Wnt signaling pathway and its downstream transcriptional activator β-catenin are involved in critical cellular processes including embryogenesis, cell proliferation, cell fate, adult stem cells differentiation, and oncogenesis (Logan and Nusse, 2004; Willert and Jones, 2006; Chien et al., 2009; Clevers and Nusse, 2012; Polakis, 2012; Saito-Diaz et al., 2013). Mutations in β-catenin or its regulatory factors, such as APC or AXIN2/conductin, result in aberrant activation of the Wnt signaling pathway and are found in a variety of human cancers including breast, colorectal, ovarian, lung, melanoma, prostate, adrenal carcinoma, and leukemias (Clevers and Nusse, 2012; Polakis, 2012). *APC* or *AXIN2* mutations lead to disruption of the β-catenin degradation complex and, consequently accumulation of β-catenin in the nucleus promoting the transcriptional expression of Wnt target genes (Korinek et al., 1997; Morin et al., 1997; Angers and Moon, 2009). Mutations of serine residues involved in ubiquitination of β-catenin show a similar outcome (Morin et al., 1997).

In the absence of Wnt-signaling, β-catenin is recognized by the ubiquitin/proteasome machinery and degraded in the cytoplasm (Stamos and Weis, 2013). In contrast, active Wnt-signaling leads to β-catenin association with Transducin Beta-like protein 1 (TBL1) and Transducin Beta-like related protein 1 (TBLR1), with subsequent translocation to the nucleus where it drives transcription of Wnt-regulated genes (Choi et al., 2011). Recent studies showed interaction of β-catenin with TBL1 protein family is critical for protecting β-catenin from ubiquitin-mediated degradation (Dimitrova et al., 2010) and Wnt/β-catenin-mediated transcription (Li and Wang, 2008).

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TBL1 and its highly homologous family member TBLR1 are F-box/WD-40 repeat containing scaffold proteins that associate with members of coactivator or corepressor complexes including NCoR/SMRT and NFκB through its N-terminus hydrophobic pockets (Li and Wang, 2008; Perissi et al., 2008). In response to Wnt pathway activation, TBL1 and TBLR1 are SUMOylated and disassociated from the NCoR/SMRT corepressor complex. TBL1/TBLR1 subsequently binds β-catenin and together translocate to the nucleus (Choi et al., 2011). The direct, physical interaction between TBL1/TBLR1 and β-catenin (Li and Wang, 2008) inhibits SIAH1-mediated polyubiquination and subsequent β-catenin degradation in the nucleus (Dimitrova et al., 2010). Depletion of TBL1/TBLR1 significantly inhibits Wnt/β-catenin-induced gene expression and blocks growth of tumor cells *in vitro* and *in vivo* (Li and Wang, 2008). Last, TBL1, but not TBLR1, interacts with TCF7L2, an interaction enhanced upon Wnt activation (Li and Wang, 2008), suggesting that the formation of TBL1/β-catenin complex is a key regulatory point downstream of Wnt pathway activation (Choi et al., 2011).

The development of an inhibitor targeting the TBL1-β-catenin complex may improve the conventional treatment of several cancers that harbor activating mutations in the Wnt pathway (Jung and Park, 2020). Currently, most active compounds targeting the Wnt-pathway work by inhibition of Wnt signaling at the receptor level, or inhibition of other cytoplasmic members of the Wnt pathway including tankyrase and porcupine, or by stimulating the activity of a β-catenin destruction complex (He et al., 2004; Mikami et al., 2005; Grandy et al., 2009; Wang et al., 2013; Koo et al., 2015; Arques et al., 2016; Wu et al., 2016; Zhong et al., 2016). Direct targeting of β-catenin has been difficult largely due to the lack of selective high affinity binding sites (Luu et al., 2004; Grossmann et al., 2012). We recently developed a small molecule inhibitor, BC-2059 (Tegavivint, Iterion Therapeutics), which potently inhibits the Wnt-signaling pathway *in*

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vitro and *in vivo* (Soldi et al., 2015). This new compound, an anthracene-9, 10-dione dioxime, inhibited the growth of several cancer cell lines *in vitro*, and promoted the reduction of nuclear β-catenin, resulting in the inhibition of Wnt-mediated gene expression (Soldi et al., 2015). Further, BC-2059 treatment inhibited colon tumor growth *in vivo*, suggesting BC-2059 as a candidate for treatment of a wide spectrum of epithelial and hematological malignancies dependent on Wnt pathway (Soldi et al., 2015). In this study, we further investigate the mechanism of action of BC-2059, demonstrating direct binding in the N-terminus hydrophobic pocket of TBL1 and TBLR1, which are involved in the complex formation with β-catenin, NCoR/SMRT or NFκB. In addition, we show that BC-2059 preferentially promotes the displacement of β-catenin but not the other complex partners of TBL1.

Materials and Methods

Cell culture, plasmids, bacterial protein expression and purification

Human colon cancer cell line HCT15 (ATCC), STF3A (generously donated by Dr. David Virshup, Duke NUS Graduate Medical School), Notch CSL reporter HEK-293, GLI reporter NIH3T3, TEAD Reporter MCF7 cell lines (BPS Bioscience), H2030 (ATCC), COR-L95 (Millipore Sigma) were cultured in RPMI (Invitrogen) containing 10% fetal bovine serum (FBS) (GIBCO) and antibiotics (penicillin and streptomycin, Invitrogen) at 37°C in a 5% CO2 condition. Human full-length TBL1 cDNA (Origene) and the PCR produced N-terminus region of TBL1 (1-90) peptide were subcloned into pET151D plasmid for bacterial expression (Invitrogen Life Technologies). Human full-length β-catenin cDNA (Origene) and the PCR produced armadillo domain (AD) of β-catenin (residues 133-467) were subcloned into pGEX-

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PP-GST plasmid (Invitrogen Life Technologies). Both plasmids contain a precision protease cleavage site after the HIS or GST tag. Recombinant proteins were expressed in E. coli BL21 (DE3) star strain (Invitrogen Life Technologies). Bacteria was grown at 37°C in ZYP-5052 media (Studier, 2005) until they reached A600 nm of 1.0 and then the temperature was lowered to 30°C to facilitate the auto-induction for 16 hours. Purification of His-tagged and GST-tagged proteins was performed by HisTrap FF and GSTrap FF column, respectively (GE Life Science). Affinity purification was followed by ion exchange (Mono S/ Mono Q, GE Life Science) and size exclusion chromatography (GE Life Science). Protein purity was assessed in SDS-PAGE coomassie stain. N-terminus region of TBL1 (55-142) and TBLR1 (1-90) peptide (TBL1_90NT) was sub-cloned into pcDNA4HISMAX plasmid for mammalian expression (Invitrogen Life Technologies). Recombinant GSP2 protein was purchased from Novus Bio. The TBL1_90NT was mutated using a QuickChange site-directed mutagenesis kit (Stratagene) to generate TBL1_90NT.V60, TBL1_90NT.Ile39, and TBL1_90NT.V60.Ile39. The choice of Val60 and Ile39 was based on the in silico optimal docking analysis results. Mutations of the amino acid residues V60L and I39F were created to affect the binding without causing a change in the non-polar character of the pockets. Primer design in Supporting Information Table 1. PCR primer design for mutagenesis and validation is available free of charge via http://pubs.acs.org.

Saturation and competition binding assay with biotinylated BC-2059

For the saturation binding assay, a high affinity binding ELISA 96-well plate (Maxisorp, Nunc) was coated with an antibody against TBL1 (Santa Cruz Biotech) or β-catenin (Cell Signaling Technologies) diluted to a final concentration of 2.0 µg/mL in carbonate/bicarbonate coating buffer. Sealed plate was incubated overnight at 4°C. Next day, the plate was washed 3 times with PBS containing 0.05% Tween-20 and blocked for 1 hour at room temperature (RT) in PBS

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containing 1% BSA. 1 μg/mL of full-length recombinant human TBL1 or full-length recombinant human β-catenin diluted in binding buffer (Tris HCl 50 mM, pH 7.5, NaCl 250 mM, MgCl 10 mM) were added and the sealed plate was incubated at RT for 2 h. After washing the plate 3 times with PBS containing 0.05% Tween-20, 100 μL of biotinylated BC-2059 was added at increasing concentration (0.1 nM to 10 μM) and incubated for 2 hours at RT. Following washes, the bound compound was detected by streptavidin-HRP conjugated antibody (Cell Signaling Technologies). ELISA was developed by addition of ABTS HRP substrate (SIGMA) and the optical density (OD) for each well was read with a microplate reader set to 405 nm. For homologous competitive assay, a single concentration of 50 nM of biotinylated BC-2059 (hot) was added to the wells containing full length TBL1 recombinant protein or TBL1_90NT (aa 1-90 of the N-terminus of the protein) in the presence of various concentrations of BC-2059 (cold, non-biotinylated; range of 0.1 nM to 10 μM) diluted in binding buffer. The sealed plate was incubated for 2 hours at RT and then processed as described above. TBL1 and TBL1_90NT/β-catenin saturation assay was performed as described above. High affinity binding ELISA 96-well plate coated with antibody against TBL1 was incubated with 1 μg/mL of full-length recombinant human TBL1 or TBL1_90NT peptide diluted in binding buffer (Tris HCl 50 mM, pH 7.5, NaCl 250 mM, MgCl 10 mM) at RT for 2 hours. After washing the plate 3 times with PBS containing 0.05% Tween-20, 100 μL of β-catenin was added at increasing concentration (0.1 nM to 10 μM) and incubated for 2 hours at RT. The bound protein was detected by anti-β-catenin antibody (Cell Signaling Technologies) followed by secondary HRP-conjugated antibody (SIGMA). For the heterologous competitive binding assay, 75 nM of β-catenin (residues 133-467) or GSP2 (full length) was used in place of the biotinylated BC-2059 and the bound protein was detected by

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anti-β-catenin antibody or anti-GPS2 antibody (Cell Signaling Technologies) followed by secondary HRP-conjugated antibody (SIGMA).

Sandwich ELISA

HCT15 cells were seeded at 70% confluency in RPMI 10% FBS (Gibco) in 100 mm tissue culture treated plates (Costar). After 24 hours, the culture media was changed and BC-2059 was added at increasing concentration (0.3 nM to 3 μM) and incubated for 6 h at 37°C. The cells were lysed in RIPA buffer (SIGMA) containing protease inhibitors cocktail (SIGMA) and the total protein concentration was determined by Quick Start Bradford Protein assay kit (Bio-Rad). To prepare the high affinity binding Sandwich ELISA plate, the 96-well Maxisorp (NUNC) was coated with 100 μL of unlabeled capture TBL1 antibody (Santa Cruz Biotech) diluted to a final concentration of 2 μg/mL in carbonate/bicarbonate coating buffer as described before. 100 μL of cell lysates were added to each well containing TBL1 antibody to capture TBL1/β-catenin complex. To determine the amount of β-catenin captured, we prepared a standard curve with recombinant β-catenin (100 fg to 100 ng), diluted in carbonate/bicarbonate coating buffer. The recombinant protein as well as cellular β-catenin from the lysate was detected by antibody against β-catenin (Cell Signaling Technologies) and secondary antibody HRP-conjugated (Sigma) diluted in the blocking buffer. ELISA was developed as described above. One-way ANOVA analysis of BC-2059 treated cells versus untreated cells was performed to assess significant difference ($P < 0.05$).

Co-immunoprecipitation and western blot analysis

Nuclear extracts were prepared from HCT15 cells using the Epigentek nuclear extraction kit (catalog no. OP-0002-1) according to the manufacturer's protocol. The total protein

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concentration was evaluated using Bradford (Bio-Rad) and the supernatants were incubated with antibodies (1-5 µg/mL) overnight at 4°C, followed by incubation with Protein A/G Sepharose (GE Healthcare) for 1 hour at 4°C. Samples were washed three times with RIPA buffer (SIGMA) and the bound proteins were eluted with SDS loading buffer at 98°C for 10 min. Samples were resolved in 4-12% SDS-PAGE (Invitrogen). Western blot analysis was performed using an enhanced chemiluminescence reagent (Thermo Scientific) or an Odyssey Imager (Li-Cor Biosciences). All antibodies used are commercially available: TBL1 (Abcam), β-catenin (Santa Cruz Biotech.), SIAH Interacting Protein antibody (Abcam), GPS2 (Cell Signaling Technologis), and p65 NFκB (Cell Signaling Technologies).

Cell viability assay

CH2030 and Cor-L95 cells were seeded in 96-well plates in triplicate at a density of 500–2000 cells per well depending on the growth curve of each cell line. After 24 hours, cells were treated with DMSO, and BC-2059 at increasing concentrations (0.001 to 10 µM). Cell viability was assessed with CellTiter-Glo (Promega) 72 hours after treatment and IC50 were calculated using GraphPad Prism v. 8.0. Absorbance values were normalized to DMSO-treated controls.

In situ Proximity Ligation Assay (PLA)

HCT15 cells were seeded onto coverslips coated with 2% gelatin (SIGMA). After 24 hours, cells were treated with 1 µM BC-2059 for 0, 3, 6 and 12 hours. The cells were fixed in ethanol and permeabilized with 0.25% Triton x-100. Slides were treated with Duo Link in Situ Assay (Olink Bioscience) according to the manufacturer's protocol. Cells were incubated with mouse antibody for TBL1 (Abcam) and rabbit antibody for β-catenin (Cell Signaling Tech). Secondary antibodies conjugated with oligonucleotides (PLA probes) were added to the slides. A solution

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containing two oligonucleotides and ligase was added to the slides to hybridize to the PLA probes. Following amplification with fluorescently labeled nucleotides in presence of polymerase, levels of associated TBL1 and β-catenin were visible as fluorescent spots and quantified by Duo Link software (Olink Bioscience).

Gene Reporter Assay

For T-cell factor (TCF) transcriptional activity assay, $4x10^4$ STF3A cells were seeded in 96-well CulturPlates (Perkin Elmer) in RPMI containing 5% FBS. After 24 hours the cells were treated with 1 μM BC-2059 for 0, 4, 6, 8, 12 and 24 h at 37°C. For Notch signaling reporter assay, $3.5x10^4$ Notch CSL reporter HEK-293 cells were seeded in 96-well CulturPlates in MEM containing 10% FBS without Geneticin and Hygromycin B. After 24 hours the cells were treated with 1 μM BC-2059 for 0, 4, 6, 8, 12 and 24 hours at 37°C. DAPT (Sellekchem)10 μM was used as a positive control. For the Hedgehog pathway, $2.5x10^4$ GLI reporter NIH3T3 cells were seeded in 96-well CulturPlates in DMEM containing 10% BCS without Geneticin. After 24 hours the medium was changed with assay medium (Optimem reduced Serum Medium (Invitrogen) containing 0.5% BCS) containing recombinant mouse Sonic Hedgehog 1 μg/mL (R&D System) and the cells were treated with 1 μM BC-2059 for 0, 4, 6, 8, 12 and 24 hours at 37°C. Cyclopamine (BPS Bioscience) 1 μM was used as a positive control. For Hippo pathway, $3.5x10^4$ TEAD Reporter MCF7 cells were seeded in 96-well CulturPlates in MEM containing 10% FBS without Geneticin. After 24 hours the cells were treated with 1 μM BC-2059 for 0, 4, 6, 8, 12 and 24 hours at 37°C. Okadoic Acid 100 nM (BPS Bioscience) was used as a positive control. After incubation, One Step Luciferase Assay Reagent (Promega) was added to each well at 1:1 ratio and incubated for 8 min at RT. The assay was evaluated by EnVision 2000 (Perkin Elmer).

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Chromatin immunoprecipitation (ChIP) assay

ChIP was performed using a ChIP assay kit (Cell Signaling Technologies) according to the manufacturer's protocol. HCT15 cells were treated with BC-2059 for 0, 6 and 12 hours and then treated with formaldehyde for 15 min. For each ChIP mixture, $4x10^6$ cells were used. After incubation with β-catenin or TBL1 antibody, the precipitated DNA samples were quantified by qPCR with ViAA 7(Applied Biosystems). Data is expressed as the percentage of input DNA. The primers used in this study are listed in Supplemental Table 1.

Cellular Thermal Shift Assay (CETSA)

HCT15 cells were seeded at 70% confluence in RPMI 10% FBS (Gibco) in 75 mm tissue culture treated flasks (Costar). After 24 hours, the medium was changed with fresh one containing 3 μM of BC-2059. After incubating for 2 hours, cells were harvested and resuspended in PBS at the final concentration of $20x10^6$ cell/mL. 50 μL of cell suspension was aliquoted into PCR tubes, and the samples heated for 3 min at increasing temperature (42ºC to 64ºC) to denature proteins. The cells were lysed by freeze/thaw rounds in liquid nitrogen and incubation at 26ºC, and centrifuged at 20000g for 20 min at 4ºC to precipitate the cell debris and aggregates from the soluble protein fraction. The soluble fractions were resolved in SDS-PAGE and analyzed by western blot with antibodies against TBL1 and β-catenin.

Gene knockdown by siRNA and TBL1 rescue

COR-L95 cells were seeded in tissue culture treated 6-well plate (Genesee Scientific) and transfected with TBL1 human siRNA oligo duplex (Origene) according to the manufacturer's protocol. After 24 hours, $2x10^3$ cells/well were transferred to tissue culture treated 96-well plate (Genesee Scientific,). After 24 hours the cells were treated with DMSO and BC-2059 at IC_{50}

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concentration (0.004 μM). Cell viability was assessed with CellTiter-Glo (Promega) 72 hours after treatment and plotted using GraphPad Prism v. 8.0. Absorbance values were normalized to DMSO-treated controls. H2030 cells were seeded in tissue culture treated 6-well plate (Genesee Scientific) and transfected with TBLR1 human siRNA oligo duplex (Santa Cruz Technologies) according to the manufacturer's protocol. After 24 hours $2x10^3$ cells/well were transferred to tissue culture treated 96 well plate (Genesee Scientific) and drug treatment performed at IC_{50} concentration (0.1 μM) as described above.

HCT15 cells were seeded in tissue culture treated 6-well plate (Genesee Scientific) and transfected with TBL1 human siRNA oligo duplex (Origene) according to the manufacturer's protocol. For TBL1 rescue assay, HCT15 cells were first transfected with TBL1 human siRNA oligo duplex. The cells were then transfected again with TBL1_90NT (aa 1-90) cloned in pcDNA3.2/GW/D-TOPO plasmid (Invitrogen). After 24 $2x10^3$ cells/well were transferred to tissue culture treated 96-well plate (Genesee Scientific) and treated with increased concentration of BC-2059 for 72 hours, followed by CellTiter-Glo (Promega) incubation to evaluate cell viability as described above.

qPCR

H2030 and COR-L95 cells were seeded at 1 X 10^6 cells in 2 mL of appropriate medium in 6-well tissue culture treated plates (Genesee Scientific). To quantify gene expression, after 24 hours total RNA was extracted (Qiagen RNeasy Mini Kit) and quantified by spectroscopy (Thermo Scientific; Nanodrop ND-8000). Samples were then reverse transcribed to cDNA using a high capacity cDNA reverse transcription kit (Thermo Scientific) and the MJ Research thermal cycler. cDNA was amplified, detected, and quantified using SYBR green reagents (Thermo Scientific)

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and the ViiA 7 Real-Time PCR System (Applied Biosystems). Data were normalized to GAPDH expression. A list of primers used in this study are provided in Supplemental Table 2.

Polyubiquitination of β-catenin

Approximately $2x10^6$ HCT 15 cells were seeded in 100 mm tissue culture treated plates (Genesee Scientific). At 75 % confluence, the cells were treated with 10 mM LiCl, the universal activator of Wnt signaling, in presence/absence of BC-2059 for 0, 3, 6, and 12 hours. Harvested cells were subjected to cytosolic/ nuclear extraction using Epigentek nuclear extraction kit (catalog no. OP-0002-1) according to the manufacturer's protocol. Both nuclear and cytosolic fractions were processed for enrichment of polyubiquitinated protein conjugates with the Ubiquitin Enrichment Kit (Thermo Scientific) according to the manufacturer's protocol. Samples were resolved in 4-12% SDS-PAGE (Invitrogen). Western blot analysis was performed using β-catenin antibody (BD Biosciences) and Odyssey Imager (Li-Cor Biosciences).

Statistical analysis

Two-tailed student's paired T-tests with 95% confidence intervals were performed using GraphPad Prism v 8.0. Symbols for significance; **=$p<0.001$; ***=$p<0.0001$. All the in vitro experiments were performed in triplicate and repeated at least three times.

Results

BC-2059 selectively binds TBL1 and TBLR1

Interaction between TBL1/TBLR1 and β-catenin in response to Wnt signaling activation has been well studied (Dimitrova et al., 2010; Choi et al., 2011), and previous reports suggested the

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N-terminus of TBL1 (residues 1-142) interacts with a portion of the β-catenin armadillo domain (residues 133-467) (Dimitrova et al., 2010). *TBL1* and *TBLR1* are highly homologous (85% identity), with TBLR1 missing 1-55 residues in the N terminus of the protein (Fig. 1A), and the remaining residues (56-142) share 94.38 percentile identity. In silico docking prediction suggested that BC-2059 binds the hydrophobic pockets at the N-terminus of TBL1/TBLR1 (Fig. 1B). These pockets have non-polar character, and are involved in protein-protein interaction with members of the NCoR complex and the NFκB subunit p65 (Oberoi et al., 2011; Ramadoss et al., 2011). To determine whether β-catenin binds TBL1/TBLR1 in the same pockets, mutations were made in the sidechains of TBL1_90NT, a peptide that covered TBL1 56-142 residues corresponding to the 1-90 residues of TBLR1, and tested for their ability to bind to β-catenin in co-purification assays. β-catenin binds TBL1_90NT peptide with an affinity similar to that measured with full length TBL1 (Fig. 1C). Mutations in Val 60 (Valine to Leucine) and/or Ile 30 (Isoleucine to Phenylalanine) in TBL1_90NT sidechains caused a significant reduction in the amount of β-catenin bound to the protein (Fig. 1D). Saturation and homologous competitive assays performed in a cell-free system confirmed that BC-2059 selectively binds TBL1 with high affinity (Fig. 2A-B). Further, we show that BC-2059 interacts with TBL1/β-catenin complex in HCT15 cells. In a cell thermal shift assay (CETSA), BC-2059 treated cells show that both proteins are maintained in a soluble state in the presence of drug (Fig. 2C), whereas TBL1 and β-catenin proteins precipitated in the absence of drug. Together these data support the hypothesis that β-catenin and BC-2059 share the same binding site in the hydrophobic pockets at the N-terminus of TBL1 and TBLR1.

BC-2059 binding to TBL1 results in disruption of TBL1/β-catenin complex.

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To investigate whether the binding of BC-2059 results in displacement of β-catenin from the hydrophobic pocket of TBL1, we performed heterologous competitive assays using recombinant proteins in the presence or absence of increasing concentrations of BC-2059. As shown in Fig. 3A, the amount of recombinant β-catenin associated with TBL1 was reduced in the presence of BC-2059 in a dose-dependent manner, with a Kd=0.0097 μM. Similar results were observed when nuclear lysates from BC-2059 treated HCT15 cells were analyzed by sandwich ELISA for the TBL1/ β-catenin complex (Fig. 3B). The amount of endogenous β-catenin bound to TBL1 was decreased in response to treatment with BC-2059 in a dose-dependent manner . In response to Wnt signaling activation, TBL1 is SUMOylated and associates with β-catenin (Choi et al., 2011). To verify whether the BC-2059 effect on TBL1/β-catenin complex was a result of the inhibition of SUMOylation of TBL1, we treated HCT15 cells with or without LiCl to promote Wnt activation in the presence of BC-2059 and measured the level of SUMOylated TBL1. As shown in Fig. 3C, BC-2059 does not affect the amount of SUMOylated TBL1 in presence of active Wnt. We next confirmed the ability of BC-2059 to interfere with the TBL1-β-catenin complex formation in intact cells using co-immunoprecipitation and in situ proximity ligation assays (PLA). As shown in Fig. 3D, co-immunoprecipitation of BC-2059 treated HCT15 cell nuclear lysates showed a reduction of β-catenin associated with TBL1 after 6-hour treatment. Importantly, in situ PLA, which provides exact spatial information to reveal protein-protein interactions in cells, demonstrated a significant reduction in the signal generated by TBL1 and β-catenin probes (Fig. 3E). Although both TBL1 and TBLR1 have low tissue specificity and are generally expressed in all tumors, there are cancer cell lines that express exclusively TBL1 or TBLR1. To verify whether BC-2059 efficacy was different in cells that selectively express TBL1 or TBLR1, we performed cytotoxicity assays on COR-L95 and H2030 lung cancer cell lines,

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which exclusively express TBL1 and TBLR1, respectively (Fig. 4B). As shown in Fig. 4A, the IC_{50} of H2030 cell line is around 0.1 μM, while the IC_{50} measured in COR-L95 cell line is approximately 0.004 μM. While both lines demonstrated sensitivity to BC-2059, the lower IC_{50} in COR-L95 may suggest cells reliant on the TBL1- β-catenin complex as more sensitive. siRNA knockdown of TBL1 and TBLR1 in these cell lines showed BC-2059 loss of efficacy, confirming the interaction of the drug with both the proteins (Fig. 4C). Further, we investigated the effect of BC-2059 in HCT15 cells in which TBL1 was knocked down by siRNA and then re-expressed. As show in Supplemental Figure S1, the IC_{50} of TBL1 siRNA transfected HCT15 cells treated with BC-2059 increased by 1.5 log folds compared to the IC_{50} of the cells in which TBL1 expression was rescued. Together these data indicate BC-2059 as a direct binder of TBL1 and TBLR1, which results in disruption of β-catenin interaction with TBL1/TBLR1 heterotetramer in cancer cells.

BC-2059 preferentially targets the TBL1/β-catenin complex

Our data have shown that the first 90 amino acids in the N-terminus of TBL1 are sufficient for binding to β-catenin and for the activity of BC-2059 to disrupt the complex. The N-terminus of TBL1 also interacts with NCoR/SMRT corepressor complex (Oberoi et al., 2011) as well as NFκB coactivator complexes (Li and Wang, 2008; Ramadoss et al., 2011). To investigate if BC-2059 disrupts TBL1 interaction with other complexes, we analyzed the levels of NCoR/SMRT and NFκB co-precipitated with TBL1 after BC-2059 treatment. As shown in Fig. 5A, BC-2059 induces a decrease in β-catenin levels, however it fails to affect the interaction of TBL1 with the NCoR/SMRT complex subunit GPS2 (Oberoi et al., 2011). In addition, GPS2 failed to displace biotinylated BC-2059 bound to TBL1 in a heterologous competition assay (Fig. 5B). Similarly, the interaction of TBL1 with p65, a subunit of NFκB (Ramadoss et al., 2011), was not

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significantly affected by BC-2059 treatment (Fig. 5A). Together, these data suggest that BC-2059 specifically impacts the association of TBL1 with β-catenin.

BC-2059 interaction with TBL1 does not affect other stem cell pathways

Wnt pathway cross-talks with the Notch, Sonic Hedgehog, and Hippo stem cell pathways is well studied, with implications for therapeutic interventions in cancers (Maeda et al., 2006; Varelas et al., 2010; Peignon et al., 2011; Ann et al., 2012; Attisano and Wrana, 2013; Song et al., 2015; Borggrefe et al., 2016; Deng et al., 2018). β-catenin is involved in the tumorigenesis activity of several stem cell signaling pathways including Notch signaling in colorectal cancer (Peignon et al., 2011), promoting transcriptional activity of Hedgehog and Hippo pathways in carcinogenesis, and progression of various human malignant tumors (Maeda et al., 2006). To assess BC-2059 selectivity, we analyzed the transcriptional activity of Wnt, Notch, Hippo and Hedgehog stem cell pathways using luciferase gene reporter assays. Our data show that BC-2059 inhibits the transcriptional activity of TCF7L2/LEF after 3 hours of treatment. Notch and Hedgehog pathways were only partially inhibited and only after longer exposure to the compound (12 hours), while the Hippo pathway was not affected by BC-2059 treatment (Fig. 5C). Together, these data suggest that BC-2059 is selective for TBL1/β-catenin dependent canonical Wnt-pathway.

BC-2059 treatment affects Wnt-signaling dependent gene expression and promotes β-catenin ubiquitination.

TBL1 and β-catenin are required co-factors for TCF7L2/LEF-dependent transcriptional activation of gene expression (Li and Wang, 2008). Previously we have shown that treatment with BC-2059 efficiently inhibits TCF7L2 activity (Soldi et al., 2015). Here we investigated the

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effect of BC-2059 on Wnt-dependent gene expression. We analyzed TBL1 and β-catenin recruitment to the Wnt-regulated element (WRE) regions of MYC and AXIN2, using chromatin immunoprecipitation (ChIP). The ChIP-enriched DNA from BC-2059 treated HCT15 cells were quantified by qPCR using specific primers for AXIN2 and MYC WREs. Compared with the untreated cells, a decrease was observed for enrichment of TBL1 and β-catenin at the AXIN2 and MYC WREs (Fig. 6A).

We previously showed that BC-2059 treatment results in reduction of β-catenin cellular levels suggesting that the protein may be degraded in response to the treatment (Soldi et al., 2015). To test this hypothesis, we measured the presence of polyubiquitinated β-catenin after treatment with BC-2059 in the HCT15 cell line. Nuclear β-catenin underwent ubiquitination after 3 hours of treatment with BC-2059 (Fig. 6B). Moreover, β-catenin pull-down showed increased levels of SIAH1 Interacting Protein (SIP), a subunit of SIAH1 E3 ligase (Dimitrova et al., 2010), after incubation with BC-2059 suggesting that the treatment with the drug may promote ubiquitination of β-catenin SIAH1-mediated (Supplemental Figure 2).

Discussion

Given the pleiotropic involvement of Wnt signaling in cancer, targeting the pathway through its crucial effector, β-catenin, is an attractive therapeutic approach. In the last few decades, extensive research efforts in this direction resulted in clinical trials in hematological and solid tumors (Tabatabai et al., 2017). However, significant challenges have hampered the approval of drugs that target this pathway through β-catenin. Wnt-β-catenin signaling is crucial in stem cells maintenance and regeneration of tissues, and there is significant crosstalk between Wnt pathway

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and other stem cell pathways such as Notch, Hippo and Sonic Hedgehog (Maeda et al., 2006; Varelas et al., 2010; Peignon et al., 2011; Ann et al., 2012; Attisano and Wrana, 2013; Song et al., 2015; Borggrefe et al., 2016; Deng et al., 2018). As such, inhibition of the Wnt pathway may affect the normal somatic stem cells compartment and the tissue homeostasis. Several inhibitors targeting members of Wnt pathway (tankyrase, porcupine, disheveled, Frizzled receptor, and others) have been developed (Krishnamurthy and Kurzrock, 2018), however none advanced to clinical use (Liu et al., 2013; Masuda et al., 2015; Zhang et al., 2017). One reason for the unsuccessful outcome of these therapeutics is that many of these inhibitors target β-catenin phosphorylation and degradation pathway. However, β-catenin is often mutated at the serine residues in cancer cells resulting in defective phosphorylation, accumulation of β-catenin, and activation of Wnt-dependent gene expression (Gao et al., 2018). Thus, therapies that directly target β-catenin or downstream signaling pathways may result in better inhibition of Wnt signaling.

The complex structure of β-catenin and lack of obvious druggable pockets makes it a difficult and elusive target for drug development. To date, several β-catenin signaling pathway inhibitors that aim to disrupt β-catenin activity and its interaction with transcription factors are under investigation (Shin et al., 2017; Yan et al., 2017; Krishnamurthy and Kurzrock, 2018). Small molecule compounds such as PKF118-310, PKF222-185, PKF115-584 and CPG049090 are capable of disrupting β-catenin/TCF7L2 complex presumably through direct binding with β-catenin on TCF interaction site (Barker and Clevers, 2006). These compounds, however, lack specificity due to the fact that β-catenin utilizes the same residues for binding with TCF, APC, or E-cadherin. PKF222-185 and PKF115-584 have been found to interfere with TCF7L2/DNA

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complex and all four of the compounds promote the disruption of β-catenin binding to APC, which in normal tissues may promote cancer formation.

Recent findings revealed the important role played by TBL1 and TBLR1 for the activation of several Wnt targets. Li and Wang reported that TBL1/TBLR1 and β-catenin recruit each other to several WREs following activation of the Wnt pathway (Li and Wang, 2008). Depletion of TBL1/TBLR1 dramatically reduces β-catenin recruitment to WREs. Further, Dimitrova *et al*., reported that TBL1 may play a crucial role in stabilizing β-catenin/TCF complex (Dimitrova et al., 2010). These reports clearly indicate a key role of the TBL1/TBLR1/β-catenin complex in the Wnt pathway and suggest that strategies targeting TBL1/TBLR1/β-catenin complex might have therapeutic value.

Our compound BC-2059 disrupts β-catenin interaction with TBL1/TBLR1 resulting in reduced β-catenin protein levels and inhibition of Wnt-dependent gene expression. Recent reports have highlighted that by depleting nuclear β-catenin and inhibiting TCF7L2-induced *MYC* levels, BC-2059 exerts synergistic lethality with BET protein antagonists and overcomes resistance to JAK inhibitor or BET inhibitor in post-Myeloproliferative Neoplasm secondary AML cells (Saenz et al., 2019; Saenz et al., 2020). Evidence suggests that BC-2059 inhibitory effect results in nuclear β-catenin degradation through the SIAH1 pathway (Dimitrova et al., 2010; Fiskus et al., 2015). In contrast to PKF222-185, PKF115-584, and CPG049090, we show that BC-2059 selectively targets the TBL1/β-catenin complex. Experimental binding affinity analyses show that BC-2059 binds at the N-terminus of TBL1/TBLR1 and disrupt the interaction between TBL1/TBLR1/β-catenin. The N-terminal LiSH and F-Box domain of TBL1/TBLR1 is known to interact with transcription inhibitor complex NCoR/SMRT and transcription activator NFκB; however, we were unable to detect any change in TBL1 interaction with GPS2 or the p65 subunit of NFκB

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after BC-2059 treatment. Moreover, treatment with BC-2059 did not affect other stem cell pathways (Notch, Hippo and Hedgehog pathways), which cross-talk with Wnt-β-catenin signaling, potentially reducing the risk of damaging the normal somatic stem cells function, tissues homeostasis, and regenerative processes. Given the high mutation rate observed in the members of Wnt signaling pathway, development of novel therapeutics that target downstream effectors such as TBL1 and TBLR1 clearly represent an advantage. Collectively, our data show that BC-2059 selectively disrupts TBL1/TBLR1/β-catenin association leading to degradation of β-catenin and its unique mechanism of action positions BC-2059 as a promising anti-cancer agent with potential for an innovative approach to treat Wnt-driven cancers.

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Footnotes

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Syndax. Equity from LSK BioPharma, Salarius Pharmaceuticals, Iterion Therapeutics, ConverGene, and Stingray Therapeutics. Honoraria from Exelixis, Loxo Oncology, Natera Inc, Hengrui Therapeutics, Tarveda Therapeutics, Dracen Pharmaceuticals, and Barricade Therapeutics. H Proterus Therapeutics. H.V. is employee and shareholder of Arrien Pharmaceuticals and Proterus Therapeutics, and shareholder of Stingray Therapeutics. R.S. holds stock in Iterion Therapeutics and Salarius Pharmaceuticals. M.R.K. and A.W. are employees and shareholders of Stingray Therapeutics. T.T. is shareholder of Stingray Therapeutics. K.N.B. holds stock in Iterion Therapeutics and Salarius Pharmaceuticals. S.K.H. is employee and shareholder of Iterion Therapeutics and Noble life Sciences. The other authors declare that they have no conflict of interest with the contents of this article. The authors declare that the financial support provided in no way impacted the design, execution or interpretation of the provided work.

Figure legends

Figure 1. **BC-2059, TBL1 and β-catenin interaction.** A. TBL1, TBLR1 and TBL_90NT schematics. B. BC-2059 structure and in silico optimal docking prediction analysis. C. Saturation assay TBL1_90NT/ β-catenin. Recombinant TBL1full length and TBL1_90NT were immobilized on MAXISORP ELISA plate and incubated with increased concentrations of recombinant β-catenin. The percentile of recombinant β-catenin bound was measured at 405nm.

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D. β-catenin binding site in TBL1. HCT 15 cells were transfected with 6xHis-tagged TBL1_90NT (first N-terminus 90 aminoacids) wild type and construct carrying mutations in Val60, Ile 39 or both. After 48h cell lysates were subjected to immunoprecipitation with α-6xHIS antibodies and resolved in SDS-PAGE. Levels of β-catenin were evaluated by western blot analysis.

Figure 2. BC-2059 binds TBL1. A. Recombinant TBL1 and recombinant β-catenin were immobilized on MAXISORP ELISA plate and incubated with increased concentrations of biotinylated BC-2059. The percentile of biotinylated compound bound was measured at 405 nm. B. Homologous Competitive assay. 100 nM biotin-linked BC-2059 was added to the wells containing TBL1 recombinant protein, in presence of increasing concentrations of BC-2059 (range of 0.1 nM to 10 μM) and incubated at RT for 2 hours. Following incubation with streptavidin-HRP conjugate antibody the ELISA assay was developed by addition of ABTS HRP substrate and read with plate reader set to 405 nm wavelength. C. CETSA: HCT15 cells were incubated with 3 μM BC-2059 for 2 hours at 37 °C, followed by CETSA at 50°C to 64°C, three min each T°. After centrifugation, soluble proteins were resolved by SDS-PAGE and the relative amount measured by densitometry.

Figure 3. BC-2059 effect on TBL1/β-catenin complex. A. Recombinant TBL1 was immobilized on a MAXISORP ELISA plate and incubated with increased concentrations of BC-2059 in the presence of 50 ng of β-catenin. Following incubation with α-β-catenin and secondary antibody HRP-conjugated, an ELISA assay was developed by the addition of ABTS HRP. The bound β-catenin was measured at 405 nm. B. Sandwich ELISA for evaluation of β-catenin levels in TBL1 nuclear extracts after 6 hours treatment with increased concentrations of BC-2059. C. TBL1 SUMOylation. HCT15 were incubated in medium +/- 20 mM LiCl for 24 h, then the

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medium was changed with fresh one containing 2 μM BC-2059 +/- 20 mM LiCl. The cells were incubated at 37°C for 1, 3 and 6 hours. OD values were normalized against the untreated cells at each time point. D. Immunoprecipitation of TBL1 from HCT15 colon cancer cells treated with 0.5 μM BC-2059 for 0, 3, 6, 12, and 24 hours. Western blot showed co-precipitation of β-catenin levels. Antibody α-TBL1 was used to verify equal loading. E. In situ PLA. HCT15 colon cancer cells were seeded on a coverslip coated with 2% gelatin, and treated with 0.5 μM BC-2059 for 0 and 6 hours. The slides were treated with Duo Link in Situ assay to verify the co-localization and association of β-catenin and TBL1. Following amplification with fluorescently labeled nucleotides in presence of polymerase, β-catenin and TBL1 were visible as a fluorescent spot and analyzed by fluorescence microscopy.

Figure 4. Cytotoxicity of BC-2059 in COR-L95 and H2030. **A.** COR-L95 and H2030 cells were treated with BC-2059 at increasing concentrations (0.001 – 10 μM) for 72 hours. The percentile of viable cells was evaluated by Cell Titer Glo and the IC_{50} calculated using GraphPad Prism v. 8.0. Absorbance values were normalized to DMSO-treated controls. B. qPCR of COR-L95 and H2030 for TBL1 and TBLR1 expression. C. Cytotoxicity of BC-2059 in TBL1 and TBLR1 siRNA transfected COR-L95 and H2030. COR-L95 and H2030 were transfected with siRNA to knock down respectively TBL1 and TBLR1 expression or scramble control. After 48 hours, the cells were treated with BC-2059 at IC_{50} dose (COR-L95, 0.004 μM; H2030, 0.1 μM). After 72 hours, the percentile of viable cells was evaluated by Cell Titer Glo. Two-tailed student's paired T-tests with 95% confidence intervals were performed; **=P<0.0013, ***=P<0.0006. The bar diagram depicts the mean of values from three independent experiments.

Figure 5. BC-2059 preferentially targets the TBL1/β-catenin complex. A. Western blot analysis of TBL1/GPS2 complex and TBL1/p65 NFκB subunit complex levels from HCT15

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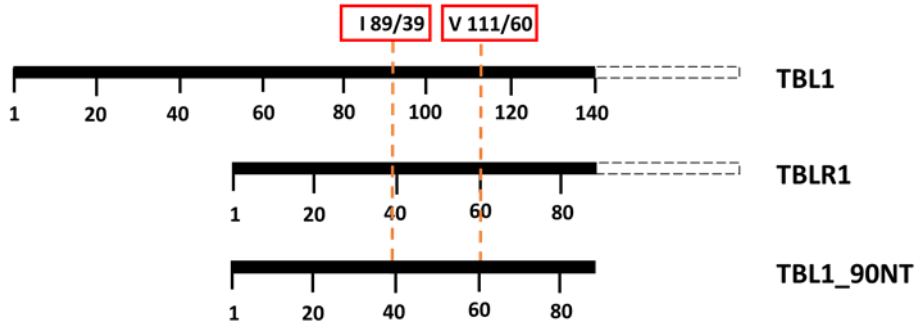
cells treated with BC-2059 (0.5 μM) for 0, 3, 6, 12 hours. Antibody α-TBL1 was used to verify equal loading. B. Heterologous Competitive assay. 100 nM biotin-linked BC-2059 was added to the wells containing TBL1 recombinant protein, in presence of increasing concentrations of GPS2 (range of 0.1 nM to 100 nM) and incubated at RT for 2 hours. Following incubation with streptavidin-HRP conjugate antibody the ELISA assay was developed by addition of ABTS HRP substrate and read with plate reader set to 405 nm wavelength. C. BC-2059 interaction with TBL1 does not affect other stem cell pathways. STF3A cells transfected with TCF/LEF reporter, HEK293 cells transfected with NOTCH CSL reporter, NIH3T3 transfected with Hedgehog GLI reporter and MCF7 transfected with TEAD reporter were treated with 1 μM BC-2059 for 0 and 6 hours. Luciferase activity measured after incubation shown TCF/LEF transcriptional activity drastically decreased. In contrast, Hedgehog GLI and NOTCH CSL transcriptional activity was partially inhibited after incubation with BC-2059, and TEAD transcriptional activity was not inhibited.

Figure 6. BC-2059 effect on Wnt-signaling dependent gene expression and β-catenin polyubiquitination. A. ChIP analysis of HCT15 cells treated with 0.5 μM BC-2059 for 6 and 12 hours. Chromatin-bound lysates were immunoprecipitated using specific antibodies for TBL1 or β-catenin, and IgG for negative control, followed by qPCR with primers that flanked the promoter regions of the Wnt target genes cMYC and AXIN2. B. Polyubiquitination of β-catenin. HCT 15 cells were treated with 0.5 μM of BC-2059 for 0, 3, 6, and 12 hours. Nuclear and cytosolic fractions were processed for enrichment of polyubiquitinated protein conjugates with the Ubiquitin Enrichment Kit and resolved in SDS-PAGE for β-catenin.

Figure 1

A



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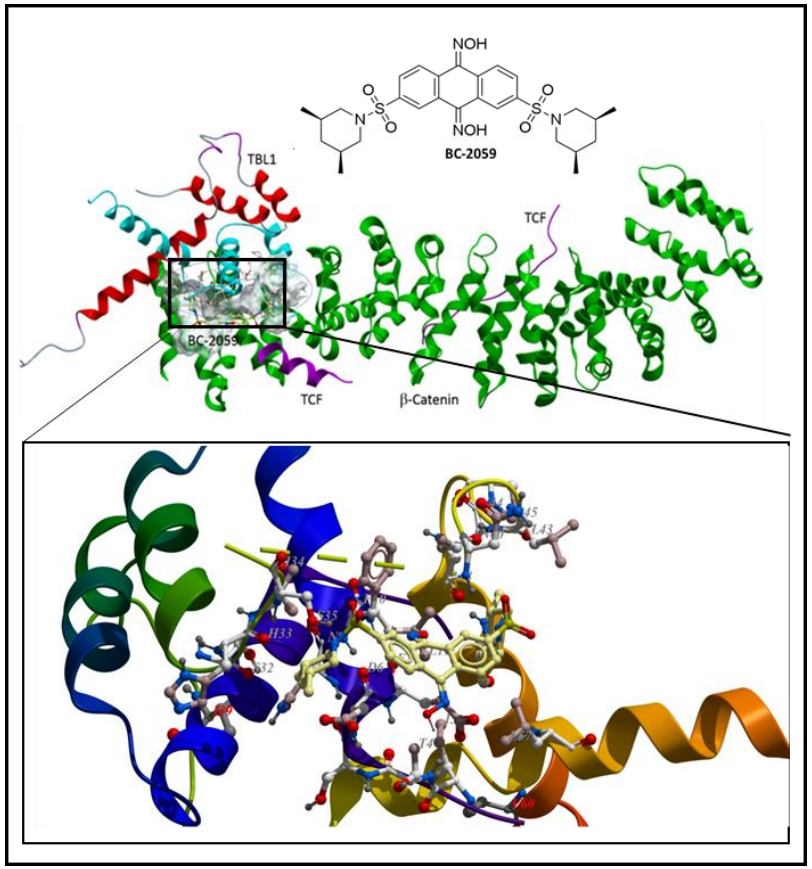

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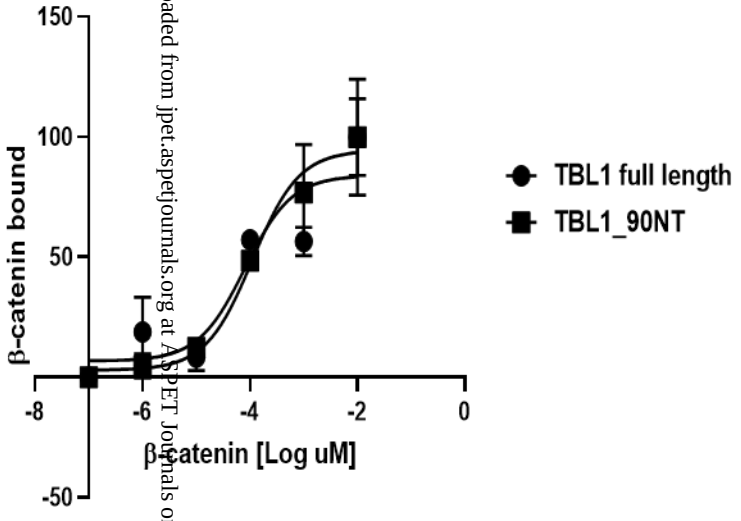


	TBL1 full length	TBL1_90NT
Kd [μM]	0.00008096	0.0001109

D

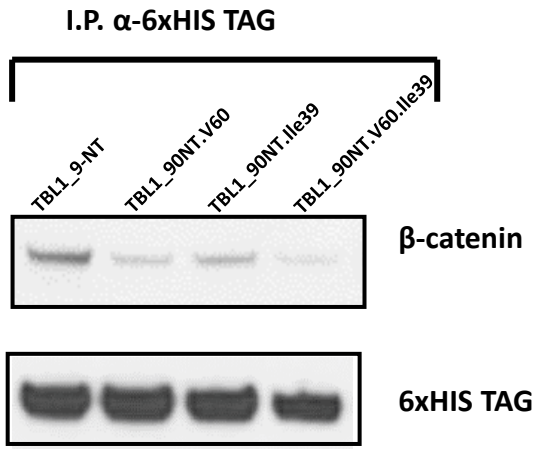
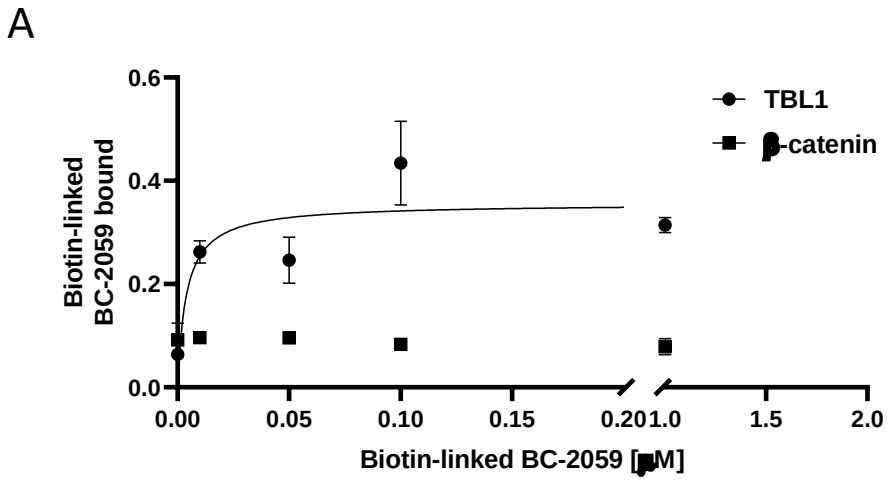
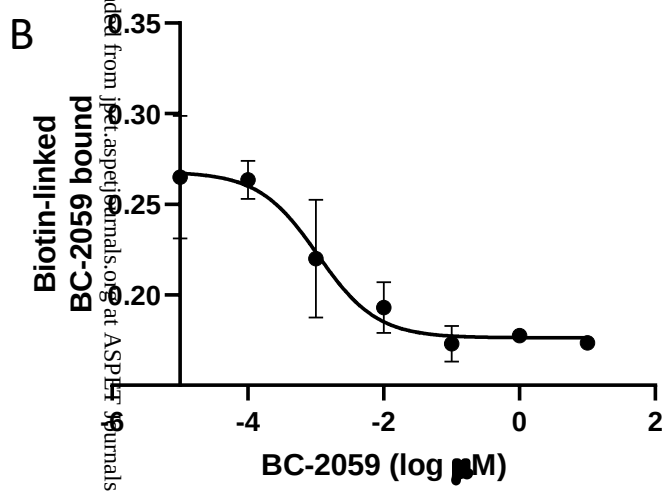


Figure 2



	TBL1	β-catenin
Kd [μM]	0.003941	NA



BC-2059	
Kd [μM]	0.00107

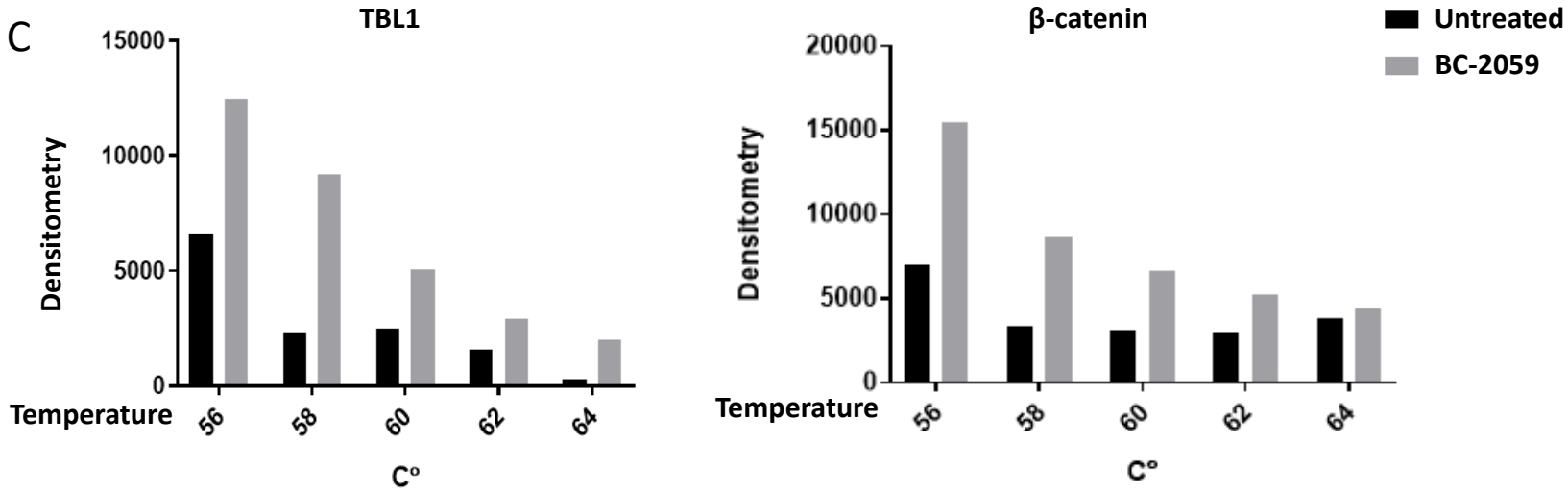


Figure 3

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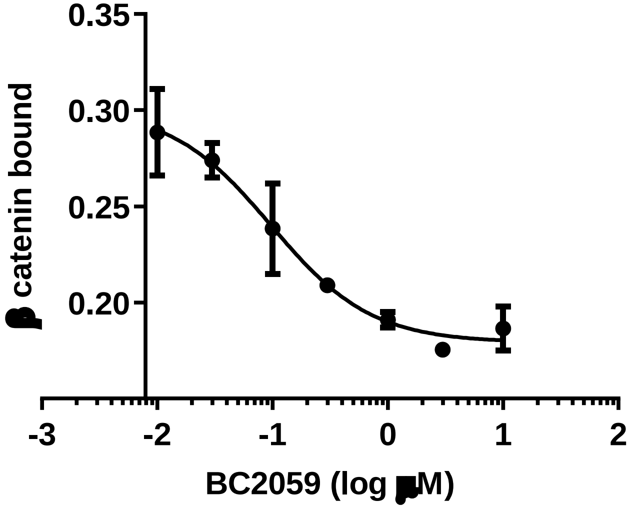
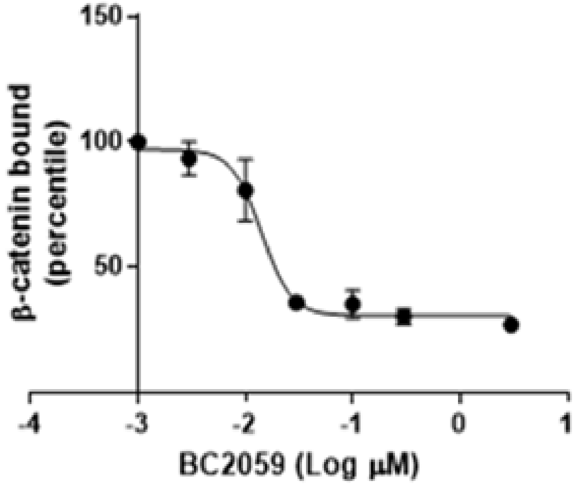


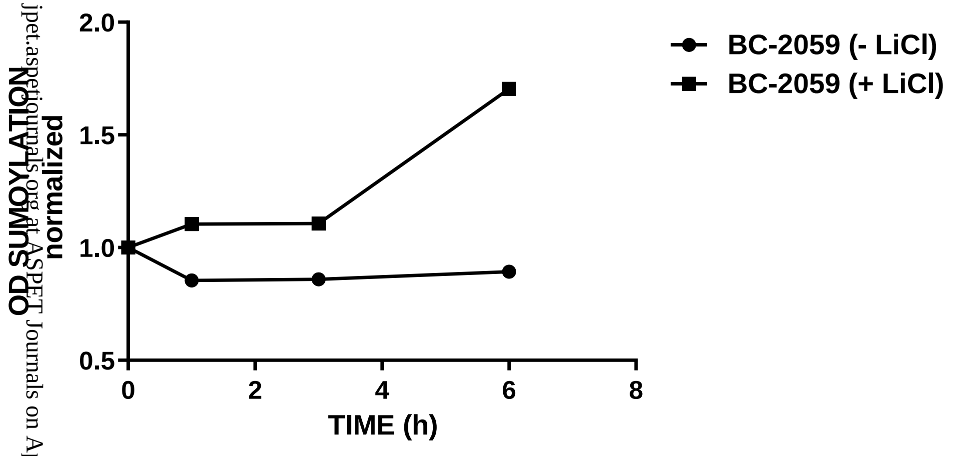
	β-catenin
Kd [μM]	0.0097

B



	HCT 15
Kd [μM]	0.01417

C



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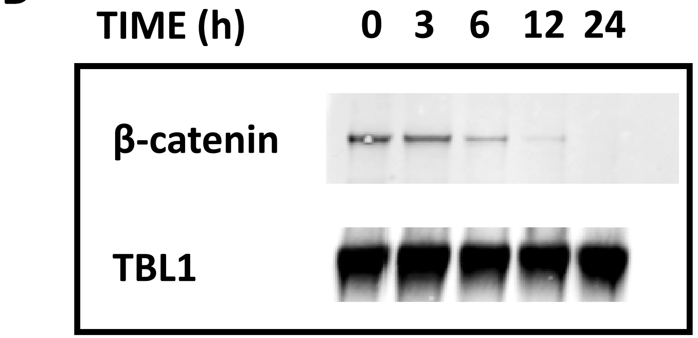

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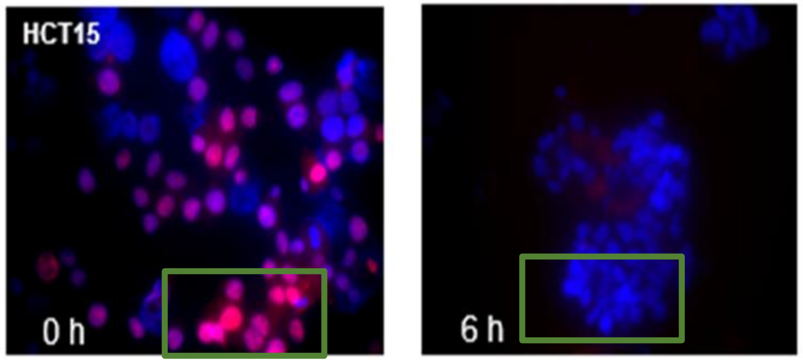
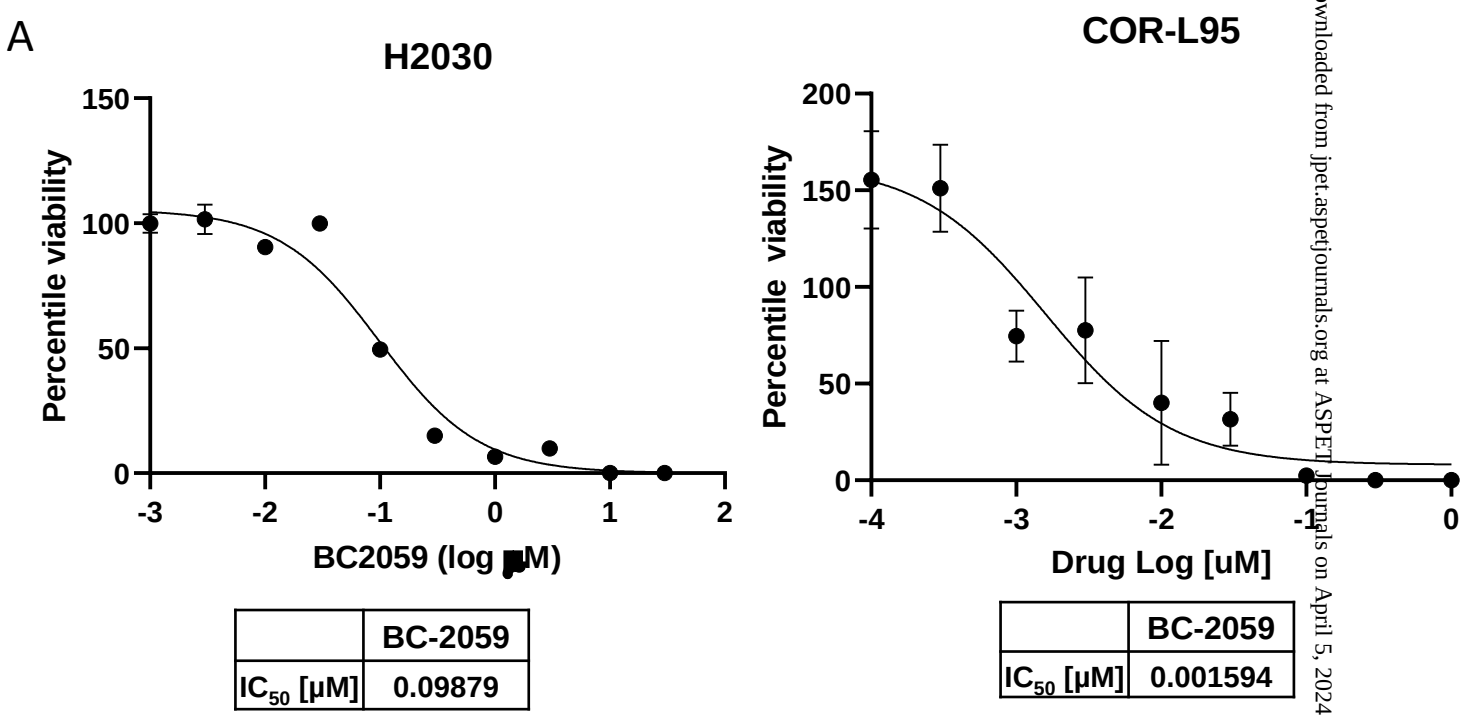
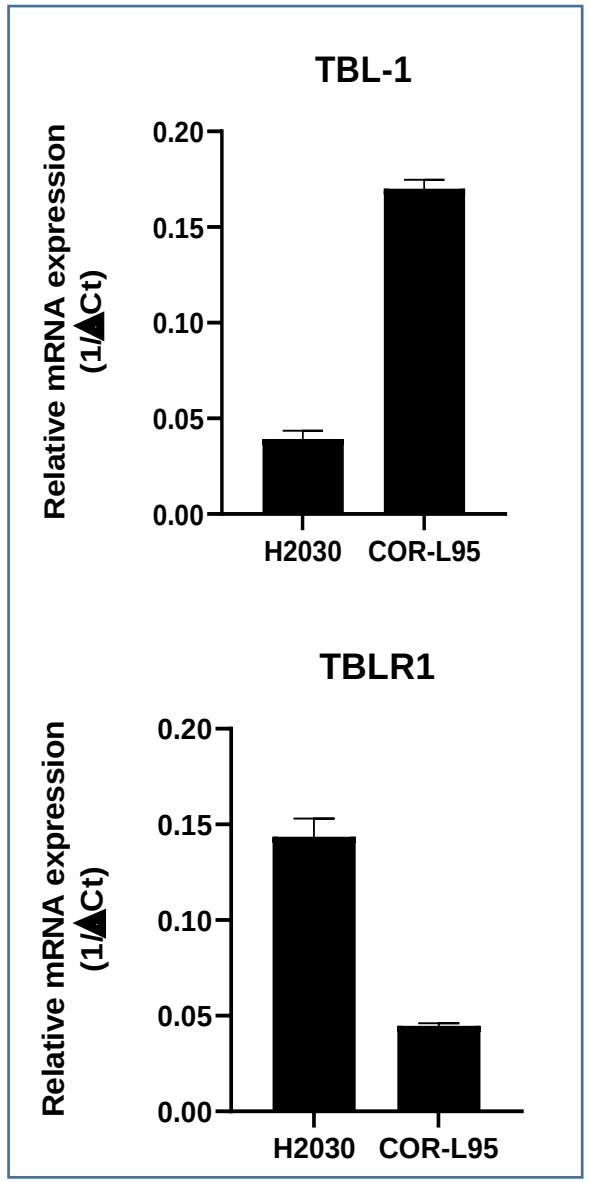


Figure 4





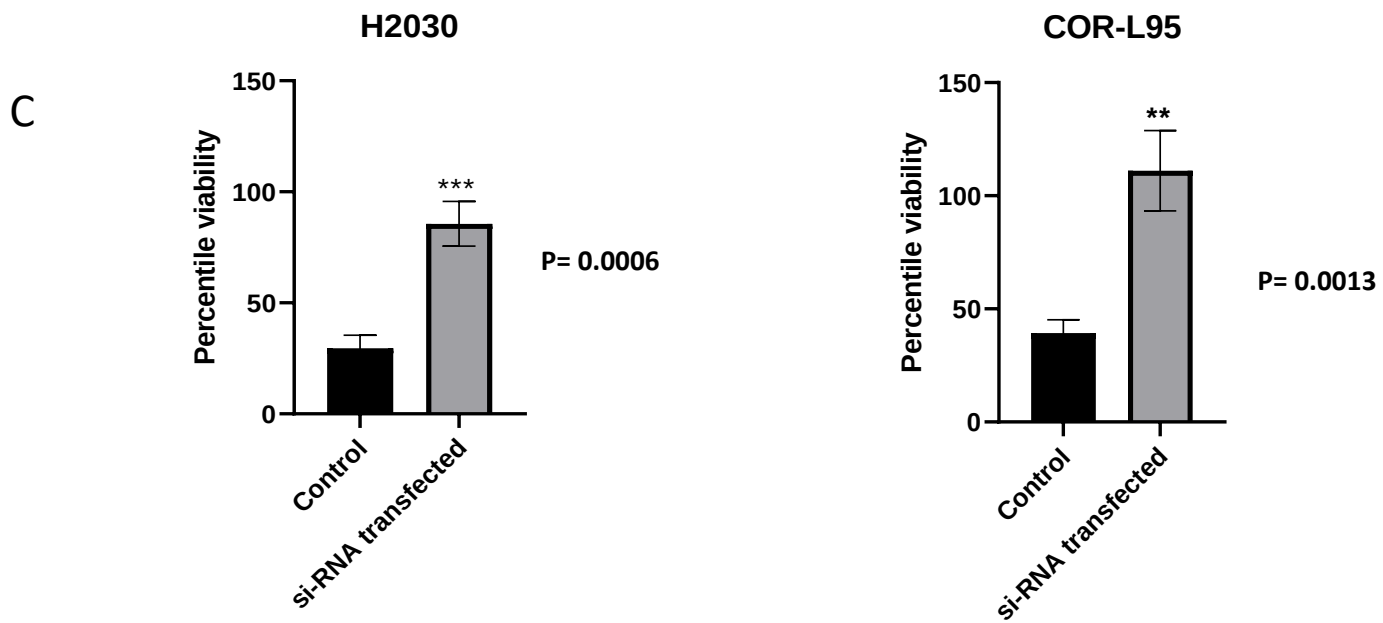
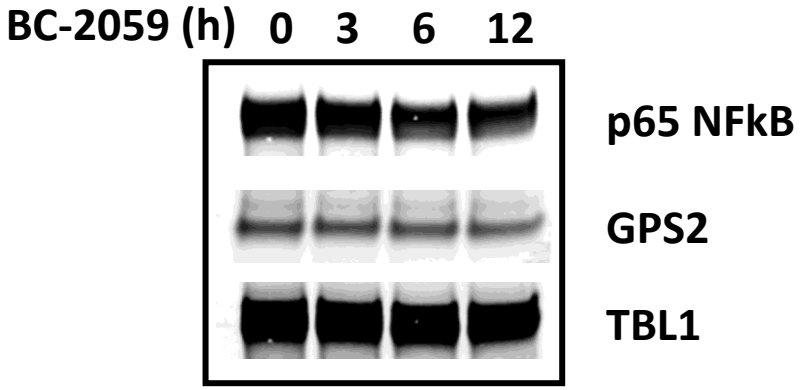


Figure 5

A



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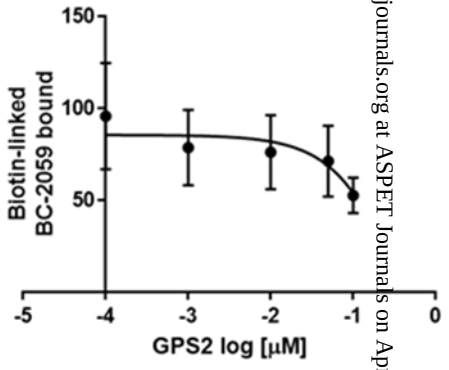
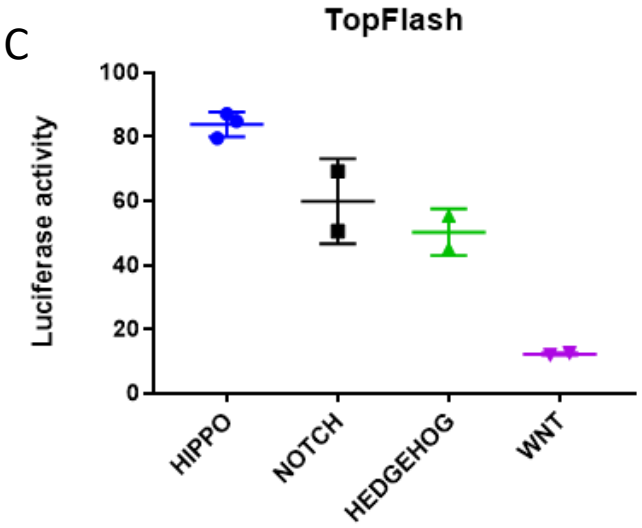


	Biotin-linked BC-2059
Kd [μM]	0.6805

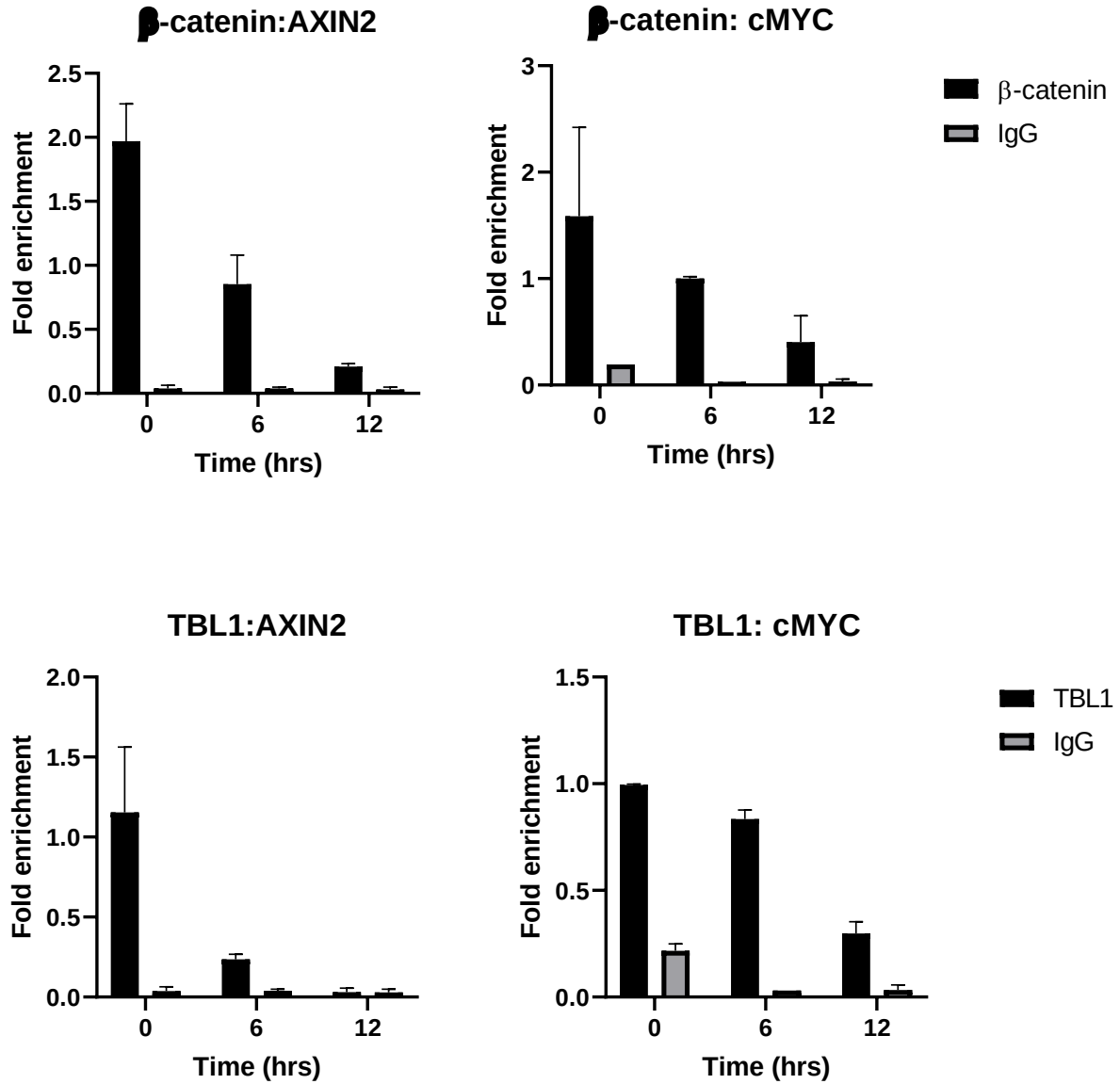
C



Percentile LUC activity at 6h			
WNT	HEDGEHOG	NOTCH	HIPPO
12.31025	50.31435845	59.94426	83.42354

Figure 6

A



B