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The identification of guanosine-5'-triphosphate binding protein 2 (GTPBP2) as a potential drug target for diseases involving excessive Wnt signaling

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Jonathan Manit Wyrick

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We, the thesis committee for the above candidate for the

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Abstract of the Thesis

The identification of guanosine-5'-triphosphate binding protein 2 (GTPBP2) as a potential drug target for diseases involving excessive Wnt signaling

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Canonical Wnt signaling is involved in the regulation of many cellular behaviors dependent upon context, including the induction of cell proliferation, promotion of cell survival, repression of apoptosis, and regulation of cell differentiation. Perturbations in the Wnt signaling pathway have been shown to be present in a various amount of human diseases, such as cancers of the colon and breast, leading to aberrant cellular activity. As such, recent studies have focused on the inhibition of the Wnt signaling pathway in hopes of restoring normal cellular behaviors in diseases with excessive Wnt signaling activity. Our lab has discovered that the large GTPase, guanosine-5'-triphosphate binding protein 2 (Gtpbp2), is essential for Wnt signaling, axis formation, and induction of organizer gene expression in *Xenopus* embryos, demonstrating that Gtpbp2 negatively regulates cytoplasmic Axin protein levels, a rate limiting component of the β-catenin destruction complex. The goal of this research was to investigate the mammalian homolog GTPBP2 to determine if its pivotal role in Wnt signaling expands to mammalian species. Co-immunoprecipitation assays in Hek293t cells revealed physical interactions between GTPBP2 and Axin and glycogen synthase kinase β (GSK3B), and in order to determine the

effects of GTPBP2 knockdown in mammalian cells, *GTPBP2* shRNAi expressing Hek293t cells were generated using lentiviral infection. As many cancer related Wnt signaling pathway mutations require low Axin protein levels, the determination whether GTPBP2 has a negative regulatory effect on Axin in mammalian cells may reveal the possibility that inhibitors of GTPBP2 might serve as a potential therapeutic for these Wnt-related diseases.

DEDICATION

"Oh the places you'll go."

Dr. Seuss

To my father, Hal Atherton Wyrick I,

who I carry in spirit.

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List of Abbreviations

Gtpbp2	GTP-binding protein 2 (*Xenopus*)
GTPBP2	GTP-binding protein 2 (Human/Mouse)
GTPBP1	GTP-binding protein 1
DC	Destruction Complex
CKIα	Casein Kinase I α
GSK3b	Glycogen Synthase Kinase 3 β (Human/Mouse)
Gsk3b	Glycogen Synthase Kinase 3 β (*Xenopus*)
APC	Adenomatosis Polyposis Coli
Dvl	Dishevelled (*Xenopus*)
Dsh	Dishevelled (Human)
LEF/TCF	Lymphoid Enhancer-binding Factor/Transcription Factor
FZD	Frizzled
EF1α	Elongation Factor 1 α
CNS	Central Nervous System
CRC	Colorectal Cancer
TNBC	Triple Negative Breast Cancer
HEK293T	Human Embryonic Kidney 293
dnGsk3b	Dominant Negative Glycogen Synthase Kinase 3 β (Kinase Dead)
COIP	Co-immunoprecipitation
TCL	Total Cell Lysate
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
Tnks2	Tankyrase 2
shRNAi	Short Hairpin RNA
PAC	Puromycin N-Acetyl Transferase
Luc	Firefly Luciferase
qPCR	Quantitative Polymerase Chain Reaction
HPRT1	Hypoxanthine Phosphoribosyltransferase 1
luc2	Firefly Luciferase
hRluc	Renilla Luciferase
SRB	Sulforhodamine B Colorimetric Assay

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The following work was only made possible with the training, guidance, and patience provided by Dr. William Q. Gillis, Dr. Gerald H. Thomsen, and Dr. Bernadette Holdener. I have taken so much from this experience, and for that I am forever in your debt.

Introduction

The canonical Wnt signaling pathway is a key, regulatory pathway for many important cellular behaviors that are often inappropriately regulated in many diseases. Its regulation and activation leads to the differential expression of downstream target genes that instruct cell behaviors such as cell proliferation, differentiation, and virtually all aspects of embryonic development [1, 2]. Like all cell signaling pathways, Wnt signaling is composed of many key protein components that, if mutated or in other ways perturbed, can lead to an increase or decrease in the pathway's activity and consequential aberrant activity of downstream events. The hallmark of canonical Wnt signaling is its involvement in the regulation of cytoplasmic β-catenin protein levels, the major transcriptional activator of canonical Wnt signaling target genes [3].

In the absence of Wnt ligand, cytoplasmic β-catenin is continually targeted for destruction by the β-catenin destruction complex; the DC is composed of two serine/threonine kinases, casein kinase I α (CKIα) and glycogen synthase kinase 3 β (Gsk3b), as well as the scaffolding proteins Axin and adenomatosis polyposis coli (APC) [4-6]. The constitutive phosphorylation of β-catenin by CKIα and Gsk3b primes β-catenin for polyubiquitination by β-Trcp and and subsequent proteasomal degradation [6, 7]. Therefore, in the absence of Wnt signaling, cytoplasmic β-catenin protein levels remain relatively low due to its constitutive destruction, preventing β-catenin nuclear translocation and activation of downstream target genes (Figure 1 A).

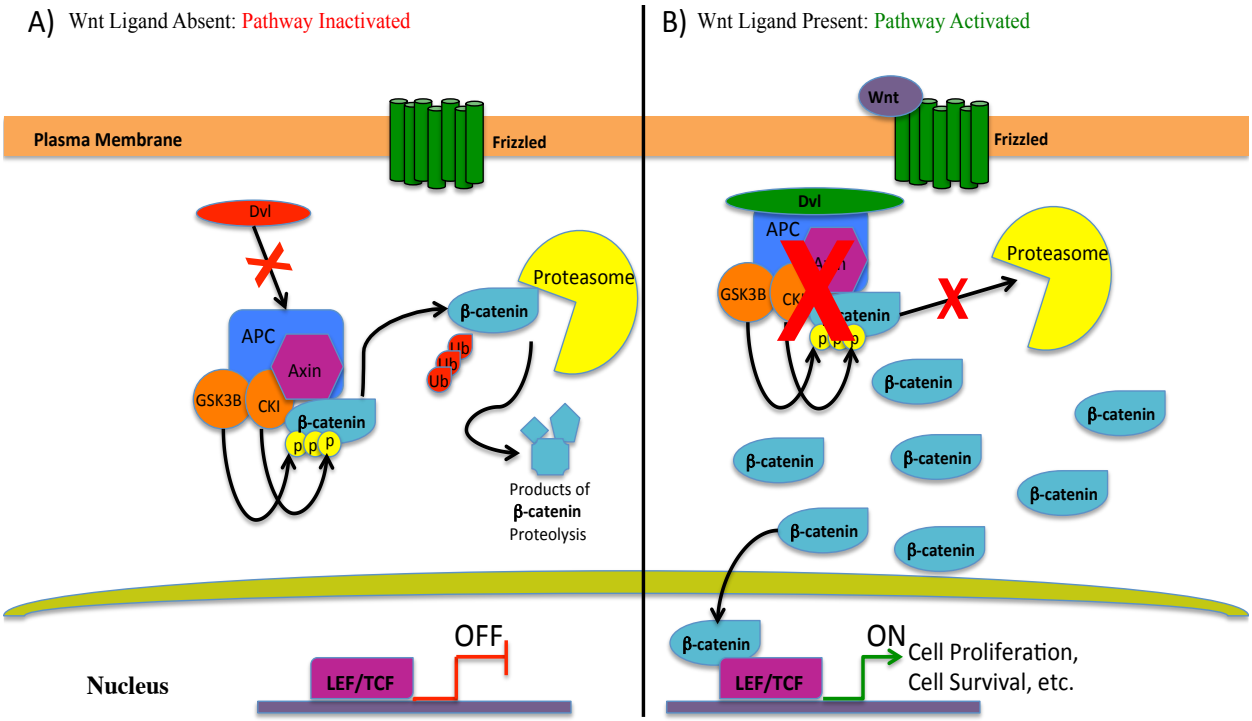

Figure 1: General schematic of the canonical Wnt signaling pathway and its key pathway components. A) In the absence of Wnt ligand, β-catenin is constituently phosphorylated by components of the β-catenin destruction complex (DC). This leads to subsequent ubiquitination and degradation of β-catenin by the proteasomal destruction pathway, preventing nuclear translocation and activation of downstream target genes. B) In the presence of Wnt ligand, Dvl is activated and acts to inhibit the phosphorylation of β-catenin by the DC, resulting in cytoplasmic accumulation and nuclear translocation of β-catenin, subsequent association with LEF/TCF, and activation of downstream target genes, leading to cell proliferation, cell survival, and many other cellular behaviors.

The binding of Wnt ligand to the Frizzled and Lrp5/6 receptors result in Dishevelled (Dvl) mediated inhibition of the DC and therefore inhibition of the phosphorylation and destruction of β-catenin. This inhibition results in the cytoplasmic accumulation of β-catenin and subsequent translocation to the nucleus [3, 8]. Following nuclear translocation, β-catenin forms a complex with LEF/TCF and can subsequently activate downstream targets of the Wnt signaling pathway, leading to the regulation of various cellular behaviors (Figure 1 B) dependent on the differential expression of these downstream target genes [9, 10].

Canonical Wnt signaling can regulate many cellular behaviors dependent upon context, including the induction of cell proliferation, promotion of cell survival, repression of apoptosis, and regulation of cell differentiation [1, 2]. Genes responsible for these cellular behaviors are

activated in a β-catenin-dependent manner, including c-myc, which encodes a potent cell proliferation and survival inducing transcriptional activator. β-catenin has the capacity to activate genes that promote cell survival and proliferation, as well as genes that repress the ability of a cell to induce its own self-destruction when needed. As such, it is not surprising that mutations that activate canonical Wnt signaling, including those preventing the degradation of cytoplasmic β-catenin, can result in the activation of cell proliferation and cell survival, two hallmarks of cancer, and have been found to promote human degenerative diseases and many diverse cancers [11, 12]. For instance, many colon cancers have mutations in components of the DC, including truncations of the scaffolding protein APC, or mutations in β-catenin itself, and although these mutations have been rarely found in breast cancers, there is strong evidence present that indicates the stabilization of cytoplasmic β-catenin in human breast tumors [13, 14]. Because canonical Wnt signaling activates genes that promote cellular division, and there is evidence of cytoplasmic stabilization of β-catenin in these diseases, treatments that result in the reduction of Wnt signaling have begun to receive significant attention in recent research. Establishing a better understanding of the proteins involved and the roles they play in the destruction of β-catenin can therefore possibly reveal future therapeutic targets for diseases with excessive Wnt signaling [15].

The functions of the large GTPase, guanosine-5'-triphosphate binding protein 2 (GTPBP2), until recently have remained largely uncharacterized. Early work had identified *GTPBP2* as a paralog of the interferon-inducible *GTPBP1*, both of which are distantly related to the translation factor EF1α (elongation factor 1 alpha). However, these studies were restricted to a cursory analysis of sequence conservation and gene expression of GTPBP2 in both humans and mice (over 99% identical) [16]. Expanding upon the identification of GTPBP2, recent work has

discovered that GTPBP2 is involved in the rescue of stalled ribosomes in the central nervous system (CNS). Mice with a loss of function mutation in a nuclear encoded, CNS-specific tRNA for the AGA codon show an increase in ribosome stalling at AGA codons during mRNA translation of genes vital for neurogenesis. The complete knockout of GTPBP2 in these mice exacerbated ribosome stalling at AGA codons, and resulted in neurodegeneration and death at 8 to 9 weeks of age; however, the absence of GTPBP2 revealed no increase in ribosome stalling at non-AGA codons, such as the well-studied pause in *Xbp1* [17]. Furthermore, in mice without this tRNA mutation, the absence of GTPBP2 revealed no increase in ribosome stalling, suggesting GTPBP2's specificity for ribosomes stalled at the AGA codon [17].

The Thomsen lab had previously identified Gtpbp2 as a novel binding partner to Smad1, revealing involvement in the normal induction of BMP signaling in the early *Xenopus* embryo. In its absence, serious defects in axis patterning are present, delineating characteristics of Gtpbp2 that associate it with embryonic development [18]. However, many of these defects, including reduced head structures and a reduction in the expression of organizer genes, such as *siamois* and *nodal-related* 3, were not consistent with decreased BMP signaling, which led to additional and yet unpublished analyses in the Thomsen lab examining if Gtpbp2 regulates additional signaling pathways [16].

The Thomsen lab has identified a critical requirement for Gtpbp2 in canonical Wnt signaling during *Xenopus* embryogenesis. Biochemical analyses involving overexpressed and tagged proteins in *Xenopus* embryos revealed that Gtpbp2 can interact with several components of the Wnt pathway, including Axin and Gsk3b, key components of the DC, as well as Dishevelled (Dsh). Genetic epistasis experiments demonstrated that Gtpbp2 plays a vital role in the inhibition of the DC, as Gtpbp2 is required for induction of Wnt targets from overexpression

of a kinase-dead Gsk3 mutant, but not for overexpression of a phospho-stable β-catenin mutant. The epistasis data of Gtpbp2 knockdown suggested that the inhibition of β-catenin-dependent Wnt signaling by Gtpbp2 occurs at the level of β-catenin phosphorylation.

The goal of this project was to investigate the comparative role of GTPBP2 in mammalian cells to determine if its pivotal role in Wnt signaling in *Xenopus* expands to mammalian species. Colorectal cancers are the most common cause of non-smoking-related cancer deaths in the western world, and more than 90% of colorectal cancers have an activating mutation of the canonical Wnt signaling pathway, leading to the stabilization and accumulation of β-catenin in the nucleus [19, 20]. Although excessive Wnt signaling is found in only a fraction of breast cancers, nearly all clinical biopsies reveal abnormally high levels of nuclear β-catenin in tumors surveyed from individuals with triple negative breast cancer (TNBC) — the cause of more than one-quarter of all deaths due to breast cancer [21, 22]. If GTPBP2's essential role in the mechanism of Wnt signaling inhibition is elucidated in mammalian cells, it can illuminate GTPBP2 as a potential therapeutic target in these two deadly cancers.

Materials and Methods

Cell Culture

Human Embryonic Kidney 293 (Hek293T) cells, a cell line known to be responsive to Wnt, were cultured in 10% Fetal Bovine Serum (FBS), 1% penicillin (100U/mL), and 1% streptomycin (100U/mL) Dulbecco's modified Eagle's medium (DMEM). Cells were kept with 5% CO_2 at 37° C, and the growth media was changed every 48-72 hours, as necessary, to maintain a proper growth environment. Cells were split and passaged once having reached near confluency to maintain a stable cell line.

DNA Plasmids

Plasmids for GTPBP2 were previously described [18], and remaining plasmids were generous gifts from Ken-ichi Takemaru (Stony Brook University), Peter Klein (University of Pennsylvania) and Sergei Sokol (Mount Sinai School of Medicine), including pCS2MT-xDsh (myc-dsh) [23], Xg134 pCS2MT-Gsk3 (myc-gsk3) [24], Xg137 pCS2-dnGsk3 (dngsk3) [25], pCS2-Axin MtFu1 (myc-axin) [26].

Antibodies

Antibodies from monoclonal supernatants (mouse anti-Myc 9E10, 1:100) were generously donated (J. Hsieh) and others were purchased from the following and used at the recommended dilutions for western blotting and/or immunoprecipitation: rabbit anti-HA (RHGT-45A, 1:500), ICL; rabbit anti-Axin1 (C95H11, 1:1000), Cell Signaling Technology; iRDye800 conjugated affinity purified anti-mouse IgG (goat) (610-131-121, 1:5000), Rockland Immunochemicals; Alexa-fluor 680 goat anti-rabbit (A-21076, 1:5000), Molecular Probes.

Transfections

Transfection of Hek293T cells was performed using linear Polyethylenimine (PEI) at 1 mg/mL. For co-immunoprecipitation (COIP) experiments, cells were cultured in 6-well plates and received no more than a total of 2 μg of plasmid DNA in the transfection reagent for each well. The transfection reagent was prepared in 100 μL of 0.15M NaCl and 4 μL of PEI and incubated at room temperature for 15 minutes prior to addition. Cells were incubated with the transfection reagent in 5% CO_2 at 37° C overnight.

For dual-luciferase reporter assay experiments, Hek293T cells were cultured in a 24-well plate and upon reaching near confluency, received no more than 1 μg of plasmid DNA in the transfection reagent for each well. The transfection reagent was prepared in 25 μL of 0.15M

NaCl and 1 μL of PEI and incubated at room temperature for 15 minutes prior to addition. The cells were incubated with the transfection reagent in 5% CO_2 at 37° C overnight.

Co-Immunoprecipitation of Gtpbp2 and components of the Wnt signaling pathway

For co-immunoprecipitation experiments, Hek293T cells cultured in 6-well plates were co-transfected with expression plasmids for HA-GTPBP2, Myc-Gsk3b, Myc-Axin and/or c-Myc using linear PEI (1mg/mL). Each culture well received 1 μg of plasmid DNA for HA-Gtpbp2 and either Myc-Gsk3b, Myc-Axin, or c-Myc. Cells were harvested following a 37° C, 5% CO_2 overnight incubation by a short incubation (~5 min) with 1mL of cold 5mM EDTA/PBS and then collected by pipetting. The cells were then pelleted by centrifugation at 3,000 x g for 3 minutes, and lysed by vigorous pipetting in 200 μL of cold Nonidet P-40 (NP40) lysis buffer (10 mM Tris-Cl pH8.0, 137 mM NaCl, 10% Glycerol, 1% NP40, and complete protease and phosphatase inhibitor tablets [Roche]). To help prevent any unwanted protein degradation, each sample was maintained on ice in all subsequent steps.

To obtain cell lysates in the absence of plasma membrane and other unwanted cellular constituents, each sample was centrifuged at 14,000 rpm for 10 minutes at 4° C and the supernatant was obtained for analysis. For analysis of protein expression in the total cell lysate (TCL) of each sample prior to antibody incubation, 20 μL of each cell lysate was removed and maintained at 4° C. The remaining 180 μL of each cell lysate sample was then incubated on an orbital rocker with mouse anti-Myc (9E10) antibody overnight at 4° C to immunoprecipitate any Myc-tagged proteins. NEB anti-IgG magnetic beads were blocked by suspending the beads in untransfected Hek293T cell lysate and were incubated on an orbital rocker for 1 hour at 4° C, subsequently washed with cold PBS, using a magnetic microcentrifuge tube rack to retain the

magnetic beads. Blocked beads were then split into equal aliquots among cell lysates incubated with the mouse anti-Myc antibody and incubated for 1 hour on a rotary rocker at 4° C. The immunocomplexes were then washed three times in cold NP40 lysing buffer utilizing a magnetic microcentrifuge tube rack to retain the immunocomplexes attached to the magnetic beads and then eluted by heating for 5 minutes at 95° C in 25 μL of denaturing sample buffer (0.1% beta-mercaptoethanol, 0.0005% Bromophenol blue, 10% Glycerol, 2% SDS, 63 mM Tris-HCl pH 6.8). The 20 μL of total cell lysate samples were also diluted in 20 μL of 2x denaturing sample buffer and heated for 5 minutes at 95° C. All of the samples were then separated through SDS-PAGE in an 8% polyacrylamide gel and transferred to a nitrocellulose membrane for immunodetection.

For immunodetection, the nitrocellulose membrane was blocked in 5% BSA/PBS solution for 1 hour and incubated overnight at 4° C while rotating in the presence of mouse anti-myc (1:100) and rabbit anti-HA (1:500) primary antibodies in a 5% BSA and 0.2% Tween-20 PBS solution. Following overnight incubation with primary antibodies, the nitrocellulose membrane was washed in 0.1% Tween-20/PBS four times in 10 minute increments with gentle shaking and then incubated with mouse anti-rabbit and rabbit anti-mouse IR secondary antibodies (1:5000 Alexa 680 and 1:5000 IRdye800) in a 1% Casein, 0.2% Tween-20, 0.01% SDS PBS solution protected from light while rotating for 1 hour at room temperature. The membrane was then washed in 0.1% Tween-20/PBS four times in 10-minute increments in a covered container with gentle shaking and scanned on a Licor Odyssey Classic Imager for immunoblot imaging.

Overexpression of Gtpbp2 and analysis of Axin levels

Hek293T cells were grown in 12-well plates and transfected when confluent with 1 μg of DNA expression plasmids for XA-Gtpbp2, Human-GTPBP2, mCherry, or Tankyrase 2 (Tnks2) in two replicates with 25 μL of 0.15M NaCl and 1 μL of PEI. Cells were incubated overnight in 5% CO_2 at 37° C and harvested in 750 μL of 5mM EDTA and replicates were combined for lysis in NP40 lysis buffer. Cell lysates were collected for analysis of endogenous Axin protein expression levels by centrifugation at 14,000 rpm for 10 minutes at 4° C, and 20 μL of each cell lysate was diluted in 20 μL of denaturing buffer. Each sample was then separated through SDS-PAGE in an 8% polyacrylamide gel and transferred to a nitrocellulose membrane.

For immunodetection, the nitrocellulose membrane was blocked in 5% BSA/PBS solution for 1 hour and incubated overnight at 4° C while rotating in the presence of rabbit anti-axin1 (1:1000) and rabbit anti-HA (1:500) primary antibodies in a 5% BSA and 0.2% Tween-20 PBS solution. Following overnight incubation, the membrane was washed in by gentle shaking in 0.1% Tween-20/PBS and incubated while rotating with mouse anti-rabbit (1:5000, Alexa 680) secondary antibody for 1 hour at room temperature. Following secondary antibody incubation, the membrane was washed in a covered container with 0.1% Tween-20/PBS and scanned on a Licor Odyssey Classic Imager for immunoblot imaging.

Gtpbp2 shRNAi Cell Line Generation

The expression of short-hairpin RNAs (shRNAi) can be used to reduce the expression of target mRNAs in mammalian cells via endogenous mechanisms. Using the Invitrogen Block-it software analysis, following specific generation guidelines, three shRNAi sequences were designed to target the human *GTPBP2* mRNA in different regions of the nucleotide sequence (Table 1) [25].

Sequence Start Site	shRNAi Sequence
461	5'-CGAGAGCGAGAAGTGGATTATGATA-3'
846	5'-CCACCATCTTTGGCCTCACATCATA-3'
1184	5'-GACCTCCTCAAAGTCTTTCTGAATA-3'

Table 1: shRNAi sequences targeting *GTPBP2* generated by Invitrogen Block-it software analysis. With the use of the analysis of the sequence of *GTPBP2* by Invitrogen Block-it software, three shRNAi sequences were designed in order to target *GTPBP2* for its subsequent reduction of expression.

To create Hek293t cell lines that stably express shRNAi that target *GTPBP2*, we used a retrovirus expressing these shRNAi along with a selectable gene for puromycin resistance, puromycin N-acetyl-transferase (PAC), using the pSiren-RetroQ plasmid (Clonetech). Forward and reverse oligonucleotides containing the sense and antisense sequences for the target sequence, separated by a hairpin loop terminator, followed by a terminator, and flanked by the appropriate EcoRI and BamHI restriction enzyme sites were designed (Table 2). Equal volumes of 100 μM forward and reverse oligonucleotides were mixed and annealed by heating at 95° C for 30 seconds, 72°C for 2 minutes, 37° C for 2 minutes, and 25° C for 2 minutes. The resulting double-stranded oligonucleotides were then linearized with EcoRI and BamHI and then ligated into the pSiren-RetroQ vector, digested with EcoRI and BamHI and treated with shrimp alkaline phosphatase (Roche).

DNA	DNA Sequence
461 Forward	5'-GATCCCGAGAGCGAGAAGTGGATTATGATAGCTTCCTGTCACTATCATAATCCACTTCTCGCTCTCGTTTTTTG-3'
461 Reverse	5'-AATTCAAAAAACGAGAGCGAGAAGTGGATTATGATAGTGACAGGAAGCTATCATAATCCACTTCTCGCTCTCGG-3'
846 Forward	5'-GATCCCCACCATCTTTGGCCTCACATCATAGCTTCCTGTCACTATGATGTGAGGCCAAAGATGGTGGTTTTTTG-3'
846 Reverse	5'-AATTCAAAAAACCACCATCTTTGGCCTCACATCATAGTGACAGGAAGCTATGATGTGAGGCCAAAGATGGTGGG-3'
1184 Forward	5'-GATCCGACCTCCTCAAAGTCTTTCTGAATAGCTTCCTGTCACTATTCAGAAAGACTTTGAGGAGGTCTTTTT G -3'
1184 Reverse	5'-AATTCAAAAAAGACCTCCTCAAAGTCTTTCTGAATAGTGACAGGAAGCTATTCAGAAAGACTTTGAGGAGGTCG-3'

Table 2: Forward and reverse oligonucleotide sequences generated for the purpose of insertion into the pSiren-RetroQ vector for subsequent use in lentiviral infection of Hek293T cells. Forward and reverse oligonucleotides containing the sense and antisense sequences for the *GTPBP2* target sequence, separated by a hairpin terminator, followed by a terminator, and flanked by appropriate EcoRI and BamHI restriction enzyme recognition sites were generated in order for insertion into the pSiren-RetroQ vector.

The generation of stable *GTPBP2* shRNAi cell lines was done as described by Clonetech Laboratories Inc. 12 separate 6 cm plates of near confluent Gp2-293 cells were transfected with 1 μg each of pSiren-RetroQ, expressing shRNAi for either 461, 846, 1184, or for the firefly *luc2* (to act as a negative control for shRNAi expression), and pVSV-G envelope plasmids using PEI in replicates of three. The cell media was then changed following an overnight incubation in 5% CO_2 at 37° C, and then incubated again in 5% CO_2 at 37° C for two days for sufficient accumulation of retroviral particles. The retroviral-particle-containing media was collected from the incubating cells and filtered using a 0.22 μm syringe filter (Sardst 831826001) and Polybrene (Fischer NC9515805) was added to the viral supernatants, which were used to transduce Hek293t cells. After a 24-hour incubation with the different viral supernatants, the media was replaced with fresh media containing 4 μM/mL of puromycin (Gemini 400128P) for selection of properly infected Hek293T cells. Cells were maintained under puromycin selection for two weeks, replacing the media with fresh puromycin containing media whenever necessary.

Following puromycin selection, cells infected with the same shRNAi expressing lentivirus were pooled for use as a stable cell line for subsequent experiments and named for the shRNAi in which they express.

Quantitative Polymerase Chain Reaction

For Quantitative PCR (qPCR) was used to determine if the stable *GTPBP2* shRNAi cell lines generated by lentiviral infection had a sufficient knockdown of *GTPBP2*. Each cell line (Luc, 461, 846, and 1184) was grown in three separate wells of a 12-well plate until confluent. The cells were harvested by gently peeling the cells off of each well with 750 μL of cold 5mM EDTA/PBS, pelleted by centrifugation at 3,000 x g for 4 minutes, and lysed by vigorous pipetting in TRIzol in order to isolate total RNA for the use of cDNA generation. Following a 5-minute incubation to solubilize all samples at room temperature, 200 μL of chloroform was added to each sample to promote phase separation for RNA isolation. Each sample was then vortexed, incubated at room temperature for 3 minutes, and centrifuged at 10,000 x g for 10 minutes at room temperature, producing two phases containing either RNA or proteins, separated by a DNA interface. The phase containing the RNA was collected and treated with 500 μL of isopropanol, to precipitate the RNA, and incubated at room temperature for 10 minutes. Precipitated RNA of each sample was pelleted and collected following centrifugation at 10,000 x g for 10 minutes at 4° C, washed with 1 mL of 75% ethanol, and allowed to air dry for 10 minutes.

In order to remove any possible DNA contaminants, each pellet was treated with a DNase solution, composed of DepC-treated water, DNase buffer, RNase-Free DNase (Invitrogen), and RNase Out RNase inhibitor, incubated at 37° C for 20 minutes for proper DNA degradation to occur, and brought up to 150 μL with a 500 mM ammonium acetate 10 mM EDTA solution. 150

μL of a phenol-chloroform-isoamyl alcohol solution was added to each sample for the isolation of RNA. Samples were centrifuged at 10,000 x g for 4 minutes to separate extraction phases and the layer containing the RNA was collected. 375 μL of 100% RNase-free ethanol was added to each extraction and each solution was incubated overnight in -80° C for RNA precipitation.

Precipitated RNA was pelleted by centrifugation at 4° C for 20 minutes, washed with 75% ethanol, air dried, and resuspended in 15 μL of DepC-treated water for subsequent cDNA generation. 250 ng of random hexamer primers and a 10 mM mix of dNTPs was added to1 μg of RNA from each solution and incubated with 200 units of Protoscript II reverse at room temperature for 10 minutes, 42° C for 50 minutes for the reaction to proceed, and finally at 70° C for 15 minutes to terminate the reaction. Each sample of cDNA was then brought up to 200 μL with RNase-free water to use as a template for qPCR analysis in with primers for *GTPBP2* (gtpbp2-F-5'GGATGAAATCTACACAGTACCAGAG-3', gtpbp2-R-5'- CATACTCTCAGCT CCAGGAAG-3') and *HPRT1* as a reference gene (hprt1-F-5'-ATGGACAGGACTGAACGTCT TGCT-3', hprt1-R-5'-TTGAGCACACAGAGGGCTACAATG-3'). Graphs represent the average and standard deviation of the three biological replicates, and for each replicate, the average levels of two technical replicates of *GTPBP2* was normalized to the average levels of two technical replicates of the *HPRT1* reference transcripts.

Preliminary rounds of qPCR of the above primers for *GTPBP2* resulted in qPCR data that suggested an increase in *GTPBP2* levels, something that should not have occurred. Reviewing the melting temperatures of each replicate and seeing amplification products in the negative controls suggested faulty primers and possible DNA contamination, and therefore new primers for *GTPBP2* were designed (gtpbp2-F-5'-CCAGGGAGAGCTGGACAAT-3', and gtpbp2-R-5'-

CGGGGCAGTATGATGT GAG-3') in order to increase the efficiency of qPCR replication and reduce the amount of possible primer dimerization.

Super-TopFlash Dual-Luciferase Reporter Assay

Hek293t cells grown in a 24-well plate were transfected with 0.025 μg and 0.0025 μg of DNA expression plasmids for the synthetic firefly luciferase (*luc2*) and the *Renilla reniformis* luciferase (*hRluc*), respectively, along with 0.25 μg of DNA expression plasmids for mCherry, Gsk3b, dnGsk3b (dominant negative Gsk3b), or Dvl in 3-well replicates [27]. Of the 24 wells of the plate, 9 wells were transfected with 0.025 μg and 0.0025 μg of DNA expression plasmids for *luc2* and *hRluc*, respectively, and 0.25 ug DNA expression plasmid for mCherry to test the efficiency of Wnt signaling activation of three differently aged Wnt-conditioned cell medias, while another 3 wells were transfected with DNA expression plasmids for both luciferases as well as for mCherry, a negative control, to be treated with unconditioned media.

Following a 37°C, 5% CO_2 overnight incubation, 9 wells that were transfected with the *luc2, hRluc*, and mCherry expression plasmids were replaced with three Wnt-conditioned medias of differing ages in 3-well replicates. The cell media of the remaining wells transfected with the luciferase plasmids and either mCherry, Gsk3b, dnGsk3b, or Dvl was replaced with fresh, unconditioned media. The cells were incubated in the newly replaced medias for 4 hours in order to allow proper cell signaling to occur.

The cells of each well were harvested after incubation in the newly replaced medias in 500 μL of 5mM EDTA/PBS by gently peeling them off each well. The cells were then pelleted by centrifugation at 3,000 x g for 3 minutes, and each cell pellet was then lysed by vigorous pipetting in 100 μL of cold "passive lysis buffer," provided by the Promega Dual Luciferase

Assay kit, and maintained on ice in all subsequent steps to help prevent any unwanted protein degradation. To collect lysates, the cells were centrifuged at 14,000 rpm for 10 minutes at 4° C, and the supernatants were obtained for analysis.

To activate the reporter genes for luminescence analysis in a luminometer, 10 μL of each lysate was obtained, the dual-luciferase assay protocol from Promega was followed, and the ratio of *luc2*:*hRluc* luminescence was utilized for relative, normalized Wnt signaling activity. The average of each 3-well replicate ratio was used to examine overall signaling activity.

To determine if shRNAi driven knockdown of GTPBP2 inhibits Wnt signaling, each of the generated shRNAi cell lines (Luc, 461, 846, and 1184) were grown in 24-well plates in 6-well replicates of each cell line and transfected with 0.025 μg of *luc2* and 0.0025 μg of *hRluc* DNA expression plasmids. 3-wells of each of the cell lines were incubated in Wnt-conditioned media and the other remaining 3-wells of each cell line were incubated in unconditioned media. The cells were incubated for 4 hours in 37° C, 5% CO_2, harvested for cell lysate analysis, and analyzed for relative *luc2* luminescence as described above. The average of each 3-well replicate *luc2*:*hRluc* luminescence of both the cells incubated in Wnt-conditioned media and unconditioned media was used to examine overall signaling activity.

Results

GTPBP2 co-immunoprecipitates with components of the destruction complex: Axin and Gsk3b

In effort to determine the role of GTPBP2 in the inhibition of the canonical Wnt signaling, the biochemical interactions of GTPBP2 with Wnt pathway components were examined. Epistasis data for Gtpbp2 and biochemical analyses in *Xenopus* embryos revealed that Gtpbp2 acts at the

level of the DC and can physically interact with Axin and Gsk3b, and the presence of these interactions in mammalian cells would suggest GTPBP2's role in Wnt signaling expands to mammalian species. Hek293t cell lysates of cells transfected with expression plasmids for HA-GTPBP2 and either Myc, Myc-Gsk3b, or Myc-Axin were analyzed to indicate possible physical interactions between the GTPBP2 and Gsk3b and/or Axin. Transfections with HA-GTPBP2 and c-myc served as a negative control to indicate that GTPBP2 was not interacting with the domains of the protein tag in the case of any co-immunoprecipitation with the Myc-tagged proteins; it allowed for the determination that specific protein-protein domain interactions between GTPBP2 and the target proteins themselves were the cause of any co-immunoprecipitation, rather than an interaction of HA-GTPBP2 with the myc-tag of Myc-Gsk3b or Myc-Axin.

Protein expression of HA-GTPBP2, Myc-Axin, and Myc-Gsk3b plasmids was confirmed in the TCL lanes by western immunodetection (Figure 2). The protein bands analyzed in the detection had molecular masses of approximately 66 kDa, 94 kDa, 47 kDa and for HA-GTPBP2, Myc-Axin, and Myc-Gsk3b, respectively. Axin and Gsk3b immunoprecipitated in all cell lysates, evidenced by bands corresponding to their proper, approximate molecular weights. Because c-Myc does not physically interact with GTPBP2, there was an absence of a molecular weight band for GTPBP2 in the lane representing lysates in which c-Myc was immunoprecipitated, as expected. There was, however, a band of corresponding molecular weight for GTPBP2 that appeared in both the cell lysates in which Axin and Gsk3b was pulled down by immunoprecipitation, allowing for co-immunoprecipitation interpretation (Figure 2).

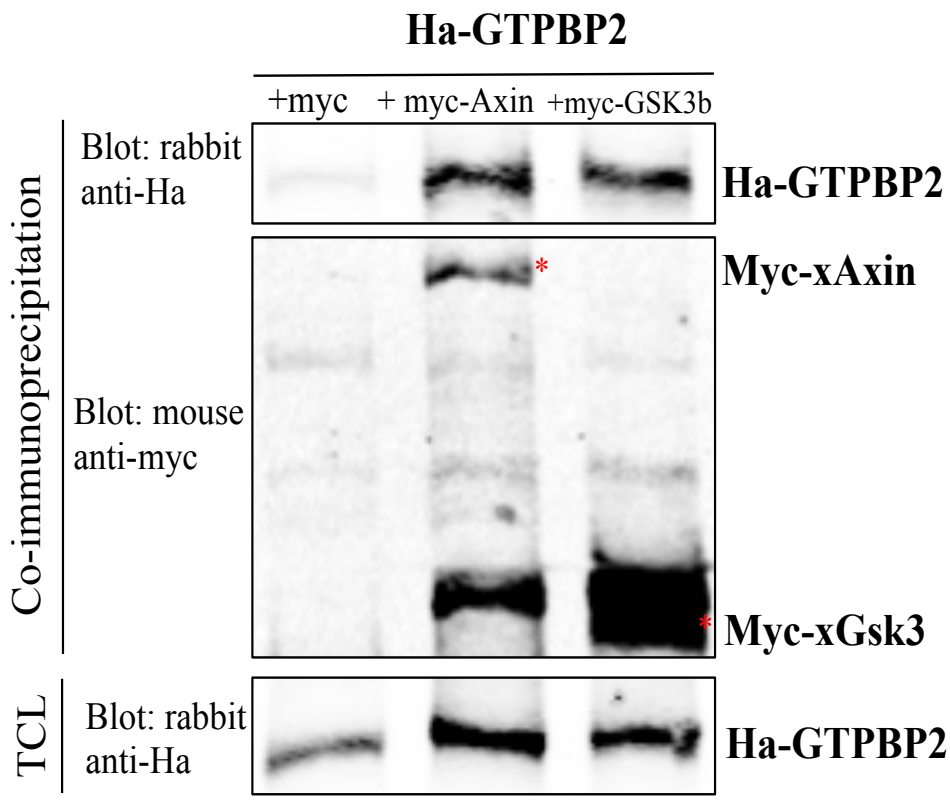

Figure 2: GTPBP2 co-immunoprecipitates with Axin and GSK3. HEK293T cells were co-transfected with DNA expression plasmids encoding HA-GTPBP2, Myc, Myc-xGSK3, and/or Myc-xAxin and lysed after two days of incubation. Lysates were incubated with anti-Myc antibody overnight, purified using anti-IgG magnetic beads, and blotted with mouse anti-myc and rabbit anti-Ha antibodies (the image represents one blot represented in separate detection channels).

To strengthen the results of this co-immunoprecipitation, HA-GTPBP2 was immunoprecipitated with anti-HA antibodies, rather than the myc-tagged proteins, in multiple experimental replicates to determine if the reverse co-immunoprecipitation was possible. Multiple replications of anti-HA pull downs failed to produce a sufficient signal in an immunoblot for interpretation; there was an absence of HA-tagged protein (i.e. GTPBP2) pull-down, which would therefore prevent any proteins interacting with them to be co-immunoprecipitated (data not shown). To determine if HA-GTPBP2 interacted with endogenous Axin, an anti-HA antibody pull-down was repeated and immunoblotted for endogenous Axin, however, these replications also failed to produce a sufficient signal in an immunoblot for interpretation (data not shown).

Overexpression of GTPBP2 and endogenous Axin levels

The results from previous experiments utilizing morpholino knockdown of Gtpbp2 translation in *Xenopus* embryos suggested that Gtpbp2 acts as a negative regulator of Axin. Therefore, we hypothesized that the overexpression of GTPBP2 may result in the decrease of endogenous Axin levels in mammalian cells. We therefore set out to test this hypothesis by overexpressing GTPBP2 in Hek293t cells and analyzing the consequential levels of endogenous Axin. Basal levels of Axin expression should have been seen in the lane for cells transfected with mCherry, and the lane for cells transfected with Tnks2 should have shown lowered levels of endogenous axin relative to mCherry transfected cells. If GTPBP2 is involved in the negative regulation of Axin, lanes for cells transfected with XA-Gtpbp2 and Human-GTPBP2 should have shown a result similar to the lane of cells transfected with Tnks2. After four repetitions of experimental trials with multiple attempts at troubleshooting problems that came about, a sufficient immunoblot for interpretation of Axin levels in response to the overexpression of GTPBP2, was unable to be produced (data not shown).

GTPBP2 shRNAi Knockdown

In order to investigate the effects of GTPBP2's loss-of-function in the regulation of Axin protein levels in mammalian cells, we created lines of Hek293t cells that stably express shRNAi that target *GTPBP2*. Because complete knockout of GTPBP2 could possibly be lethal to mammalian cells or cause self-sustaining cells to reduce their rates of replication, a cell line that has a reduced level of GTPBP2 expression, rather than a complete knockout, was desired. Cells that endogenously express *GTPBP2* shRNAi were generated in order to investigate these effects. Two of the *GTPBP2* shRNAi cell lines (461 and 1184) showed a decrease in *GTPBP2* levels, in comparison to basal expression levels of *GTPBP2* (Luc), however, it appeared that the level of

GTPBP2 in the 846-cell line had an apparent increase (Figure 3). After analyzing the melting curves for each replicate, low and multiple melting temperature curves were indicative of primer dimerization and possible contamination with other DNA plasmids in multiple samples, respectively.

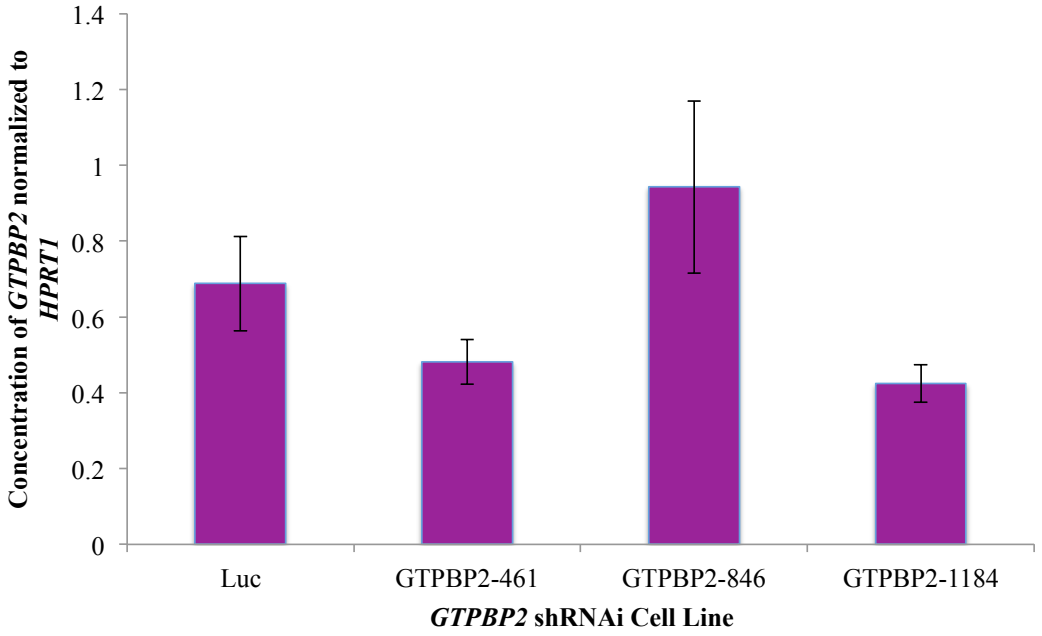

Figure 3: The effect of shRNAi lines on *GTPBP2* expression in Hek293t. Three Hek293t cell lines were infected with retroviral DNA encoding shRNAi for *GTPBP2* with different targeting locations of *GTPBP2*, numbered with the corresponding first nucleotide of the mRNA target location (461, 846, and 1184). Three cultures of each cell line were analyzed for *GTPBP2* concentrations by RT-qPCR, and the average concentrations for each sample were normalized to the average concentration of the *HPRT1* housekeeping gene. Each bar represents the average relative concentrations of *GTPBP2* between two technical replicates of three biological replicates with their standard error.

New primers for GTPBP2 were designed to prevent any possible primer dimerization that might have occurred in the first set of primers, and following a thorough process to decontaminate the work area, in the case of DNA contamination, another qPCR analysis was done on the same cell lines, revealing lower levels of *GTPBP2* in all *GTPBP2* shRNAi cell lines in comparison to the Luc negative control. Although all lines showed a decrease in *GTPBP2*, the cell line with the most significant reduction in *GTPBP2* expressed shRNAi that targets *GTPBP2* beginning at nucleotide 461. The replicates of this cell line contained the smallest standard

deviation and an overall approximate 72% reduction in the expression of *GTPBP2* in comparison to the Luc control (Figure 4).

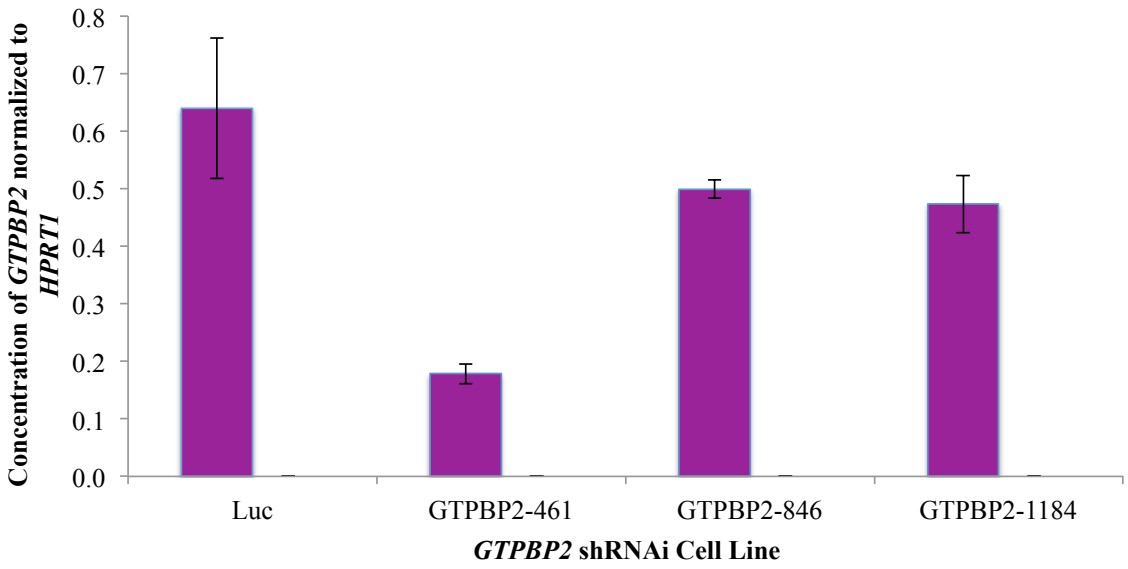

Figure 4: The effect of shRNAi lines on *GTPBP2* expression post efforts of decontamination. Hek293T cells were infected with retroviral DNA encoding shRNAi for *GTPBP2* with different targeting sequences of *GTPBP2*. Three cultures of each cell line were analyzed for *GTPBP2* concentrations by qPCR, and the average concentrations for each sample were normalized to the average concentration of the *HPRT1* housekeeping gene. Each bar represents the average relative concentrations of *GTPBP2* between two technical replicates of three biological replicates with their standard error.

Relative Wnt signaling pathway activity by Wnt8 in shRNAi mediated knockdown of GTPBP2

In order to determine the effects that shRNAi driven *GTPBP2* knockdown has on Wnt signaling, a dual-luciferase reporter assay was used to establish relative activity of the canonical Wnt signaling pathway following induced activation in the *GTPBP2* shRNAi lines. We first tested the *GTPBP2* shRNAi cell lines using a conditioned cell media from cells expressing Wnt8, a known pathway activator. In the presence of Wnt8, the cell line expressing *luc2* shRNAi should have been relatively high, due to the luciferase protein's nonexistent involvement in the Wnt signaling pathway as well as its lack of presence in Hek293T cells. If GTPBP2 knockdown is required for Wnt signaling activation, *GTPBP2* shRNAi cell lines should show a decrease in pathway activity in response to Wnt8 conditioned media as well as a decreased basal activity in

unconditioned media compared to the Luc control. In contrast, however, incubation of the *luc2* shRNAi cells in media with or without the presence of Wnt8 resulted in relatively similar activity levels; the Wnt-Luciferase reporter was not transactivated (Figure 5).

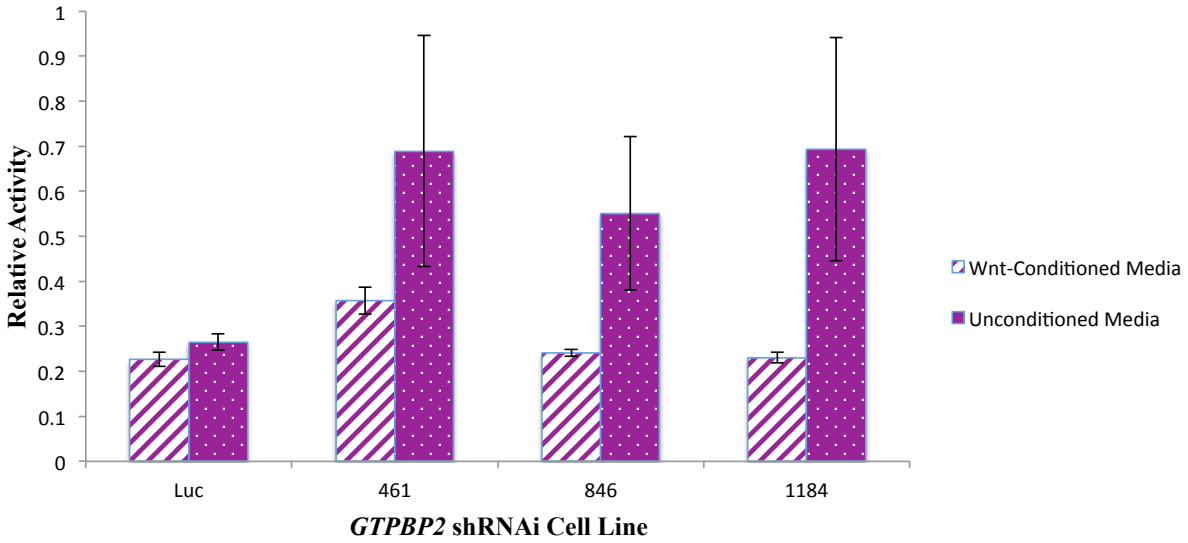

Figure 5: Relative activity of Wnt signaling in shRNAi cell lines. Each shRNAi cell line (Luc, 461, 846, 1184) was transfected with different DNA expression plasmids for *luc2* and *hRluc* reporter genes and incubated with either Wnt-conditioned cell media or unconditioned cell media to determine the activity of the canonical Wnt signaling pathway by a dual-luciferase reporter assay. Relative activity is measured by the ratio of *luc2:hRluc* luminescence and presented as the average of three replicates with their standard error.

Determining the basal levels of luciferase reporter activity in Wnt8 activated cells

Because the results of the dual-luciferase assay of shRNAi cell lines were uninterpretable, Hek293T cells were transfected with known activators and repressors of the Wnt signaling pathway to determine the efficiency of the reporter assay reagents as well as to test the efficacy of different Wnt8 conditioned cell medias for future use, in the case that the age or storage of the media resulted in their inefficiency in eliciting a proper response. mCherry plays no role in the Wnt signaling pathway, and therefore cells transfected with DNA expression plasmids for mCherry should not show an increase in relative pathway activity, in which it was observed (Figure 6A). Wnt8 is a known activator of the canonical Wnt signaling pathway;

therefore, cells treated with three different Wnt8 conditioned cell medias should have resulted in sharp peaks in the relative activity of the Wnt signaling pathway.

However, each Wnt8 conditioned media failed to elicit such a response in Hek293T cells (Figure 6B-D). In the absence of Wnt8, increasing the concentration of Gsk3b should decrease the activity of the pathway, ultimately being lower than the mCherry control in the absence of Wnt8, because of the increased phosphorylation and subsequent degradation of β-catenin. It was observed that the activity of the pathway was lower in this condition than that of the mCherry negative control, as expected (Figure 6E).

Introducing a dominant negative Gsk3b (dnGsk3b), that has lost its kinase activity due to a mutation, or increasing the levels of Dvl should theoretically allow for an increase Wnt signaling activity, due to the decrease in the number of active DCs and subsequent β-catenin degradation. Although increasing the levels of Dvl resulted in a sharp increase in relative activity as expected (Figure 6G), the introduction of dnGsk3b failed to also show a sharp increase (Figure 6F).

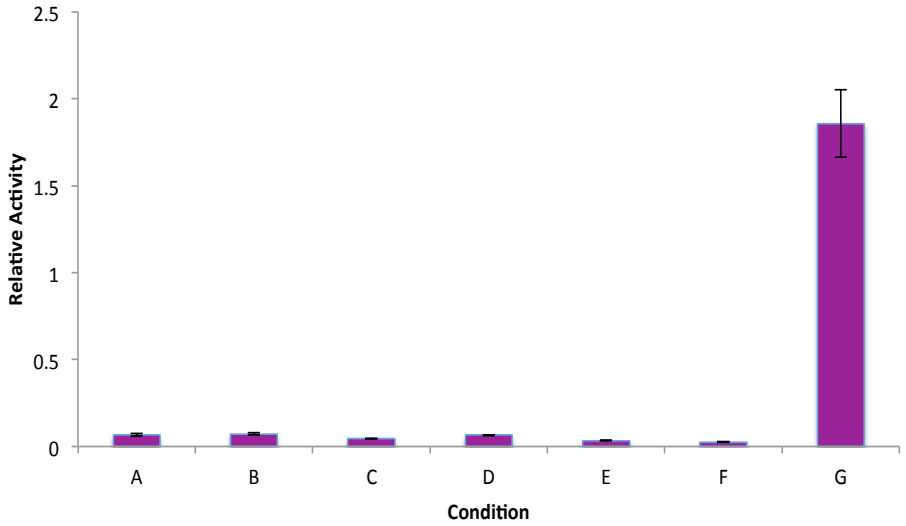

Figure 6: Relative activity of Wnt signaling in transfected Hek293t cells. Hek293t cells were transfected with different DNA expression plasmids and incubated with either Wnt-conditioned cell media or unconditioned cell media to determine the activity of the canonical Wnt signaling pathway by a dual-luciferase reporter assay. All cells were transfected with plasmids expressing *luc2* and *hRluc* reporter genes. Relative activity is measured by the ratio of *luc2:hRluc* luminescence and presented by the average of three replicates and their standard error. A) Cells also transfected with mCherry in unconditioned media. B) Cells also transfected with mCherry in Wnt-conditioned media 1. C) Cells also transfected with mCherry in Wnt-conditioned media 2. D) Cells also transfected with mCherry in Wnt-conditioned media 3. E) Cells also transfected with Gsk3b in unconditioned media. F) Cells also transfected with dnGsk3b in unconditioned media. G) Cells also transfected with Dvl in unconditioned media.

Discussion

The goal of this thesis work was to determine if GTPBP2 plays a role in regulating canonical Wnt signaling in mammalian cells, expanding upon earlier work done in *Xenopus* embryos. To this end, I have examined biochemical interactions of GTPBP2 with Wnt pathway components, as well as examined the effect of both gain and loss of function of GTPBP2 in mammalian cells.

This showed that GTPBP2 physically interacts with Axin and Gsk3b in mammalian cells, via co-immunoprecipitation of overexpressed and tagged components. This evidence was generated via overexpression of an HA-tagged version of GTPBP2 with myc-tagged Axin and Gsk3b, similar to previous experiments done in frogs, and suggests that GTPBP2 may be working at the level of the DC. I also attempted to examine if HA-GTPBP2 could co-

immunoprecipitate with endogenous Axin protein in Hek293t cells, however, the signal was not sufficient to determine a result, suggesting either the conditions need to be optimized, or there is a true lack of interaction. To distinguish between these results, additional experiments incorporating a positive control would need to be done (e.g. APC or other components known to co-immunoprecipitate with endogenous Axin protein).

Previous work in *Xenopus* embryos revealed that Gtpbp2 knockdown resulted in elevated levels of Axin. I attempted to test if Gtpbp2 has a similar negative regulatory role in Axin stability in mammalian cells via overexpression of GTPBP2, predicting that increasing GTPBP2 in mammalian cells could decrease endogenous Axin levels. However, overexpression of GTPBP2 did not appear to affect the levels in Hek293t cells. This suggests that either GTPBP2's regulation of Axin is rate-limited by another component, that GTPBP2 may not play a conserved role in regulating Axin in mammalian cells, or that the appropriate experimental conditions are not yet found to reveal an effect. As overexpression of *gtpbp2* via injection of mRNA also has no noticeable effect on Axin levels in *Xenopus* embryos (data not shown), ideally we would test the effect of GTPBP2 loss of function on Axin levels in mammalian cells. The inability to obtain such data, however, did not prevent the investigation of the effects that shRNAi mediated knockdown of GTPBP2 has on the Wnt signaling pathway.

In order to examine GTPBP2 loss of function in mammalian cells, we attempted knockdown of *GTPBP2* levels by creating three cell lines that stably express shRNAis targeting *GTPBP2.* To test the knockdown of endogenous *GTPBP2*, we used qPCR to screen for *GTPBP2* mRNA levels in these three cell lines. The initial results obtained were variable, which we realized might be a technical issue by analyzing the melting curve temperatures of the qPCR results for trials utilizing the first set of primers for *GTPBP2* and concluding the presence of

possible primer dimerization and/or contamination. Decontamination efforts and the generation of new *GTPBP2* primers resulted in qPCR data which found ~20-300% reduction in *GTPBP2* expression levels across the three *GTPBP2* shRNAi cell lines. This reduction in *gtpbp2* of the cell lines suggests that the lentiviral infection used to generate stable cell lines that actively repress the expression of *GTPBP2* was successful, that the cell lines could further be utilized in experiments to test the effects that knockdown of GTPBP2 has on the Wnt signaling pathway, and that further experimentation with 461-cell line would have the most severe decrease in *GTPBP2* levels.

In order to test if *GTPBP2* shRNAi knockdown could elicit Wnt signal transduction in Hek293t cells, I attempted to use Wnt8 conditioned media to activate Wnt signaling in these lines and probe for differences in induction using the TopFlash luciferase reporter assay. However, the lack of an increase and relative similarity between both conditions was indicative that insufficient Wnt8 activation occurred from the Wnt8 conditioned media. Without the proper control, and the significant variation between replicates of the *GTPBP2* shRNAi cell lines, the results of the assay could not be properly interpreted, and the lack of response in all cells to the Wnt8 conditioned media left the efficacy of assay reagents and Wnt8 conditioned media in question.

In order to determine the efficacy of the reagents used, I proceeded to test if activation or repression could be seen from several different preparations of conditioned media, as well as the transfection of Wnt pathway reagents, including the overexpression of Dvl, wildtype Gsk3b, and dominant-negative Gsk3b. I found that the overexpression of Dvl resulted in a strong induction of the TopFlash reporter, suggesting that the luciferase reporters and the luciferase detection reagents were working as expected. However, I found little to no transactivation in response

three different preparations of Wnt8 conditioned media, nor from overexpression of dnGsk3b. The lack of appropriate activity levels in the cells treated with three Wnt8 conditioned medias and cells thought to overexpress dnGsk3b suggested that the conditioned medias were inefficient in eliciting activation of the Wnt signaling pathway and that the DNA expression plasmid for dnGsk3b did not transfect properly or was not sufficient to induce a response.

Studies of GTPBP2 in mice have revealed that GTPBP2 plays a CNS-specific role in the rescue of ribosomes that are stalled at AGA codons during neurogenesis due to a loss of function mutation in a CNS-specific tRNA for AGA. However, in mice wild-type or heterozygous for this tRNA, GTPBP2 knockout displays no significant phenotype, suggesting the compensation of GTPBP2 loss by another similar acting protein and/or mechanism [17]. These studies on GTPBP2 in mice failed to investigate GTPBP2's role in signaling pathways however, and if it were shown that the Wnt signaling pathway is affected in any significant way by loss of GTPBP2, as it is shown in *Xenopus*, phenotypes would be severe, as seen in Wnt knockout mice [28-30].

Gtpbp2 was shown to be essential in the negative regulation of Axin in *Xenopus* embryos. The determination of its essential involvement in the negative regulation of Axin in human cells could prove to be crucial in developing treatments of diseases and cancers that involve excessive Wnt signaling. Axin is crucial for β-catenin scaffolding, leading to the proper association of all DC protein components. Unlike its sister components of the DC, Axin is maintained at a relatively low endogenous concentration, providing a limit on the rate of β-catenin phosphorylation by the DC; all components of the DC can associate together in the absence of Axin, however, the DC will be unable to target β-catenin for destruction without this crucial protein component [31, 32], and it has further been discovered that the overexpression of

Axin is sufficient to completely block β-catenin stabilization and target gene expression, further elucidating the implication that Axin is a rate-limiting component of the DC and illuminating the mechanistic importance of Axin turnover [26, 33]. Because Axin is thought to be rate limiting in the destruction of β-catenin, increasing Axin stability could lead to an overall increase in β-catenin destruction and subsequent reduction in signaling, reestablishing normal activity levels [31].

Results indicative of GTPBP2's binding of Axin suggests GTPBP2's multifunctionality due to its coexisting function in the rescue of stalled ribosomes in the CNS and gives rise to the hypothesis that GTPBP2 binds to Axin in order to aid in its negative regulation [17]. Although the mechanism and pathway in which GTPBP2 acts to do so has yet to be established, this involvement in Axin turnover would therefore help to prevent the accumulation of Axin and subsequent increase in the number of active of DCs. Many human disease-related mutations in the canonical Wnt signaling pathway have been identified, most of which resulting in the improper stabilization of cytoplasmic β-catenin levels. In the case of diseases and cancers with excessive activity of the Wnt signaling pathway, such as 90% of CRCs and nearly all TNBC tumors, it is possible that GTPBP2 assists in the negative regulation of Axin, preventing proper degradation of β-catenin due to the lack of sufficient numbers of DCs [15, 16]. Consequently, this would lead to the accumulation of nuclear β-catenin and activation of downstream target genes (Figure 7).

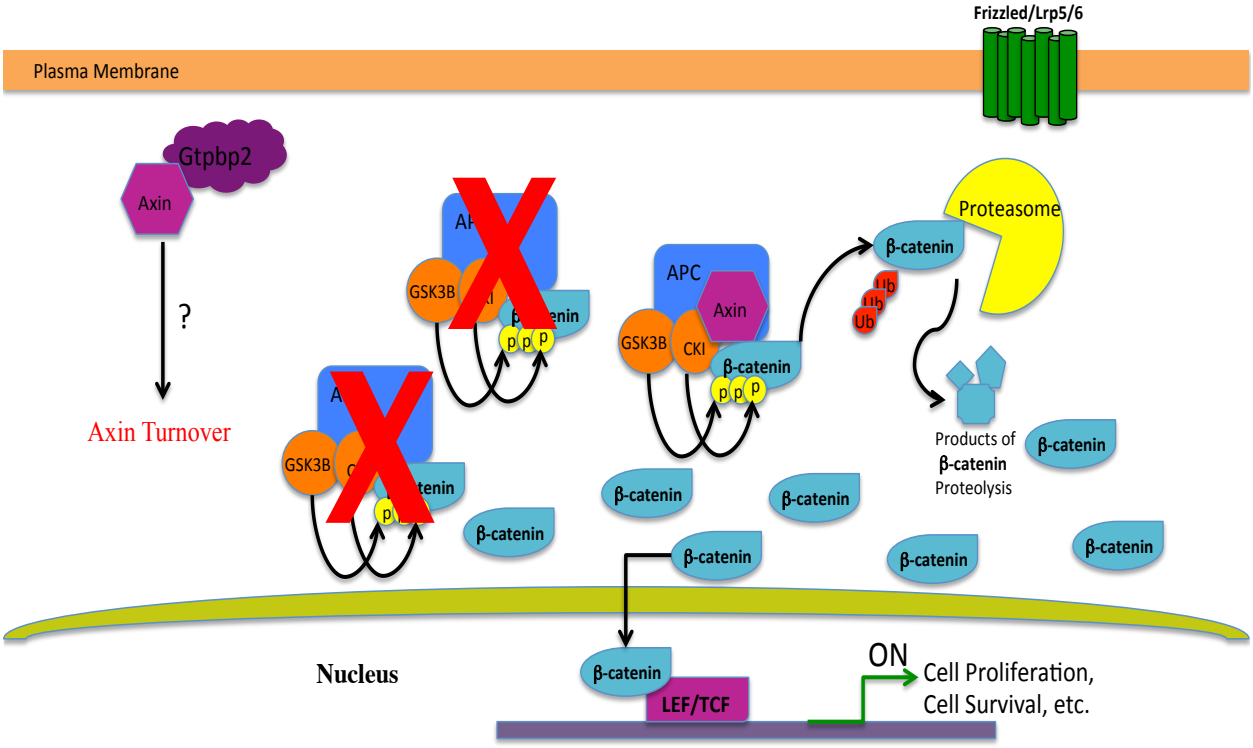

Figure 7: Hypothesized schematic of GTPBP2's role in DC inhibition by promoting Axin turnover, resulting in a decrease in active DC. GTPBP2 interacts with and promotes Axin turnover, lowering cytoplasmic levels of Axin. The decrease in Axin protein levels leads to incomplete association of destruction complex components and insufficient destruction of β-catenin, resulting in the nuclear translocation and subsequent activation of target genes.

In a vast majority of CRCs, a mutation in APC (a tumor suppressor gene) plays a rate-limiting role in the destruction of β-catenin by the DC. Truncating mutations of APC are autosomal dominant, and lead to the expression of both truncated and normal APCs. The truncation does not prevent APC from binding to the components of the DC; however, it prevents proper activity of the DC, even in the presence of all components, and because Axin is maintained relatively low in the cell, binding to an inactive DC sequesters Axin away from wild-type DCs, further increasing the rate-limitation of Axin [34, 35]. In the contrast to the mutations present in the Wnt pathway components in CRCs, excessive Wnt signaling present in TNBCs appears to be the result of autocrine Wnt signaling, leading to the need of therapeutic targets downstream of the Wnt receptor [21].

Further investigation into GTPBP2's role in negatively regulating Axin could illuminate GTPBP2 as a therapeutic target for diseases that are associated with excessive Wnt signaling. Now having a stable *GTPBP2* shRNAi cell line, the extent of which endogenous Axin protein levels are increased should be investigated. An increase in Axin levels of these cell lines would further suggest *GTPBP2* shRNAi efficiency as well as GTPBP2's role in Axin regulation being extended to the mammalian class. Preventing the turnover of Axin, therefore increasing its endogenous levels, could allow for an increase in the concentration of available Axin for association with the DC, leading to an increase in the concentration of active DCs and a consequential increase in β-catenin destruction (Figure 8).

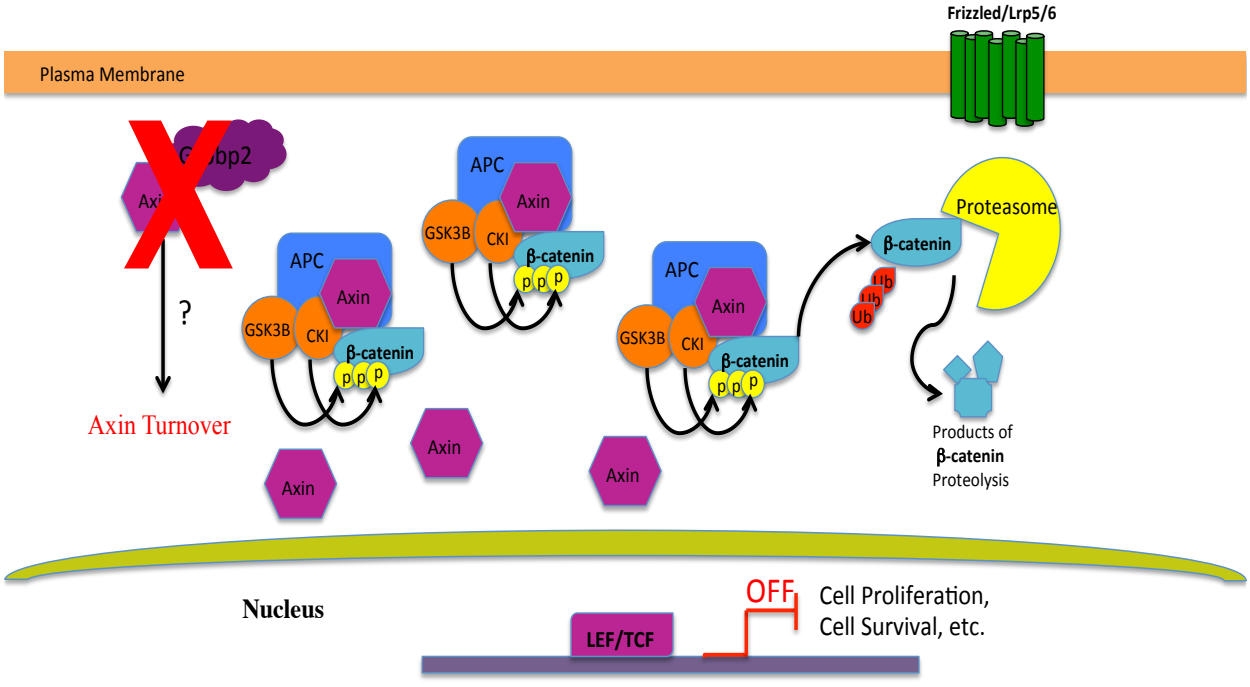

Figure 8: GTPBP2 as a potential therapeutic target in diseases with excessive Wnt signaling. Its involvement in Axin turnover illuminates GTPBP2 as a potential therapeutic target for treatment of diseases associated with aberrant Wnt signaling. Inhibition of GTPBP2 removes one mechanism of Axin turnover, leading to the accumulation of cytoplasmic Axin protein levels. The increase in Axin protein levels increases the amount of available cytoplasmic Axin for association with destruction complex components and therefore increases the number of active DCs for sufficient destruction of β-catenin. Increased destruction of β-catenin prevents nuclear translocation and activation of target genes.

Determining the effects of GTPBP2 knockout on the Wnt signaling pathway, specifically cytoplasmic Axin levels, in GTPBP2 knockout mice could offer crucial information in regards to

GTPBP2's involvement in Wnt signaling; if GTPBP2 knockout mice display an increase in Axin levels and consequential decrease in Wnt signaling, it would provide evidence for GTPBP2's role in the Wnt signaling pathway. After establishing if shRNAi-driven GTPBP2 knockdown is sufficient to increase levels of endogenous Axin, the generation of CRC and TNBC cell lines expressing *GTPBP2* shRNAi and subsequent sulforhodamine B (SRB) assays to determine if GTPBP2 knockdown and consequential Axin stabilization decreases cell proliferation could further illuminate GTPBP2 as a potential therapeutic target. If the extension of Gtpbp2's role in Wnt signaling in *Xenopus* is present in mammalian cells, the inhibition of GTPBP2 would allow for more Axin to associate with active DCs in the case of CRCs and increase the level of fully associated DCs in the case of TNBC, ultimately resulting in the decrease in β-catenin signaling and hopeful suppression of cell proliferation, tumorigenicity, and metastatic ability.

References

1. Logan, C.Y. and R. Nusse, *The Wnt signaling pathway in development and disease.* Annu Rev Cell Dev Biol, 2004. **20**: p. 781-810.
2. Morin, P.J., *beta-catenin signaling and cancer.* Bioessays, 1999. **21**(12): p. 1021-30.
3. Tolwinski, N.S. and E. Wieschaus, *A nuclear function for armadillo/beta-catenin.* PLoS Biol, 2004. **2**(4): p. E95.
4. Grimes, C.A. and R.S. Jope, *The multifaceted roles of glycogen synthase kinase 3beta in cellular signaling.* Prog Neurobiol, 2001. **65**(4): p. 391-426.
5. Kimelman, D. and W. Xu, *beta-catenin destruction complex: insights and questions from a structural perspective.* Oncogene, 2006. **25**(57): p. 7482-91.
6. Liu, C., et al., *Control of beta-catenin phosphorylation/degradation by a dual-kinase mechanism.* Cell, 2002. **108**(6): p. 837-47.
7. Aberle, H., et al., *beta-catenin is a target for the ubiquitin-proteasome pathway.* Embo j, 1997. **16**(13): p. 3797-804.
8. Gao, C. and Y.G. Chen, *Dishevelled: The hub of Wnt signaling.* Cell Signal, 2010. **22**(5): p. 717-27.
9. Behrens, J., et al., *Functional interaction of beta-catenin with the transcription factor LEF-1.* Nature, 1996. **382**(6592): p. 638-42.
10. Huber, O., et al., *Nuclear localization of beta-catenin by interaction with transcription factor LEF-1.* Mech Dev, 1996. **59**(1): p. 3-10.
11. Schmidt, E.V., *The role of c-myc in cellular growth control.* Oncogene, 1999. **18**(19): p. 2988-96.
12. Sierra, J., et al., *The APC tumor suppressor counteracts beta-catenin activation and H3K4 methylation at Wnt target genes.* Genes Dev, 2006. **20**(5): p. 586-600.
13. Clevers, H., *Wnt/beta-catenin signaling in development and disease.* Cell, 2006. **127**(3): p. 469-80.
14. Howe, L.R. and A.M. Brown, *Wnt signaling and breast cancer.* Cancer Biol Ther, 2004. **3**(1): p. 36-41.
15. Bilir, B., O. Kucuk, and C.S. Moreno, *Wnt signaling blockage inhibits cell proliferation and migration, and induces apoptosis in triple-negative breast cancer cells.* J Transl Med, 2013. **11**: p. 280.
16. Kudo, H., et al., *Mouse and human GTPBP2, newly identified members of the GP-1 family of GTPase.* Biochem Biophys Res Commun, 2000. **272**(2): p. 456-65.
17. Ishimura, R., et al., *RNA function. Ribosome stalling induced by mutation of a CNS-specific tRNA causes neurodegeneration.* Science, 2014. **345**(6195): p. 455-9.
18. Kirmizitas, A., et al., *Gtpbp2 is required for BMP signaling and mesoderm patterning in Xenopus embryos.* Dev Biol, 2014. **392**(2): p. 358-67.
19. Giles, R.H., J.H. van Es, and H. Clevers, *Caught up in a Wnt storm: Wnt signaling in cancer.* Biochim Biophys Acta, 2003. **1653**(1): p. 1-24.
20. Kongkanuntn, R., et al., *Dysregulated expression of beta-catenin marks early neoplastic change in Apc mutant mice, but not all lesions arising in Msh2 deficient mice.* Oncogene, 1999. **18**(51): p. 7219-25.
21. Bafico, A., et al., *An autocrine mechanism for constitutive Wnt pathway activation in human cancer cells.* Cancer Cell, 2004. **6**(5): p. 497-506.

22. O'Toole, S.A., et al., *Therapeutic targets in triple negative breast cancer.* J Clin Pathol, 2013. **66**(6): p. 530-42.
23. Sokol, S.Y., *Analysis of Dishevelled signalling pathways during Xenopus development.* Curr Biol, 1996. **6**(11): p. 1456-67.
24. Yost, C., et al., *GBP, an inhibitor of GSK-3, is implicated in Xenopus development and oncogenesis.* Cell, 1998. **93**(6): p. 1031-41.
25. Yost, C., et al., *The axis-inducing activity, stability, and subcellular distribution of beta-catenin is regulated in Xenopus embryos by glycogen synthase kinase 3.* Genes Dev, 1996. **10**(12): p. 1443-54.
26. Zeng, L., et al., *The mouse Fused locus encodes Axin, an inhibitor of the Wnt signaling pathway that regulates embryonic axis formation.* Cell, 1997. **90**(1): p. 181-92.
27. Veeman, M.T., et al., *Zebrafish prickle, a modulator of noncanonical Wnt/Fz signaling, regulates gastrulation movements.* Curr Biol, 2003. **13**(8): p. 680-5.
28. Fritzsch, B., et al., *Development of midbrain and anterior hindbrain ocular motoneurons in normal and Wnt-1 knockout mice.* J Neurobiol, 1995. **27**(4): p. 457-69.
29. Korinek, V., et al., *Two members of the Tcf family implicated in Wnt/beta-catenin signaling during embryogenesis in the mouse.* Mol Cell Biol, 1998. **18**(3): p. 1248-56.
30. van Amerongen, R. and A. Berns, *Knockout mouse models to study Wnt signal transduction.* Trends Genet, 2006. **22**(12): p. 678-89.
31. Lee, E., et al., *The roles of APC and Axin derived from experimental and theoretical analysis of the Wnt pathway.* PLoS Biol, 2003. **1**(1): p. E10.
32. Salic, A., et al., *Control of beta-catenin stability: reconstitution of the cytoplasmic steps of the wnt pathway in Xenopus egg extracts.* Mol Cell, 2000. **5**(3): p. 523-32.
33. Huang, S.M., et al., *Tankyrase inhibition stabilizes axin and antagonizes Wnt signalling.* Nature, 2009. **461**(7264): p. 614-20.
34. Fodde, R., *The APC gene in colorectal cancer.* Eur J Cancer, 2002. **38**(7): p. 867-71.
35. Smith, K.J., et al., *The APC gene product in normal and tumor cells.* Proc Natl Acad Sci U S A, 1993. **90**(7): p. 2846-50.