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Cell Biology of Canonical Wnt Signaling

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Keywords

Wnt-STOP, GSK3, endocytosis, receptor trafficking, lysosome, macropinocytosis

Abstract

Wnt signaling has multiple functions beyond the transcriptional effects of β-catenin stabilization. We review recent investigations that uncover new cell physiological effects through the regulation of Wnt receptor endocytosis, Wnt-induced stabilization of proteins (Wnt-STOP), macropinocytosis, increase in lysosomal activity, and metabolic changes. Many of these growth-promoting effects of canonical Wnt occur within minutes and are independent of new protein synthesis. A key element is the sequestration of glycogen synthase kinase 3 (GSK3) inside multivesicular bodies and lysosomes. Twenty percent of human proteins contain consecutive GSK3 phosphorylation motifs, which in the absence of Wnt can form phosphodegrons for polyubiquitination and proteasomal degradation. Wnt signaling by either the pharmacological inhibition of GSK3 or the loss of tumor-suppressor proteins, such as adenomatous polyposis coli (APC) and Axin1, increases lysosomal acidification, anabolic metabolites, and macropinocytosis, which is normally repressed by the GSK3-Axin1-APC destruction complex. The combination of these cell physiological effects drives cell growth.

Contents

INTRODUCTION

Wnt signaling is a fundamental pathway across animal life that is central to cellular processes during development (Nusse & Clevers 2017). In vertebrate embryogenesis, Wnt signaling coordinates the formation of the primary head-to-tail axes, organogenesis, asymmetrical stem cell division, and regeneration (Loh et al. 2016, Niehrs 2012). Wnt signaling drives a cell growth program that can become inappropriately activated in cancer (Galluzzi et al. 2019). While Wnt pathway studies have traditionally focused on the transcriptional activity of β-catenin, recent work reveals that Wnt signaling is also a major regulator of proteasomal degradation, endocytosis, and lysosomal activity.

THE CANONICAL WNT PATHWAY ACTS THROUGH β-CATENIN STABILIZATION AND OTHER OUTPUTS

In the absence of Wnt ligand activation, β-catenin is continually degraded by a complex of proteins known as the destruction complex comprised of adenomatous polyposis coli (APC), Axin, glycogen synthase kinase 3-beta (GSK3-β), and casein kinase 1 (CK1) (Nusse & Clevers 2017). The assembly of destruction complexes is essential for coordinating the Wnt pathway through the regulation of β-catenin (Stamos & Weis 2013). Within the destruction complex, CK1 primes the phosphorylation of β-catenin by GSK3 at multiple sequential residues in the N-terminal domain, generating a phosphodegron site that is recognized by the E3 polyubiquitin ligase β-transducin repeat–containing protein, leading to the polyubiquitination and proteasomal degradation of β-catenin (Logan & Nusse 2004, MacDonald et al. 2009). The canonical Wnt pathway is activated by a set of secreted Wnt ligands that bind to the coreceptor proteins Frizzled

(Fz) and low-density lipoprotein receptor–related protein 5/6 (Lrp5/6) at the plasma membrane, resulting in β-catenin stabilization. The accumulation of β-catenin in the cytoplasm is followed by the nuclear transport and activation of the target genes of the T cell factor/lymphoid enhancer factor transcription factors, such as c-Myc, cyclin D, and Axin2 (Behrens et al. 1996, Logan & Nusse 2004). The noncanonical branch of Wnt signaling regulates cell polarity and directional movement through Fz receptor interactions with other receptor partners such as receptor tyrosine kinase (RTK), RTK-like orphan receptor, and protein tyrosine kinase 7, activating non-β-catenin effectors such as calcium/calmodulin-dependent kinase II, protein kinase C, heterotrimeric G-proteins, sarcoma family kinase, and c-Jun N-terminal kinase [reviewed in Niehrs (2012)].

This review focuses on new research unveiling key cell biological effects of Wnt that drive a growth program beyond β-catenin transcriptional activity. We examine plasma membrane receptor trafficking, the formation of signalosomes, the sequestration of cytosolic GSK3 in multivesicular bodies (MVBs), the Wnt-stabilization of proteins (Wnt-STOP), macropinocytosis, and lysosomal acidification and activation.

ENDOCYTOSIS AND WNT SIGNALING

Regulation of Wnt Receptor Endocytosis

The Wnt signal transduction cascade is initiated at the level of the cell surface receptors Fz and Lrp5/6 (**Figure 1**). Lrp5/6 receptors have a single transmembrane domain, and phosphorylation of its intracellular domain triggers signal transduction (Niehrs & Shen 2010). Fz, the first class of Wnt receptors identified, is comprised of 10 family members in humans (Bhanot et al. 1996, Xu & Nusse 1998). Fz receptors have the classic seven transmembrane domains of G-protein-coupled receptors (GPCRs) in which a highly conserved extracellular cysteine-rich domain (CRD) is essential for binding Wnt ligands (Hsieh et al. 1999, Janda et al. 2012). The intracellular domain of Fz is responsible for orchestrating cytoplasmic signaling through binding partners such as the conserved KTXXXW motif of Dishevelled (Dvl), a player in both canonical and noncanonical Wnt signaling (Colozza & Koo 2021, Gammons & Bienz 2018). The recruitment of Dvl to Fz at the plasma membrane is essential for canonical activity, as the destruction complex proteins Axin, CK1, and GSK3 are also recruited to stabilize newly translated β-catenin protein that translocates into the nucleus (MacDonald et al. 2009). Dvl promotes endocytosis at the plasma membrane through the formation of oligomers that cluster Wnt-Fz receptor complexes (Bilić et al. 2007, Niehrs 2012). Upon exposure to Wnt ligands, Fz forms complexes with its coreceptor Lrp5/6, which together trigger Wnt pathway activity (**Figure 1**).

Recent research has uncovered that the availability of Wnt receptors in the plasma membrane is exquisitely regulated by endocytosis. The first endocytic regulator was Dickkopf1 (Dkk1), a secreted glycoprotein that blocks Wnt signaling by binding to both Lrp5/6 and Kringle-containing transmembrane protein 1 (Kremen1), forming a ternary complex that triggers the removal of Lrp5/6 from the plasma membrane by endocytosis (Glinka et al. 1998, Mao et al. 2002) (**Figure 1**).

More recently, a trimeric module composed of R-spondin (roof-plate spondin or RSPO), the leucine-rich repeat–containing G-protein-coupled receptor (Lgr), and transmembrane ubiquitin ligases was discovered to operate as a feedback loop for Wnt signaling activity (Hao et al. 2012, Koo et al. 2012) (**Figure 1**).

Niehrs and colleagues (Kazanskaya et al. 2004) had described that RSPO, a secreted protein, was an activator of canonical Wnt but its mechanism had remained elusive (Kim et al. 2005). RSPOs drive cell proliferation, as evidenced in colorectal cancers with gain-of-function RSPO translocations (Seshagiri et al. 2012, Han et al. 2017). In addition, RSPO2 is secreted by the apical

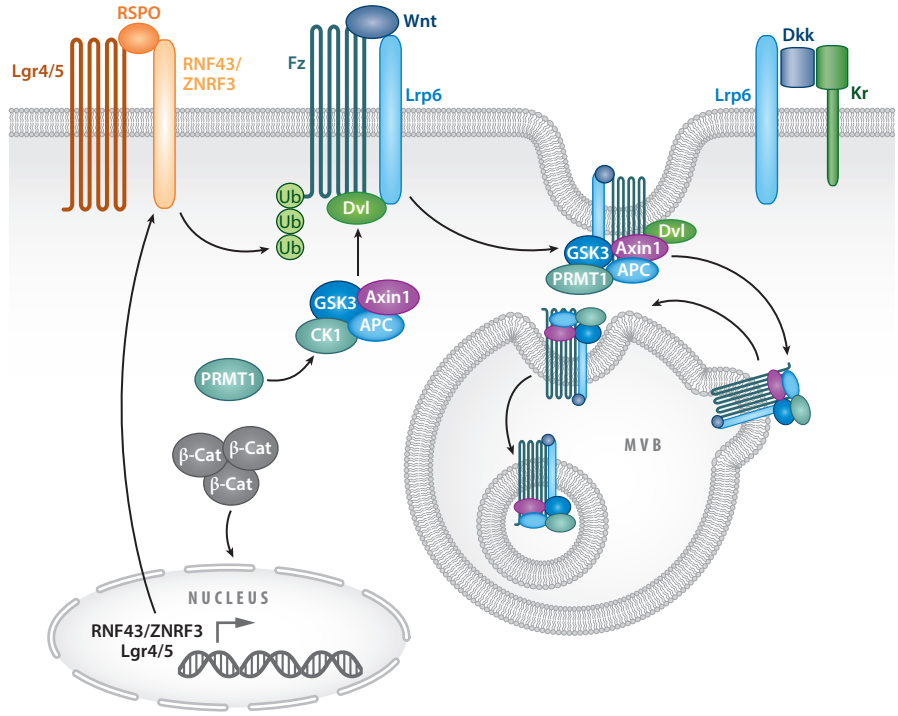

Figure 1

In the absence of Wnt ligands, β-catenin (β-Cat) is continually degraded by glycogen synthase kinase 3 (GSK3) phosphorylation in destruction complexes comprised of adenomatous polyposis coli (APC), Axin1, and casein kinase 1α (CK1). During pathway activation, Wnt ligands bind to the membrane receptors low-density lipoprotein receptor–related protein 5/6 (Lrp5/6) and Frizzled (Fz). Dishevelled (Dvl), the destruction complex, and protein arginine methyltransferase 1 (PRMT1) are recruited to the cytoplasmic tails of the activated receptors. This results in the rapid sequestration of the complex into late endolysosome or multivesicular body (MVB) vesicles within 5 min. PRMT1 activity and the endosomal sorting complexes required for transport (ESCRT) machinery that forms MVBs are essential for Wnt signaling. To mitigate excessive Wnt signaling, β-Cat transcriptional activity leads to the expression of the transmembrane E3 ligases ring finger protein 43 (RNF43) and zinc finger protein 3 (ZNRF3) that catalyze the ubiquitination (Ub) of Wnt receptors and target them for removal by endocytosis. This negative feedback loop is counteracted by secreted proteins from the R-spondin (RSPO) family that bind to leucine-rich repeat–containing G-protein-coupled receptors 4/5 (Lgr4/5) in the plasma membrane, causing the auto-ubiquitination of the E3 polyubiquitin ligases RNF43/ZNRF3 and their removal from the plasma membrane by endocytosis. RSPO1–4 are potent activators of canonical Wnt signaling by increasing the availability of Fz and Lrp5/6 receptors. Dickkopf 1 (Dkk) is a Wnt antagonist that binds to both Lrp5/6 and Kremen (Kr), leading to the removal of Lrp5/6 from the plasma membrane.

ectodermal ridge of limb buds, and its loss-of-function mutations cause complete absence of the four limbs (tetra-amelia) in humans (Szenker-Ravi et al. 2018).

Lgr4/5/6 are orphan seven-transmembrane GPCRs that were first identified as markers of intestinal stem cells in which Wnt signaling is highest (de Lau et al. 2011). Subsequent studies revealed that Lgr4/5/6 are receptors for the secreted RSPO1–4 ligands (Carmon et al. 2011, de Lau et al. 2011, Glinka et al. 2011).

Ring finger protein 43 (RNF43) and zinc finger protein 3 (ZNRF3) are transmembrane E3 polyubiquitin ligases that ubiquitinylate Fz and Lrp5/6 receptors, triggering their endocytosis,

removal from the cell surface, and degradation without signaling (Chen et al. 2013, Hao et al. 2012, Zebisch et al. 2013). As indicated in **Figure 1**, the transcription of RNF43-ZNRF3 is activated by nuclear β-catenin and establishes an important negative feedback mechanism that regulates the duration of the Wnt signal (Hao et al. 2012, Koo et al. 2012).

In the presence of RSPO ligands, Lgr4/5/6 bind to the RNF43-ZNRF3 E3 ligases and trigger their removal from the plasma membrane by endocytosis. The endocytic removal of these polyubiquitin ligases results in an increase in Wnt receptor levels and hypersensitivity to Wnt signals. An additional regulatory layer is provided by the deubiquitinase USP42, which protects ZNRF3 in the ternary complex of RSPO-Lgr-ZNRF3 from auto-ubiquitination and clearance from the plasma membrane, thus counteracting this sophisticated endocytic control feedback mechanism (Giebel et al. 2021).

Loss-of-function mutations in RNF43 and ZNRF3 are frequently found in cancer cells (Galluzzi et al. 2019), and the targeted gene inactivation of these two E3 polyubiquitin ligases in the flank of *Xenopus tropicalis* causes ectopic limb formation through Wnt activation (Szenker-Ravi et al. 2018). The endocytosis of RNF43 and ZNRF3 via their RSPO ligands can occur even in the absence of Lgr4/5/6, through heparan sulfate proteoglycans or other coreceptors (Dubey et al. 2020, Lebensohn & Rohatgi 2018, Park et al. 2018, Szenker-Ravi et al. 2018). In conclusion, Wnt receptor endocytosis through the RSPO-RNF43-ZNRF3 module potently regulates the levels of β-catenin signaling required for physiological conditions such as stem cell maintenance.

Therapeutic Opportunities

Wnt-targeted therapies in cancer have been severely limited by off-target effects such as skeletal bone disorders (Galluzzi et al. 2019). New therapeutic windows have recently emerged by distinguishing whether the genetic alterations driving aberrant Wnt signaling in different cancers occur through ligand-dependent or -independent mechanisms.

In colorectal carcinoma (CRC), loss-of-function mutations in RNF43 (found in 10% of such cancers) or gain-of-function of RSPO2/3 (8%) are frequent drivers that operate through ligand-dependent mechanisms (Hinze et al. 2020). These tumor cells have an intact Wnt signal transduction pathway and require Porcupine, a membrane-bound O-acyltransferase responsible for the palmitoylation and secretion of Wnt ligands that is targeted by inhibitors such as LGK974 and IWP-L6 (Herr & Basler 2012, Zimmerli et al. 2017). Axin2, a general marker of canonical Wnt signaling (Jho et al. 2002), can be used to classify ligand-dependent tumors that express lower Axin2 levels (Kleeman et al. 2020). Axin2 levels, complementing tumor genomics, are a cost-effective method of enabling precision medicine approaches in the clinic.

Archetypical drivers of the ligand-independent Wnt pathway in cancer include gain-of-function stabilizing mutations in β-catenin that prevent phosphorylation and inactivating mutations in destruction complex proteins. Loss-of-function APC mutations are found in 80% of colorectal carcinomas (Cancer Genome Atlas Netw. 2012, Zehir et al. 2017). In these cases, reengaging the destruction complex function represents a novel strategy for inhibiting tumor growth (Dow et al. 2015, O'Rourke et al. 2017). Tankyrase 1 and 2 (TNKS1/2) are members of the poly-ADP ribose polymerase family of proteins that lead to the degradation of Axin1/2 (Huang et al. 2009, Riffell et al. 2012). Treatment with a small molecule inhibitor of TNKS, XAV939, stabilized Axin1 and decreased Wnt signaling in tumors resulting from APC loss-of-function mutations (Huang et al. 2009). Importantly, the type of APC mutation present is a predictive marker of which tumors are vulnerable to Axin1 activation by TNKS inhibition, the most sensitive being APC mutations within the destruction complex–binding region (Schatoff et al. 2019). Understanding the molecular players in Wnt pathway biology will be increasingly important for precision cancer treatment.

New Secreted Regulators of Wnt Signaling and the Spemann Organizer

Wnt ligands are secreted cysteine-rich growth factors that operate as the initial step of paracrine Wnt signal transduction. The Wnt family of proteins consists of 19 glycoproteins and is tightly regulated by a complex network of secreted proteins (Niehrs 2012). Secreted Fz-related proteins (sFRPs, which include Frzb and Crescent), Cerberus, and Wnt-inhibitory factor 1 are secreted factors that bind to and antagonize Wnt ligands in the extracellular space (Hsieh et al. 1999, Leyns et al. 1997, Malinauskas et al. 2011, Piccolo et al. 1999). Secreted sFRP proteins antagonize Wnt function through a Wnt-binding CRD domain found in Fz receptors (Cheng et al. 2011, Leyns et al. 1997, Uren et al. 2000). In *Xenopus* embryos, sFRPs such as Frzb and Crescent bind Wnts and not only inhibit them but also allow their diffusion through the extracellular space and signaling at a distance (Mii & Taira 2009). Notum is a secreted lipase that inactivates Wnt ligands by removing the Wnt palmitoylation adducts extracellularly (Zhang et al. 2015). Klotho is a transmembrane protein and coreceptor for endocrine fibroblast growth factors that also binds to Wnt ligands in its extracellular domain and inhibits canonical Wnt activity (Liu et al. 2007). Another secreted regulator is Norrin, which activates Fz receptors and β-catenin signaling independently of Lrp5/6 or Wnt ligands (Xu et al. 2004).

Sclerostin is a secreted protein that antagonizes Wnt signaling by impeding the interactions between Fz and Lrp5/6 (Joiner et al. 2013). Antibody-targeted therapies toward sclerostin represent the most successful application of how understanding the molecular basis of Wnt signaling can be used to generate tissue-specific therapies. Humanized monoclonal anti-sclerostin inhibitory antibodies such as romosozumab result in successful outcomes for patients with osteoporosis (McClung & Grauer 2014). Further, romosozumab increased the efficacy of the parathyroid hormone mimic tetriparatide and of bisphosphonates in the treatment of low bone mineral density in postmenopausal women (Cosman et al. 2016, Langdahl et al. 2017, Saag et al. 2017).

As first shown for Dkk1 (Mao et al. 2002), the removal of Lrp5/6 from the plasma membrane by endocytosis results in powerful inhibition of Wnt signaling. The family of Wnt antagonists that function in this manner has been recently expanded with the identification of angiopoietin-like 4 (Angpt4) and Bighead (Ding et al. 2018, Kirsch et al. 2017). Angpt4 was first identified in mammals as a soluble inhibitor of lipoprotein lipase, an enzyme responsible for the transport of triglycerides from blood plasma to tissues. However, in the *Xenopus* embryo it functions as a Wnt antagonist that triggers Lrp5/6 removal by endocytosis via a Lrp6-Angpt4-syndecan ternary complex (Kirsch et al. 2017). Bighead is a secreted protein present in fish and frogs but lost in mammals, with sequence similarities to the proregion of myostatin, also known as growth differentiation factor 8. The overexpression of Bighead induces similar phenotypes to those of Dkk1 in *Xenopus* embryos by promoting Lrp5/6 endocytosis, while its knockdown causes small heads through the activation of canonical Wnt (Ding et al. 2018). Remarkably, Dkk1, Angpt4, and Bighead—as well as other Wnt antagonists such as Frzb, Crescent, and Cerberus—were all originally cloned from the *Xenopus* Spemann organizer, a signaling region of the gastrula embryo that orchestrates body axis formation (De Robertis & Kuroda 2004). Regulation by extracellular antagonists appears to be critical for embryonic patterning by Wnt signals.

WNT-INDUCED STABILIZATION OF PROTEINS

Wnt-STOP Regulates the Degradation of Many Proteins

Wnt signaling has emerged as a global mediator of protein homeostasis through its regulation of GSK3. Twenty percent of human proteins contain three or more consecutive putative GSK3 phosphorylation sites (Taelman et al. 2010). In the absence of Wnt signaling, GSK3 is

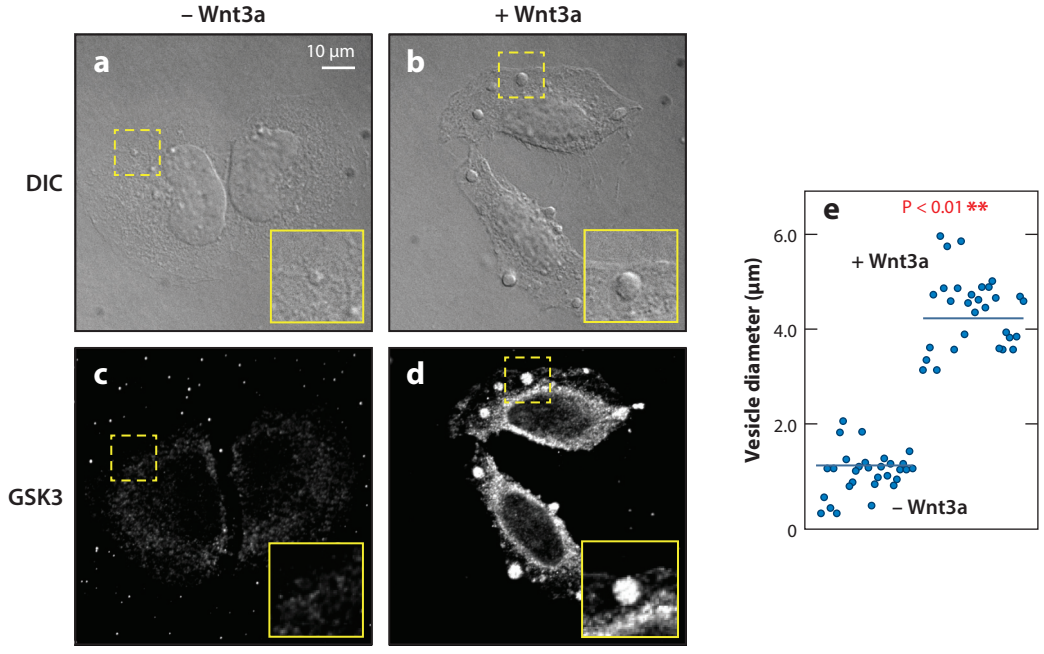

Figure 2

Wnt ligands induce the rapid formation of vesicular structures consistent with macropinocytosis. (*a–d*) Cytosolic glycogen synthase kinase 3 (GSK3) is sequestered in large endolysosomes or multivesicular bodies (MVBs) within 20 min, decreasing the proteasomal degradation of phosphodegrons dependent on GSK3 phosphorylation and causing Wnt-stabilization of proteins (Wnt-STOP). Vesicles were visualized by differential interference contrast (DIC) microscopy. To show that GSK3 was located inside membrane-bounded organelles, an in situ protease protection assay with digitonin-permeabilized cells was used. Digitonin dissolves cholesterol patches in the plasma membrane, making cytosolic proteins accessible to digestion by added proteinase K, while MVB membranes are resistant to digitonin treatment, therefore protecting their contents from digestion. (*e*) Vesicle quantification by DIC microscopy after 20 min of Wnt3a treatment illustrates the large size of the vesicular structures formed in HeLa cells. Figure adapted with permission from Albrecht et al. (2019); copyright 2019 Proceedings of the National Academy of Sciences.

constitutively active and phosphorylates S/T-XXX-S/T[PO_3] sites, forming phosphodegrons recognized by polyubiquitin ligases that mark many cytosolic proteins for degradation in proteasomes (Patel & Woodgett 2017). After Wnt3a treatment, cytosolic GSK3 substrates are stabilized, and the half-life of total proteins in HeLa cells is extended by 25% (Taelman et al. 2010). Active GSK3 from the cytosol is incorporated into membrane-bounded endolysosomal MVBs through microautophagy (**Figure 1**), a process mediated by the endosomal sorting complexes required for transport (ESCRT) machinery that internalizes intraluminal vesicles. Cytosolic GSK3 and its substrates such as Axin1 become entrapped together with the Wnt-Fz-Lrp6 complex (Taelman et al. 2010, Vinyoles et al. 2014). **Figure 2** shows how the addition of Wnt3a induces (within minutes) large endosomal vesicles of 3 µm or more, which sequester GSK3 inside membrane-bounded late endosomes or MVBs. Visualized by electron microscopy, Wnt signaling results in a marked increase in lysosomes and MVBs, underscoring the importance of membrane trafficking in Wnt signaling (Taelman et al. 2010) (**Figure 3**). Inhibiting the ESCRT machinery blocks this sequestration and therefore canonical Wnt signaling (Dobrowolski & De Robertis 2011, Dobrowolski et al. 2012). While targeting plasma membrane receptors to MVBs normally downregulates signaling, in the case of Wnt-Fz-Lrp6, the sequestration of GSK3 and Axin1 bound to the ternary receptor complex inside MVBs is required for maintaining signaling.

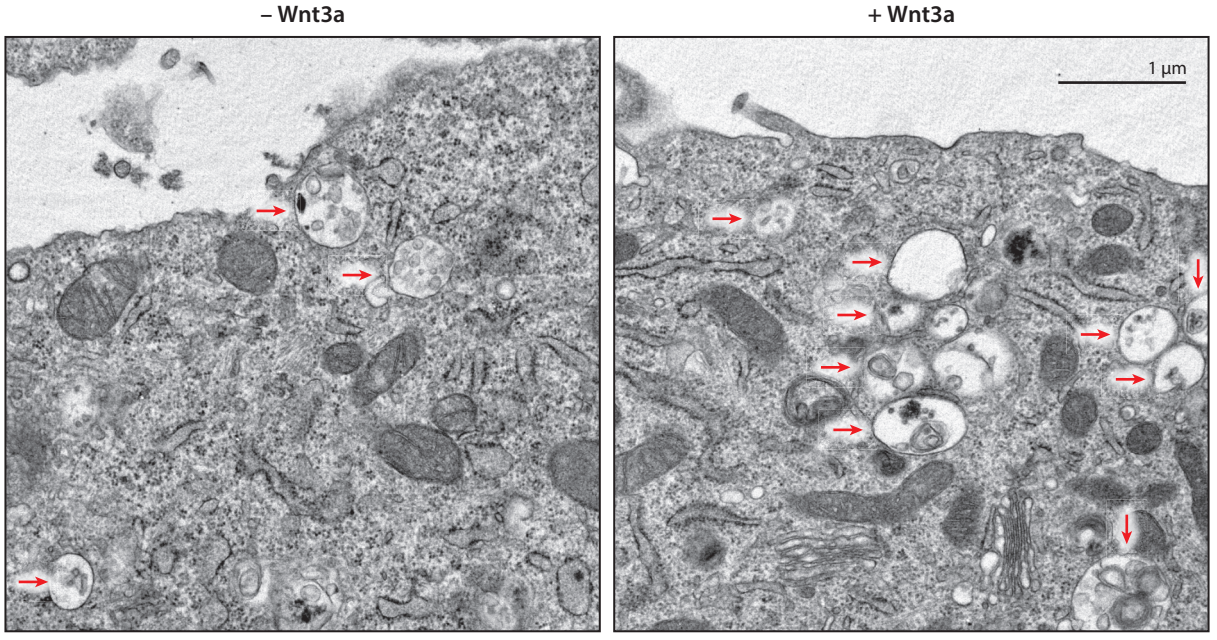

Figure 3

Wnt3a treatment (12 h) leads to increased numbers of lysosomes and multivesicular bodies, indicated by red arrows, in 3T3 fibroblasts. Readers are directed to Taelman et al. (2010) for experimental details. Unpublished transmission electron micrographs provided by D. D. Sabatini at the New York University Medical School.

The proteasomal and lysosomal degradation machineries, which represent the two major protein catabolic systems of the cell, had long been thought to be largely independent (De Duve & Wattiaux 1966, Hershko & Ciechanover 1998). During Wnt activation, many GSK3 phosphorylation substrates [such as Smad4, β-catenin, and protein arginine methyl transferase 1 (PRMT1)] are translocated into endolysosomes even after lysine (Lys)48-linked polyubiquitin modification (which is typically targeted to and degraded in proteasomes), resulting in the transient reduction of free cellular ubiquitin levels and the further inhibition of proteasomal degradation (Arias & Cuervo 2020, Kim et al. 2015). The main effect of Wnt-STOP, however, is to stabilize GSK3 cytosolic substrates by inhibiting the formation of phosphodegrons (Acebron et al. 2014).

Wnt-STOP and the Cell Cycle

Wnt signaling is maximal during the G2 and M phases of the cell cycle due to the ability of the cyclin 7/cyclin-dependent kinase (CDK) complex to phosphorylate and activate the C-terminus of the Lrp6 receptor (Davidson et al. 2005). Wnt-STOP inhibits GSK3, causing a multitude of GSK3 targets (Taelman et al. 2010) to become stabilized, thus increasing biomass and cell size precisely at the moment cells prepare to divide (Acebron et al. 2014). The misregulation of GSK3 has been proposed to play a role in the pathogenesis of many diseases, ranging from Alzheimer disease to cancer (Patel & Woodgett 2017). Wnt-STOP is also important in mitotic chromosome separation (Lin et al. 2021) and sperm maturation (Koch et al. 2015). Further research in the rapidly developing Wnt-STOP field will help to understand the integration of signaling pathways during the cell cycle. **Table 1** summarizes some of the multiple physiological effects of Wnt signaling reviewed here.

Table 1 Activation of the Wnt signaling pathway has multiple cellular physiological outcomes

Cell behavior	Treatment	Cellular impact	Physiological function	Reference(s)
Canonical Wnt and nuclear β-catenin accumulation	Wnt ligand	GSK3 and destruction complex sequestration in multivesicular bodies	Sustained β-catenin transcriptional signaling	Nusse & Clevers 2017, Taelman et al. 2010
Wnt-STOP	Wnt ligand	Stabilization of many GSK3 targets	Biomass accumulation for cell division	Acebron et al. 2014, Koch et al. 2015, Lin et al. 2021, Taelman et al. 2010
Macropinocytosis	Wnt ligand	Nutrient scavenging	Growth and proliferation	Redelman-Sidi et al. 2018, Tejeda-Muñoz et al. 2019
Lysosomal acidification	Wnt ligand	Nutrient digestion	Growth and proliferation	Albrecht et al. 2020a,b
Macropinocytosis and lysosome activation	APC mutation	Constitutive nutrient scavenging and digestion	Colorectal carcinoma	Tejeda-Muñoz et al. 2019
Macropinocytosis and lysosome activation	Axin1 mutation	Constitutive nutrient scavenging and digestion	Hepatocellular carcinoma	Albrecht et al. 2020a

Abbreviations: APC, adenomatous polyposis coli; GSK3, glycogen synthase kinase 3; Wnt-STOP, Wnt-stabilization of proteins.

ARGININE METHYLATION IS REQUIRED FOR CANONICAL WNT SIGNALING

Recent work has uncovered an unexpected requirement for arginine methylation by PRMT1 in Wnt signal transduction (Albrecht et al. 2018). During Wnt signaling, PRMT1 is rapidly recruited to the cytoplasmic tails of the Wnt receptor complex to methylate substrates of GSK3 such as Smad4. Within minutes, PRMT1 becomes sequestered with GSK3 and its many substrates inside MVBs, depleting free PRMT1 from the cytosol (Albrecht et al. 2018). The inhibition of methylation by PRMT1 blocks Wnt-induced endocytosis and canonical Wnt signaling (Albrecht et al. 2018, 2019). Multiple inhibitors of arginine methylation are currently in clinical trials (Li et al. 2019) and are expected to inhibit Wnt signaling and have combinatorial effects in the treatment of Wnt-driven cancers.

MACROPINOCYTOSIS

Canonical Wnt Induces Macropinocytosis

Macropinocytosis is a clathrin-independent cell drinking mechanism that internalizes extracellular fluids via large vesicles of over 200 nm in diameter (Commisso et al. 2013, Doherty & McMahon 2009, Recouvreux & Commisso 2017). Macropinocytosis provides a mechanism for ingesting concentrated nutrient packages of amino acids and sugars from plasma glycoproteins at much higher concentrations than could be ingested when these nutrients are soluble in serum via plasma membrane transporters (Palm & Thompson 2017). Macropinocytic uptake requires the activation of p21-activated kinase (Pak) (Dharmawardhane et al. 2000), which induces the actin cytoskeleton to form plasma membrane ruffles, actin tent poles, and macropinocytic cups (Condon et al. 2018, Swanson & King 2019). Additional regulators of the actin cytoskeleton involved in macropinocytosis are Ras, phosphoinositide 3-kinase, the PI(3,4,5)P3 phosphatase PTEN, phospholipase C, and small GTPases such as Rac, CDC42, Arf6, and Rab5 (Araki et al. 1996, Bar-Sagi & Feramisco 1986, Hodakoski et al. 2019, Yoshida et al. 2018). Some cells such as macrophages have constitutive macropinocytosis, ingesting one third of their volume per hour and recycling it back to the extracellular milieu (Lewis 1931). The activation of RTK receptors such as epidermal growth factor receptor (EGFR) triggers transient macropinocytosis via Ras-Pak activation that lasts for

only about 10 min (Haigler et al. 1979, West et al. 1989), while Wnt-induced macropinocytosis is sustained (see below).

A major advance in cancer research was the realization that activating point mutations in Kras drive sustained macropinocytosis in pancreatic ductal carcinoma cells, conferring on them the ability to grow on bovine serum albumin (BSA) in the absence of serum (Commisso et al. 2013, Palm et al. 2015). Since then, macropinocytosis has been found to drive growth in a wide array of solid tumors such as non–small cell lung cancer, bladder cancer, and others (Hodakoski et al. 2019, Zhang & Commisso 2019).

We found that Wnt3a treatment rapidly increased the endocytosis and lysosomal degradation of BSA-Dequenched (BSA-DQ), a reagent that fluoresces after degradation by lysosomal proteases (Albrecht et al. 2018). Subsequent work from both the Glickman lab and our own showed that this resulted from sustained Wnt-induced macropinocytosis (Redelman-Sidi et al. 2018, Tejeda-Muñoz et al. 2019). The modern gold standard for visualizing macropinocytosis is by following the endocytosis of a fluorescent dextran with a hydrated diameter of >200 nm (TMR-Dextran 70 kDa), which can be blocked by a derivative of the common diuretic amiloride known as EIPA (ethyl-isopropyl amiloride) (Commisso et al. 2013). EIPA inhibits the Na^+/H^+ exchange pump, acidifying the submembranous cytoplasm and blocking actin polymerization (Koivusalo et al. 2010) (**Figure 4**).

Redelman-Sidi et al. (2018) discovered that small interfering RNAs that increased Wnt signaling, such as the knockdown of Kremen1 or Dkk1, increased bacterial phagocytosis in bladder cancer cells. Phagocytosis and macropinocytosis are similar phenomena, and it was found that a Wnt-induced β-catenin transcriptional program caused macropinocytosis that could be blocked by EIPA and Pak1 inhibition. Tejeda-Muñoz et al. (2019) found that the addition of Wnt3a induced sustained macropinocytosis and GSK3 sequestration. Remarkably, this effect

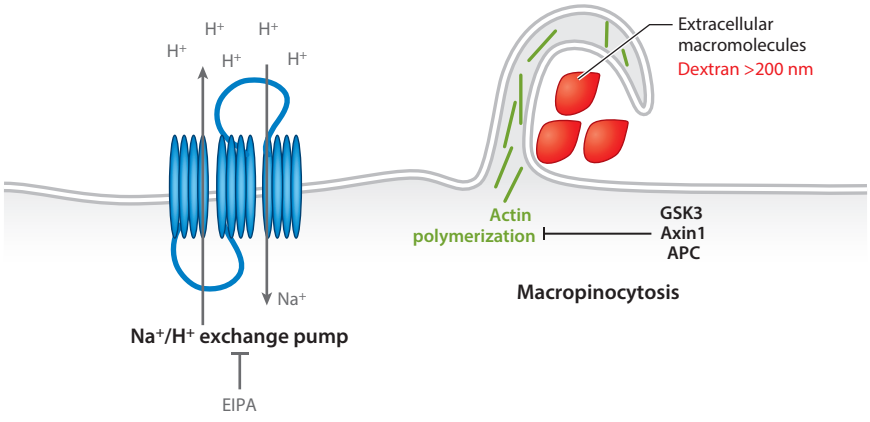

Figure 4

Macropinocytosis leads to the massive uptake of fluid from the surrounding environment via actin-driven polymerization. Membrane remodeling and subsequent vesicular macropinosome structures can be inhibited by blocking the Na^+/H^+ exchange pumps in the plasma membrane using EIPA (ethyl-isopropyl amiloride). The inhibitor acidifies the submembranous cytoplasm and prevents actin polymerization, which is the driving force of macropinosome cups that engulf extracellular macromolecules (*red*) in a non-receptor-mediated manner. GSK3 (glycogen synthase kinase 3) and the tumor suppressors Axin1 and APC (adenomatous polyposis coli) of the β-catenin destruction complex act in concert to repress basal cellular macropinocytosis in the absence of Wnt. Macropinocytosis is commonly defined experimentally by the uptake of Dextran of >200 nm that can be inhibited by EIPA.

was rapid, taking place within minutes, and did not require new protein synthesis. Wnt-induced macropinocytosis can occur through the β-catenin transcriptional loop, but the cellular actin machinery is also poised to respond to Wnt by activating Pak1 even in the absence of new protein synthesis and β-catenin activation (Tejeda-Muñoz et al. 2019).

Sustained Macropinocytosis

During signaling, receptor-mediated endocytosis takes place in most cases through the micropinocytosis of small vesicles of <100 nm that are visible through the electron microscope (Doherty & McMahon 2009, Nichols & Lippincott-Schwartz 2001). A long-standing controversy in the Wnt field surrounds the role of the receptor-mediated endocytosis of Wnt-Fz-Lrp6 receptor complexes in pathway activation (Blitzer & Nusse 2006, Niehrs 2012, Rim et al. 2020, Yamamoto et al. 2006). A recent study suggested that the depletion of either clathrin- or caveolin-mediated endocytosis did not affect β-catenin accumulation or target gene transcription, and that discrepancies among previous reports could be attributed to off-target effects of micropinocytosis inhibitors (Rim et al. 2020). Previously, we suggested that micropinocytosis might first deplete cytoplasmic GSK3 before inducing macropinocytosis but did not specifically investigate the involvement of the clathrin or caveolin machineries (Albrecht et al. 2020a, Tejeda-Muñoz et al. 2019). In light of the report of Rim et al. (2020), we now propose a revised model in which the Wnt-Fz-Lrp6 receptor complex forms within the macropinocytic cup itself (**Figure 5**). There, it triggers a local transient inhibition of GSK3 activity in the plasma membrane signalosome (Bilić et al. 2007, Cselenyi et al. 2008) sufficient to initiate Pak1-mediated macropinocytosis. Macropinocytosis does not require either clathrin or caveolin. In the next step, the ESCRT machinery sequesters GSK3 and Axin1 to achieve sustained canonical Wnt signaling and membrane trafficking into lysosomes (**Figure 5**). The ESCRT machinery and MVB formation is essential for Wnt signaling (Dobrowolski & De Robertis 2011) and to sustain macropinocytosis (Tejeda-Muñoz et al. 2019). A recent study using in vivo peroxidase proximity labeling with ascorbate peroxidase (APEX2) fused to Lrp6 confirmed that multiple ESCRT components rapidly associate with the Lrp6-APEX2 receptor after Wnt3a treatment (Colozza et al. 2020).

The mechanism by which Pak1 is activated by Wnt remains an enigma. Kras is recruited to Lrp6-APEX2 within minutes of the Wnt3a ligand addition to cells, providing a possible mechanism for macropinocytosis activation (Colozza et al. 2020). Wnt-STOP stabilization could also play a role in Pak1 regulation as Pak1–4, DOCK1–10 (guanine nucleotide exchange factors that activate CDC42) (Taelman et al. 2010), and Ras (Jeong et al. 2012) contain multiple GSK3 phosphorylation sites. A proximity labeling study using Fz9b-APEX2 demonstrated the requirement of EGFR-mediated phosphorylation for receptor endocytosis and canonical Wnt signaling, providing an additional connection between Wnt, endocytosis, and the RTK-Ras pathway (Grainger et al. 2019). The integration of the Wnt and RTK-Ras pathways in macropinocytosis is still hypothetical; in addition, keep in mind that while EGF-induced macropinocytosis lasts for only a few minutes, Wnt-induced macropinocytosis is sustained for hours and days (Albrecht et al. 2020a, Haigler et al. 1979, Taelman et al. 2010, West et al. 1989).

GSK3 and the β-Catenin Destruction Complex Repress Macropinocytosis

How does Wnt signaling have such diverse physiological effects as β-catenin stabilization, macropinocytosis, Wnt-STOP, and endosomal trafficking? The common link is the activity of GSK3 and its regulation by the destruction complex. GSK3 inhibition by either lithium chloride (LiCl), CHIR99021, or a dominant-negative GSK3 (DN-GSK3) is sufficient to rapidly trigger

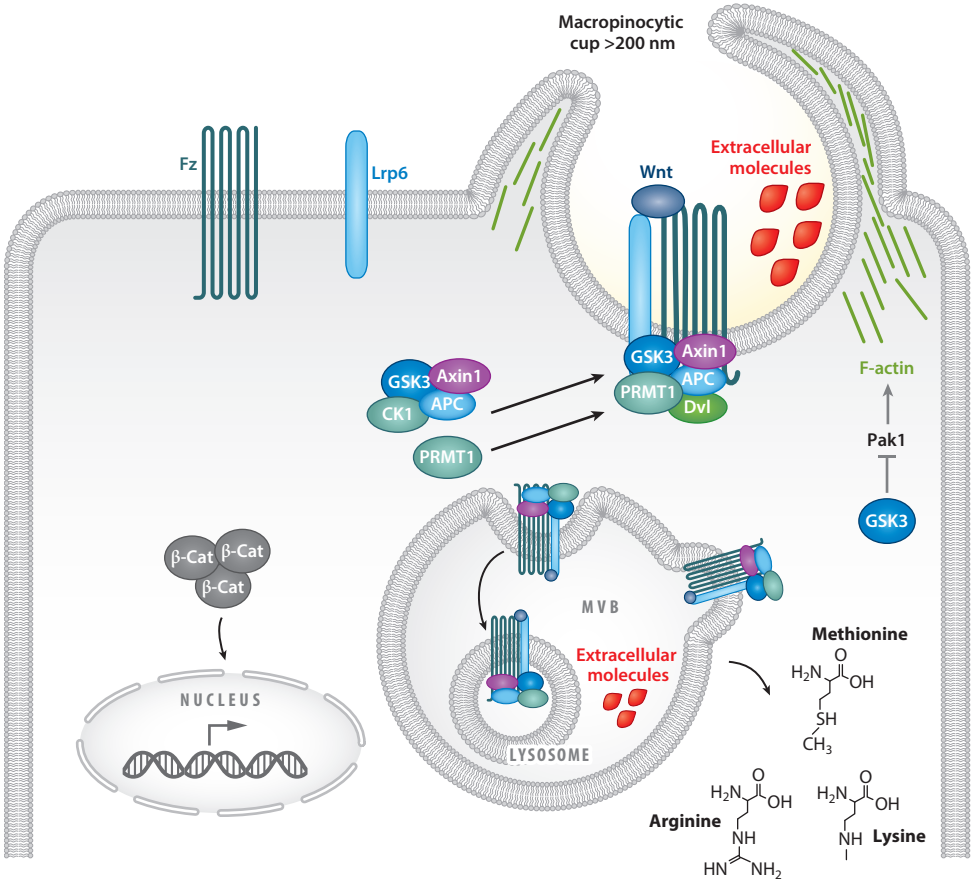

Figure 5

During canonical Wnt signaling, macropinocytic cups engulf extracellular macromolecules from the surrounding medium, internalize Wnt-Fz-Lrp6 receptor complexes, and promote the sequestration of GSK3 (*dark blue*) and Axin1 (*purple*) inside the intraluminal vesicles of MVBs. Macropinosome formation is driven by cortical actin polymerization downstream of the Pak1 kinase, which is a Wnt-STOP target. The sequestration of cytosolic GSK3 and Axin1 in endolysosomes leads to the stabilization of newly translated β-Cat and many Wnt-STOP GSK3 substrates. In the absence of Wnt signals, the destruction complex proteins repress macropinocytosis. When APC or Axin is mutated in cancer, GSK3 is unable to repress the actin machinery, resulting in a prodigious amount of nutrient uptake by macropinocytosis that fuels growth and proliferation. Abbreviations: β-Cat, β-catenin; APC, adenomatous polyposis coli; CK1, casein kinase 1α; Dvl, Dishevelled; Fz, Frizzled; GSK3, glycogen synthase kinase 3; Lrp6, low-density lipoprotein receptor–related protein 6; MVB, multivesicular body; PRMT1, protein arginine methyltransferase 1; Wnt-STOP, Wnt-stabilization of proteins.

macropinocytosis via Pak activation of the actin machinery, even in the absence of new protein synthesis and to the same levels as Wnt3a (Albrecht et al. 2020a). In vivo, inhibition of GSK3 by microinjection of LiCl, but not NaCl, into the *Xenopus* blastula cavity caused the endocytosis of TMR-Dextran in surrounding cells (Albrecht et al. 2020a).

The canonical Wnt pathway is constitutively activated by mutation of the destruction complex proteins Axin1 and APC, which are major tumor suppressors. GSK3 activity is increased by its

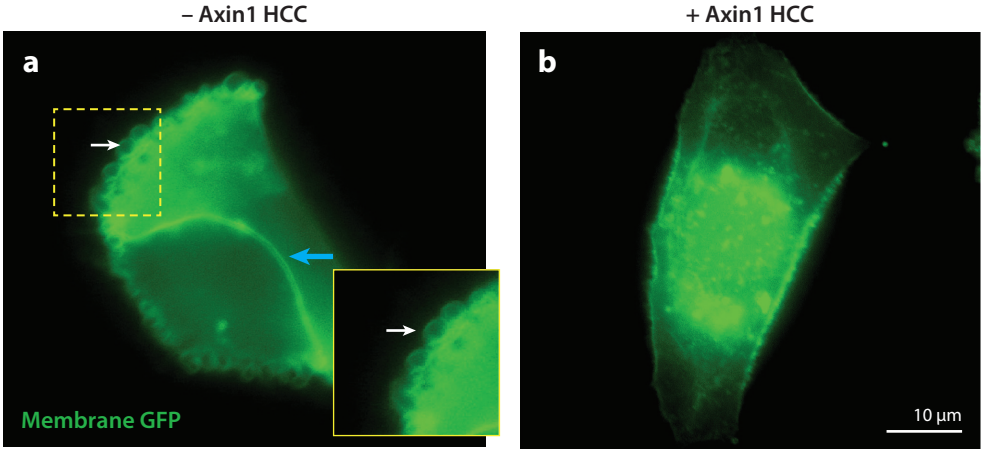

Figure 6

Loss-of-function mutations in β-catenin destruction complex proteins are prevalent tumor suppressors and lead to macropinocytotic membrane remodeling for nutrient uptake. (*a*) Axin1 mutation fuels the growth of the hepatocellular carcinoma (HCC) Alexander cell line through macropinocytosis, here visualized as large vesicles demarcated by membrane green fluorescent protein (GFP) labeling (see inset). Note that in the absence of Axin1, vesicular structures form around the apical region (*white arrows*) but are absent in basolateral regions where cells contact each other (*blue arrow*). (*b*) Restoration of full-length Axin1 was sufficient to block macropinocytosis in this cell line, even after it had undergone 40 years of in vitro growth. For additional information, readers are referred to **Supplemental Movie 1**, which illustrates the dynamic nature of macropinocytosis. Micrographs adapted with permission from Albrecht et al. (2020a); copyright 2020 Elsevier.

Supplemental Material >

binding to Axin1 (Kim et al. 2013). To test the role of Axin1 in membrane trafficking, we developed a hepatocellular carcinoma (HCC) model cell line in which full-length Axin1 was permanently reconstituted at physiological levels. These HCC cells lack exon 4 of the adaptor protein Axin1 (Alexander et al. 1976), which contains the binding sites to GSK3. As shown in **Figure 6** and **Supplemental Movie 1**, Axin1 mutant cells develop large macropinocytic vesicles in the apical membrane, which are eliminated simply by the reconstitution of Axin1, in spite of the 40-year propagation of these cells in culture (Albrecht et al. 2020a).

Another very useful system is the SW480 CRC cell line reconstituted with full-length APC (Faux et al. 2004). The original SW480 cells display the prodigious amounts of macropinocytosis that are required to maintain high levels of nuclear β-catenin signaling and are repressed in SW480+APC (Tejeda-Muñoz et al. 2019). In a mouse in vivo inducible shAPC system, depletion of APC caused EIPA-sensitive TMR-Dextran uptake by colonic instillation as well as by macropinocytic entry of intestinal bacteria (Redelman-Sidi et al. 2018). Increased uptake of colonic luminal microbiota is an initiating event in colon cancer progression (Bullman et al. 2017). APC knockdown also causes ligand-independent Lrp6 endocytosis in cultured cells (Colozza et al. 2020, Saito-Diaz et al. 2018). While GSK3 itself is not traditionally regarded as a tumor suppressor, inactivating mutations in GSK3α/β are found in acute myeloid leukemia and Hodgkin lymphoma as well as in skin, colon, liver, and kidney cancers (Cancer Genome Atlas Netw. 2012).

The main conclusion of these studies on Wnt, GSK3 inhibitors, Axin1, and APC is that cellular macropinocytosis is normally repressed by GSK3 activity. Treatments that decrease GSK3 trigger the actin machinery of the macropinocytosis pathway even in the absence of protein synthesis. Because the role of the destruction complex goes well beyond the degradation of β-catenin, it will be of great interest to identify the full panoply of target proteins phosphorylated by GSK3.

LYSOSOMES AND CELL METABOLISM

Wnt Signaling Requires Multivesicular Body and Lysosomal Activity

Lysosomes are the catabolic center of the cell (De Duve & Wattiaux 1966). Originally appreciated for their janitorial services, we now know that lysosomes are dynamic regulators of global homeostasis and contribute to the progression of many diseases (Appelqvist et al. 2013). Lysosomal degradation is responsible for breaking down proteins, polysaccharides, and complex lipids into building blocks that can be recycled. Both intracellular and extracellular materials are delivered into lysosomes, where they are degraded by 60 different types of acid hydrolases (Perera et al. 2019). The lysosomal lumen is kept acidic by the vacuolar H^+-ATPase (v-ATPase), a proton pump that regulates most stages of endosomal membrane trafficking (Doherty & McMahon 2009).

Inhibition of v-ATPase, for example with bafilomycin A1, blocks Wnt signaling (Cruciat et al. 2010). Chloroquine, a weak base that is protonated and accumulated in lysosomes, causing their alkalinization, can also block Wnt signaling at high concentrations (Dobrowolski et al. 2012). However, at low doses both bafilomycin A1 and chloroquine expand the MVB or late endosome compartment, causing hypersensitivity to Wnt treatment in an ESCRT-dependent manner (Dobrowolski et al. 2012).

The Lrp6 receptor complex binds to the prorenin receptor (Cruciat et al. 2010) (also called ATP6AP2), which, through the transmembrane protein 9 (TMEM9) adaptor (Jung et al. 2018), is also bound to v-ATPase. These adaptor proteins are localized to the MVB compartment and are essential for Wnt signaling (Jung et al. 2018). Microinjection of TMEM9 messenger RNA induces axial duplication in *Xenopus* assays. TMEM9 is overexpressed in colon cancer through a positive feedback transcriptional loop with β-catenin, driving the degradation of APC in lysosomes. In mice, inhibiting v-ATPase with bafilomycin A1 decreased growth of colorectal cancer xenografts (Jung et al. 2018). Combinatorial approaches that target lysosomes and macropinocytosis may provide unexpected therapies for aberrant Wnt signaling in cancer.

Wnt Increases Lysosomal Acidification and Catabolism

Wnt-stimulated macropinocytosis leads to a remarkable increase in the degradation of extracellular proteins such as ovalbumin-DQ and BSA-DQ in lysosomes (Albrecht et al. 2018, 2020a). The engulfed proteins are not recycled to the outside but rather are directed into lysosomes by mechanisms not yet understood. Macropinocytosis also drives striking increases in the activity of lysosomal enzymes such as cathepsin D and β-glucosidase (Albrecht et al. 2020a,b). Preexisting lysosomes are rapidly acidified after Wnt treatment, and this can be assayed using the pH-sensitive LysoSensor dye, as illustrated using a GSK3 inhibitor in **Figure 7**. Wnt-induced lysosomal acidification requires macropinocytosis, as it can be blocked by EIPA or the Pak1 inhibitor IPA-3 (Albrecht et al. 2020a). Remarkably, lysosomal activation occurs within minutes and does not require new protein synthesis and, therefore, is not the result of newly synthesized β-catenin. The molecular mechanism of the Wnt-GSK3-regulated lysosome acidification is unknown but is likely to involve the reported interactions between Lrp6 and the prorenin receptor/TMEM9/v-ATPase machinery (Cruciat et al. 2010, Jung et al. 2018).

A recent report illustrates how endolysosomal trafficking can affect Wnt signaling in cancer in the absence of Wnt pathway mutations (Rodgers et al. 2021). Phosphoinositoids regulate endolysosome traffic, and, in the case of breast estrogen-receptor-positive cancers, the increased expression of a phosphatase (INPP4B) that converts PI(3,4)P2 into phosphatidylinositol 3-phosphate [PI(3)P] facilitates GSK3 translocation into lysosomes. The INPP4B phosphatase binds to the late endosome regulator Rab7, promoting the formation of PI(3)P, which in turn

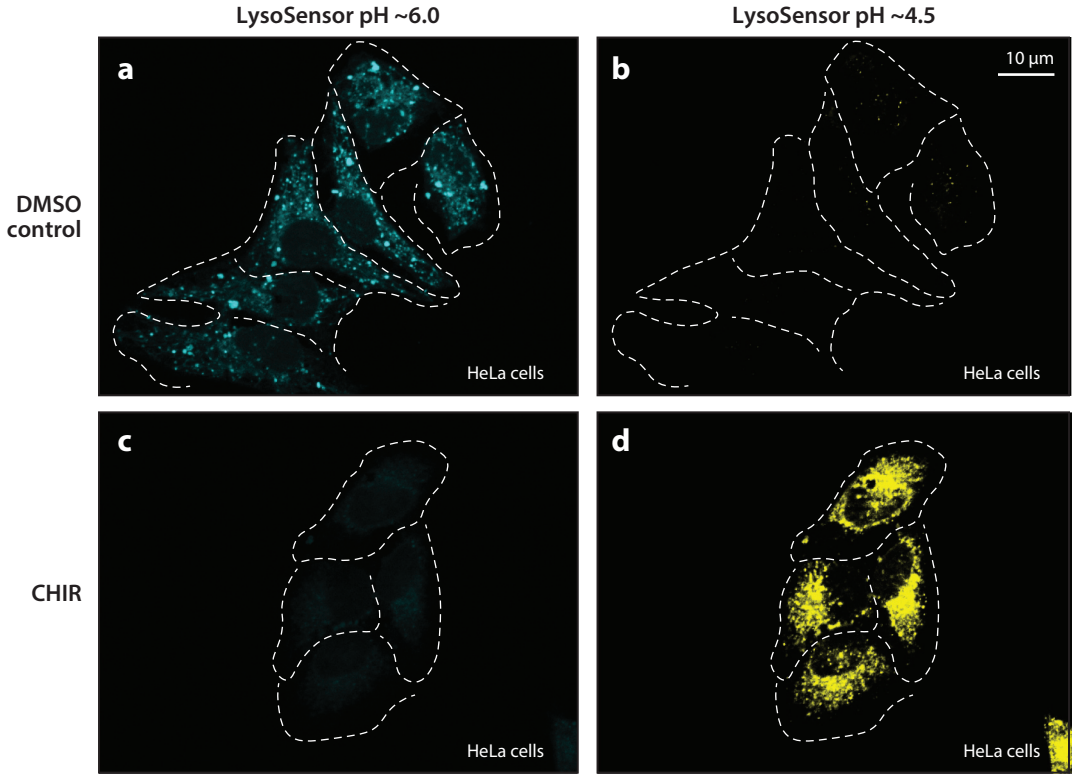

Figure 7

Inhibition of GSK3 (glycogen synthase kinase 3) induces rapid changes in lysosomal pH. Acidification was measured using the ratiometric cell-permeable LysoSensor reagent in HeLa cells. Control cells treated with DMSO (dimethyl sulfoxide) have a higher pH (~6.0) that fluoresces in the blue channel. In contrast, the GSK3 inhibitor CHIR99021 (CHIR) decreases pH (~4.5), as revealed by a shift to the yellow channel. Acidification is rapid (20 min in this example), takes place in the absence of new protein synthesis, and requires macropinocytosis, as it is inhibited by EIPA (ethyl-isopropyl amiloride). Similar effects were found with the addition of Wnt3a. Figure adapted with permission from Albrecht et al. (2020b); copyright 2020 Elsevier.

leads to the increased sequestration of GSK3 and the destruction complex into lysosomes through the ESCRT machinery (Rodgers et al. 2021). The resulting sequestration of GSK3 in lysosomes drives cell proliferation through increased Wnt/β-catenin signaling via a membrane trafficking mechanism.

By increasing the lysosomal enzyme degradation of extracellular proteins, activation of the Wnt pathway leads to the availability of free cellular metabolites that fuel growth (**Figure 5**). Metabolomic studies showed increased levels of arginine, methionine, and lysine within 60 min of Wnt3a addition to HeLa cells (Albrecht et al. 2020a). Interestingly, methionine levels are increased during growth phases of the cell cycle when Wnt activity is highest (Lee et al. 2017), and arginine and lysine levels have been linked to the progression of tumor cell proliferation (Kanarek et al. 2020). Metabolic reprogramming is universal across malignancies, with tumors displaying distinct metabolic requirements based on genetic mutations, microenvironments, and tissue of origin (Pate et al. 2014, Pavlova & Thompson 2016). These studies underscore that elucidating the molecular mechanisms of Wnt, lysosomes, and metabolism could also lead to new therapeutic disease targets.

CONCLUSIONS AND PROSPECTS

Wnt-GSK3 signaling has a wide range of effects in cell physiology (**Table 1**). The Wnt pathway is choreographed by membrane trafficking, which explains its many distinct physiological outcomes. Emerging literature supports a new model of Wnt stimulation in which the inhibition of the proteasomal degradation of a subset of GSK3 target proteins by cell cycle–dependent Wnt-STOP and the stimulation of macropinocytosis and lysosomal degradation of extracellular nutrients are parts of a coordinated cell growth program. The cell is poised for these rapid responses, which take place even in the absence of new protein synthesis. Great strides are being made in understanding the mechanisms of Wnt signaling using new genetic- and systems-based technologies. Novel APEX2 fusion protein technology is revealing the intricate regulation of Wnt coreceptors at the plasma membrane and during their travels through the endosomal system to MVBs and the lysosome. There is much more to the Wnt canonical pathway than β-catenin stabilization and transcriptional regulation. In particular, studies on endocytosis will illuminate the multiple roles of canonical Wnt in cell biology.

DISCLOSURE STATEMENT

The authors are not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

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