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Analysis of the role of Wnt signaling activation in ciliogenesis

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Table of Contents

Summary

Wnt signaling pathways play an important role in a variety of cellular processes such as embryonic development, tissue homeostasis, and regeneration. Abnormal activation or inactivation of Wnt signaling leads to several diseases. Primary cilia functions as a key center of signaling transduction in vertebrate cells and, therefore, defects of primary cilia result in disorganized signaling transduction. Previous studies found that the primary cilia have been implicated in negative regulation of the canonical Wnt signaling. On the other hand, numerous Wnt components are found localized at the centrosome or basal body and involved in primary cilium formation. However, whether and how Wnt signaling regulates primary cilia formation is still poorly understood. In my study, I observed Wnt/β-catenin signaling is active under primary cilia-inducing conditions by using the RPE1-7TGC Wnt reporter cell line. However, Wnt signaling's high activation could regulate primary cilia formation. Moreover, Wnt signaling highly activation-induced longer cilia formation. Because canonical Wnt signaling activates the transcriptional co-factor β-catenin and LEF/TCF family of transcription factors to trigger gene expression, I also knockdown of TCF7 and found that Wnt/LRP6 signaling inhibits ciliogenesis independently of β-catenin transcriptional regulation. By directly inhibiting GSK3β activity, I observed Wnt signaling suppresses cilia formation through GSK3β but in an indirect way. Because the downstream events of Wnt signaling also include β-catenin dependent signaling events, I observed Wnt signaling activated the downstream Wnt/TOR signaling. Moreover, inhibition of Wnt/TOR signaling by using rapamycin treatment rescued the cilia loss phenotype upon activation of Wnt signaling. Furthermore, I observed activation of Wnt/TOR signaling attenuated autophagy activity. Since autophagy degrades centriolar satellite OFD1 a negative regulator of primary cilia formation to promote ciliogenesis, I also depleted the autophagy targeting gene OFD1. Depletion of OFD1 rescued the cilia loss phenotype upon activation of Wnt signaling. Based on these results, I proposed that activation of Wnt

signaling suppresses ciliogenesis towards autophagy by inhibiting degradation of OFD1 at the centriolar satellites.

Zusammenfassung

Wnt-Signalwege spielen eine wichtige Rolle bei einer Vielzahl zellulärer Prozesse wie der Embryonalentwicklung, der Gewebehomöostase und der Regeneration. Eine abnorme Aktivierung oder Inaktivierung der Wnt-Signalübertragung führt zu verschiedenen Krankheiten. Primäre Zilien fungieren als Schlüsselzentrum der Signaltransduktion in Wirbeltierzellen, so dass Defekte der primären Zilien zu einer gestörten Signaltransduktion führen. Frühere Studien haben gezeigt, dass die primären Zilien in die negative Regulierung der kanonischen Wnt-Signalübertragung involviert sind. Andererseits sind zahlreiche Wnt-Komponenten am Zentrosom oder Basalkörper lokalisiert und an der Bildung von Primärzilien beteiligt. Ob und wie der Wnt-Signalweg die Bildung von Primärzilien reguliert, ist jedoch noch wenig bekannt. In meiner Studie habe ich beobachtet, dass der Wnt/β-Catenin-Signalweg unter Bedingungen, die zur Bildung von Primärzilien führen, aktiv ist, indem ich die Wnt-Reporterzelllinie RPE1-7TGC verwendete. Die hohe Aktivierung des Wnt-Signals könnte jedoch die Bildung primärer Zilien regulieren. Außerdem führte die hohe Aktivierung des Wnt-Signals zu einer längeren Zilienbildung. Da die kanonische Wnt-Signalisierung den Transkriptions-Cofaktor β-Catenin und die LEF/TCF-Familie von Transkriptionsfaktoren aktiviert, um die Genexpression auszulösen, habe ich auch TCF7 ausgeschaltet und festgestellt, dass die Wnt/LRP6-Signalisierung die Zilienbildung unabhängig von der β-Catenin-Transkriptionsregulation hemmt. Durch direkte Hemmung der GSK3β-Aktivität konnte ich beobachten, dass das Wnt-Signal die Zilienbildung durch GSK3β unterdrückt, allerdings auf indirekte Weise. Da die nachgeschalteten Ereignisse der Wnt-Signalisierung auch β-Catenin-abhängige Signalereignisse umfassen, habe ich beobachtet, dass die Wnt-Signalisierung die nachgeschaltete Wnt/TOR-Signalisierung aktiviert. Darüber hinaus rettete die Hemmung der Wnt/TOR-Signalisierung durch die Behandlung mit Rapamycin den Phänotyp des Zilienverlusts bei Aktivierung der Wnt-Signalisierung. Darüber hinaus konnte ich

beobachten, dass die Aktivierung des Wnt/TOR-Signals die Autophagie-Aktivität abschwächt. Da die Autophagie den zentriolären Satelliten OFD1, einen negativen Regulator der primären Zilienbildung, abbaut, um die Zilienbildung zu fördern, habe ich auch das auf die Autophagie abzielende Gen OFD1 ausgeschaltet. Die Deletion von OFD1 rettete den Phänotyp des Zilienverlusts bei Aktivierung der Wnt-Signalübertragung. Auf der Grundlage dieser Ergebnisse schlug ich vor, dass die Aktivierung der Wnt-Signalübertragung die Zilienbildung in Richtung Autophagie unterdrückt, indem sie den Abbau von OFD1 an den zentriolären Satelliten hemmt.

1. Introduction

1.1 Canonical Wnt signaling

Wnt signaling includes Canonical and non-canonical Wnt signaling functions in a variety of cellular processes such as embryonic development, tissue homeostasis, and regeneration(MacDonald et al., 2009)(Taelman et al., 2010). Impaired Wnt signaling causes developmental diseases and also drives oncogenesis in several human cancers (Kim et al., 2017). The well-studied Wnt pathway is the canonical Wnt pathway, which controls the developmental gene expression programs by regulating the activity of transcriptional co-factor β-catenin. Canonical Wnt signaling is regulated by three multiprotein complexes including the β-catenin destruction complex (Degradosome), the Wnt signalosome, and the Wnt enhanceosome (Figure 1.1). The transcriptional response to Wnt activation is regulated by the nuclear endpoint of β-catenin binding factors (Taelman et al., 2010). However, beyond the β-catenin transcriptional regulation of canonical Wnt signaling, β-catenin-independent pathways have also received a lot of attention recently (Acebron and Niehrs, 2016)(Prossomariti et al., 2020).

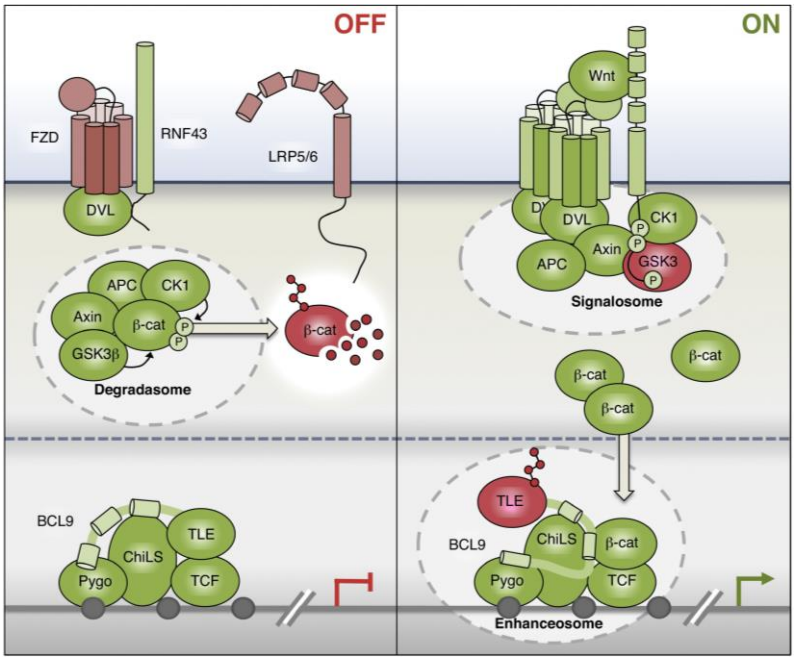

Figure 1.1 Overview of multiprotein complexes in Wnt signaling transduction. Three

multiprotein complexes include Degradosome, Signalosome, and Enhanceosome regulate the activity of Wnt signaling. This cartoon is adapted from (Gammons and Bienz, 2018).

1.2 The β-catenin destruction complex

As the heart of the canonical Wnt signaling pathways, the β-catenin destruction complex functions in the absence of Wnt signaling to decrease the level of β-catenin in the cells. In the Wnt-off state, the β-catenin destruction complex is formed in the cytoplasm, the key components of this complex include cytoplasmic β-catenin, glycogen synthase kinase (GSK3), the tumor suppressor adenomatous polyposis coli gene product (APC), and casein kinase (CK1) (Figure 1.2). In this protein complex, the flexible N-terminus of cytosolic β-catenin is first phosphorylated at Ser45 by CK1a, and then by a serine/threonine kinase GSK3 at Ser33, Ser37, and Thr41 (kimelman and xu 2006). Phosphorylated β-catenin creates a binding site for E3 ubiquitin ligase β-Trcp, which ubiquitylates β-catenin and causes its degradation by the proteasome (Davidson and Niehrs, 2010). Loss-of-function mutations in β-catenin destruction complex genes or gain-of-function β-catenin mutations can lead to the erroneous transcriptional of Wnt target genes causing diseases (Cadigan and Waterman, 2012)(Letamendia et al., 2001).

At the center of the β-catenin destruction complex is the scaffolding protein Axin which shares a common goal of ensuring β-catenin phosphorylation and degradation. Since Axin concentration is the least abundant protein in the β-catenin destruction complex, the number of the β-catenin destruction complex present in a cell may depend on the Axin protein level (Lee et al., 2003). Importantly, Axin can interact with all other components of the β-catenin destruction complex as well as Axin itself. The N-terminal of Axin contains a regulator of G protein signaling (RGS) responsible for binding APC and the C-terminal contains the DIX domain for mediating oligomerization (Kimelman and Xu, 2006)(Liu et al., 2011). Indeed, both Axin and APC are tumor suppressor genes and are key components for the function of the β-catenin destruction complex (Gammons and Bienz, 2018). APC contains

three repeats with the sequence serine-alanine-methionine- proline (SMAP) to mediate the interaction with Axin. Furthermore, the APC is a very large protein that is mutated in nearly 90% of colorectal cancer (Najdi et al., 2011). However, the roles of APC for the assembly of the β-catenin destruction complex are still not completely understood. Indeed, structural studies found that APC contains two different binding sites with β-catenin, three 15 amino acid (AA) repeats bind to β-catenin and seven 20AA repeats also bind to β-catenin. These two different binding sites in APC show different β-catenin binding affinity depending on whether APC is unphosphorylated or phosphorylated (Figure 1.2) (Ha et al., 2004).

GSK3, which is involved in a diverse array of cellular processes is another important component of the β-catenin destruction complex. The C-terminus of GSK3 binds to the central region of Axin and leaves the GSK3 active site free to phosphorylate β-catenin (Dajani et al., 2003). Therefore, inhibition of GSK3-mediated phosphorylation of β-catenin is commonly considered to be the major mechanism that the canonical Wnt signaling uses to prevent β-catenin degradation. In addition to GSK3, CK1 also binds to Axin and phosphorylates β-catenin at Ser45 to enhance GSK3 phosphorylation at the N-terminal residues (Huang and He, 2008)(Kimelman and Xu, 2006).

Protein phosphatase 2A(PP2A) is a three-subunit serine/threonine phosphatase protein complex that provides a positive role in the Wnt signaling pathway (Bajpai et al., 2004). PP2A was also reported as a target of IQ-1 and sodium selenate which is the activator of Wnt signaling (Jin et al., 2017). However, although strong evidence supports that PP2A is a part of the β-catenin destruction complex, the mechanism of PP2A in the complex is still uncertain.

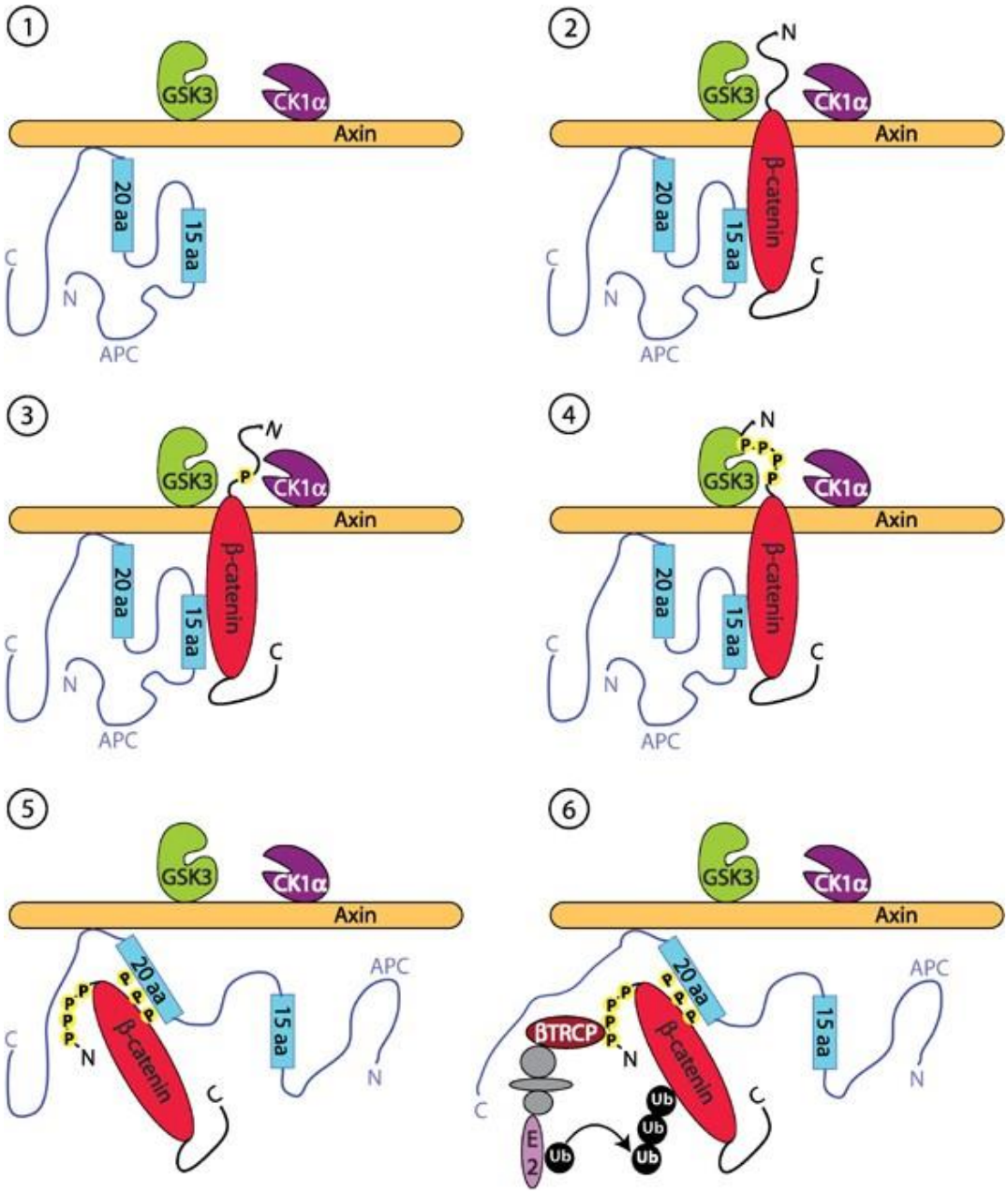

Figure 1.2 The working model of the β-catenin destruction complex. Schematic presentation of the β-catenin destruction complex. The cartoon shows how β-catenin is degraded by the destruction complex. The figure is taken from (Kimelman and Xu, 2006).

1.3 The Wnt signalosome and its regulation

Another important protein complex of the canonical Wnt signaling is the Wnt signalosome (Figure 1.3). Over the past 20 years, the identification of cell surface receptors for WNT ligands has been increasingly focused on. Wnt receptors, as well as co-receptors, include members of the Frizzled (FZD) family and low-density

lipoprotein receptor-related proteins 5 (LRP5) and LRP6 coreceptors, receptor Tyrosine kinase-like orphan (ROR), protein Tyrosine kinase (PTK7), and so on(Van Amerongen et al., 2008)(Ahn et al., 2011) (Kamizaki et al., 2021) (Peradziryi et al., 2012). FZD receptors and LRP5/6 coreceptors are two distinct receptor families that are critical for the activation of Wnt/β-catenin signaling. FZDs are the major WNT receptors and have a large extracellular Cys-rich region that is important for mediating WNT binding (Niehrs, 2012). In terms of coreceptors, they are cell-surface proteins that can enhance the complex formation and downstream signaling by associating with a ligand and its primary receptor (Niehrs, 2012). To activate Wnt/β-catenin signaling, Wnt-FZD forms a ternary complex with LRP5/6, while in PCP signaling this ternary complex engage ROR or PTK7 (Grumolato et al., 2010)(Nishita et al., 2010)(Niehrs, 2012). As the first identified Wnt receptors, FZD receptors class comprises 10 family membranes in humans (Bhanot et al., 1996). Like other G protein-coupled receptors (GPCRs), FZD receptors are also transmembrane proteins and can dimerize and be coupled to the heterotrimeric G protein(Koval et al., 2011). Further regulation of FZD receptors can occur by intracellular binding to the scaffold DVL proteins which are cytoplasmic partners. In general, FZDs engage DVL proteins by their conserved KTXXXW motif. As the key component of Wnt signaling, DVL proteins are recruited by FZD receptors at the plasma membrane to prevent the constitutive destruction of cytosolic β-catenin and co-recruit cytoplasmic transducers Axin, CK1, and GSK3 binding proteins (Kikuchi et al., 2011)(Gammons et al., 2016). DVL proteins form oligomers that cluster Wnt-FZD receptor complexes and regulate the downstream of canonical Wnt signaling (Bilić et al., 2007).

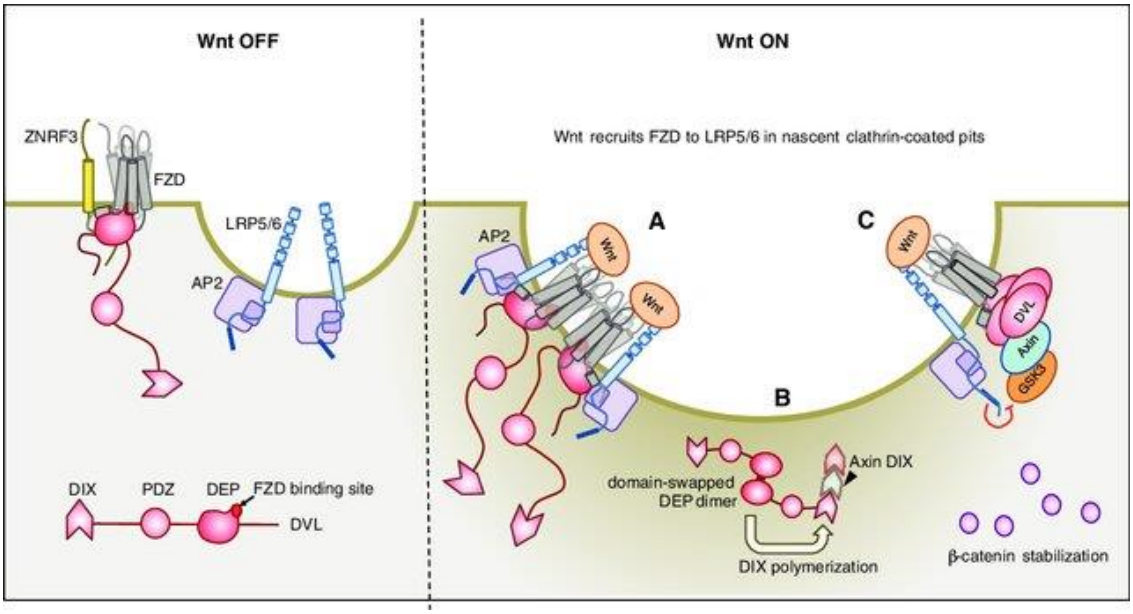

Figure 1.3 Model of Wnt Signalosome Assembly. Wnt OFF (left): Without Wnt ligands. Wnt ON (right): In the presence of Wnt ligands. The figure is taken from (Gammons et al., 2016).

1.4 The Wnt enhanceosome

The Wnt enhanceosome is a multiprotein complex that enables β-catenin binding to gain access to target genes. The complex includes a dimer of LIM-domain protein, a tetramer of SSDP, Groucho/TLE, BCL9/Legless, and so on (Figure 1.4). The same as the β-catenin destruction complex, the Wnt enhanceosome is based on multivalent and partial particles between individual components. However, the core components of this multiprotein complex are still the same upon Wnt signaling activation. The only changes upon Wnt activation may be the conformation of proteins in the complex which could affect the binding to β-catenin. However, there are very few studies that focus on the conformation change of the complex. Therefore, more research is needed to be done on enhanceosomes, especially on changes when β-catenin binds to the enhanceosome and changes the morphology of the components (Gammons & Bienz, 2018).

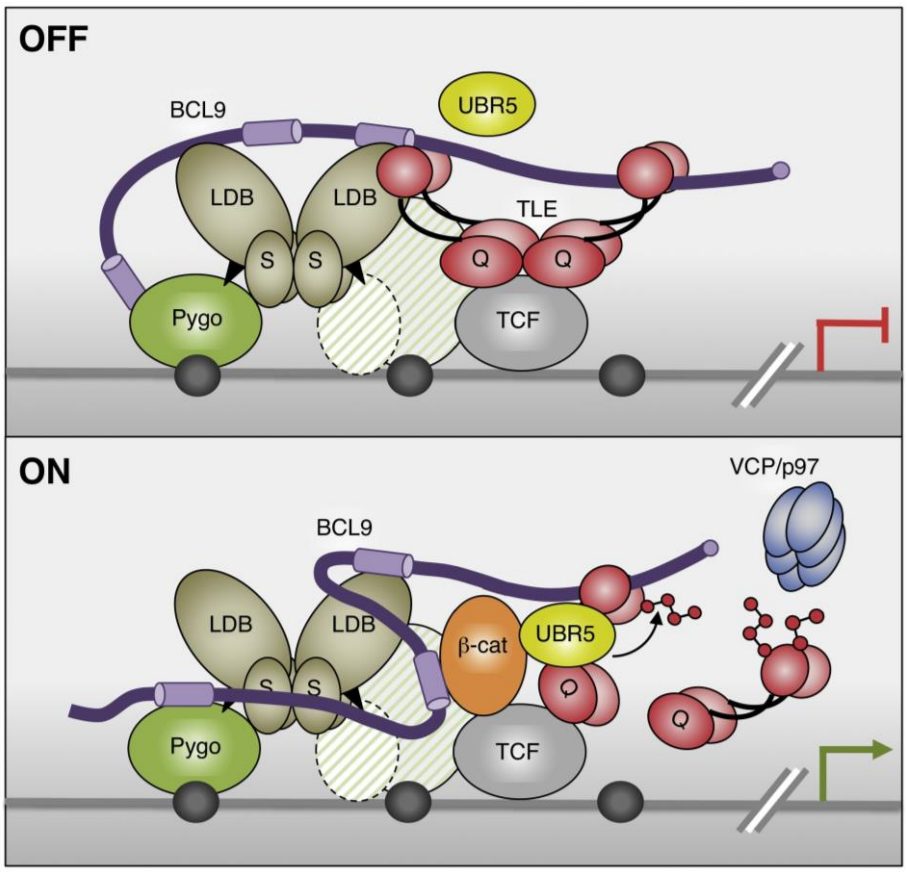

Figure 1.4 Overview of the Wnt enhanceosome. In the Wnt-OFF state, TLE binds to TCF by its Q domain. However, in the Wnt-ON state, β-catenin is captured by BCL9/Legless scaffold and binds to TCF. This cartoon is adapted from (Gammons and Bienz, 2018).

1.5 β-catenin-independent Wnt/LRP6 signaling

Compared to the β-catenin-dependent signaling, β-catenin-independent signaling is more diverse and less well studied (Anastas and Moon, 2013). Activation of canonical Wnt signaling causes the suppression of GSK3β up to 70% of the cellular GSK3 activity in multivesicular bodies as well as subsequent blocking of poly-phosphorylation and poly-ubiquitination of target proteins (Taelman et al., 2010). This Wnt-dependent stabilization of proteins (Wnt/STOP) has been identified as a new β-catenin-independent pathway (Acebron et al., 2014). Wnt/STOP signaling has been suggested to affect cell division, chromosomal stability, endolysosomal biogenesis, and so on (Acebron and Niehrs, 2016).

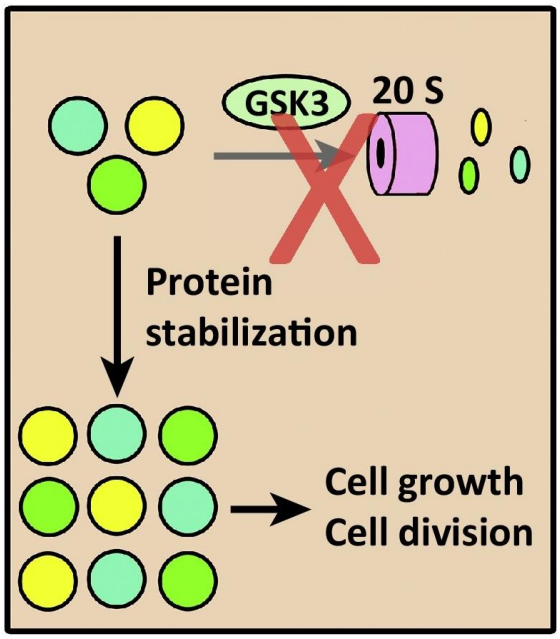

Figure 1.5 Overview of Wnt/STOP signaling. Wnt/STOP signaling stabilizes proteins that are targeted by GSK3 to regulate cell growth and cell division. This cartoon is adapted from (Acebron and Niehrs, 2016).

In addition to Wnt/STOP signaling, GSK3-mediated phosphorylation activates tuberous sclerosis complex 2 (TSC2) which leads to inactivation of the mammalian target of rapamycin (mTOR) signaling. mTOR signaling is made of two functionally multiprotein complexes mTORC1 and mTORC2 (Jain et al., 2014). mTOR signaling regulates anabolic and catabolic metabolism and plays an important function in transducing nutrient signals to cell responses, such as cell growth, metabolism, and proliferation (Yuan et al., 2012)(Saxton and Sabatini, 2017). In general, the activation of canonical Wnt signaling blocks phosphorylation of TSC2 by GSK3β, and then mTOR is activated to stimulate protein translation during the G1 phase (Inoki et al., 2006). However, another study found canonical Wnt signaling upregulates DEPTOR which is a negative regulator of mTORC1 to mediated mTORC1 suppression (Wang et al., 2018).

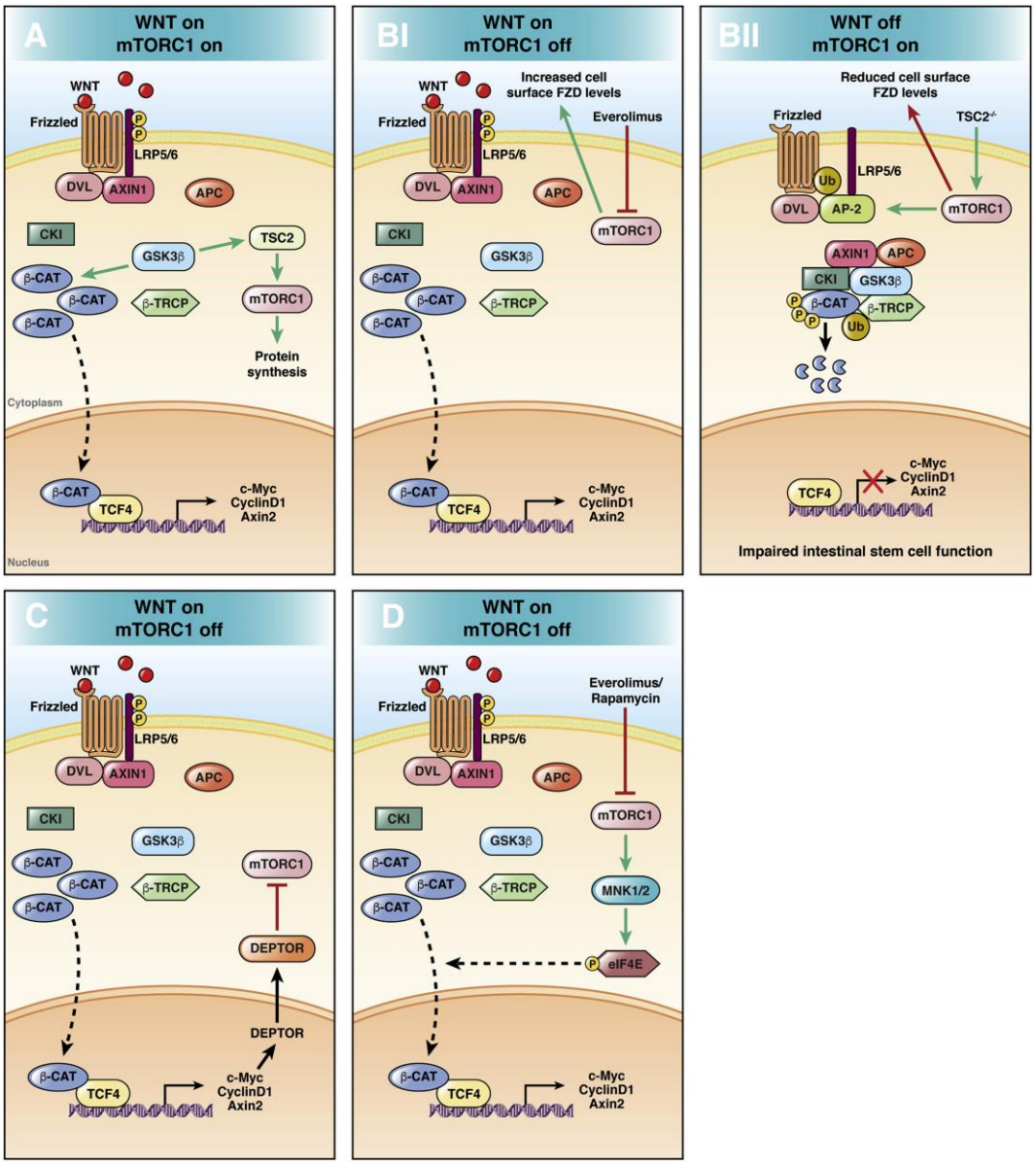

Figure 1.5 Overview of canonical Wnt signaling and mTOR signaling interconnections. (A) The canonical Wnt signaling regulates mTORC1 to modulate translation. (BI) mTORC1 inhibition induces canonical Wnt signaling activation. (BII) Activation of mTORC1 negatively regulates canonical Wnt signaling. (C) Canonical Wnt signaling switches off mTOR signaling. (D) mTORC1 inhibition leads to increased eIF4E phosphorylation and is then associated with nuclear β-catenin. The cartoon is taken from (Prossomariti et al., 2020).

1.6 Canonical Wnt signaling in disease and therapy

As important conserved signaling for development and tissue homeostasis, abnormal activation includes high activation and destruction of Wnt signaling leading to a variety of types of diseases, including cancer, osteoporosis, chronic wounds, and so on(Huang et al., 2019)(Katoh and Katoh, 2017)(Zhan et al., 2017). In cancers, the

Wnt/β-catenin pathway was observed to be highly activated (Patel et al., 2019). The activation of Wnt/β-catenin signaling causes the loss of APC function, which is a negative regulator of cell proliferation. Therefore, inhibitors of the Wnt/β-catenin pathway have therapeutic value in many cancer treatments, and several inhibitors which play a role in different steps of the Wnt/β-catenin signaling have been identified. These inhibitors can be divided into two categories, the Wnt-receptor complex inhibitors, and β-catenin destruction complex inhibitors. The Wnt-receptor complex can be divided into the sFRP class and the DKK1 class (Figure 1.6). Because of the members of the sFRP class include the sFRP family, WIF-1 and Cerberus, the antagonists of the sFRP class not only block the canonical Wnt signaling, but also inhibit the noncanonical Wnt signaling such as Wnt/PCP signaling. However, the DKK1 class inhibits Wnt signaling by binding to the LRP5/6 complex. It specifically inhibits the canonical Wnt signaling. Since the β-catenin destruction complex plays an important role in the regulation of Wnt/β-catenin signaling, enhancing the activity of the β-catenin destruction complex is another way to inhibit Wnt/β-catenin signaling. Several small molecules can be used to enhance the activity of the β-catenin destruction complex such as XAV939, G007-LK and so on (Norum et al., 2018)(Huang et al., 2009)(van Kappel and Maurice, 2017).

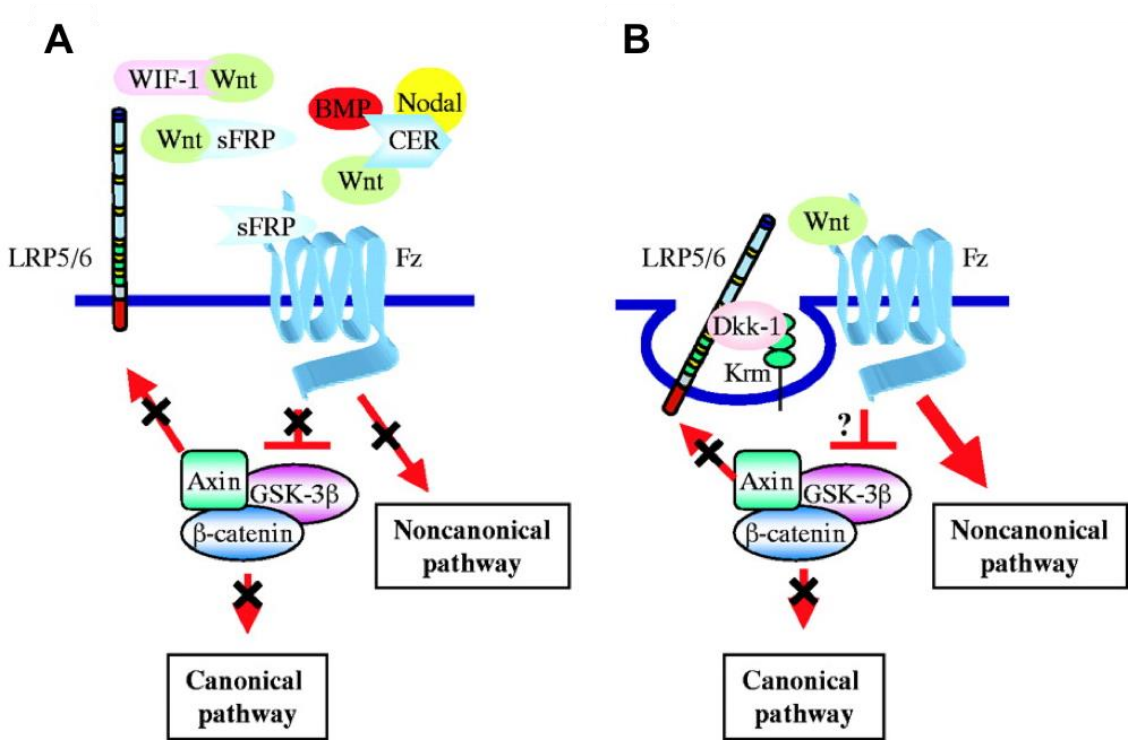

Figure 1.6 Regulation of the Wnt-receptor complex inhibitors. (A)The sFRP class prevents Wnt signaling from binding to its receptors. (B) The Dkk-1 class binds to LRP5/6 and blocks the formation of the LRP5/6-Wnt-Frizzled complex. This cartoon is adapted from (Kawano, 2003).

High activation of Wnt signaling causes a variety of types of diseases. However, abnormal inactivation of canonical Wnt signaling has been also found in many types of diseases. For example, hair growth disorders, pigmentary disorders, bone diseases, and so on (Figure 1.7). Currently, much research has focused on the inhibitors of the Wnt signaling pathway. However, the activators of the Wnt signaling pathway have been rarely studied (Huang et al., 2019). The way to activate Wnt signaling can be divided into three categories, Wnt proteins or Wnt mimics, inhibition of Wnt inhibitors, and disruption of β-catenin degradation (Bonnet et al., 2021). However, safety and efficacy need to be considered when it is used for regenerative medicine and therapy.

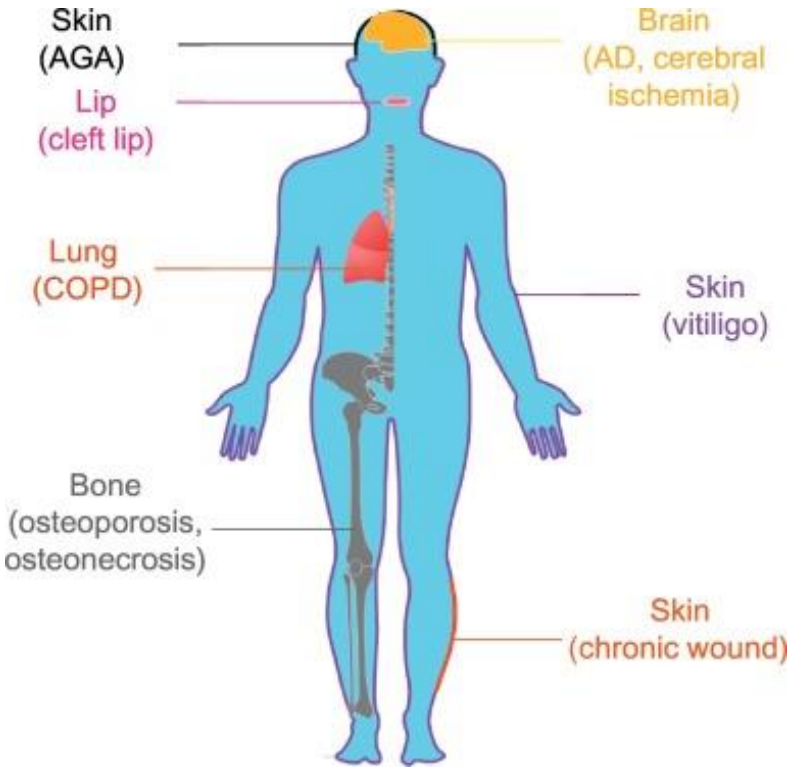

Figure 1.7 Overview of diseases caused by abnormal inactivation of the Wnt signaling pathway. Androgenetic alopecia(AGA); Alzheimer's disease(AD); Chronic obstructive pulmonary disease(COPD). The figure is adapted from (Huang et al., 2019)

1.7 Wnt surrogate

To activate the canonical Wnt signaling pathway, currently adding Wnt3a conditioned media (Wnt3a-CM) or GSK3 inhibitor is the common method. However, Wnt3a protein as the hydrophobic protein is not stable under serum starvation conditions (Janda and Garcia, 2015). In parallel, serum-stabilized Wnt3a-CM is used as a substitute in the Wnt signaling study. However, conditioned media contains undefined factors, and the quality of conditioned media is also hard to control which makes the experimental variables (Seino et al., 2018). On the other hand, directly inhibiting GSK3 also can activate the canonical Wnt signaling. However, the activation of the canonical Wnt signaling only happens at the downstream pathway of the Wnt signaling, thus, this also may miss the important phenotype which is regulated by the upstream of the Wnt signaling pathway. Importantly, the inhibitor causes off-target

effects which could bring unknown phenotypes to the experiment (Jope and Johnson, 2004).

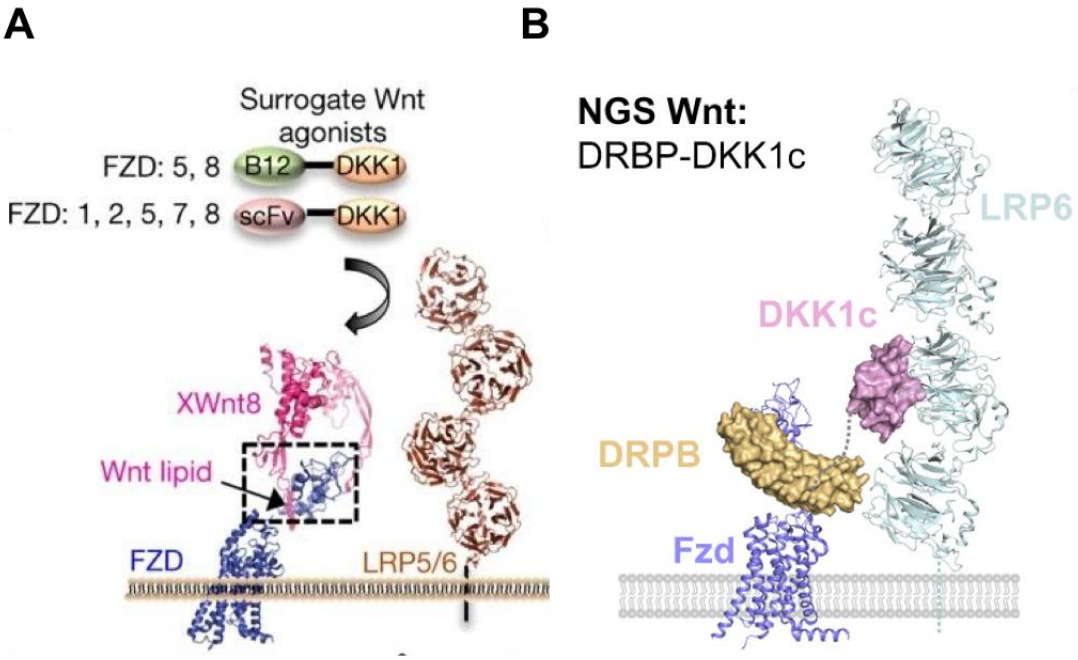

Figure 1.8 Overview of two generations of Wnt surrogate. (A) First-generation Wnt surrogate which is heterodimerization of FZD and LRP5/6. (B) The second generation of Wnt surrogate which is the Fzd subtype binds to DRPB is fused with LRP5/6 binds to DKK1c. Cartoon A is adapted from (Janda et al., 2017). The cartoon B is adapted from (Miao et al., 2020).

Janda et. al. developed a wnt surrogate that links the FZD and LRP5/6-binding modules into a single polypeptide chain. This single polypeptide chain elicited a characteristic of Wnt/β-catenin signaling response in an FZD-selective fashion (Figure 1.8 A). However, this molecule lacked Fzd selectivity that was weakly potent. To figure out this problem, they further developed a new generation of 'tetrameric' Wnt surrogate which is the Fzd7/8 subtype (activating Fzd 1, 2, 5, 7, and 8) Wnt surrogate (Figure 1.8 B). The new generation of Wnt surrogates induced much stronger canonical Wnt signaling at 5 nM concentration than the 250 nM first-generation surrogate. However, the second generation of Wnt surrogates may still hide some phenotype that activates Wnt signaling through Wnt/Fzd/Lrp complexes. More experiments need to be done to validate this hypothesis.

1.8 Primary cilia

Primary cilia also called non-motile cilia are single microtubule-based hair-like sensory organelles that project from the apical surface of the cells into the extracellular. Major insights into the function of the primary cilium came when analyzing the flagellum in *Chlamydomonas* in 1990 that observed the existence of a conserved intraflagellar transport (IFT) system. This system is important for organelle biogenesis (Kozminski et al., 1993). After then, a lot of studies have been learned on the function of primary cilia and found its role as an important organelle for signal transduction pathways. These signal transduction events include Ca^{+} flux in the kidney, growth, differentiation, memory, learning, and so on (Wang and Dynlacht, 2018)(Goetz and Anderson, 2010)(Chavali et al., 2014)(Einstein et al., 2010).

1.9 Ciliary structure

As a microtubule-based structure, primary cilia are formed from the basal body which is derived from the distal end of the mother centriole (Malicki and Johnson, 2017)(Nigg and Stearns, 2011). The mother centriole is distinguished from the daughter centriole by the presence of fibrous distal and subdistal appendages(Paintrand et al., 1992). Once the basal body is docked to the plasma membranes or the ciliary vesicle, the axoneme begins to be assembled. Upon the basal body is the “Y-shaped” linkers structure and the ciliary necklace which is a characteristic structure of the transition zone (TZ) (Figure 1.9). TZ structures connect the outer doublets of microtubules to the plasma membrane and the ciliary necklace. The upper part of the cilium which extends from the TZ and is continuous with the plasma membrane is the axoneme. Although the axoneme is also enclosed by a ciliary membrane, the lipid and protein composition of the axoneme are distinct from other parts of the primary cilia.

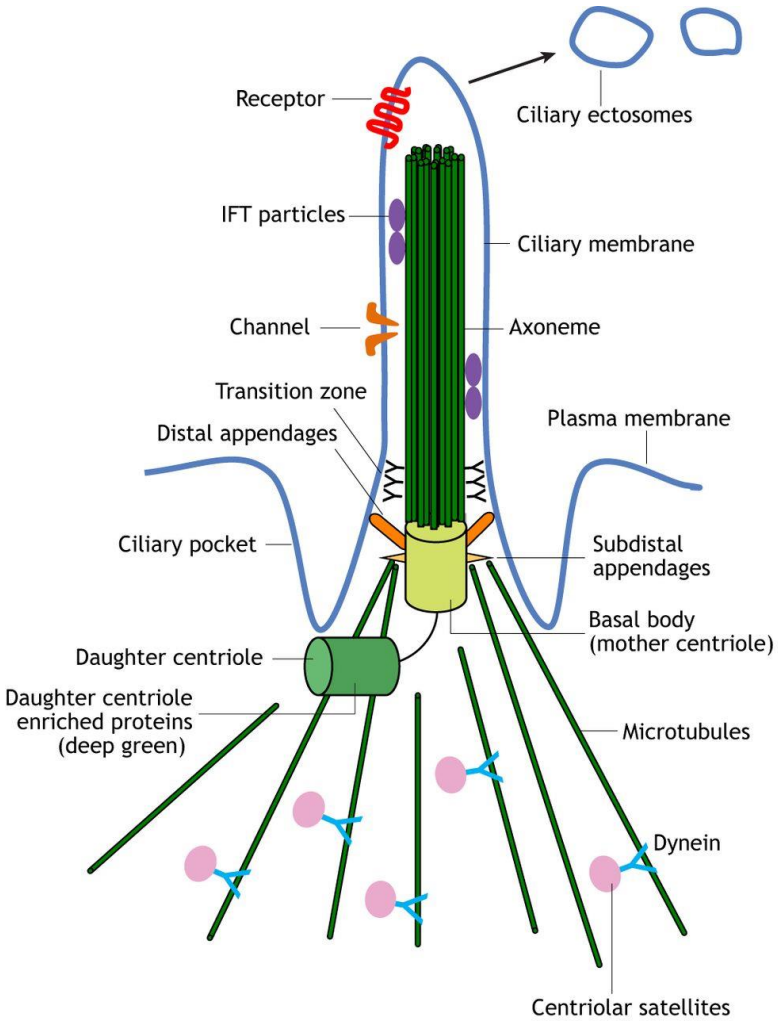

Figure 1.9 Structure of the primary cilium. The key structure of a primary cilium is composed of a ciliary axoneme extending from the basal body which is a microtubule-organized structure derived from the mother centriole. The figure is adapted from (Wang and Dynlacht, 2018).

1.10 The multiple phases and regulation of cilium assembly and disassembly

Ciliogenesis is defined as the time of cilia formation which is restricted to the stage of the cell cycle. Primary cilia assembly happens when the cell exits mitosis to the G0/G1 phase, and disassembly starts at the S/G2 phase (Sánchez and Dynlacht, 2016). The primary cilia assembly can be divided into several different successive stages. First, when the cell exits the cell cycle in response to mitogen deprivation or after receiving developmental signals, the mother centriole docks at the plasma

membrane of the cell (Piprek et al., 2019)(Wheatley et al., 1996). Second, the small cytoplasmic vesicles such as Rab11 and Rabin8 which originated from the Golgi, and the recycling endosome begin to accumulate in the distal end of the mother centriole. To help the Golgi-derived vesicles attach to the mother centriole, cells need a mature mother centriole that can recruit pericentriolar material and appendage proteins. Among them, these distal appendages (DAs) are important for vesicle docking and late for initiating the centriole-to-basal-body transition (Schmidt et al., 2012). After DAs assembled through the sequential recruitment of CEP83, CEP89, SCLT1, CEP164, and FBF1 proteins, the mother centriole converts into a basal body for primary cilium formation(Schmidt et al., 2012)(Kobayashi et al., 2014)(Spektor et al., 2007)(Lo et al., 2019). Pre-ciliary vesicles (PCVs) are transported via microtubule-actin networks to the distal end of the mother centriole. These vesicular fusions then produce a membrane cap on the distal tip of the mother centriole as the primary ciliary vesicles (SOROKIN, 1962). This is followed by a reorganization of the cytoskeleton to help the docking of the mother centriole to the plasma membrane of the cell. Then CEP97-CP110 complex which functions as a negative regulator of primary cilia formation should be removed (Tsang et al., 2008)(Spektor et al., 2007). Since the ciliary compartment cannot synthesize the protein for axonemal assembly, IFT complexes can help to carry out the ciliary precursors from the basal body to the distal end of the axonemal (Webb et al., 2020)(Rosenbaum and Witman, 2002). So IFT is recruited to the ciliary base, while Rab8a is recruited to the mother centriole to help ciliary membrane extension (Wang et al., 2018a). Movement in the anterograde direction of the axonemal is regulated by kinesin-2 motors. Accordingly, dynein-2 regulates the movement for the retrograde direction (Webb et al., 2020)(Scholey, 2008).

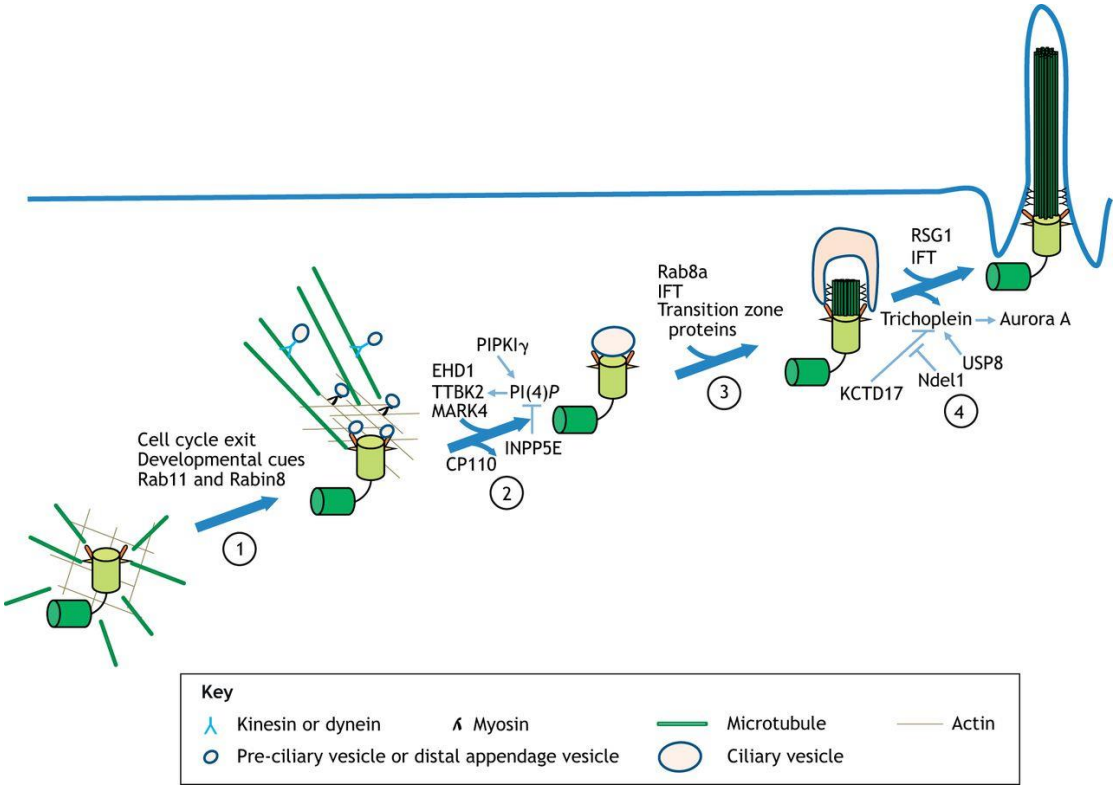

Figure. 1.10 An overview of the initiation process of ciliogenesis. Primary cilia formation is a well-organized event. (1) Pre-mature CV are transported to mother centriole that is recruited by the Rab11-Rabin8 complex. (2) EHD1-regulated expansion of CV at the DA and removal of the CP110-CEP97 inhibitory complex from the basal body. (3) The TZ protein forms the cap-like structure or ciliary vesicle (CV). (4) The ciliary axoneme is assembled and extensional which is regulated by IFT complex that is mediated by TTBK2 and DA proteins. The suppression factor of cilium disassembly, which is mediated by the Aurora A kinase, is also required for primary cilia formation. The figure is adapted from (Wang and Dynlacht, 2018).

In contrast to primary cilia assembly, understanding the mechanisms of how cilium disassembly processes are regulated is still less known and fragmented (Sánchez and Dynlacht, 2016). A previous study found primary cilia disassemble in a biphasic manner. The first wave mainly occurs in the G1 phase shortly after mitotic stimulation of resting cells, and the second wave occurs before mitosis (Figure 1.10). After the restimulation of serum and the cell cycle re-entry has been triggered, the primary cilia begin to detach from the proximal end of the cilia by several mechanisms (Figure 1.10). Cell cycle re-entry causes cilium disassembly by

activation of AuroraA (AurA) which is activated by multiple different signaling pathways and proteins, such as non-canonical Wnt signaling, Ca+ signaling, PDGFRβ signaling, and so on. Activation of AurA leads to phosphorylate histone deacetylase (HDAC6) and triggers tubulin deacetylation(Plotnikova et al., 2012). Importantly, Nde1-Trichoplein and Pitchfork (Pifo) play a role to activate AurA pathway activity (Inaba et al., 2016). Moreover, the second depolymerizing kinesin KIF24 can also regulate axonemal microtubule disassembly. At the S/G2 phase, NEK2 and kinase PLK1 activate KIF24 at the basal body and block primary cilia re-formation (Kim et al., 2015)(Miyamoto et al., 2015). Furthermore, recent studies found that actin mediates exocytosis is another cilium disassembly pathway that happens when the cilia retrieval pathways are disrupted at the ciliary tip (Nager et al., 2017).

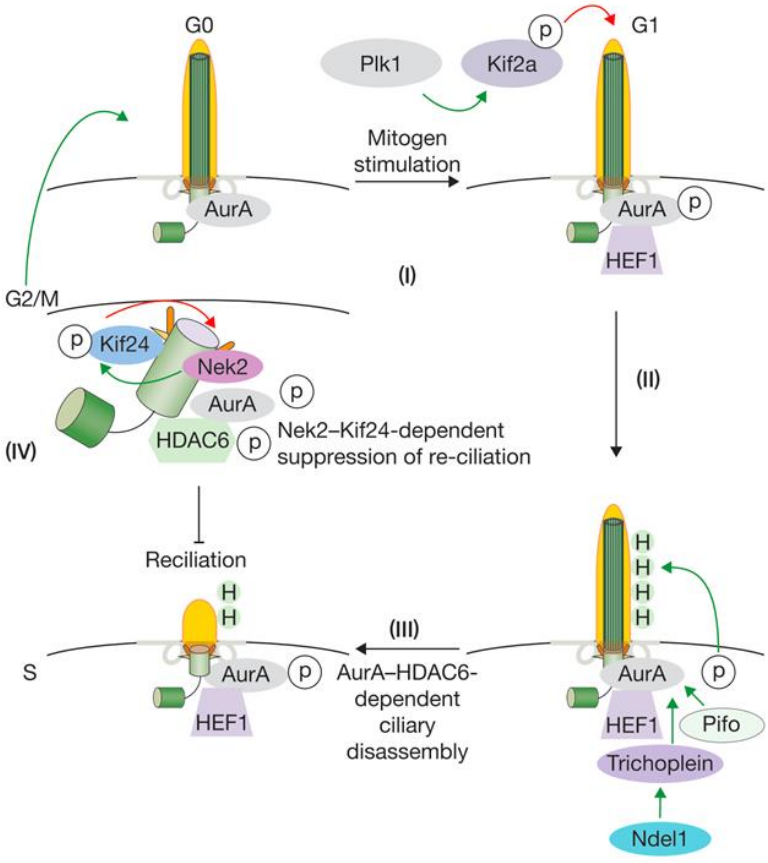

Figure. 1.10 An overview of primary cilia disassembly pathways. (I). PLK1-dependent KIF2a causes depolymerization of acetylated tubulins. (II). The AurA-HEF1 pathway is regulated by mitogen stimulation, Nde1-Trichoplein, and Pifo. (III). AurA activates HDAC6

that causes cilia disassembly. (IV). The second depolymerizing kinesin promotes microtubule de-polymerization and the Kif24-NEK2-dependent pathway blocks cilia reformation. The figure is adapted from (Wang and Dynlacht, 2018).

1.11 Ciliopathies

As an important organelle, primary cilia serve as a complex signaling hub to regulate signaling in cells (Kilander et al., 2018)(Baala et al., 2007). Therefore, primary cilia are essential for cell signaling during development and tissue maintenance. Dysfunction or impaired primary cilia cause a group of human diseases referred to as ciliopathies which is a kind of development problem (Mora-Garcia & Sakamoto, 1999). The ciliopathies that have been reported include JBTS, nephronophthisis (NPHP), Senior-Loken syndrome (SLS), Oro-facial-digital symptom type I (OFDS1), asphyxiating sternal dysplasia (Jeune syndrome), autosomal dominant polycystic kidney disease (ADPKD), autosomal recessive polycystic kidney disease (ARPKD), Leber congenital amaurosis (LCA), MKS, Bardet-Biedel syndrome(BBS) and Usher syndrome (US) and so on (Wheway et al., 2018)(Ishikawa and Marshall, 2011)(Tammachote et al., 2009)(Youn and Han, 2018)(Sharma et al., 2008). Different ciliopathies present different effects which cause several developmental problems, such as birth, kidney, brain, eyes, and so on. Patients with JBTS have hyperextension of the superior cerebellar peduncle, deep interpeduncular fossa, and atrophy of the cerebellar vermis. Patients with BBS present experience problems with obesity, polydactyly, retinopathy, and other symptoms; Patients with MKS are born dead because of occipital brain expansion. Importantly, there are some common features between these disorders: low muscle tone, polycystic kidney, agenesis of the corpus callosum, mental retardation, and hypopnea (Nigg and Raff, 2009)(Novarino et al., 2011). The complexity of the cilia phenotype makes clinical diagnosis and treatment extremely difficult, therefore, the study of primary cilia formation, maintenance, and functional regulation is important for the diagnosis and treatment of cilia (Novarino et al., 2011).

1.12 Primary cilia, signaling pathway, and development

In healthy tissue, primary cilia govern several core signaling pathways, including Hedgehog (Hh) signaling, Hippo signaling, TGF-beta, Notch, GPCR, mTOR, TGF-beta, Wnt signaling, and so on (Wheway et al., 2018)(Oh and Katsanis, 2013)(Schou et al., 2015)(Aznar and Billaud, 2010). Hh signaling is the best known of the function signaling that is regulated by primary cilia. On one hand, Primary cilia can function as both positive and negative switches of the Hedgehog pathway. Previous studies found that ciliary components regulate anterior/posterior patterning which is also important for cilia-dependent Hedgehog signaling during brain development (Wheway et al., 2018). Another study found ciliary components are also important for cilia-dependent Hedgehog signaling during molar development (Nakatomi et al., 2013). However, the Hedgehog pathway may also influence the formation and maintenance of primary cilia in a feedback manner (Jacob et al., 2011). Another study found many core components of the Hedgehog pathway localize at the primary cilia including receptors and co-receptors such as Gli proteins, which are transcriptional effectors of the Hedgehog signaling pathway, are important for primary cilia formation (Hui and Angers, 2011).

Wnt signaling is another of the most important developmental signaling pathways also regulated by primary cilia. The cilium is suggested to act as a switch between the canonical Wnt pathway and the noncanonical Wnt signaling. The key protein that regulates the switch between both pathways was Inversin (Simons et al., 2005). Simons et al. found that primary cilia have been implicated in negative regulation of the canonical Wnt signaling (Simons et al., 2005). Inversin mutation caused impaired cilia formation and hyperactive canonical Wnt responses by downregulation of BBS as well as ciliary-associated genes. However, the role of Inversin in primary cilia formation shown is somewhat controversial in different experimental conditions. Phillips's study found Inv and Dvl both localized at the basal body. His model suggested that Inversin regulates Wnt signaling by reducing

the cytoplasmic Dvl. However, knockout of Inversin in mice still had primary cilia of normal length (Phillips et al., 2004). Importantly, some studies also suggested primary cilia do not affect Wnt signaling. For example, the Ift88 defect zebrafish lack primary cilia. However, the intensity of canonical and non-canonical Wnt signaling behaved the same as normal zebrafish. The role of primary cilia in regulating Wnt signaling appeared to be somewhat controversial in different experimental conditions. More importantly, these studies used impaired basal body or primary cilia such as knockdown BBS1, BBS4, BBS6 in cell lines or knockout Kif3a, IFT88, and ofd1 in mouse or zebrafish models to measure the Wnt signaling activity (Lienkamp et al., 2012)(Corbit et al., 2008). One explanation why the intensity of canonical Wnt signaling behaved the same in these zebrafish and mouse lack cilia mutants might be the ciliary transition zones remain functional that still can regulate Wnt signaling intensity (May-Simera and Kelley, 2012). However, the details of how primary cilia regulate Wnt signaling are yet poorly understood and data occasionally conflict (Berbari et al., 2009; Ross et al., 2005; Simons et al., 2005).

In addition to Hh signaling and Wnt signaling, primary cilia are also reported to regulate PDGF signaling. PDGFRα is localized on the cilium and its phosphorylation causes the activation of the downstream cascades that regulate cell cycle progression and cell migration (Christensen et al., 2008).

Current research suggests that primary cilia regulate a variety of signaling pathways to control tissue development and organ function. However, the mechanism of how primary cilia coordinate some signaling pathways is still not clear, and some signaling such as Hh, Wnt signaling is considered to have bona fide ciliary pathways (Cellular signaling by primary cilia in development, organ function). So, answering these questions is important for us to better understand the link between primary cilia and development.

1.13 Wnt components and ciliogenesis

Many studies reported that several Wnt signaling components are present in cilia or basal bodies (Bryja et al., 2017). The Dickkopf (DKK) family consists of five subgroups Dkk1, Dkk2, Dkk3, Dkk4, and soggy (Glinka et al., 1998). Among them, DKK1 is the most important one that plays an essential role in vertebrate development such as head induction, bone formation, and so on (Morvan et al., 2006) (Mahua, 2001). DKK1 is also reported as a negative component in the feedback loop induced by Wnt signaling in normal tissues (Niida et al., 2004). In the feedback loop, DKK1 binds to LRP5/6 to induce LRP endocytosis and prevent the signal cascade. Recently, there is one report that found DKK1 can also activate the Wnt-PCP pathway (Johansson et al., 2019). There was another report that found induction of Dkk1 led to a significant reduction in cilia length in zebrafish Kupffer's vesicle during development stages before the 3-somite (Caron et al., 2012).

The CK1 family consists of 7 isoforms in mammals. However, only CK1δ was detected at the centrosome and functioned to promote ciliogenesis (Greer et al., 2014; Greer & Rubin, 2011). In Greer's study, they found two mechanisms of CK1δ that involves in regulating primary cilia formation, one pathway is the centrosomal function and another pathway is CK1δ interacts with AKAP450 which can regulate the trafficking of multiple factors that are important for ciliary transport (Greer et al., 2014).

Another important kinase of Wnt signaling is GSK3 which contains two structurally similar isoforms GSK3α and GSK3β in mammals. A fraction of GSK3β was localized to the centrosome (Wakefield et al., 2003)(Hoeflich et al., 2000). It has also been shown to play an important role in regulating microtubule assembly, stability, and dynamic (Zhou & Snider, 2005). In renal cysts, GSK3β was subjected to inhibitory phosphorylation which exhibits decreased frequencies of primary cilia (Thoma et al., 2007). In another research, GSK3β has been identified as a promoter

during the assembly of the ciliary membrane and initiating ciliogenesis after mitotic exit (Zhang et al., 2015).

As a co-recruit of cytoplasmic transducers CK1, Axin, and GSK3 binding proteins. The researcher also found that DVL was detected as crucial for the basal body in multi-ciliated cells (Park et al., 2008). The most recent study identifies a new signaling cascade for the ciliary disassembly in which DVL2 interacts with PLK1 to initiate primary Cilia disassembly (Lee et al., 2012). All these results showed that Wnt signaling may control ciliogenesis. In most cases, the canonical WNT signaling appears to be connected to problems with basal body docking and vesicular transport. However, it should be noted that, although evidence is strong for the participation of Wnt component proteins, not all studies provide sufficient mechanistic findings.

2. Aim of my study

Wnt signaling is one of the most important developmental signaling pathways that control cell fate decisions and tissue patterning. Primary cilia serve as signaling hubs to maintain human health. As primary cilia have emerged as a key center of signaling transduction in vertebrate cells. Therefore, defects of primary cilia result in a group of developmental and degenerative diseases. Primary cilia have been implicated in negative regulation of the canonical Wnt signaling. In parallel, numerous Wnt molecules are involved in primary cilium formation. This raises questions that whether Wnt signaling affects primary cilia formation. The previous study found Wnt3a protein promotes primary cilia formation (Kyun et al., 2020). However, Bernatik et al. reported that primary cilia formation doesn't rely on Wnt/β-Catenin signaling (Bernatik et al., 2021). The question of whether and how Wnt signaling regulates ciliogenesis is still a matter of debate and this question was the focus of my study.

To investigate how Wnt signaling affects primary cilia formation, a Wnt-reporter cell line was used in this study to analyze the activity of canonical Wnt signaling. I also used this reporter cell line to measure the intensity of canonical Wnt signaling upon serum starvation to analyze whether primary cilia can break canonical Wnt signaling.

To answer how Wnt signaling regulates ciliogenesis, I analyzed the localization of some important Wnt components at the centrosome. Moreover, I aimed to identify which branch of the Wnt signaling pathway regulates primary cilia formation and which step of ciliogenesis Wnt signaling affects. With these notions, my final goal was to provide novel insights on how Wnt signaling regulates primary cilia biogenesis.

3. Results

3.1 Wnt/β-catenin signaling is active in ciliated RPE1 cells

Several previous studies suggest that primary cilia may play a role in inhibiting Wnt/β-catenin signaling. However, these studies used an impaired basal body or primary cilia (X. He, 2008). To test the hypothesis that primary cilia inhibit Wnt signaling, I used Human telomerase-immortalized retinal pigmented epithelial (hTERT-RPE1; herein RPE1) cells, which are commonly used as the model cell line to study molecular mechanisms of ciliogenesis (Spalluto et al., 2013). I aimed to compare Wnt signaling activity in the RPE1 cell population having different percentages of primary ciliated cells. To measure the Wnt signaling intensity, first, I generated a stable cell line carrying the Wnt reporter 7TGC (Fuerer and Nusse, 2010). Briefly, this Wnt reporter contains eGFP under the control of Wnt-responsive promoter (7xTCF) in addition to mCherry, which is under control of SV40 enhancer and early promoter and used for selection of cells carrying the reporter construct. In general, RPE1 cells form primary cilia after serum starvation in a time-dependent manner. Thus, I compared the levels of Wnt/β-catenin signaling intensity after 16h and 24h of serum starvation with or without Wnt activation (Figure 3.1 A and B). To activate the Wnt signaling pathway, I have used the Wnt3a conditioned medium (Wnt3a-CM) produced from stably transfected mouse L-cells expressing Wnt3a ligand (This medium was obtained from Dr. Sergio P. Acebrón). Wnt3a-CM specifically activated the Wnt reporter 7TGC, as no Wnt activation was observed in the control conditioned medium (Co-CM) (Figure 3.1 A). Interestingly, when treating the RPE1-7TGC cells with Wnt3a-CM after serum starvation for 24h, I observed that Wnt/β-catenin signaling still can be activated in RPE1 cells (Figure 3.1 A). Although the levels of 7TGC activation gradually increased over time with Wnt3a-CM treatment, the percentage of ciliated cells was similar for 16h and 24h of serum

starvation in Wnt3a-CM treated cells (Figure 3.1 B and C). Thus, I concluded that Wnt activity is not down-regulated in RPE1-7TGC ciliated cells.

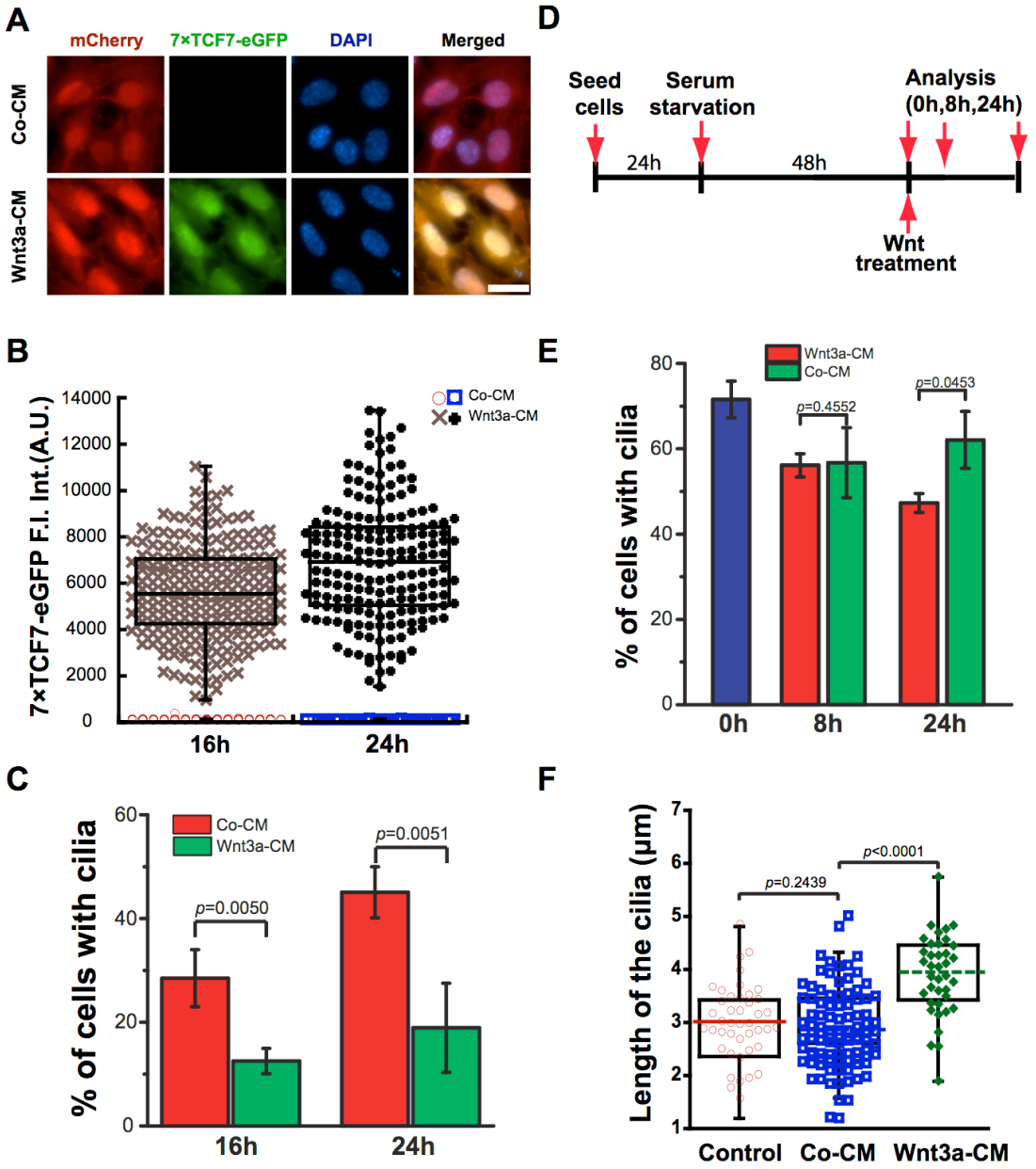

Figure 3.1 Wnt/β-catenin signaling is active in ciliated RPE1 cells. (A) The intensity of canonical Wnt signaling in RPE1-7TGC cells after 24h incubation with Wnt3a-CM in serum starvation condition. RPE1-7TGC cells expressed mCherry and the addition of Wnt3a-CM led to the expression of eGFP. Scale bar 20 μm. (B) The activity of Wnt/β-catenin signaling after 16h and 24h serum starvation with Co-CM or Wnt3a-CM treatment. (C) Box graph shows the percentage of ciliated cells in Co-CM or Wnt3a-CM treatment RPE1-7TGC cells in (B) Bar graph from three independent experiments. (D) Experimental scheme for the effects of

Wnt3a-CM after primary cilia formation in RPE1 cells. (E) Wnt signaling significantly caused cilia loss after 24h treatment with Wnt3a-CM after primary cilia formed in RPE1 cells. (F) Wnt3a-CM treatment significantly promotes the length of cilia to grow longer after primary cilia are formed in RPE1 cells for 48h.

To further confirm the result that Wnt activity is not down-regulated in RPE1-7TGC ciliated cells. I tested the ciliation in RPE1-7TGC cells by adding Wnt3a-CM after serum starvation for 48h (Figure 3.1 D and E). Clearly, after 48h serum starvation in RPE1-7TGC cells over 70% of the cells can form cilia (Figure 3.1 D and E). However, the percentage of the ciliated cell after adding Wnt3a-CM to the ciliated cells for 8 h and 24 h, I found that Wnt3a-CM treatment significantly reduced ciliated cells compared to the control conditioned medium (Co-CM) treatment after 24 h treatment (Figure 3.1 D and E). Moreover, RPE1-7TGC cells with Wnt3a-CM treatment formed fewer cilia than with Co-CM treatment, implying Wnt/β-catenin signaling may inhibit the formation of primary cilia (Figure 3.1 C). Interestingly, the length of cilia under Wnt3a-CM treatment was longer than the Co-CM treatment, indicating there is an unknown connection between the length of cilia and Wnt cell signaling (Figure 3.1 F). Taken together, my data shows that Wnt/β-catenin signaling may inhibit the formation of primary cilia.

3.2 The activities of canonical Wnt signaling in RPE1 cells upon Wnt3a-CM treatment

My previous data indicated that canonical Wnt signaling may inhibit the formation of primary cilia. To confirm this hypothesis, I tested whether canonical Wnt signaling regulates primary cilia formation. To figure out this question, I first activate Wnt signaling in RPE1 cells and then remove serum from the culture to induce cells to form primary cilia. To answer how much time canonical Wnt signaling can be activated in RPE1 cells, I tested the activity of canonical Wnt signaling in RPE1-7TGC cells upon Wnt3a-CM treatment (Figure 3.2 A and B).

After adding the Wnt3a-CM to the culture for 4h, I observed the weak eGFP signaling in RPE1-7TGC cells meaning the canonical Wnt signaling was slightly activated in RPE1 cells. Then the intensity of the signaling increased as time went on (Figure 3.2 B). All the RPE1 cells showed strong eGFP signaling after 6h incubation with Wnt3a-CM. These results indicate that canonical Wnt signaling can be highly activated in RPE1 cells after 6h treatment with Wnt3a-CM.

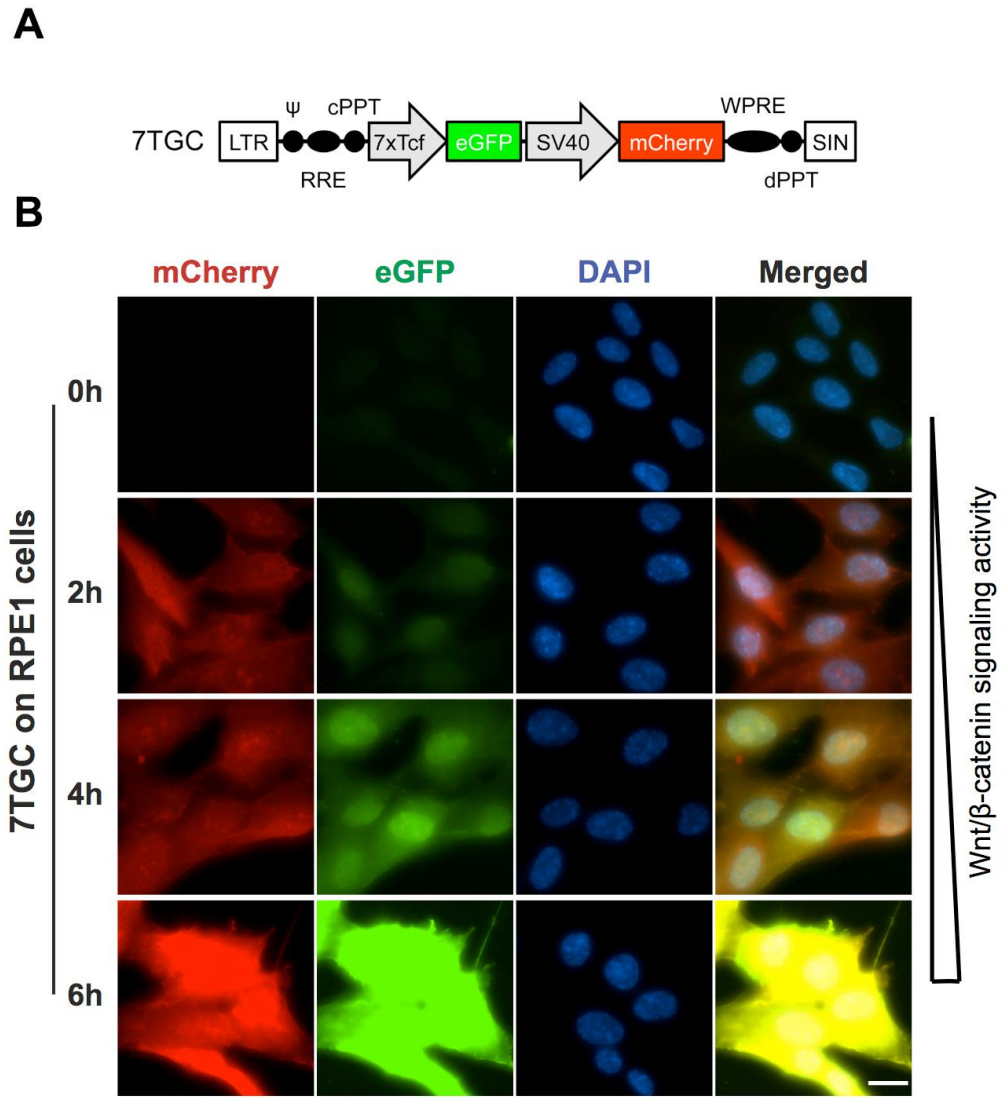

Figure 3.2 The activity and intensity of canonical Wnt signaling in RPE1-7TGC cells upon Wnt3a-CM treatment. (A) Schematic description of the7xTcf-eGFP//SV40-mCherry (7TGC) lentiviruses (Addgene: 24304) adapted from (Fuerer and Nusse, 2010). (B) RPE1 infected with the 7TGC lentivirus cells expressed mCherry, and the addition of Wnt3a-CM led to the expression of eGFP after 4h incubation. Scale bar 20 µm.

3.3 Wnt signaling suppresses cilia formation upon Wnt3a-CM treatment in RPE1 cells

Since my previous results implied Wnt/β-catenin signaling may inhibit the formation of primary cilia, to confirm this hypothesis, I examined ciliation in RPE1 cells upon serum starvation with Wnt3a-CM treatment (Figure 3.3 A). I confirmed a significant reduction of ciliated cells upon Wnt3a-CM treatment, whereas Co-CM did not affect cilia formation in RPE1 cells (Figure 3.3 C and D). Moreover, Wnt/β-catenin signaling was highly activated according to the western blot result showing stabilization of β-catenin protein levels (Figure 3.3 B). Next, I asked whether primary cilia formation occurs in a delayed manner in the presence of Wnt3a-CM. To answer this question, I studied the time course of primary cilia formation upon Wnt3a-CM treatment in more detail. The percentage of cells with cilia in Wnt3a-CM treatment was less than in Co-CM treatment at all-time points (Figure 3.3 E and F). After keeping the culture for 24h, Wnt3a-CM treatment suppressed primary cilia to less than 25%, suggesting Wnt signaling could regulate primary cilia formation (Figure 3.3 F).

One previous report found that Wnt3a stimulation promotes primary ciliogenesis (Kyun et al., 2020). However, Wnt3a-CM was added to the culture after serum starvation in this report. Since the experiment in the publication setup was different from my experiment, to further confirm that Wnt signaling suppresses primary cilia formation. I also analyze the ciliation in RPE1 cells with different time schemes for Wnt3a-CM addition (Figure 3.3 G and H). Wnt signaling significantly suppresses primary cilia formation in both different setups in the first 30h of serum starvation. Moreover, the percentage of cells with cilia when Wnt3a-CM was added before serum starvation was less than in culture when Wnt3a-CM was added 6h after serum starvation (Figure 3.3 G and H). After 30h of serum starvation, no different between Co-CM and Wnt3a-CM treated cells was observed (Figure 3.3 G and H). Together,

these results suggest that Wnt signaling functions as a negative regulator of primary cilia formation.

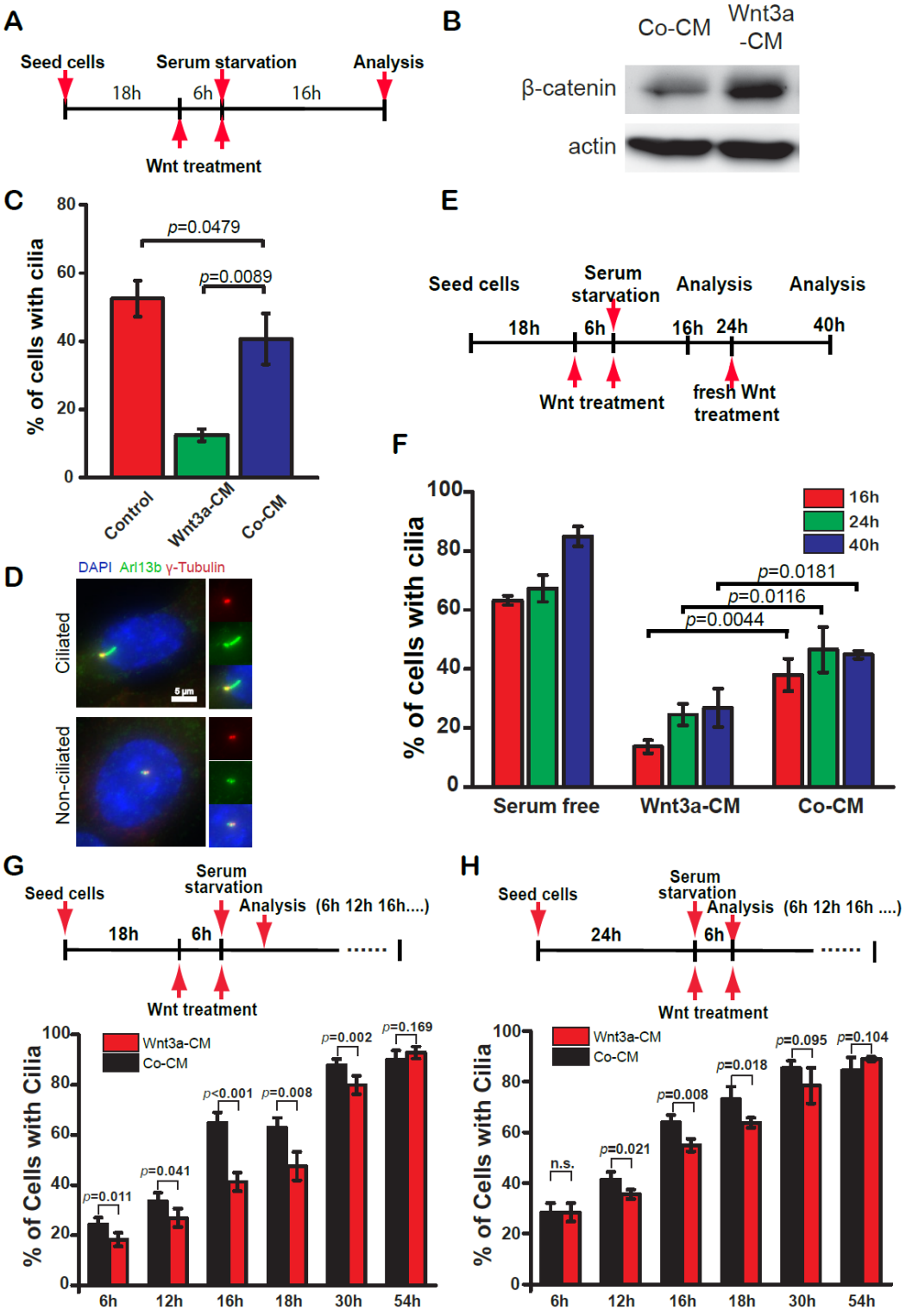

Figure 3.3 Wnt/β-catenin signaling suppresses primary cilia formation. (A) Experimental scheme for the effects of Wnt signaling on primary cilia formation in RPE1

cells. (B) Western blot analysis of saponin lysed RPE1 pellet of cytoplasmic β-catenin after 16h Co-CM or Wnt3a-CM treatment. (C) Quantification of ciliation in control, Co-CM, and Wnt3a-CM treatment cells after indicated times of serum starvation. The bar graph indicates the average from three independent experiments. (D) Images of ciliated and non-ciliated cells upon treatment of RPE1 cells with Co-CM or Wnt3a-CM after serum-starved for 16 h. The basal body was stained with γ-tubulin (red). The ciliary membrane was stained with Arl13b (green) and DNA was stained with DAPI (blue). Enlargements are shown to the right. Scale bar, 5μm. (E) Experimental scheme for the time course effects of Wnt signaling on primary cilia formation in RPE1 cells. (F) Quantification of ciliation in control, Co-CM, and Wnt3a-CM treatment cells with the different time course of serum starvation. The bar graph indicates the average from three independent experiments. (G) Experimental scheme for the effects of Wnt signaling on primary cilia formation in RPE1 cells (up). Quantification of ciliation in Co-CM and Wnt3a-CM treatment RPE1 cells after indicated times of serum starvation. The bar graph indicates the average from three independent experiments(down). (H) Experimental scheme for the effects of Wnt signaling on primary cilia formation in RPE1 cells (up). Quantification of ciliation in Co-CM and Wnt3a-CM treatment RPE1 cells after indicated times of serum starvation. The bar graph indicates the average from three independent experiments(down).

3.4 Wnt signaling suppresses cilia formation upon Wnt3a-CM treatment in NIH3T3 and IMCD3

To exclude a cell line-specific effect of Wnt signaling activation on primary cilia biogenesis, I also examined primary cilia formation upon Wnt3a-CM treatment in NIH3T3 cells, which is another ciliated cell line from mouse (Figure 3.4 A). Similar results were obtained in NIH3T3 cells upon Wnt signaling activation, supporting the suppressor function of Wnt signaling in primary cilia formation (Figure 3.4 B and C). The length of cilium upon Wnt3a-CM treatment was also the same as the Co-CM treatment (Figure 3.4 B and C). Because cilia formation in RPE1 cells and NIH3T3 cells need serum removal, I also examined ciliation in IMCD3 cells, another mouse cell line that can form cilia without serum starvation, to exclude conditional serum causes side effects upon Wnt3a-CM or Co-CM treatment (Figure 3.4 E). Similar results were also obtained in RPE1 and IMCD3 cells, as Wnt3a-CM treatment

suppressed primary cilia formation after 24h treatment. Importantly, I also treated IMCD3 cells with Wnt3a-CM for a longer time. After 48h treatment with Wnt3a-CM, IMCD3 cells still formed fewer cilia than the Co-CM treatment (Figure 3.4 F and G). Furthermore, I also analyzed the length of cilia under Wnt3a-CM and Co-CM treatment and observed no significant difference (Figure 3.4 D and H). These results suggest that the negative effect of Wnt signaling activation on ciliogenesis is not limited to RPE1 cells.

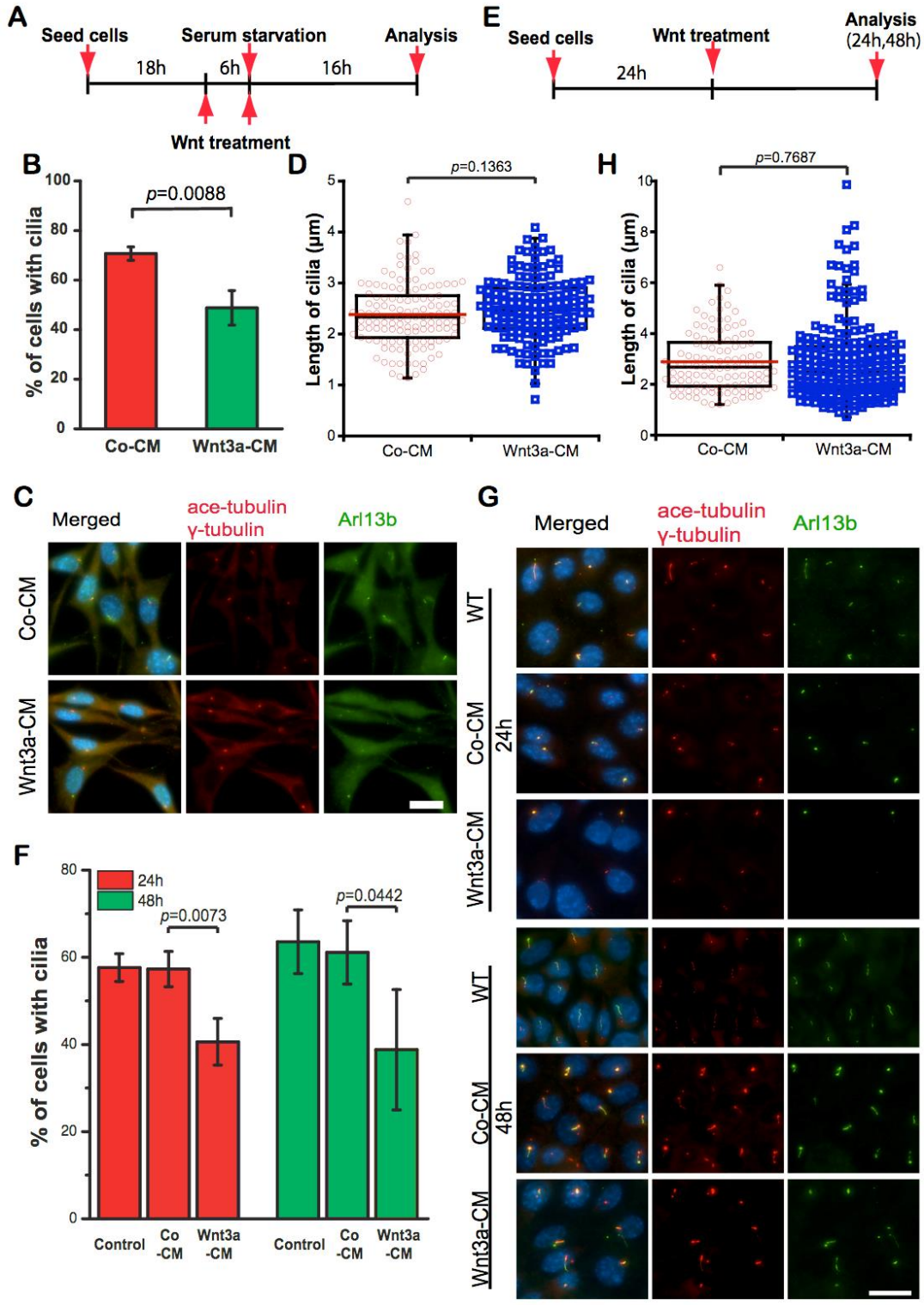

Figure 3.4 Wnt signaling suppresses primary cilia formation in NIH3T3 and IMCD3 cells. (A) Experimental scheme for the effects of Wnt signaling on primary cilia formation in NIH3T3 cells. (B) Quantification of ciliation in Co-CM and Wnt3a-CM treatment NIH3T3 cells after indicated times of serum starvation. The bar graph indicates the average from three independent experiments. (C) Images of ciliated and non-ciliated cells upon treatment

of NIH3T3 cells with Co-CM or Wnt3a-CM after serum-starved for 16 h. Scale bar, 20μm. (D) Box/dot plots show quantification of ciliary length in Co-CM or Wnt3a-CM treatment NIH3T3 cells in (B) Bar graph from three independent experiments. (E) Experimental scheme for the effects of Wnt signaling on primary cilia formation in IMCD3 cells. (F) Quantification of ciliation in Co-CM and Wnt3a-CM treatment IMCD3 cells after seeding cells for 24h and 48h. (G) Images of ciliated and non-ciliated cells upon treatment of IMCD3 cells with Co-CM or Wnt3a-CM after seeding cells for 24h and 48h. Scale bar, 20μm. (H) Box/dot plots show quantification of ciliary length in Co-CM or Wnt3a-CM treatment IMCD3 after seeding cells for 24h and 48h in (F) Bar graph from three independent experiments.

3.5 Wnt signaling suppresses primary cilia formation independent of cell cycle regulation

As primary cilia formation requires a G0/G1 cell cycle, and previous studies found that β-catenin oscillates during the cell cycle and peaks at the G2/M phase (Orford et al., 1999). To exclude the possibility that the loss of cilia was caused by cell cycle perturbation upon high activation of Wnt/β-catenin signaling, the cell cycle process was analyzed by Fluorescence-activated cell sorting (FACS) and IF staining. The Wnt3a-CM treatment did not significantly change the cell cycle compared to the Co-CM, and most of the cells in both treatments stayed in G0/G1 phase, which is the cell cycle stage in primary cilia assembly (Figure 3.5 A). I also investigated the levels of Retinoblastoma (Rb) protein which is responsible for a major G1 checkpoint and blocks S-phase entry and cell growth. pRb becomes increasingly phosphorylated during progression through G1 and keeps hyperphosphorylated until late mitosis (Giacinti and Giordano, 2006). In cycling cultures, no significant difference was observed between Co-CM and Wnt3a-CM treatment for the percentages of cells in the G1, S, and G2/M phase of the cell cycle determined by FACS (Figure 3.5 A). However, a slight decrease in the percentage of G1 phase cells was observed for Wnt3a-CM treated cells when Rb was used as a marker (Figure 3.5 B and C). Importantly, under serum starvation conditions, the activation of Wnt/β-catenin signaling did not affect the cell cycle process in RPE1 cells and over

80% of the cells were in the G1 phase (Figure 3.5 D and E). These results suggest that Wnt/β-catenin signaling inhibition of primary cilia formation was not a consequence of a lack of G1-phase arrest.

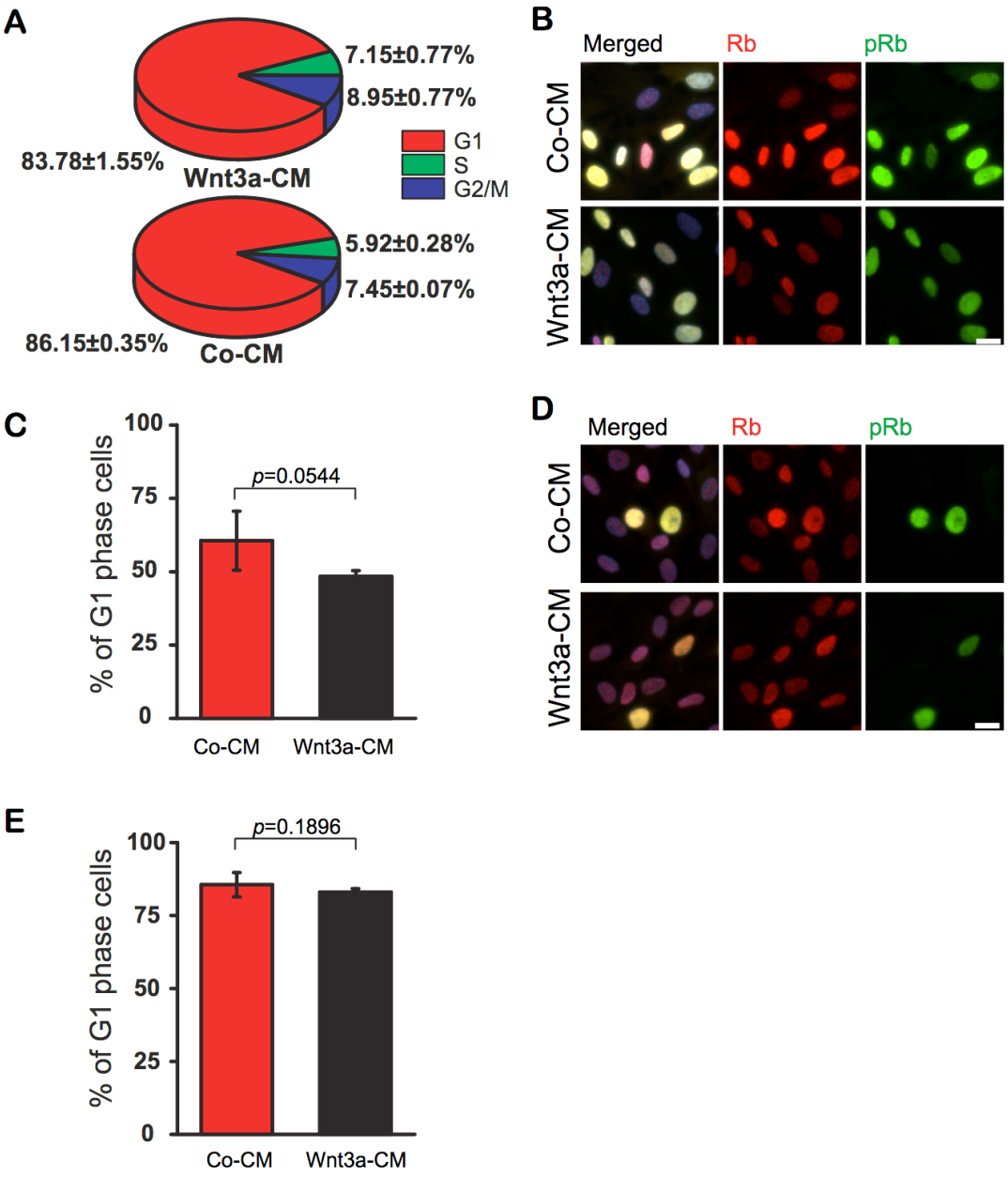

Figure 3.5 Wnt signaling suppresses primary cilia formation independent of cell cycle regulation. (A) Pie charts show the percentage of RPE1 cells in G1, S, and G2/M phases of the cell cycle by FACS analyze the DNA content analysis of cells after 16h of serum starvation upon Co-CM and Wnt3a-CM treatment. (B) Images of Rb and pRb staining cells upon treatment of RPE1 cells with Co-CM or Wnt3a-CM for 16h. RPE1 cells were stained with Rb (red). pRb

(green) and DNA were stained with DAPI (blue). Scale bar, 10μm. (C) Quantification of G1 phase cells in Co-CM and Wnt3a-CM treatment RPE1 cells for 16h. The bar graph indicates the average from three independent experiments. (D) Images of Rb and pRb cells upon treatment of RPE1 cells with Co-CM or Wnt3a-CM after serum-starved for 16h. RPE1 cells were stained with Rb (red). pRb (green) and DNA were stained with DAPI (blue). Scale bar, 10μm.(E) Quantification of G1 phase cells in Co-CM and Wnt3a-CM treatment RPE1 cells after 16h serum starvation. The bar graph indicates the average from three independent experiments.

3.6 Wnt surrogate suppresses cilia formation in RPE1 cells

Since Wnt signaling is subdivided into the canonical and noncanonical pathways, to further confirm canonical Wnt signaling is involved in controlling primary cilia formation in RPE1 cells, I made use of the Wnt surrogate as a way to activate the Wnt pathway (Janda et al., 2017). Wnt surrogate performs a characteristic of Wnt/β-catenin signaling response in an FZD-selective fashion (Figure 3.6 A and B) (Janda et al., 2017). . Importantly, the Wnt surrogate is a water-soluble Wnt agonist that links antagonistic FZD and LRP5/6-binding modules into a single polypeptide chain to activate the Wnt signaling. To produce the Wnt surrogate, I first produced the retrovirus and infected the HEK293T cells to make a stable cell line that can constantly secret the Wnt surrogate to the medium. Then I purified the Wnt surrogate from the cell culture and then test by Dr. Sergio P. Acebrón in HEK293T cells. The activity of Wnt signaling with 5μm Wnt surrogate is similar to the Wnt3a-CM with a 1:10 ratio (Figure 3.6 A). Similar to the published paper, RSPO3 strongly potentiated the activity of Wnt signaling (Fig 3.6 A). Importantly, I also tested the Wnt activities in RPE1 cells with the Wnt surrogate. The same as the Wnt3a-CM, I also observed the weak eGFP signaling after adding Wnt surrogate to the culture for 4h. However, the intensity of Wnt signaling upon Wnt surrogate treatment was weaker than upon Wnt3a-CM treatment. Since the Wnt surrogate can activate Wnt signaling in RPE1 cells, I used the purified Wnt surrogate to identify whether the surrogate can also suppress cilia formation in RPE1 cells. After treating the cells with 20 μM Wnt surrogate for 16h, Wnt surrogate significantly suppressed cilia formation, compared

with the cells without treatment (Fig 3.6 C). As the Wnt surrogate links the LRP5/6-binding modules and antagonistic FZD into a single polypeptide chain, only the canonical Wnt signaling can be activated with the Wnt surrogate. These results further confirm that Wnt/LRP6 signaling suppresses cilia formation at the early stage of the cilia formation.

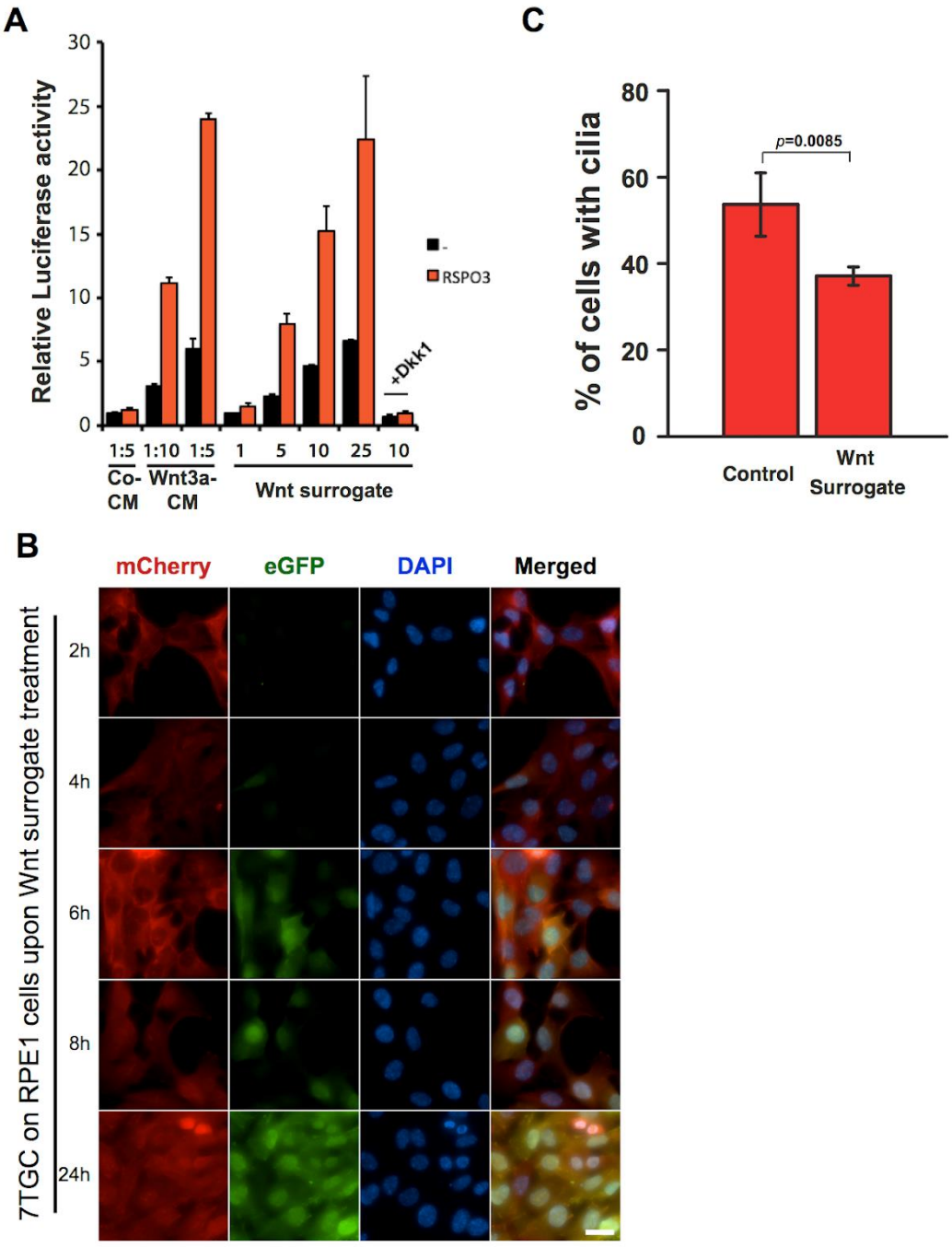

Figure 3.6 Wnt/LRP6 signaling suppresses cilia formation. (A) Relative luciferase activity in HEK293T cells by Wnt3a-CM and Wnt surrogate treatment (This data was obtained from Dr.

Sergio P. Acebrón). (B) RPE1 infected with the 7TGC lentivirus cells expressed mCherry, and the addition of Wnt surrogate led to the expression of eGFP after 6h incubation. (C) Wnt surrogate significantly suppressed cilia formation in the early time of cilia formation.

3.7 Inhibition of Wnt/LPR6 signaling rescue the cilia loss phenotype

DKK1 is the Wnt antagonist which triggers LRP5/6 endocytosis and prevents the formation of the LRP5/6-FZDs complex (Kawano, 2003). The previous study found that DKK1 is a negative component in the feedback loop and blocks Wnt signaling in normal tissues (Niida et al., 2004). To test the role of DKK1 during development, I added DKK1 conditioned medium (DKK1-CM) to the mouse intestinal organoids, compared to the normal condition and control conditioned medium condition, intestinal organoids can't form small crypts upon DKK1-CM treatment (Figure 3.7 A). This result suggests that DKK1-CM treatment can block Wnt/LPR6 signaling in tissues to affect intestinal organoid development. To test whether DKK1-CM rescues the cilia loss phenotype upon Wnt signaling activation, I have added DKK1-CM to RPE1 cells treated with Wnt3a-CM- however, the presence of serum in both conditioned medium suppressed primary cilia formation, making conclusions very difficult. To circumvent this problem, I transiently overexpressed the Wnt1alone in combination with a DKK1 construct in RPE1 cells to analyze the primary cilia formation. After culturing for 24h and followed by serum starvation for another 24h, overexpressing Wnt1 construct in RPE1-7TGC cells formed fewer cilia than the control condition with only empty plasmid only. However, there is no significant difference between these cells co-overexpressing the Wnt1 and DKK1 construct and the cells only overexpressing the Wnt1 construct (Figure 3.7 B and C), implying that DKK1 reverts the negative effect of Wnt1 on primary cilia formation. As DKK1 specifically inhibits the Wnt/LRP6 pathway, I confirmed that the inhibition of cilia formation in RPE1 cells was caused by the activation of Wnt/LRP6 signaling. My data suggest that Wnt/LRP6 signaling suppressed cilia formation in RPE1 cells.

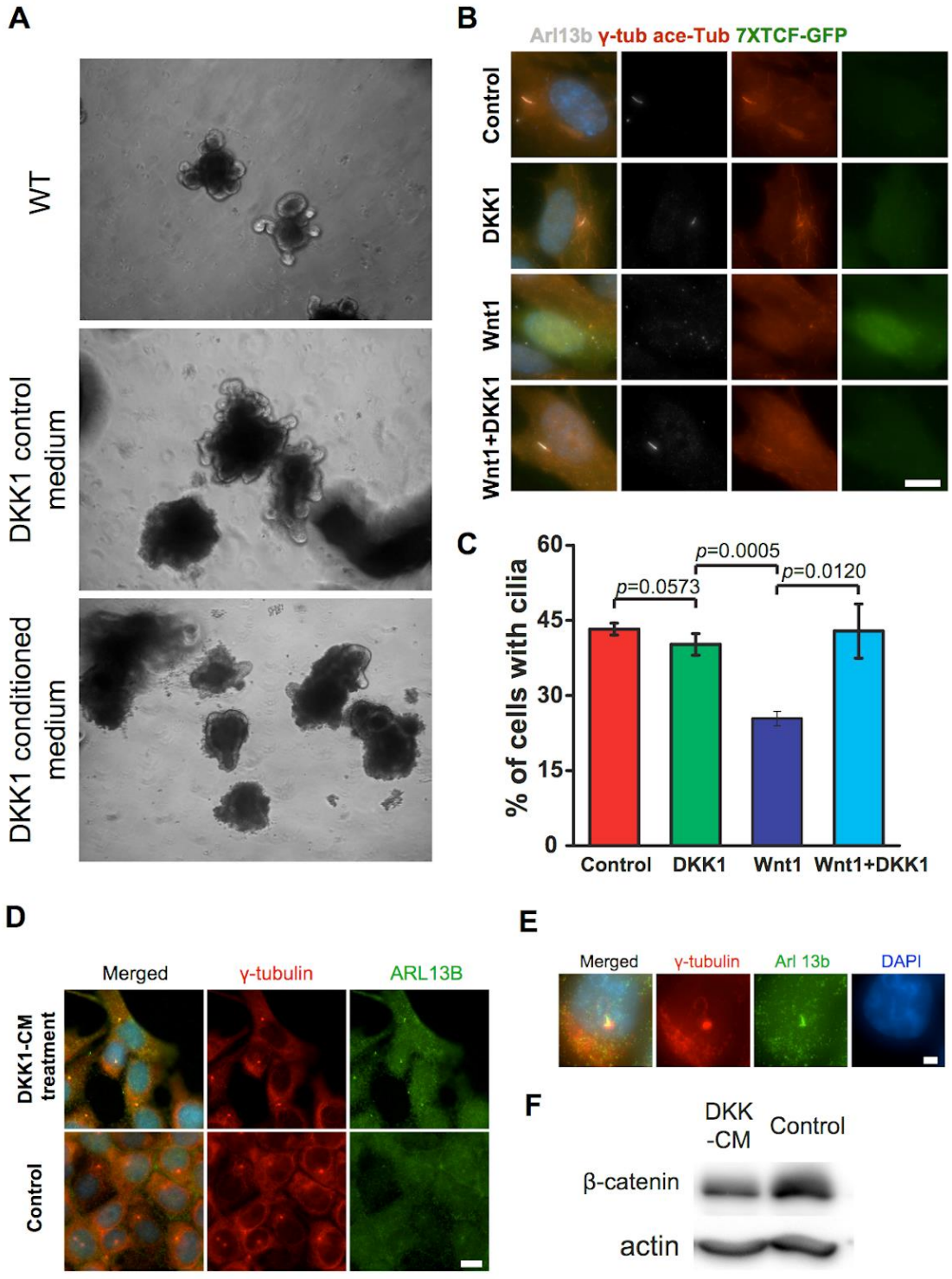

Figure 3.7 Inhibition of Wnt/LPR6 signaling rescues the cilia loss phenotype. (A) DKK1-CM treatment blocks intestinal organoids to form small crypts. (B) Representative images of cilia in RPE1-7TGC cells transiently overexpressed the Wnt1 or DKK1 construct after 16h serum starvation. Scale Bar, 10μm. (C) Quantification of ciliation in Wnt1 and DKK1 transiently overexpressed RPE1-7TGC cells after serum starvation for 16h. (D)

Image of DKK1-CM treatment-induced cilia formation in HCT116 cells. Scale bar, 10μm. (E) Images of ciliated cells upon DKK1-CM treatment in HCT116 cells after serum-starved for 24h. The basal body was stained with γ-tubulin (red). Scale bar, 2μm. (F) Western blot analysis of saponin lysed HCT116 pellet of active β-catenin upon DKK1-CM treatment.

Many studies found that ciliogenesis has been indicated to be significantly suppressed in cancer cells (Higgins et al., 2019). In parallel, the Canonical Wnt/β-catenin signaling cascade is aberrantly activated in colorectal cancer (Katoh and Katoh, 2017). Then I asked whether the defect of primary cilia in colorectal cancer is caused by the high activation of canonical Wnt signaling. After DKK1 treatment for 24h in HCT116 cells, I observed cells HCT116 can form short cilia (Figure 3.7 D and E). Moreover, β-catenin level reduced a lot upon DKK1-CM treatment according to the western blot result (Figure 3.7 F). These results suggest that the loss of cilia in HCT116 cells is caused by the high level of Wnt/β-catenin signaling.

3.8 Wnt signaling activation does not affect the maturation of the mother centriole

Since activation of Wnt/β-catenin signaling suppressed primary cilia formation, I asked which steps of ciliogenesis Wnt/β-catenin signaling affected. Ciliogenesis initiates at the mother centriole (Piprek et al., 2019)(Wheatley et al., 1996). Pre-ciliary vesicles accumulate in the vicinity of the mother centriole distal appendage protein and form a large ciliary vesicle. As distal appendage proteins are important for vesicle docking, I analyzed the level of some important distal appendage proteins such as CEP164, CEP83, TTBK2, and sub-distal appendage protein ODF2 by immunofluorescence (IF) staining upon activation of Wnt/β-catenin signaling (Figure 3.8 A-E). In comparison to cells of the control condition, the levels of these components were not significantly decreased in the Wnt3a-CM treatment. These results suggested that Wnt activation does not affect the maturation of the mother centriole.

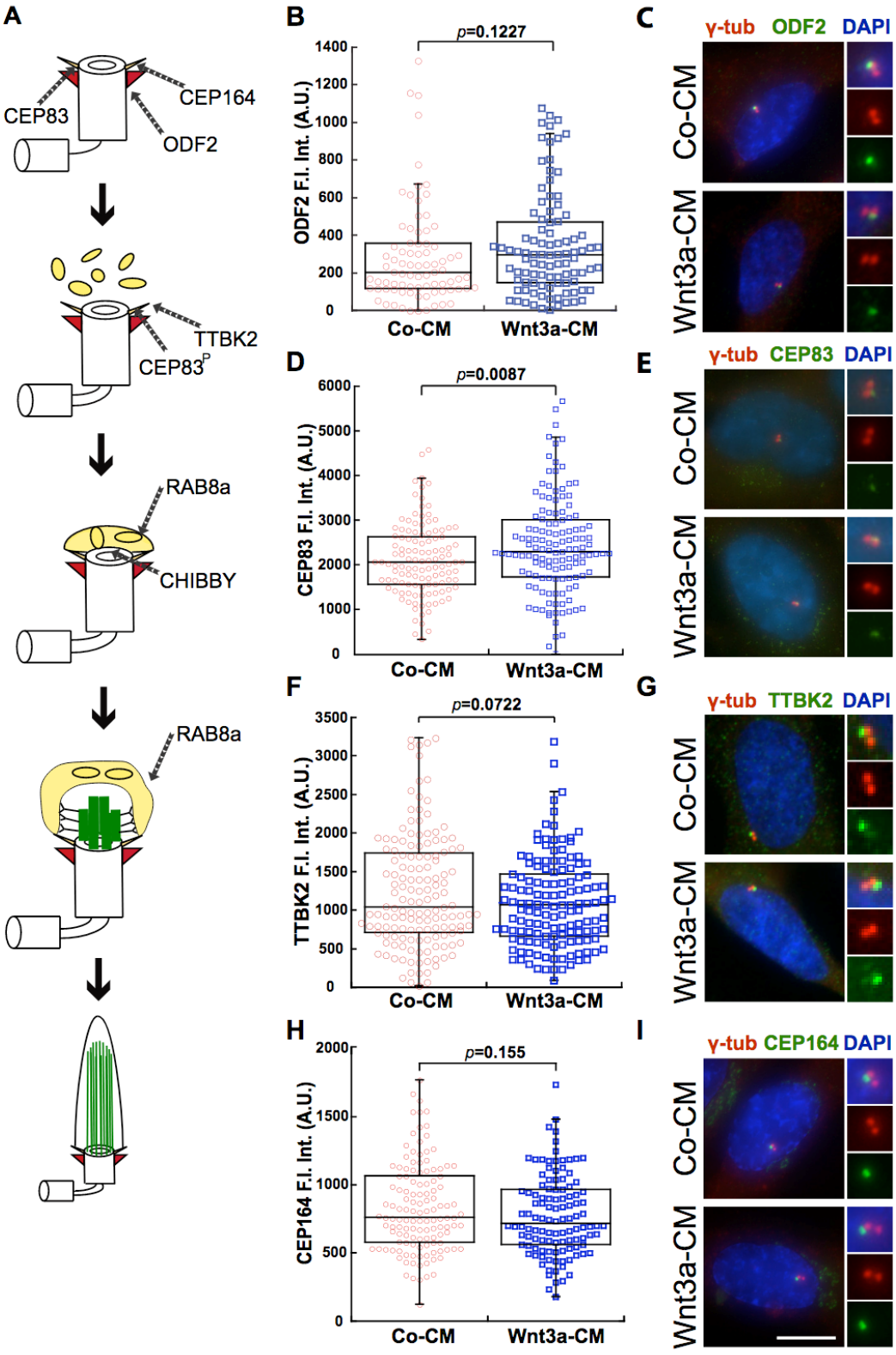

Figure 3.8 Wnt/β-catenin signaling affects ciliary vesicle formation during ciliogenesis. (A) The multiple phases and regulation of cilium assembly. Representative Box/dot plots show quantification of fluorescent intensities of ODF2 (B), CEP83 (D), TTBK2(F), and

CEP164(H) on the centrosomes in Co-CM treatment and Wnt3a-CM treatment after 16h serum starvation. (C) ODF2 (green) localization in Co-CM and Wnt3a-CM treatment RPE1 cells after serum starvation for 16 h. (E) CEP83 (green) localization in Co-CM and Wnt3a-CM treatment RPE1 cells after serum starvation for 16 h. (G) TTBK2 (green) localization in Co-CM and Wnt3a-CM treatment RPE1 cells after serum starvation for 16 h. (I) CEP164 (green) localization in Co-CM and Wnt3a-CM treatment RPE1 cells after serum starvation for 16 h. Scale bar, 20μm.

3.9 Wnt signaling affects ciliary vesicle formation during ciliogenesis

Previous studies found that CHIBBY is a nuclear β-catenin-associated antagonist that promotes the recruitment of membrane-associated RAB8 GTPase for ciliary vesicle formation and basal body docking in motile cilia(Takemaru et al., 2003)(Li et al., 2008)(Burke et al., 2014). To explore whether the role of CHIBBY in primary cilia formation, I knocked down CHIBBY in RPE1 cells to check the ciliation of the cells. Depletion of CHIBBY caused RPE1 cells to form fewer primary cilia, suggesting CHIBBY is important for primary cilia formation (Figure 3.9 E and F). Furthermore, I analyzed the intensity of the RAB8a at the centrosome upon depletion of CHIBBY (Burke et al., 2014). The enrichment of RAB8a around centrosomes was reduced in non-ciliated cells upon depletion of CHIBBY (Figure 3.9 G). Since CHIBBY is an antagonist of Wnt signaling, I hypothesized that Wnt signaling activation may affect the level of CHIBBY to regulate transcriptional activity. I further analyzed the level of CHIBBY at the centrosome upon Wnt signaling activation. However, the intensity of CHIBBY at the centrosome was not significantly decreased in Wnt3a-CM treatment from the statistical analysis (Figure 3.9 A and B). Interestingly, the enrichment of RAB8a around centrosomes was reduced in non-ciliated Wnt3a-CM treatment cells in comparison to control cells, suggesting Wnt/β-catenin signaling affects primary cilia formation by regulating the enrichment of RAB8a at the mother centrosome (Figure 3.9 G). Furthermore, Wnt3a-CM treatment reduced the intensity of RAB8a at the centrosome in CHIBBY depleted cells, suggesting Wnt signaling activation suppresses primary cilia formation by regulating the enrichment of RAB8a at the

centrosome independent of CHIBBY (Figure 3.9 C and D). Together, my data imply that activation of Wnt/β-catenin signaling affects primary cilia formation through regulating the enrichment of RAB8a at the mother centrosome, instead of affecting the assembly of appendage proteins CEP164, CEP83, TTBK2, CHIBBY, and sub-distal appendage protein ODF2.

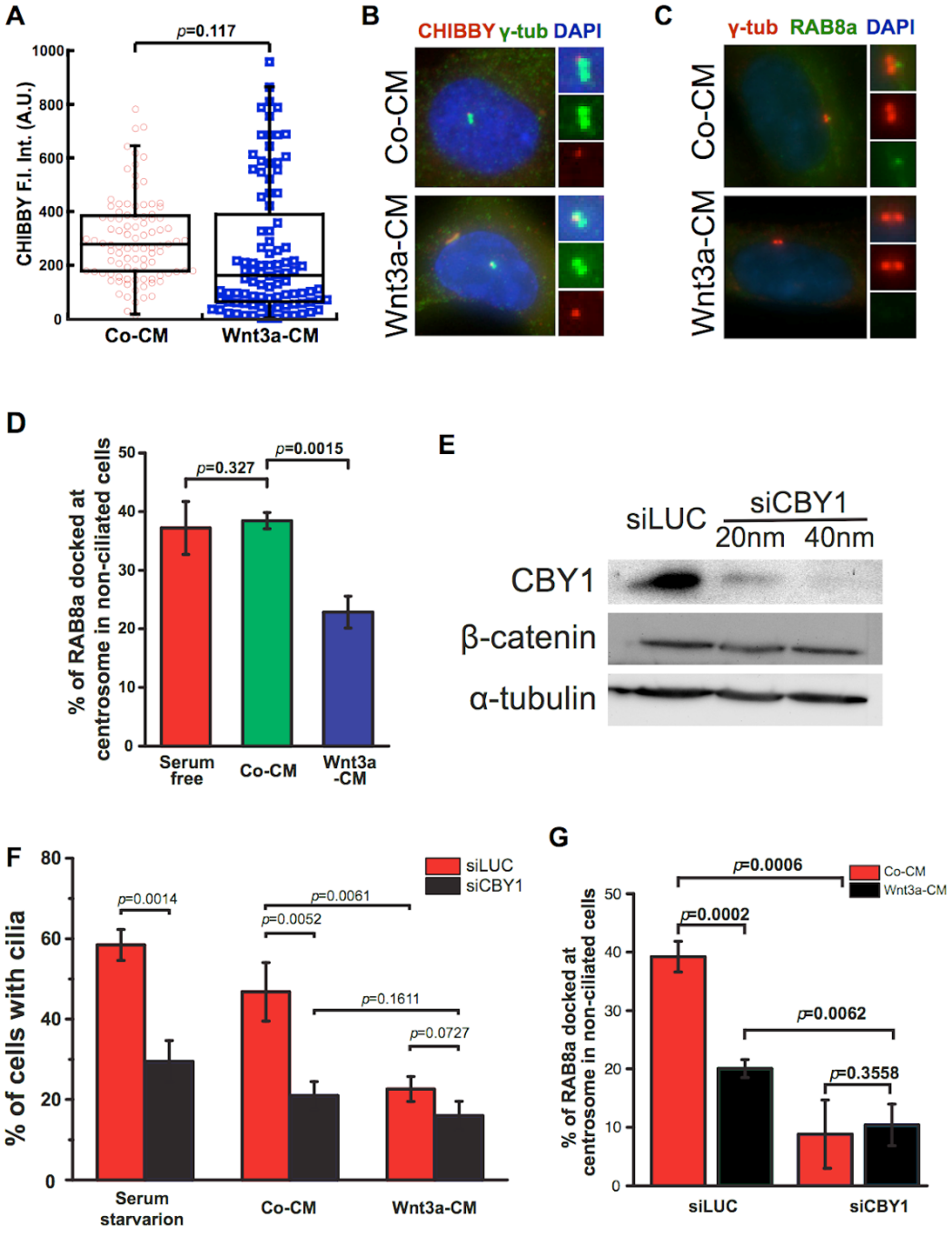

Figure 3.9 Activation of Wnt signaling affects primary cilia formation through regulating the enrichment of RAB8a at the mother centrosome. (A) Representative

Box/dot plots show quantification of fluorescent intensities of CHIBBY on the centrosomes in Co-CM treatment and Wnt3a-CM treatment after 16h serum starvation. (B) CHIBBY (red) localization in Co-CM and Wnt3a-CM treatment RPE1 cells after serum starvation for 16 h. Scale bar, 20μm. (C) RAB8a (green) localization in Co-CM and Wnt3a-CM treatment RPE1 cells after serum starvation for 16 h. (D)The percentage of cells lacking an elongated cilium that contain docked RAB8a at their mother centrioles upon Wnt3a-CM treatment after serum starvation for 16 h. (E) Western blot analysis of saponin lysed RPE1 pellet of cytoplasmic β-catenin and CHIBBY in control and CHIBBY-depleted RPE1 cells. (F) Quantification of ciliation in siLUC and siCBY1 RPE1 cells with Co-CM and Wnt3a-CM treatment after 20h serum starvation. The bar graph indicates the average from three independent experiments. (G) The percentage of cells lacking an elongated cilium that contain docked RAB8a at their mother centrioles upon Wnt3a-CM treatment in CHIBBY depleted RPE1 cells after serum starvation for 16 h.

3.10 Transcriptional regulation independent signaling suppresses the primary cilia formation

In terms of Wnt/LRP6 signaling, the downstream events include β-catenin-dependent signaling and β-catenin-independent signaling. To elucidate which downstream pathway contributed to the suppression of primary cilia formation upon activation of Wnt/LRP6 signaling, I first checked the localization of TCF7, which is the co-activator of Wnt/β-catenin signaling and binds with β-catenin to activate the transcription in the nucleus. I observed that TCF7 accumulates at the nucleus upon Wnt3a-CM treatment (Figure 3.10 A and B). Importantly, the level of cytoplasm β-catenin also increased upon Wnt3a-CM treatment, and I also observed DVL2 phosphorylation after Wnt activation (Figure 3.10 B). However, the loss of TCF7 did not affect the number of ciliated cells (Figure 3.10 B and C). The percentage of ciliated in the Wnt3a-CM treated condition is lower than the Co-CM treated condition upon depletion of TCF7 (Figure 3.10 C). These results indicated that Wnt/LRP6 signaling suppresses the primary cilia formation independent of transcription level.

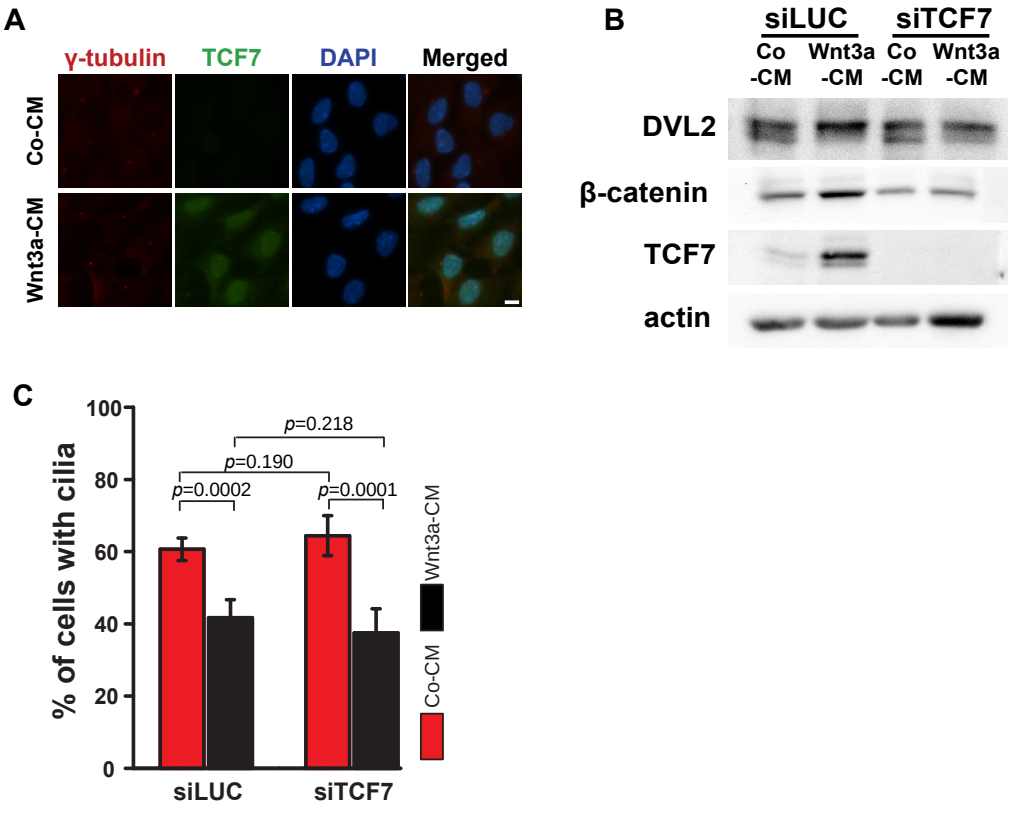

Figure 3.10 TCF7-independent signaling suppresses the primary cilia formation. (A)Image of the localization of TCF7 (green) in Co-CM and Wnt3a-CM treatment RPE1 cells. Scale bar, 5μm. (B) Western blot analysis of DVL2, saponin lysed RPE1 pellet of cytoplasmic β-catenin and TCF7 in control and TCF7-depleted RPE1 cells after 16h Co-CM or Wnt3a-CM treatment. (C) Quantification of ciliation in control and TCF7-depleted RPE1 cells upon Co-CM or Want3a-CM treatment after 16h serum starvation. The bar graph indicates the average from three independent experiments.

3.11 β-catenin is important for the primary cilia formation

To confirm the hypothesis that β-catenin-independent signaling has a function in the inhibition of primary cilia formation, I also examined depleted β-catenin upon Wnt3a-CM treatment to check the ciliation in RPE1 cells. First, I checked the localization of β-catenin in serum starvation conditions. The β-catenin is localized at cell adhesion (Figure 3.11 A and B). Unfortunately, I could not detect β-catenin co-localization at the centrosome (Figure 3.11 A and B). From my previous TCF7 western blot data, the level of cytoplasm β-catenin increased upon Wnt3a-CM treatment (Figure 3.10 B). These results indicate β-catenin has two pools in RPE1 cells: One cell membrane-bound and one cytoplasmic poll. Interestingly, the level of total β-catenin reduced a lot upon serum starvation, suggesting serum may affect the amount of β-catenin in RPE1 cells. More importantly, the amount of total β-catenin was rather high upon Co-CM and Wnt3a-CM treatment (Figure 3.11 C). As β-catenin can accumulate in the cytoplasm and then translocate into the nucleus upon Wnt signaling activation, I observed β-catenin accumulates in the cytoplasm in RPE1 cells upon Wnt3a-CM treatment in my previous data (Figure3.3 B). However, I could not observe the accumulation of total β-catenin in the Wnt activation condition upon both Wnt3a-CM treatment and Wnt-surrogate treatment, suggesting only the level of cytoplasmic, and not cell membrane-bound, β-catenin increases upon Wnt activation (Figure 3.11 D and E).

Activation of Wnt signaling causes the accumulation of β-catenin in the cytoplasm and nucleus. If Wnt signaling suppressed primary cilia formation through accumulating of β-catenin in the cytoplasm functions, knockdown of β-catenin should rescue the phenotype caused by Wnt activation. Next step, I knocked down β-catenin in RPE1 cells to check the ciliation of the cells upon Wnt signaling activation. However, after treating the cells with Wnt3a-CM for 16h of serum starvation, knockdown of β-catenin didn’t increase the number of ciliated cells, compared with

no knockdown of β-catenin cells (Figure 3.11 E and F). To exclude the unknown factors in the conditioned medium that might influence primary cilia formation, I also knockdown of β-catenin in RPE1 and treat with Wnt surrogate. Similar to Wnt3a-CM treatment, Wnt surrogate also suppressed primary cilia formation in β-catenin depleted cells (Figure 3.11 G).

To investigate whether β-catenin influences ciliogenesis, I knocked down β-catenin in RPE1 cells to check the ciliation of the cells. Depletion of β-catenin caused cells to form fewer cilia (Figure 3.11 H and I). In parallel, I also quantified the intensity of appendage protein CEP164 at the centrosome upon depletion of β-catenin. Knockdown of β-catenin decreases CEP164 levels at the centrosome (Figure 3.11 J), implying that β-catenin is required for basal body maturation. Together, all these data imply that Wnt/LRP6 signaling suppresses the primary cilia formation independent of transcriptional regulation. Importantly, my data suggest that β-catenin is important for primary cilia formation independently of Wnt signaling activation.

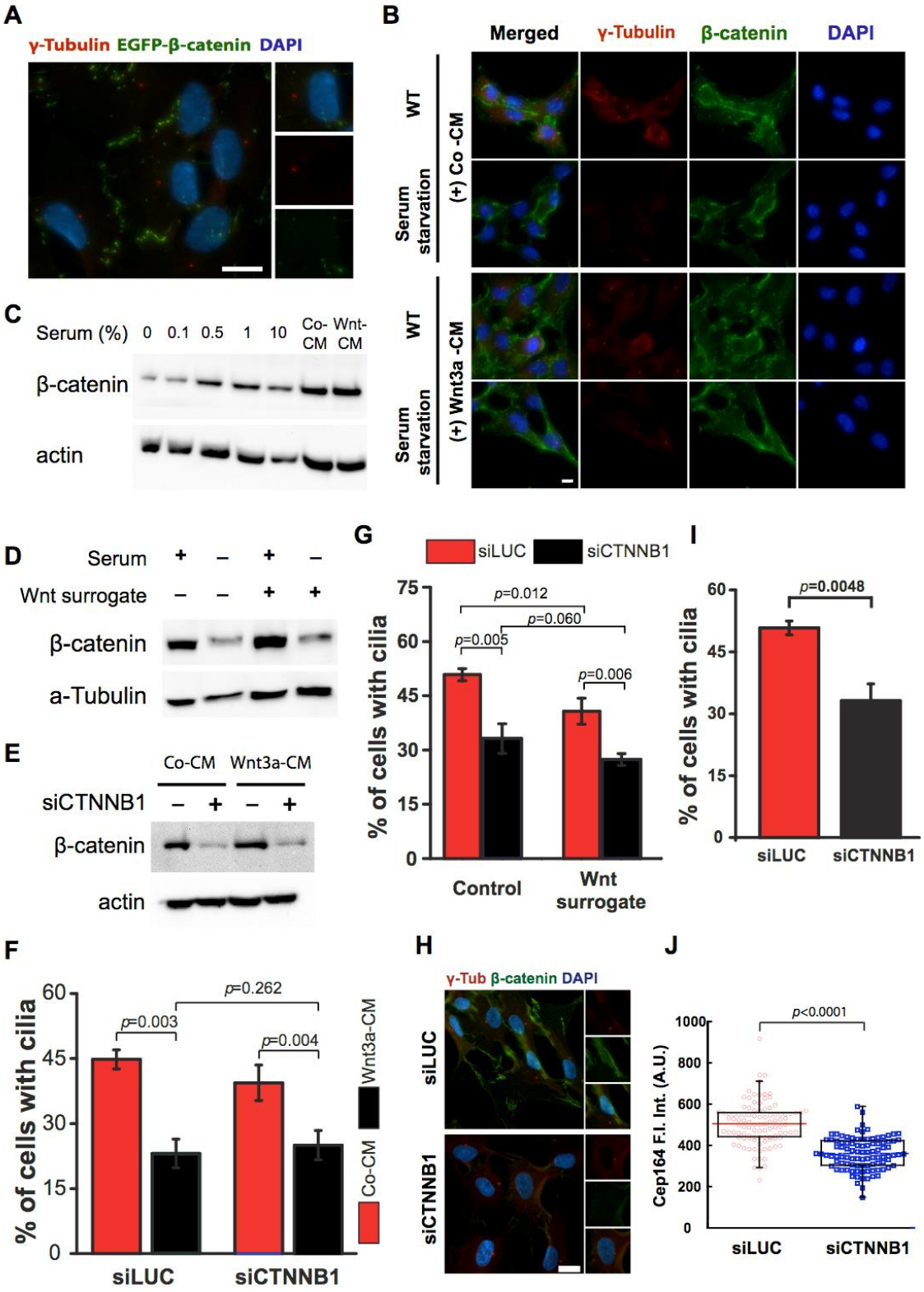

Figure 3.11 Transcriptional regulation independent signaling suppresses the primary cilia formation. (A) Endogenous EGFP-β-catenin localization in RPE1 cells. γ-tubulin (red) and DAPI (blue) serve as markers for centrosomes and nuclei, respectively. Scale bar, 20μm. (B) β-catenin (green) localization upon Wnt signaling activation. Scale bar, 10μm. (C) Western blot

analysis of total β-catenin with the different amounts of serum after 16h treatment. (D) Western blot analysis of total β-catenin after 16h Wnt surrogate treatment. (E) Western blot analysis of total β-catenin level in control and β-catenin-depleted RPE1 cells upon Co-CM or Wnt3a-CM treatment after 16h serum starvation. (F) Quantification of ciliation in control and β-catenin-depleted RPE1 cells upon Co-CM or Wnt3a-CM treatment after 16h serum starvation. The bar graph indicates the average from three independent experiments. The bar graph indicates the average from three independent experiments. (G) Quantification of ciliation in control and β-catenin-depleted RPE1 cells upon Wnt surrogate treatment after 16h serum starvation. The bar graph indicates the average from three independent experiments. The bar graph indicates the average from three independent experiments. (H) β-catenin (green) localization in control and β-catenin-depleted RPE1 cells. γ-tubulin (red) and DAPI (blue) serve as markers for centrosomes and nuclei, respectively. Scale bar, 20μm. (I) Quantification of ciliation in control and β-catenin-depleted RPE1 cells after 16h serum starvation. The bar graph indicates the average from three independent experiments. (J) Box/dot plots show quantification of fluorescent intensities of CEP164 on the centrosomes upon β-catenin-depletion.

3.12 GSK3β inhibitor BIO and CHIR99021 is sufficient to suppress ciliogenesis

GSK3β is a key enzyme in Wnt signaling. It phosphorylates β-catenin and leads it to be subsequently degraded by the proteasome. My results indicate a transcriptional regulation independent rule of Wnt/LPR6 signaling in primary cilia formation (Figure 3.12 A). Importantly, a previous study found that GSK3β can promote the assembly of the ciliary membrane and initiate ciliogenesis (Zhang et al., 2015). To examine whether Wnt/LPR6 signaling suppresses cilia formation directly through the GSK3β pathway. I investigated the effect of GSK3β inhibition on the primary cilia formation in RPE1 cells. Two inhibitors of GSK3β were used in this experiment. BIO serves as a potent, reversible, and ATP-competitive GSK-3α/β inhibitor. CHIR99021 serves as an aminopyrimidine derivative and also activates Wnt-signaling by binding secreted Wnt-protein to its receptor. Clearly, with GSK3β inhibitors' treatment, β-catenin accumulated in the cells upon serum starvation (Figure 3.12 C). Similar to a previous study (Zhang et al., 2015), the percentage of ciliated cells was significantly decreased with BIO treatment and CHIR99021 treatment, in comparison to the control condition after 16 h of serum starvation (Figure 3.12 B), suggesting that direct inhibition of

GSK3β caused primary cilia loss. Furthermore, the length of the assembled cilia in BIO and CHIR99021-treated cells was markedly longer than in control cells (Figure 3.12 D and E). However, these results are different from the previous report showing that the length of the assembled cilia in BIO and CHIR99021-treated cells was shorter than that in control cells (Zhang et al., 2015). These results suggest that GSK3β inhibition is sufficient to suppress ciliogenesis.

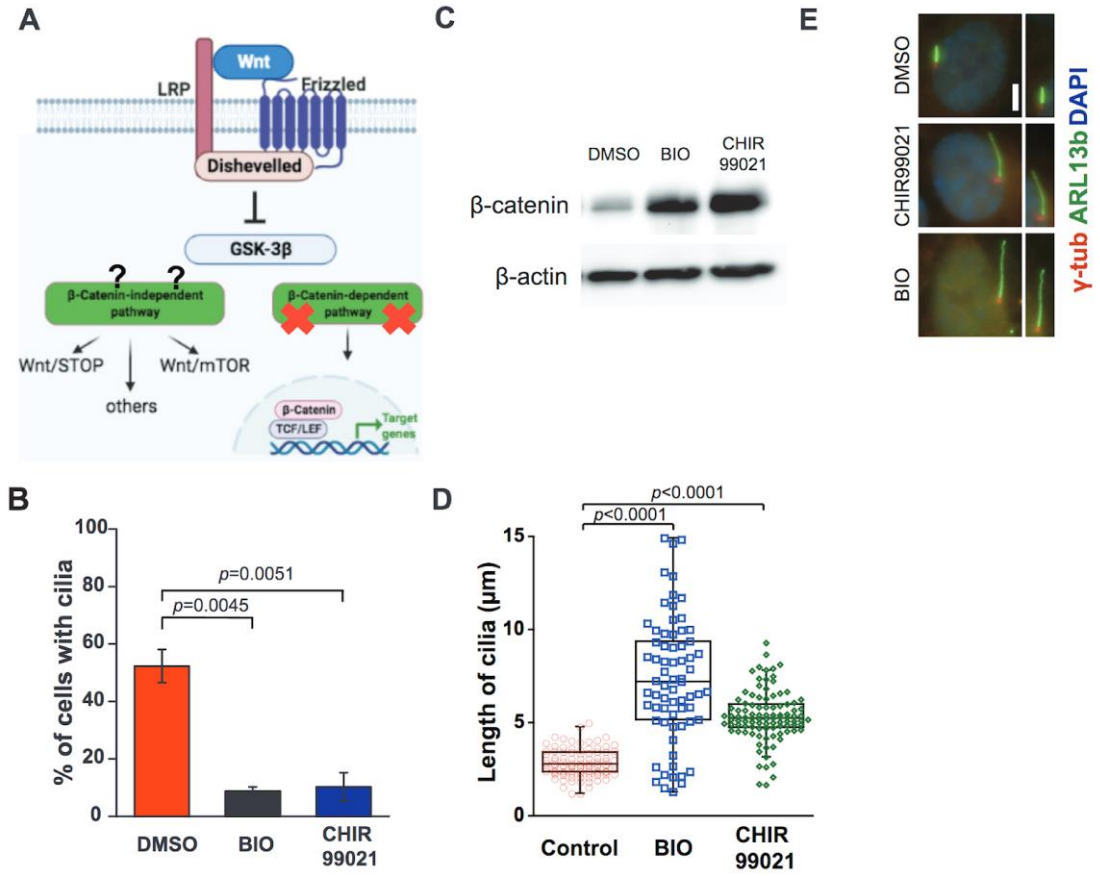

Figure 3.12 GSK3β is sufficient to suppress ciliogenesis. (A) The scheme of the β-catenin independent signaling is analyzed in this figure. (B) Quantification of ciliation in control and GSK3β inhibition with BIO and CHIR99021 RPE1 cells after 16h serum starvation. The bar graph indicates the average from three independent experiments. (C) Western blot analysis of saponin lysed RPE1 pellet of cytoplasmic β-catenin after 16h DMSO, BIO, or CHIR99021 treatment. (D) Box/dot plots show quantification of ciliary length upon control or GSK3β inhibition with BIO and CHIR99021 in (B) Bar graph from three independent experiments. (E) Representative images of the length of cilia in RPE1 cells treated with DMSO, BIO, and CHIR99021 after 16h serum starvation. Scale Bar, 5μm.

3.13 GSK3β inhibition affects the level of CEP164 and RAB8a at the centrosome to affect primary cilia formation

My previous studies showed that GSK3β inhibition is sufficient to suppress ciliogenesis. To clarify whether Wnt/LRP6 signaling suppresses primary cilia formation by directly controlling GSK3β, I further asked which steps of ciliogenesis GSK3β affected. Since my previous studies showed that the level RAB8a is decreased at the centrosome upon Wnt3a-CM treatment. I first analyzed RAB8a levels at the centrosome upon inhibition of GSK3β. Similar to Wnt3a-CM treatment, the level RAB8a at the centrosome also decreased (Figure 3.13 A and B). Then I asked whether GSK3β affects the level of appendage proteins at the centrosome. I used immunofluorescence staining to systematically observe the localization of appendage proteins upon GSK3β inhibition. In comparison to control cells, the levels of ODF2 and TTBK2 were also not significantly changed in CHIR treatment conditions (Figure 3.13 C, D, G, and H). Different from Wnt3a-CM treatment, the levels of CHIBBY that promote ciliary vesicle formation and basal body docking were significantly reduced in the CHIR99021 treatment (Figure 3.13 M). Importantly, the levels of CEP164 were significantly reduced in CHIR99021 treatment conditions (Figure 3.13 K). These results indicated that GSK3β suppressed cilia formation via deficiency of the Cep164-CHIBBY-RAB8 pathway (Figure 3.13 O).

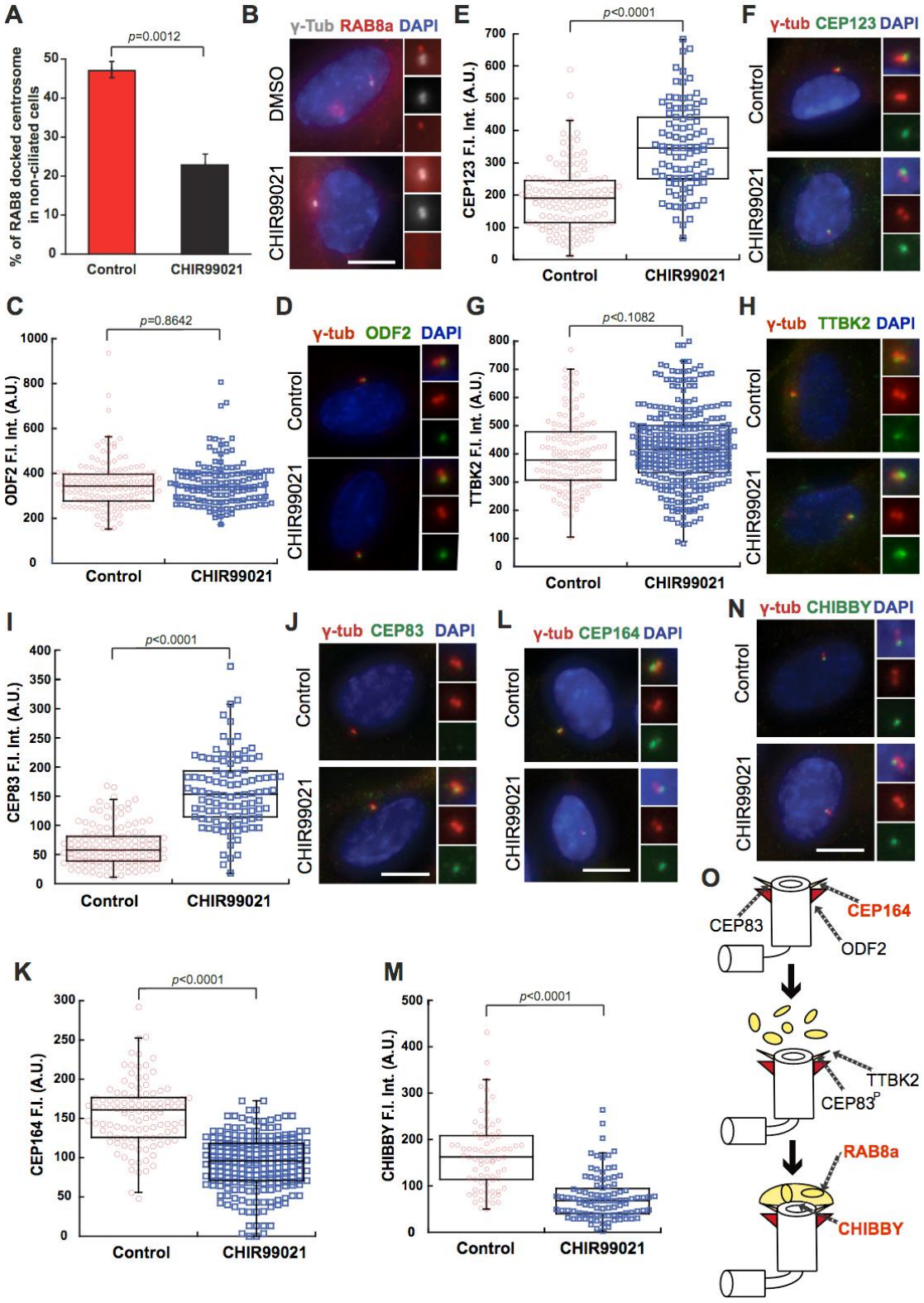

Figure 3.13 GSK3β inhibition affects the level of CEP164 and Rab8a at the centrosome to block primary cilia formation. (A) The bar graph shows the percentage of cells lacking elongated cilia that contain docked RAB8a at their mother centrioles upon control and

CHIR99021 treatment after serum starvation for 16 h, in three independent experiments. (B) Images of RPE1 stably expressing RAB8a-mRuby non-ciliated cells upon treated with DMSO or CHIR99021 after serum-starved for 16 h. Representative box/dot plots show quantification of fluorescent intensities of ODF2 (C), CEP83(E), TTBK2 (G), CEP164 (K), and CHIBBY (M) on the centrosomes in control and CHIR99021 treatment. (D) ODF2 (green) localization in control and CHIR99021 treatment RPE1 cells after serum starvation for 16 h. (I) CEP83 (green) localization in control and CHIR99021 treatment RPE1 cells after serum starvation for 16 h. (F) CEP123 (green) localization in control and CHIR99021treatment RPE1 cells after serum starvation for 16 h. (H) TTBK2 (green) localization in control and CHIR99021 treatment RPE1 cells after serum starvation for 16 h. (L) CEP164 (green) localization in control and CHIR99021 treatment RPE1 cells after serum starvation for 16 h. (N) CHIBBY (green) localization in control and CHIR99021 treatment RPE1 cells after serum starvation for 16 h. Scale bar, 20μm. (O) The model of how GSK3β rugulate primary cilia formation.

3.14 mTOR signaling activation upon Wnt3a-CM treatment regulates ciliogenesis

The downstream events of Wnt/LRP6 signaling also include β-catenin dependent signaling and β-catenin-independent events such as Wnt/mTOR signaling, Wnt/STOP signaling, and so on (Acebron and Niehrs, 2016). Next, I asked whether Wnt/LRP6 signaling suppresses cilia formation through β-catenin-independent pathways such as Wnt/STOP or Wnt/mTOR signaling. mTOR signaling is an important regulator of growth, cell metabolism, and proliferation (Foerster et al., 2017). The substrate of mTOR signaling is the protein P70-S6 kinase 1 (S6K), which becomes phosphorylated upon mTOR signaling activation (Kim and Guan, 2019). From the Western blot result, I observe the Phospho-p70 S6 Kinase (pS6K) levels increased upon Wnt3a-CM treatment in serum starvation conditions. The level of pS6K in the cycling RPE1 cells was higher than Wnt3a-CM treatment in serum starvation conditions (Figure 3.14 A). Interestingly, inhibition of mTOR signaling with rapamycin promoted primary cilia formation in cycling cells (Figure 3.14 B). This result suggested that the activity of mTOR signaling regulates primary cilia formation. Moreover, the high level of mTOR signaling activity that was activated by Wnt3a-CM suppressed primary cilia formation, suggesting Wnt/mTOR signaling could regulate

primary cilia formation. To further figure out whether Wnt/mTOR signaling is involved in the regulation of primary cilia formation, I introduced rapamycin to inhibit mTOR signaling. If the Wnt signaling suppressed cilia formation through Wnt/mTOR signaling, mTOR signaling inhibition should rescue the primary cilia formation caused by Wnt signaling activation. After treating the cells with rapamycin upon Wnt activation with Wnt3a-CM, mTOR inhibition caused an increase in the number of ciliated cells, compared with the control condition (Figure 3.14 C). However, inhibition of mTOR signaling upon Co-CM treatment in serum starvation didn't promote primary cilia formation (Figure 3.14 C). A previous study found inactivation of mTORC1 plays a role in shortening cilium length (Takahashi et al., 2018). Thus, I also analyzed the cilium length with rapamycin treatment upon Wnt activation. The length of cilia that were formed in rapamycin-treated cells was shorter than those in rapamycin-untreated cells under Wnt-inactive or Wnt-active conditions with serum-starved conditions (Figure 3.14 D). All these results suggested that Wnt signaling might regulate primary cilia formation through mTOR signaling.

To further confirm that Wnt signaling regulates primary cilia formation through mTOR signaling, I knocked down S6K which is downstream of the mTOR signaling. Apparently, knockdown of S6K promoted primary cilia formation with Wnt3a-CM treatment as well as with Co-CM treatment (Figure 3.14 E and F). Interestingly, the knockdown of S6K increased the level of ATG5 in both Co-CM and Wnt3a-CM treatment (Figure 3.14 E). ATG5 activates ATG7 and forms an E3-like ATG12-ATG5-ATG16 protein complex that is involved in the extension of the phagophore in the autophagy pathway(Collier et al., 2021). This result suggested that autophagy which is downstream of mTOR signaling may contribute to the regulation of primary cilia formation. Moreover, the length of cilia with depletion of S6K was shorter in the Co-CM treatment than in other treatments (Figure 3.14 E). However, the length of cilia with Wnt3a-CM treatment was the same as other treatments without

depletion of S6K (Figure 3.14 E). Together, all these results suggest that mTOR signaling regulates primary cilia formation downstream of Wnt signaling.

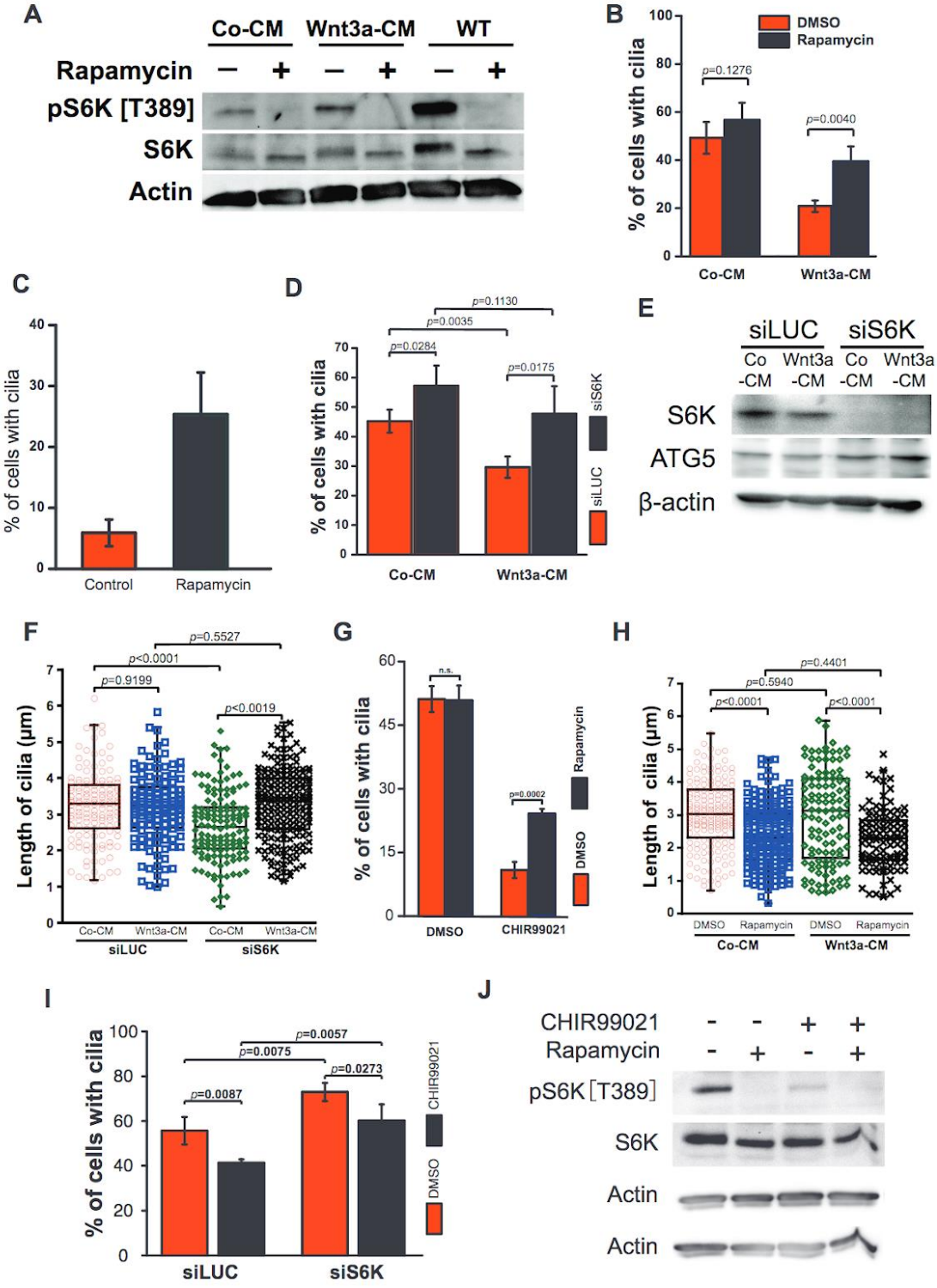

Figure 3.14 Wnt/LRP6 signaling regulates ciliogenesis through mTOR signaling. (A)

Representative western blot analysis of mTORC1 signaling activity with Co-CM or Wnt3a-CM treatment for 16 h in serum starvation condition. (B) Quantification of ciliation in RPE1 cell after 16h control or Rapamycin treatment. The bar graph indicates the average from three independent experiments. (C)Quantification of ciliation in Co-CM or Wnt3a-CM treated RPE1 cells upon control or rapamycin treatment after 16h serum starvation. The bar graph indicates the average from three independent experiments. (D) Box/dot plots show quantification of ciliary length in Co-CM or Wnt3a-CM upon control or rapamycin treatment in (C) Bar graph from three independent experiments. (E) Quantification of ciliation in control and S6K-depleted RPE1 cells after 16h serum starvation upon Co-CM or Wnt3a-CM treatment. The bar graph indicates the average from three independent experiments. (F) Western blot analysis of S6K and ATG5 in control and S6K-depleted RPE1 cells after 16h serum starvation upon Co-CM and Wnt3a-CM treatment. (G) Box/dot plots show quantification of ciliary length in control and S6K-depleted RPE1 cells upon Co-CM or Wnt3a-CM treatment in (E) Bar graph from three independent experiments. (H) Quantification of ciliation in CHIR99021 treated RPE1 cells upon control or rapamycin treatment after 16h serum starvation. The bar graph indicates the average from three independent experiments. (I) Quantification of ciliation in control and S6K-depleted RPE1 cells after 16h serum starvation upon control or CHIR99021 treatment. The bar graph indicates the average from three independent experiments. (J) Representative western blot analysis of mTORC1 signaling activity with CHIR99021 treatment for 16 h in serum starvation condition.

Next, I asked whether mTOR inhibition could also rescue the primary cilia loss phenotype caused by GSK3β inactivation. Unfortunately, Rapamycin treatment only partly rescued the cilia loss phenotype upon CHIR99021 treatment (Figure 3.14 F). Compared to the un-treatment condition, CHIR99021 treatment didn't increase the mTOR signaling activity, as estimated by the levels of pS6K on Western blotting (Figure 3.14 J). Together, these results indicated the Wnt signaling suppresses cilia formation in part through Wnt/mTOR signaling.

3.15 DVL2 has an important role in Wnt stimulation of mTOR signaling

DVL2 is a key component and acts positively in canonical Wnt signaling. To dissect mechanisms whereby Wnt suppresses primary cilia formation, I explored the roles of

DVL2 in regulating primary cilia formation. The previous study observed that DVL2 localizes at the centrosome during mitosis (Kikuchi et al., 2010). Unfortunately, I could not observe DVL2 localization at the centrosome in G0/G1 phage in RPE1 cells stably expressing low levels of DVL2 (Figure 3.15 A). Interestingly, knockdown of DVL2 caused a significant decrease in primary cilia formation, suggesting DVL2 has a role in regulating the primary cilia formation (Figure 3.15 B). Inoki et. al. found DVL2 significantly activates mTOR signaling by S6K phosphorylation, suggesting DVL2 has an important role in Wnt stimulation of mTOR (Inoki et al., 2006). I also analyzed the Wnt signaling activity and mTOR activity with the depletion of DVL2 upon Wnt3a-CM treatment. Knockdown of DVL2 significantly inactivated S6K phosphorylation (Figure 3.15 C). Interestingly, depletion of DVL2 upon Wnt3a-CM treatment only enhanced the TCF7 activity instead of the β-catenin level. These observations suggest Wnt stimulation of mTOR signaling through DVL2.

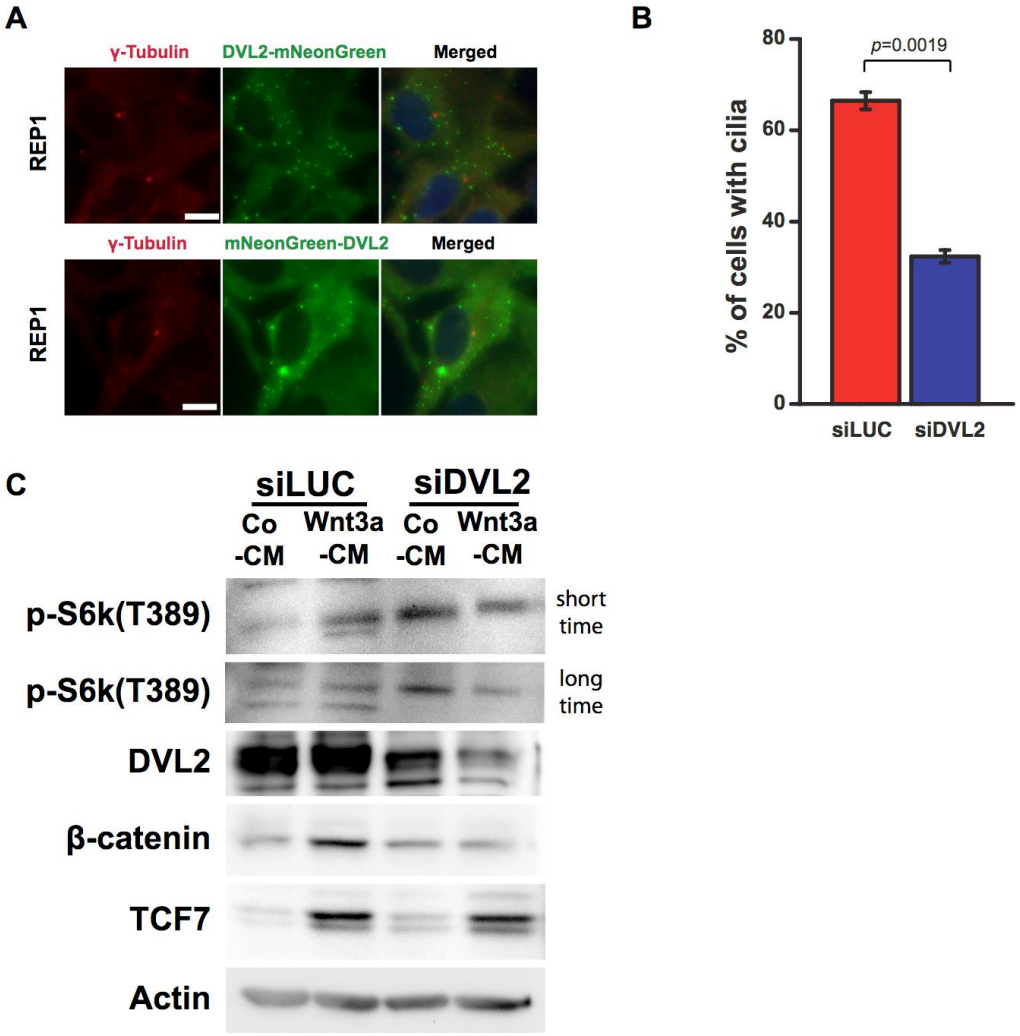

Figure 3.15 DVL2 has an important role in Wnt stimulation of mTOR signaling. (A) Representative images of localization of DVL2 at the centrosome with serum starvation. Scale Bar, 10μm. (B) Quantification of ciliation in siLUC and siDVL2 RPE1 cells after 20h serum starvation. The bar graph indicates the average from three independent experiments. (C)Western blot analysis of pS6K, DVL2, saponin lysed RPE1 pellet of cytoplasmic β-catenin and TCF7 in control and DVL2-depleted RPE1 cells after 16h Co-CM or Wnt3a-CM treatment.

3.16 Autophagy also contributes to the suppression of primary cilia formation by Wnt signaling

My previous results suggested that Wnt signaling suppresses cilia formation in part through Wnt/mTOR signaling. Autophagy is also downstream of Wnt/TOR signaling

(Noda and Ohsumi, 1998). To analyze the activity of autophagy upon Wnt activation, the traditional method is to measure the levels of protein LC3-II which localizes both inside and outside of autophagosomes membrane and is formed by phosphatidylethanolamine conjugated LC3-I (Tanida et al., 2008)(Kabeya et al., 2000). To test my hypothesis that Wnt signaling activation attenuated autophagy activity, I examined the activity of autophagy upon Wnt activation by analyzing the levels of the autophagy proteins LC3-I and LC3-II with Western blot. My results showed that LC3-II levels were similar in control and Wnt3a protein treatment conditions upon serum starvation (Figure 3.16 A). However, LC3-I levels with Wnt3a protein treatment were higher than the control condition, indicating autophagy activity decreased upon Wnt3a protein treatment (Figure 3.16 A). Moreover, I used bafilomycin A1 (BafA1) treatment which blocks fusion of lysosomal and autophagosome to increase LC3-II levels. My results showed that LC3-II levels with Wnt3a protein treatment were lower than the control condition (Figure 3.16 A). This result further confirmed that autophagy activity decreased upon Wnt activation. Together, these results suggested that Wnt signaling activation in RPE1 cells inhibited autophagy activity.

A previous study reported that autophagy promotes primary cilia formation upon serum starvation by degrading the protein OFD1. OFD1 is a centrosomal and centriolar satellite that inhibits ciliogenesis (Tang et al., 2013). The centriolar satellite OFD1 was shown to be reduced by autophagy upon serum starvation to promote ciliogenesis(Tang et al., 2013). To further test that Wnt signaling suppresses primary cilia formation through inhibition of autophagy, I analyzed the intensity of OFD1 upon Wnt3a-CM treatment by IF staining. The imaging data showed the centriolar satellite pool of OFD1 was still kept around the centrosome (Figure 3.16 B). The OFD1 intensity was higher in Wnt3a-CM treatment than in Co-CM treatment (Figure 3.16 B and C). This result suggests that Wnt signaling activation decreased autophagy

activity and kept centriolar satellite pool of OFD1 levels could most likely be the cause of less primary cilia formation. I reasoned that depleting OFD1 in RPE1 cells upon Wnt3a-CM treatment should rescue the cilia loss phenotype. To test this hypothesis, I knocked down OFD1 by siRNA and subjected the cells to Wnt3a-CM treatment, cells formed more cilia than the control condition, suggesting Wnt signaling suppressed primary cilia formation by inhibiting the degradation of OFD1 through autophagy (Figure 3.16 D). Moreover, the length of cilia became longer upon knockdown OFD1 (Figure 3.16 E). In contrast to a previous report (Kyun et al., 2020), I did not observe any significant differences for the levels of the centriolar satellite proteins CEP290 and PCM1 upon Wnt3a-CM treatment (Figure 3.16 F and G). This result implies that the transport of centriolar satellites to the centrosome is not defective. Together, these results suggest that Wnt signaling suppresses primary cilia formation might through autophagy by inhibiting the degradation of centriolar satellite OFD1.

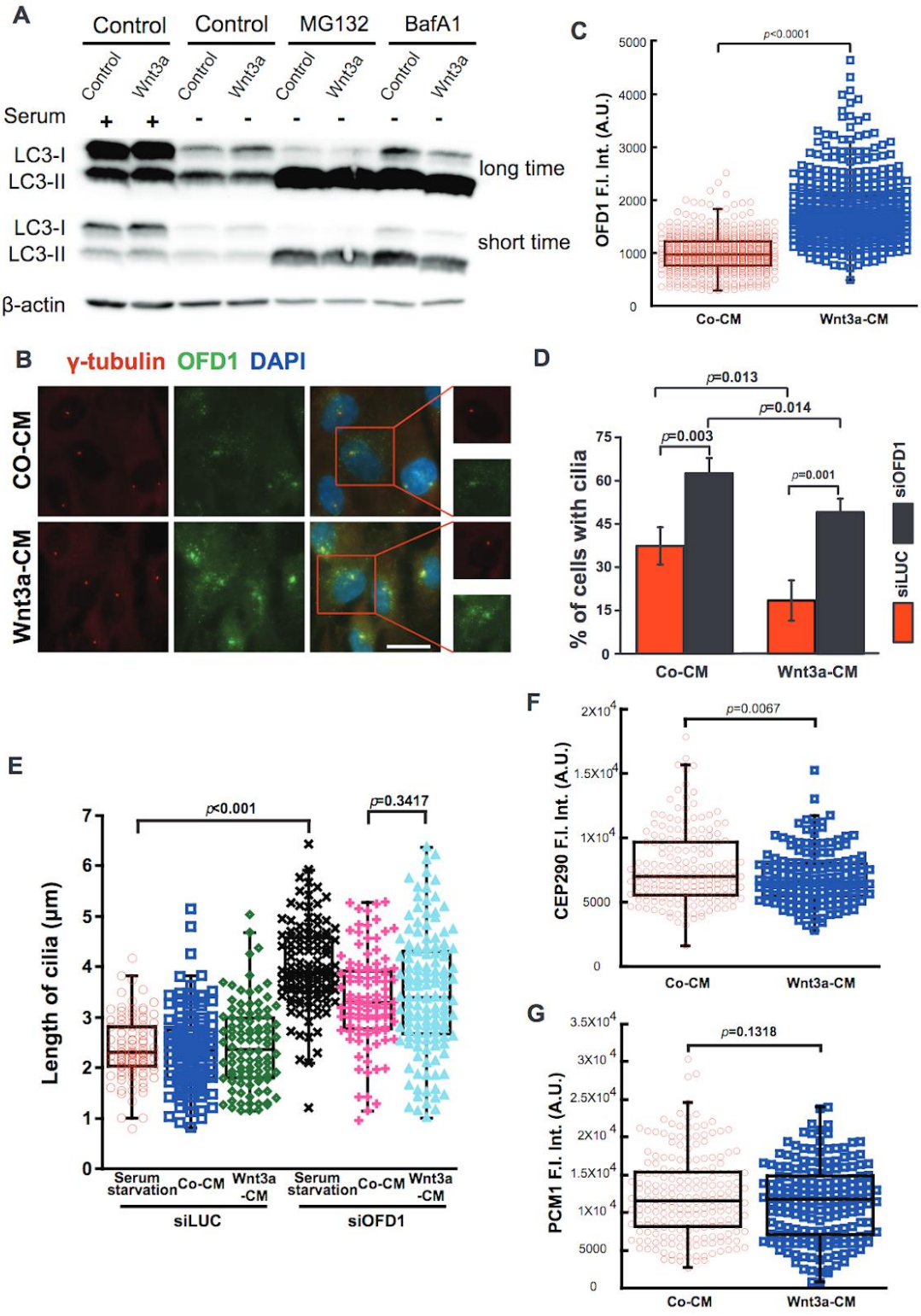

Figure 3.16 Autophagy also contributes to the suppression of primary cilia formation by Wnt signaling. (A) Representative western blot analysis of autophagy markers upon Wnt3a protein treatment for 16 h in serum starvation condition. (B) Representative images of RPE1 cells indicating OFD1(green) distribution and intensity upon Co-CM or Wnt3a-CM

treatment for 16h. The basal body was stained with γ-tubulin (red). DNA was stained with DAPI (blue). Enlargements are shown to the right. Scale bar, 20μm. (C) Representative box/dot plots show quantification of fluorescent intensities of OFD1 on the centrosomes in Co-CM and Wnt3a-CM treatment. (D) Quantification of ciliation in control and OFD1-depleted RPE1 cells after 16h serum starvation. The bar graph indicates the average from three independent experiments. (E) Box/dot plots show quantification of ciliary length upon Co-CM or Wnt3a-CM treatment with depletion of OFD1 from three independent experiments. (F) Representative box/dot plots show quantification of fluorescent intensities of CEP290 on the centrosomes in Co-CM and Wnt3a-CM treatment. (G) Representative box/dot plots show quantification of fluorescent intensities of PCM1 on the centrosomes in Co-CM and Wnt3a-CM treatment.

4. Discussion

4.1 Do primary cilia regulate Wnt signaling?

The primary cilium is an important regulator of cellular signaling. Many studies have focused on revealing how cilia regulate the Wnt/LRP6 signaling pathway (He, 2008). However, current research has focused on using mutant cell lines, for example, to investigate changes in the Wnt signaling pathway. In my study, I tried to first induce cilia formation in RPE1 cells and then add Wnt3a-CM to observe the percentage of cells with primary cilia. My results showed that for the first 8h there was no significant difference in the Wnt3a-CM treatment compared to the control group. As the conditioned medium was used in the assay, the conditioned medium contained serum which prompted the cells to lose primary cilia, and then, as the serum was consumed, primary cilia were formed again. However, my results also showed the cells became less ciliated upon Wnt3a-CM treatment after 24h treatment. More importantly, I also observed that the activity of Wnt signaling after Wnt3a-CM treatment for 24h in serum-free conditions was highly activated. All these results indicate that Wnt activity is not down-regulated in RPE1 ciliated cells.

4.2 Overactivation of canonical Wnt signaling suppresses primary cilia formation

Primary cilia is an important regulator of cellular signaling. Many studies have focused on revealing how cilia regulate the Wnt/LRP6 signaling pathway (May-Simera et al., 2018)(Veland et al., 2009). The Wnt/LRP6 signaling pathway has an important function during mitosis (Davidson and Niehrs, 2010). Canonical Wnt signaling activates the transcription factor β-catenin and LEF/TCF family to trigger gene expression which contributes to cell cycle progression, in particular at the G1/S transition. The formation of primary cilia is in the G0/G1 phase which coincides with the completion of mitosis into the next cell cycle. If there is abnormal

activation of the Wnt signaling pathway, it will inevitably affect the cell's entry into the next cell cycle. In addition to regulating β-catenin dependent transcription, recent studies have reported activation of the Wnt signaling pathway causes relatively stable expression of several proteins that are associated with inhibition of GSK3β, referred to as Wnt/STOP (Wnt-dependent stabilization of proteins) (Acebron et al., 2014). Importantly, recent studies have reported canonical Wnt pathway components might regulate the cell cycle (Bryja et al., 2017). However, relatively little research has been done on the function of the Wnt signaling pathway in interphase. The primary cilia is an important structural checkpoint for cell cycle re-entry, it is important to study the effect of the Wnt signaling pathway on cilia formation to reveal the function of the Wnt signaling pathway in interphase.

Two previous publications have focused on the effects of the canonical Wnt signaling pathway on primary cilia formation (Kyun et al., 2020)(Bernatik et al., 2021). Kyun et al. recently proposed that Wnt3a protein promotes primary cilia formation by regulating the phosphorylation of β-catenin in the cytoplasm (Kyun et al., 2020). From my results, I also observed activation of the Wnt signaling pathway inhibits primary cilia formation. Activation of Wnt/ β-catenin causes the accumulation of β-catenin. If Wnt/ β-catenin signaling regulates primary cilia formation through the accumulation of β-catenin, β-catenin depletion should rescue the cilia loss phenotype. However, depletion of β-catenin causes cilia loss, which means cytoplasmic β-catenin is important for primary cilia formation. Since Kyun et al.started adding Wnt3a-CM 6h after serum starvation (Kyun et al., 2020), in my experiment I activated the Wnt signaling pathway by adding Wnt3a-CM 6h earlier serum starvation and then induced primary cilia formation. I proposed that it might be this difference in experimental design that caused the different results that Wnt signaling has different functions on primary cilia formation. To figure out this question, I also followed the experimental setup as in the Kyun et al. publication, but I still could not observe that the canonical Wnt signaling pathway promotes cilia

formation. However, by comparing the results of the experiment setup with those of the early activation of canonical Wnt signaling, RPE1 cells formed more primary cilia under conditions of added Wnt3a-CM after serum starvation. This suggests that once PRE1 cells initiate the process of primary cilia formation, canonical Wnt signaling cannot inhibit primary cilia formation. Previous studies also indicate that primary cilia can regulate the Wnt signaling pathway (He, 2008). Together, my results also supported that canonical Wnt signaling inhibits the initial step of primary cilia formation.

Bernatik et al. recently also studied the effect of the Wnt signaling pathway on cilia formation and found that primary cilia formation doesn't rely on WNT/β-Catenin signaling (Bernatik et al., 2021). Their results found that the canonical Wnt signaling had no effect on primary cilia formation in RPE1 cells and HEK293 cells but had a slight inhibition effect on NIH3T3 cells. From my experiments, I found that RPE1 cells were able to form more primary cilia when I used the Wnt surrogate to activate canonical Wnt signaling after 16 h treatment and that the Wnt surrogate failed to inhibit primary cilia formation after extended treatment to 24h. Since the Wnt surrogate activated the Wnt signaling pathway, not as strongly as Wnt3a-CM. I proposed that the strength of canonical Wnt signaling determines whether it can inhibit the formation of primary cilia. Bernatik et al. used Wnt3a proteins, which is a hydrophobic protein, only stable for 16h under the serum-free condition. Since they analyze the activities of Wnt signaling by measuring the abundance of β-catenin after 2h with Wnt3a protein treatment and studied the ciliation after 24h treatment with Wnt3a protein in serum-free condition, it is not known whether the strength of the Wnt signaling pathway is enough to inhibit primary cilia formation. More importantly, as different cell lines respond differently to the canonical Wnt signaling pathway, The concentration of Wnt3a protein used by Bernatik et al. may not be able to strongly activate the Wnt signaling pathway in RPE1 cells and HEK293T cells, and thus the phenotype of inhibition of cilia formation by the Wnt signaling pathway

could not be observed. On the other hand, NIH3T3 may be responding more strongly to the wnt signaling pathway (Chen et al., 2007). However, to study the effect of the canonical Wnt signaling pathway on primary cilia formation, the Wnt signaling pathway needs to be activated before serum starvation. Importantly different cell types have different responses to the Wnt signaling pathway, the amount of Wnt3a protein should be optimized for different cell lines to ensure the overactivation of the Wnt signaling pathway. Taken together, my results suggest that overactivation of canonical Wnt signaling suppresses primary cilia formation.

4.3 Wnt/mTOR signaling and primary cilia formation

Since activation of canonical Wnt signaling causes the suppression of GSK3β up to 70% of the cellular total GSK3 activity, direct inhibition of GSK3β may also affect other pathways which are independent of Canonical Wnt signaling. Previous reports suggest that GSK3 can also regulate primary cilia formation via the Cep164-Dzip-Rab8a pathway (Zhang et al., 2015). In my study, I observed the defects of CEP164 and RAB8a at the centrosome upon GSK3 inhibition. However, I only observed RAB8a defect upon Wnt3a-CM treatment. My results suggest that Wnt signaling regulates primary cilia formation in the GSK3β-related pathway but is independent of GSK3β.

mTOR signaling regulates cell growth and metabolism by controlling anabolic processes and catabolic processes (Kim and Guan, 2019). mTOR is a serine/threonine-protein kinase that forms two functionally distinct complexes. mTORC1 phosphorylates S6K1, while mTORC2 phosphorylates Akt (Huang et al., 2008). The upstream of mTOR signaling includes the TSC1-TSC2 complex which is a negative regulator of mTORC1. Previous studies found that knockdown or knockout of TSC1-TSC2 complex in kidney epithelial cells of zebrafish and mice induced cells form longer cilia, however, the number of ciliated cells didn't change (Armour et al., 2012; DiBella et al., 2009). Takahashi et al. found that glucose

deprivation inactivated mTORC1 signaling promotes primary cilia formation and shortens cilium length. All these studies suggest that the mTOR signaling pathway can influence cilia formation. In my project, the signal strength of the mTOR signaling was higher in cycling cells than serum free conditions suggesting that primary cilia formation requires inhibition of mTOR signaling. However, the Wnt signaling activation caused the activation of the mTOR signalling. So, I speculate that Wnt signaling suppresses primary cilia formation through mTOR signaling. Inhibition of mTOR signalling by the addition of rapamycin could partially rescue the phenotype of inability of primary cilia formation. At the same time, to further confirm that the Wnt signalling suppressed primary cilia formation via mTOR. I also knocked down S6K1, a downstream protein of the mTOR signaling, and found that knocking down S6K1 could rescue the phenotype of loss of primary cilia formation in the presence of Wnt3a-CM. However, when I directly inhibited GSK3 activity in the absence of serum, the activity of the mTOR signaling pathway did not increase. Instead, the activity of mTOR signaling became lower compared to the control group. More importantly, PRE1 cells did not form more primary cilia when the activity of mTOR signaling was low. Taken together, my results suggest that the overactivation of canonical Wnt signaling affects ciliogenesis through the mTOR signaling pathway.

4.4 Autophagy and primary cilia formation

Previous studies have shown that activation of mTORC1 suppresses autophagy (Noda and Ohsumi, 1998)(Kim et al., 2011). In my study, I observed that Wnt3a-CM treatment caused activation of mTOR signaling in serum starvation conditions. More importantly, previous studies also found autophagy regulates the cilium growth and ciliogenesis in resting cells (Pampliega and Cuervo, 2016). So, to further explain Whether Wnt signaling regulates ciliogenesis through autophagy, I try to analyze the autophagy activity upon Wnt3a-CM treatment in serum starvation conditions. Apparently, my results showed that Wnt3a-CM treatment suppressed autophagy activity. Since autophagy degrades centriolar satellite OFD1 which is a negative

regulator of primary cilia formation to promote ciliogenesis (Tang et al., 2013). I hypothesized that Wnt3a-CM treatment might suppress primary cilia formation through autophagy by inhibiting the degradation of centriolar satellite OFD1. The intensity measurement of OFD1 around centriole indicated that Wnt signaling activation inhibited autophagy activity in serum starvation conditions and the centriole satellite OFD1 cannot be removed. Importantly, knockdown of OFD1 upon Wnt3a-CM treatment can recuse the cilia loss phenotype caused by the high activation of Wnt signaling. This result fits the previous study that autophagy promotes ciliogenesis by removing the satellite OFD1 from the centriole (Tang et al., 2013). The previous study hypothesized that rapamycin treatment might induce ciliogenesis by promoting autophagy (Takahashi et al., 2018). In my study when I used rapamycin to treat the cells upon Wnt3a-CM treatment, I also observed that RPE1 cells can form more cilia than cells without treatment. However, in the Takahashi et.al. study rapamycin treatment had no apparent effect on autophagy activity as well as the amount of the satellite OFD1 at the centrosome in RPE1 cells (Takahashi et al., 2018). I hypothesize that the mTOR activity of RPE1 cells under serum starvation conditions is low enough to promote cells to form primary cilia. More importantly, serum starvation rapidly induces autophagy activity (Mejlvang et al., 2018). So additional inhibition of mTOR activity does not help RPE1 cells to form more primary cilia. However, Wnt signaling suppresses primary cilia formation through activation of mTOR signaling to inhibit autophagy. So, rapamycin treatment recuses the RPE1 cells to form primary cilia by inhibiting mTOR signaling. Taken together, my results suggest that Wnt signaling suppresses primary cilia formation might through autophagy by inhibiting the degradation of centriolar satellite OFD1 (Figure 4.1).

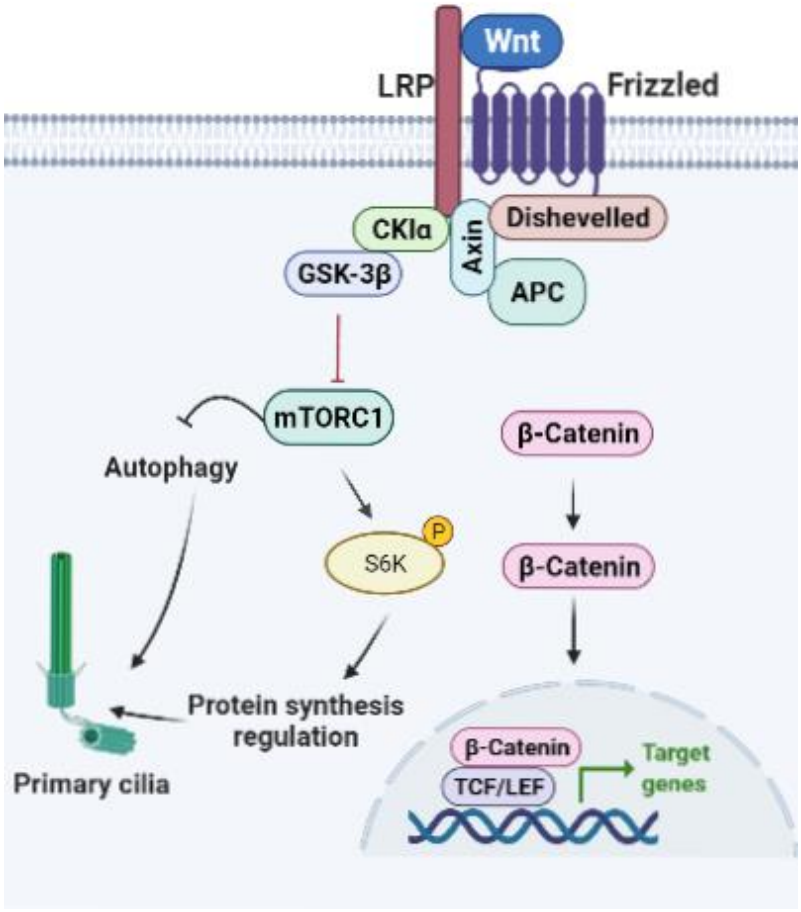

Figure 4.1 Model. Graphical abstract indicating how Wnt signaling suppresses primary cilia formation via mTOR signaling by regulating the activation of autophagy to keep the satellite protein of OFD1. It also activates the downstream of mTOR signaling to suppress cilia formation. This graph is made by BioRender (https://app.biorender.com/).

4.5 Future perspective

Abnormal activation or disruption of Wnt signaling causes many types of diseases. As the signaling hubs, primary cilia govern several important signaling pathways. In my study, I found abnormal activation of Wnt signaling suppresses primary cilia formation. To eventually understand the mechanism of how Wnt signaling suppresses primary cilia formation, happened at the centrioles need to be analyzed by mass spectrometry.

It was reported primary cilia put a brake Wnt signaling (He, 2008). In my study, I observed that Wnt activity is not down-regulated in RPE1-7TGC ciliated cells. However, to better investigate whether cilia can regulate the strength of the Wnt signaling pathway or recuse the phenotype that is caused by the high activation of Wnt signaling. Therefore, it is interesting to first optimize the conditions of the study using the Wnt3a protein or a surrogate and then to measure whether the strength of the Wnt signaling pathway changes during a dynamic process.

In my research, I found that Wnt signaling regulates cilia formation via the mTOR signaling pathway. In parallel, I found that inhibition of the Wnt signaling pathway induced primary cilia formation in colorectal cancer cell line HCT116. Due to the time limitation of the study, I was unable to test more different cancer cell lines, so it would be very interesting to select more cell lines with abnormal Wnt signaling pathways to test whether there is a problem with primary cilia formation and whether primary cilia formation is regulated via the mTOR pathway upon abnormal Wnt signaling. Furthermore, all my experiments were done with cell lines, Wnt signaling is important to maintain the 3D organoids such as intestinal, liver, kidney, and so on. It would be more interesting to study the effect of Wnt signaling on primary cilia formation in human organoids which provides unique opportunities to understand human biology and disease.

Since all my data indicates Wnt signaling regulates primary cilia formation is through GSK3β. However, GSK3 has been identified as an important player in many different pathways(Wu and Pan, 2010).it is not known whether there are other pathways by which Wnt-regulated GSK3β regulates primary cilia formation, so it is interesting to find the substrate of GSK3β. More importantly, activation of canonical Wnt signaling causes the suppression of GSK3β up to 70% of the cellular GSK3β activity. It is interesting to compare these two conditions and to eventually understand the mechanism of how Wnt signaling suppresses primary cilia formation.

5. Materials and methods

5.1 Cell culture and transfection

RPE1 (ATCC, CRL-4000) cells were grown in DMEM/F12 (Sigma-Aldrich) supplemented with 10% fetal bovine serum (FBS, Biochrom), 2 mM L-glutamine (Thermo Fisher Scientific), and 0.348% $NaHCO_3$ (Sigma Aldrich). HEK293T and GP2-293 cells (Takara Bio) were cultured in DMEM high glucose supplemented with 10% FBS. NIH3T3 cells (ATCC, CRL-1658) were grown in DMEM high glucose (Sigma Aldrich) supplemented with 10% newborn calf serum (NCS, PAN-Biotech). mIMCD3 cells were grown in DMEM/F12 (Sigma-Aldrich) supplemented with 10% FBS. All cell lines were grown with 5% CO_2 at 37°C. Wnt3a-CM and Co-CM were a gift from Dr. Sergio P. Acebrón and produced from stably transfected L-cells (ATCC, CRL-2648).

To induce cilia formation, 2×10^4-2.5×10^4 RPE1 or NIH3T3 cells were seeded on coverslips (No.1.5, Thermo Fisher Scientific) in 24-well plates for 24h and incubated in a serum-free medium for 16-48h. 2.5×10^4 mIMCD3 cells were seeded on coverslips in 24-well plates for 24h and incubated in DMEM/F12 supplemented with 0.5% FBS for 24-48h. Cell number was counted by using the Luna automated cell counter (Logos Biosystems).

RPE1 cells were transiently transfected with plasmid by either electroporation using the NEPA21 Super Electroporator (Nepa Gene) or FuGENE 6 (Promega) according to the manufacturer's protocol.

5.2 Generation of stable gene-expression cell line

The stable cell line was generated by lentivirus integration RPE1 cells with stable expression of 7TGC reporter. RPE1 doxycycline-inducible cell lines with tetON system were generated by using retrovirus and lentivirus-mediated gene integration.

To produce lentivirus, HEK293T cells were seeded in a 6-well plate one day before PEI (polyethyleneimine 25000, Polysciences) transfection by packaging plasmid of pMD2.G and psPAX2, and stably expressing gene plasmid. After 2 days, harvest 4 ml virus contained medium and then mixed with 2 ml FBS and 1 ml fresh RPE1 culture medium.

To produce retrovirus, GP2-293 cells were seeded in a 6-well plate. After 1 day, cells were transfected with pMD2.G and stably expressing genes contained plasmid by PEI. After 2 days, harvest 4 ml virus contained medium and then mixed with 2 ml FBS and 1 ml fresh RPE1 culture medium.

Two ml of prepared virus medium was added to 2×10^4 cells of the interested cell line in a 6-well plate for 48-72 h. The positively integrated cells were enriched by FACS or antibiotic selection. For Dox-induced cell lines, 50 ng/ml of doxycycline were added to the cells for 24h before FACS sorting.

5.3 FACS analysis of DNA content

Cell cycle profile of Co-CM and Wnt3a-CM treated serum-starved RPE1 cells were determined by analyzing total DNA content using propidium iodide (PI) staining. Briefly, cells were scraped off the culture plates and then fixed with 70% ice-cold ethanol and incubated with staining solution (50 µg/ml PI, 0.08% Triton X-100, 0.2 mg/ml RNase A, and 1 mM EDTA in PBS buffer). Cells were subjected to analysis on a BD FACS Canto.

5.4 Immunofluorescence cell staining and widefield microscopy

Cells were fixed with pre-cold methanol at -20°C for 5 mins. Cells expressing fluorescent protein fusions were fixed with 4% paraformaldehyde for 3 mins at room temperature followed by pre-cold methanol fixation for another 4 mins at 20°C. For

anti-acetylated-tubulin antibody staining, cells were first placed on ice for 20 min before fixation. Cells were blocked with PBS containing 3% IgG-free BSA and 0.1% Triton X-100 (PBST buffer) for 30 mins, incubated with primary antibodies in a wet and dark chamber at room temperature for 1 hour. After washing with PBS three times, cells were incubated with secondary antibodies for 45 mins at room temperature. All antibodies were diluted in PBST buffer. Coverslips were mounted with Mowiol. Images were acquired as Z-stacks by Nikon Ti2 Microscope with Plan Apo λ 60x or 40x Oil Ph3 DM objectives.

Raw images were processed to Fiji (ImageJ) prior and then exported to Adobe Photoshop and Illustrator CS3 for panel arrangement. Images of brightness and contrast were equally applied to all the images. Quantification of the protein fluorescence intensity was performed using the maximum projection of images in Fiji. An area around the centrosome of 7 square pixels was measured and around the centrosome of 10 square pixels was defined as the background. The models and schemes were created with BioRender.com. Measurement of cilia length was performed using the maximum projection of images in Fiji. Arl13B or acetylated tubulin-marked cilia were measured from the base of the centrosome to the tip, using the Measure Plugin in the Fiji software.

5.5 Live-cell imaging

RPE1 cells (4×10^4) with stably expressing fluorescent protein were seeded in a 4-divided round dish supplemented with HEPES-buffered DMEM/F12 without phenol red (Thermo Fisher Scientifics) contained 10% FBS, 1% L-glutamine, and 1% penicillin-streptomycin. After culturing for 24h, changed the serum-free medium with different treatments for Wnt intensity measurement. Images were acquired as Z-stacks by Nikon Ti-TuCam microscope every 30 mins for 24h. Z Raw images were imported to Fiji (ImageJ) before their export to Adobe Photoshop and Illustrator CS3 for panel arrangement. The models and schemes were created with

BioRender.com.

5.6 Small-interfering RNAs (siRNAs)

Transfections of siRNA were performed with Lipofectamine RNAiMAX reagent according to the manufacturer's instructions (Thermo Fisher Scientific). siRNA transfections were performed briefly as follows, 3×10^4 cells were seeded per well in a 24-well plate and analyzed 48 h after the initial transfection. 1.2×10^5 cells were reverse-transfected in one well of a 6-well plate. Detailed information about the siRNAs can be found in the table below.

The list of siRNAs

Name	Origin	Sequence (5'-3')	References
siGENOME HUMAN CTNNB1 (1499) SIRNA-SMART pool	Dharmacon	GCUGAAACAUGCAGUUGUA GAUAAAGGCUACUGUUGGA CCACUAAUGUCCAGCGUUU ACAAGUAGCUGAUAUUGAU	This study
ON-TARGET plus RPS6KB1 siRNA	Dharmacon	CAUGAACAUUGUGAGAAA GGAAUGGGCAUAAGUUGUA GUAAAUGGCUUGUGAUACU CAAAUUAGCAUGCAAGCUU	This study
ON-TARGET plus TSC2 siRNA	Dharmacon	GCAUUAAUCUCUUACCAUA CGAACGAGGUGGUGUCCUA GGAAUGUGGCCUCAACAAU GGAUUACCCUUCCAACGAA	This study
siOFD1_1	Ambion	GCUCAUAGCUAUUAAUUCAtt	
ON-TARGET plus TCF7 siRNA	Dharmacon	UCAAUCUGCUCAUGCAUUA GAUGCUAGGUUCUGGUGUA CCAAGAAGCCAACCAUCAA CCAACUCUCUCUCUACGAA	This study
ON-TARGET plus CBY1 siRNA	Dharmacon	GAAUACGGAUCCCCGACUA GGGAAUACGUUCAGUCCGA GAAAAGUGGACAUCUUAUUA GAAAAUGGCCAGUGGAUAG	This study

5.7 Western blotting

For Western blotting, to extract cytoplasmic β-Catenin and phosphorylation protein, cells were lysed in RIPA buffer (supplemented with 1x protease phosphatase inhibitor cocktail (Thermo Fisher) or cytoplasmic lysis buffer (PBS supplemented with 0.05% saponin, 10 mM β-mercaptoethanol, 2 mM EDTA, 1x protease phosphatase inhibitor cocktail). For other proteins, cells were lysed in 8 M Urea with 10mM Tris and benzonase (Sigma, 1:1000 dilution) at room temperature for 10 mins. The protein concentration was measured by Bradford reagent (Sigma). Separated proteins on the gel were transferred onto nitrocellulose membranes, then blocked with 5% milk or 5% BSA in PBST for 30 min. The membrane was incubated with primary antibodies at 4 degrees overnight. HRP-conjugated secondary antibodies were incubated with the membrane for 1h. Then the membrane was activated by ECL (homemade) and processed image with INTAS.

To detect protein (> 120kDa) by western blot, a wet immunoblot on a PVDF membrane (Ge Healthcare Amersham) was performed. The separating gel combined with 2 tissues and 6 Whatman paper equally immersed in the Borate buffer (1.25g Boric acid, 0.3725 g EDTA, to 1L with H_2O, pH8.8). The PVDF membrane was activated in Methanol for 1 min before preparing the blotting sandwich. The blotting sandwich was assembled between the two electrodes. Blotting was performed at 350 mA for 3h. The transfer process was cooled during the whole process in the cold room. The membrane was blocked and stained the same as nitrocellulose membranes.

5.8 Wnt surrogate purification

HEK293T Wnt-surrogate cells (RKS-IAT34-DKK1 Lentivirus) were grown in DMEM/F12-high glucose (Sigma) supplemented with 10% FBS. Collected the cells and supernatant after culturing the cells for 7 days. A 10x concentrated cell lysis

buffer was added to the culture and incubated on ice for 30 min. The lysate was centrifuged at 10,000xg for 20-30 min at 4°C and collected the supernatant. Add 1 ml of the 50% Ni-NTA slurry to 4 ml cleared supernatant and mix gently by shaking at 4°C for 1-2h. The lysate-Ni-NTA mixture was loaded into a column and washed twice with 4 ml wash buffer. The lysate-Ni-NTA mixture was washed with 0.5 ml elution buffer and collected the eluted protein 7 times. The fractions were analyzed by SDS-PAGE.

5.9 Antibodies

The list of primary antibodies

Gene	Species	Dilution	Company
Anti-actin	Mouse	WB 1:1000	Chemicon/Millipore ,MAB1501
ARL13B	Rabbit	IF 1:500	Proteintech 17711-1-AP
ARL13B	Mouse	IF 1:50	NeuroMabs
C3B9 (acetylated tubulin)	Mouse	IF 1:100	Self-made
Anti-Cep 164N	Guinea pig	IF 1:500	Self-made
Anti-FLAG M2	Mouse	IF 1:200	Sigma Aldrich, F1804
Anti-CEP290	Rabbit	IF 1:1000	Bethyl via Biomol
Anti-Gamma tubulin	Rabbit	IF 1:500	Sigma T5192
Anti-ODF2	Guinea pig	IF 1:500	Self-made
Anti-ODF2	Rabbit	IF 1:500	Self-made
Anti-IFT88	Guinea pig	IF 1:200	Self-made
Anti-S6K	Rabbit	WB 1:1000	Cell signaling
Anti-phospho-S6K(T389)	Rabbit	WB 1:1000	Cell signaling
Anti-LC3B	Rabbit	WB 1:1000	Cell signaling

Anti-P62 (SQSTM1)	Rabbit	WB 1:1000	MBL # PM045
Anti-actin	Mouse	WB: 1:2500	Chemicon/Millipore MAB1501
Anti-TCF1/TCF7 (C63D9)	Rabbit	WB 1:1000	Cell signalling
Anti-Phospho-LRP6 (Ser1490)	Rabbit	WB 1:1000	Cell signalling #2568
Anti-ATG5	Rabbit	WB 1:1000	Proteintech: 10181-2-AP
Anti-TSC2	Rabbit	WB 1:1000	Proteintech: 24601-1-AP
Anti-OFD1	Rabbit	WB 1:500	Self-made
Anti-CP110	Rabbit	WB 1:1000	Biomol # A301-343A
Anti-Cep123 N	Guinea pig	IF 1:500	Proteintech: 12037-1-AP
Anti-DVL2	Rabbit	WB 1:1000	Proteintech: 12037-1-AP
Anti-CEP83	Rabbit	IF 1:500	Sigma # HPA038161
Anti-β-Catenin	Rabbit	IF: 1:1000, WB 1:1000	Abcam # ab6302 IF: 1:1000
Anti-γ-tubulin clone GTU-88	Mouse	IF 1:500	Sigma Aldrich, #T6557
Anti-Chibby (Cby)	Mouse	IF 1:500	From Ryoko Kuriyama
Anti-Rb (4H1)	Mouse	IF: 1:1000	Cell signaling #9309
Anti-Phospho-Rb (Ser807/811)	Rabbit	IF: 1:1000	Cell Signaling #8516
Anti-PCM1	Rabbit	IF: 1:2000	A gift from Oliver Gruss
Anti-β-Catenin	Mouse	WB 1:2000	BD
Anti-CBY1	Rabbit	WB 1:1000	Proteintech

The list of secondary antibodies

Name	Host	Dilution	Company
Mouse Alexa 488	Goat	IF 1:500	Thermo Fisher Scientific
Mouse Alexa 594	Goat	IF 1:500	Thermo Fisher Scientific
Mouse Alexa 647	Goat	IF 1:500	Thermo Fisher Scientific
Rabbit Alexa 488	Goat	IF 1:500	Thermo Fisher Scientific
Rabbit Alexa 594	Goat	IF 1:500	Thermo Fisher Scientific
Rabbit Alexa 647	Goat	IF 1:500	Thermo Fisher Scientific
Guinea pig Alexa 488	Goat	IF 1:500	Thermo Fisher Scientific
Guinea pig Alexa 594	Goat	IF 1:500	Thermo Fisher Scientific
Guinea pig Alexa 647	Goat	IF 1:500	Thermo Fisher Scientific

5.10 List of the plasmids

Name	Description
3XFlag DVL2 (WT)	gift from Dr. Sergio P. Acebrón
pMSCV-Blast-C1-mNeongreen-DVL2	pMSCV-Blast-C1-Neongreen for Retrovirus transduction, inserted DVL2 from 3XFlag DVL2 (WT)
pMSCV-Blast-DVL2-N1-mNeongreen.	pMSCV--Blast-N1-Neongreen for Retrovirus transduction, inserted DVL2 from 3XFlag DVL2 (WT)
RKS-IAT34-DKK1 Lentivirus	Wnt surrogate, gift from Dr. Sergio P. Acebrón
pMD2G	VSV-G envelope expressing plasmid.
Wnt1 plasmid	gift from Dr. Sergio P. Acebrón
DKK1-Flag	gift from Dr. Sergio P. Acebrón
pCMV-3Tag-1A	gift from Prof. Dr. Ingrid Hoffmann

5.11 Basic techniques of molecular biology for DNA cloning

In this study, I used manufactured protocols of basic molecular biological techniques.

Name	Description	Protocol
Q5 DNA polymerase NEB)	Amplification for DNA flagments	https://international.neb.com/protocols/2013/12/13/pcr-using-q5-high-fidelity-dna-polymerase-m0491
T4 DNA ligase (NEB)	Ligating DNA fragments	https://international.neb.com/protocols/0001/01/01/dna-ligation-with-t4-dna-ligase-m0202
Restriction enzymes (NEB)	Digests DNA fragment and plasmid	https://international.neb.com/tools-and-resources/selection-charts/type-iis-restriction-enzymes
Plasmid Midi Kit (Macherey-Nagel)	Isolation of plasmid DNA from bacteria	https://www.takarabio.com/documents/User%20Manual/NucleoBond%20Xtra%20Plasmid%20DNA%20Purification%20User%20Manual_Rev_15.pdf
Plasmid Mini Kit (Qiagen)	Isolation of plasmid DNA from bacteria	http://mpheijden.tripod.com/files/Miniprep.pdf
PCR Purification Kit (Qiagen)	Cleaning up DNA fragments after PCR and gel extraction	http://2012.igem.org/wiki/images/a/a3/QIAquick_PCR-purification.pdf

5.12 Chemicals

Name	Concentration	Company
Bafilomycin A	20 nM	Sigma
MG132	10 µM	Sigma
BIO	1 µM	Sigma
CHIR99021	500 nM	Sigma
Rapamycin	100 nM	Sigma
Doxycycline		Sigma

IgG-free BSA	Jackson ImmunoResearch
Triton X-100	Sigma
Mowiol	EMD Millipore
Penicillin–streptomycin	Sigma

5.13 Statistical analyses

Data were shown as mean with standard error of the mean (SEM or SD), as indicated in the figure legends. Where indicated, Student's t-tests (two groups) were calculated using KaleidaGraph.

5.14 Primer information

The detailed information of primers used in this study can be found in Pereira's lab (COS, Heidelberg University) primer collection.

5.15 Bacteria strains

DH5α	F- 80dlacZ M15 (lacZYA-argF) U169 recA1 endA1hsdR17(rk-, mk+) phoAsupE44 -thi-1 gyrA96 relA1	Clontech Palo Alto

5.16 The list of cell lines

RPE1	Human immortalized retinal pigment epithelial cells
RPE1 Tet3G	Tet-ON 3G inducible Expression System integrated RPE1 cells
NIH3T3	Mouse immortalized fibroblast
HEK293T	Human embryonic kidney cell 293
GP2-293	Manipulated HEK293 cells for retroviral packaging cells that express gag and pol genes
HCT116	Human colon cancer cell line
Intestinal stem cell	Isolated from mouse
mIMCD3	an inner medullary collecting duct (IMCD) cell line derived in 1991 by Michael Rauchman from a mouse transgenic for the early region of SV40

6. Publications

Kurtulmus, B., Yuan, C., Schuy, J., Neuner, A., Hata, S., Kalamakis, G., Martin-Villalba, A. and Pereira, G. (2018). LRRC45 contributes to early steps of axoneme extension. J Cell Sci. doi: 10.1242/jcs.223594

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