

REVIEW ARTICLE

Dysregulation of Wnt/ β -catenin signaling by protein kinases in hepatocellular carcinoma and its therapeutic application

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Abstract

Wnt/ β -catenin signaling is indispensable for many biological processes, including embryonic development, cell cycle, inflammation, and carcinogenesis. Aberrant activation of the Wnt/ β -catenin signaling can promote tumorigenicity and enhance metastatic potential in hepatocellular carcinoma (HCC). Targeting this pathway is a new opportunity for precise medicine for HCC. However, inhibiting Wnt/ β -catenin signaling alone is unlikely to significantly improve HCC patient outcome due to the lack of specific inhibitors and the complexity of this pathway. Combination with other therapies will be an important next step in improving the efficacy of Wnt/ β -catenin signaling inhibitors. Protein kinases play a key and evolutionarily conserved role in the Wnt/ β -catenin signaling and have become one of the most important drug targets in cancer. Targeting Wnt/ β -catenin signaling and its regulatory kinase together will be a promising HCC management strategy. In this review, we summarize the kinases that modulate the Wnt/ β -catenin signaling in HCC and briefly discuss their molecular mechanisms. Furthermore, we list some small molecules that target the kinases and may inhibit Wnt/ β -catenin signaling, to offer new perspectives for pre-clinical and clinical HCC studies.

KEYWORDS

β -catenin signaling, combination therapy, hepatocellular carcinoma, protein kinase, small molecule, Wnt

Abbreviations: AMPK, AMP-activated protein kinase; APC, adenomatous polyposis coli; CDK1, cyclin-dependent kinase 1; CK1, casein kinase1; Dvl, Dishevelled; EGFR, epidermal growth factor receptor; FAK, focal adhesion kinase; FGFR, fibroblast growth factor receptor; GSK3 β , glycogen synthase kinase 3 β ; HCC, hepatocellular carcinoma; HGF, hepatocyte growth factor; JNK, Jun N-terminal kinase; LEF/TCF, lymphoid enhancer factor/T cell factor; LRP5/6, low density lipoprotein receptor-related protein5/6; MAPK, mitogen-activated protein kinase; Met, mesenchymal-epithelial transition factor; NEK2, NIMA-related kinase2; NF- κ B, nuclear factor- κ B; PAK4, P21 activated kinase4; PI3K, phosphatidylinositol 3-kinase; ROR2, tyrosine kinase-like orphan receptor2; RTK, receptor tyrosine kinase; SIK1, Salt-inducible kinase1; STAT, signal-transducer and activator of transcription; TAK1, transforming growth factor- β activated kinase1; TGF- β , transforming growth factor- β ; VEGFRs, vascular endothelial growth factor receptors; WCS, Wnt/ β -catenin signaling.

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1 | INTRODUCTION

Liver cancer is the sixth most common cancer and the fourth leading cause of cancer mortality worldwide, with approximately 841 000 new cases and 782 000 deaths annually.¹ Hepatocellular carcinoma (HCC) is the most common type and accounts for 75%-85% of all liver cancer cases, the main risk factors for HCC are hepatitis B or C virus infection, alcohol abuse, and aflatoxin-contaminated food-stuffs.¹ The clinical management of HCC mainly includes surgical therapies, tumor ablation, transarterial therapies, and systemic therapies.² Although these management strategies have developed quickly over the past decade, the prognosis is still dismal, with a 5-y survival rate of 18%. Therefore, a better understanding of the molecular mechanisms involved in HCC initiation and progression is crucial for the identification of therapeutic targets and the design of specific drugs. Excessive activation of the Wnt/ β -catenin signaling (WCS) in hepatocytes leads to uncontrollable growth, expansion, and metastasis of malignant clones,³ indicating a great potential for WCS as a drug target in HCC.

There are approximately 518 protein kinase genes in the human genome, targeting one-third of the proteins in cells. Protein kinases modulate most of the signal transduction pathways in humans, as well as most cellular processes, including metabolism, transcription, translation, cell cycle, and proliferation.⁴ Mutations, overexpression, and dysfunction of protein kinases play essential roles in the pathogenesis of cancer, and kinase has become one of the most important drug targets over the past 20 y. To date, the US Food and Drug Administration (FDA) has approved 38 kinase inhibitors, most of which are RTK inhibitors for the clinical treatment of cancer.⁵ Protein kinases have been reported to regulate the WCS in the pathogenesis of HCC and could be targeted specifically. Therefore, in this review we summarize the kinases reported that modulate the WCS in HCC; the small molecules that targeting these kinases could be applied for HCC clinical treatment.

2 | THE OVERVIEW OF WCS

The Wnt signaling pathways are mechanically complex and relatively conserved pathways associated with many physiological and pathological processes such as embryonic development, tissue self-renewal, and cancer. Currently, there are 3 summarized pathways upon Wnt stimulation: the WCS, the Wnt/ Ca^{2+} pathway and the non-canonical

planar cell polarity (PCP) pathway.⁶ Of these 3, the WCS is most well studied. The hallmark of this pathway is that it activates the transcriptional activity of β -catenin, which is the key mediator of WCS. β -Catenin in the cytoplasm is tightly regulated by the destruction complex, formed by scaffold protein Axin, APC, casein kinase 1 (CK1) and glycogen synthase kinase 3 β (GSK3 β).⁷ In the absence of Wnt ligands, the destruction complex captures and phosphorylates β -catenin, which is subsequently recognized by the E3 ubiquitin ligase β -TRCP and targeted for proteasomal degradation.⁸ When the Wnt ligand binds the heterodimeric receptor complex formed by Frizzled (Fz) and low density lipoprotein receptor-related protein5/6 (LRP5/6) at the plasma membrane, the signal is primed by LRP5/6 phosphorylation, activating and recruiting Dishevelled (Dvl) to form an aggregation platform and the LRP6 signalosome, composed of Wnt, Fz, Axin, phosphorylated LRP5/6, GSK3 β and CK1, which eventually disrupt the destruction complex and promote stabilization and cytoplasmic accumulation of β -catenin. Free β -catenin can translocate into the nucleus, and bind lymphoid enhancer factor/T cell factor (LEF/TCF) transcription factor complex to promote the transcription of Wnt target genes, including *Myc*, *cyclin D1* and *Axin2* (Figure 1).⁶⁻⁸

The WCS is inactive in the normal condition of liver, however it is frequently mutated and activated in HCC.⁹ There are multiple regulatory ways in which hepatocarcinogenesis is sustained by the WCS. For instance, the protein regulator of cytokinesis 1 (PRC1)/Wnt positive feedback loop plays a crucial role in HCC recurrence and metastasis.¹⁰ Targeting WCS is a new opportunity for precise medicine for HCC. To date, many clinical agents, such as small molecules, peptides and antibodies, have been developed to modulate the WCS in HCC. These agents target Wnt ligands, inhibit the Fz-Dvl interaction, or stabilize the β -catenin destruction complex.³ However, inhibiting WCS alone is unlikely to significantly improve HCC patient outcome due to the lack of specific inhibitors and the complexity of this pathway.¹¹ Combination with other therapies will be an important next step in improving the utility of WCS inhibitors. Protein kinase has become one of the most important drug targets in cancer, targeting WCS and its regulatory kinase together would be the preferred choice. A deeper understanding of the WCS and its regulatory kinase would provide opportunities to design better combination therapies.

3 | KINASES MODULATING WCS

The dysfunction of kinases results in significant changes in most cellular signal transduction processes, including in the WCS. In HCC,

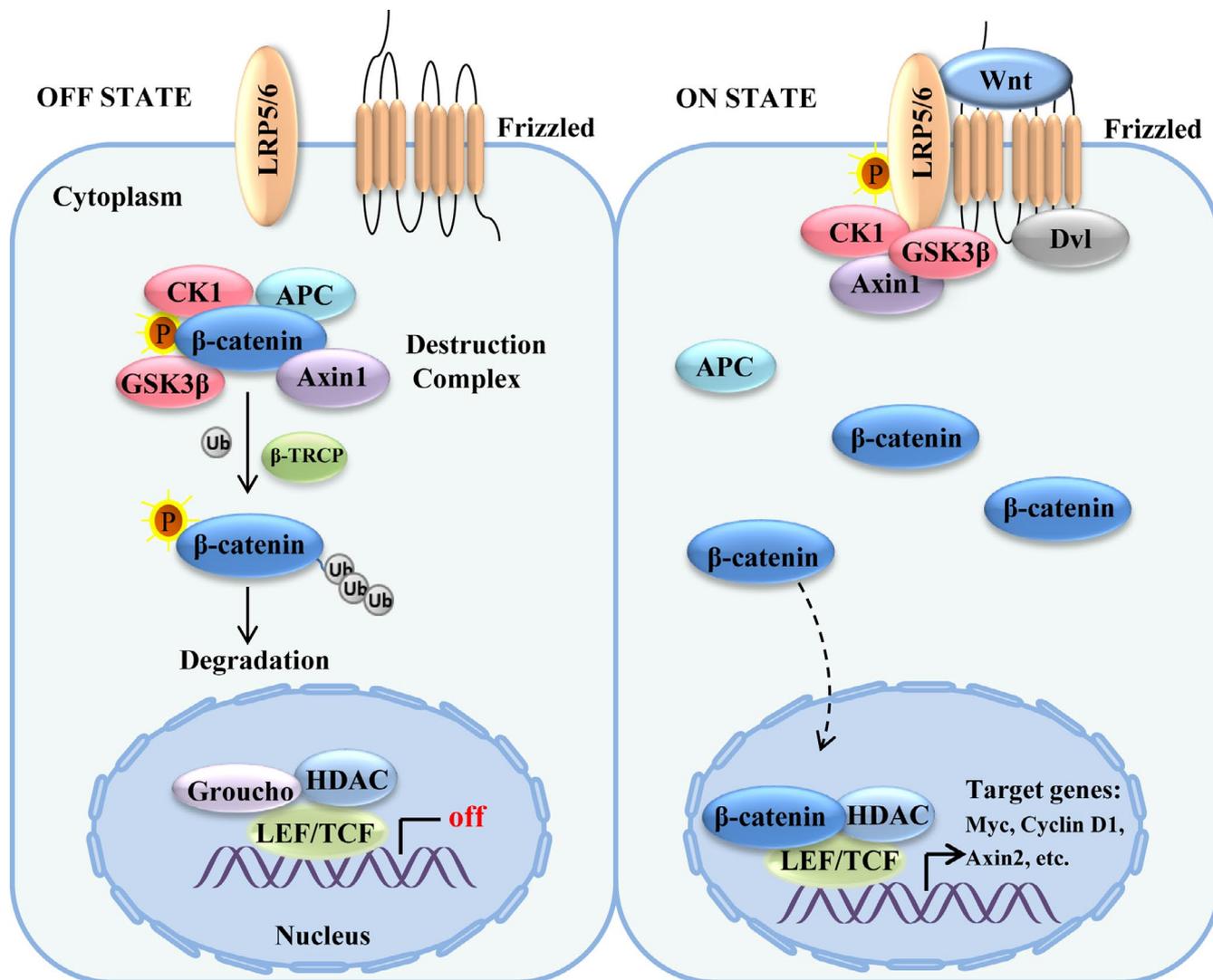


FIGURE 1 Wnt/β-catenin signaling. Wnt/β-catenin signaling is inactive without binding of Wnt ligands (Wnt off). In the cytoplasm, the destruction complex, which is composed of proteins APC, GSK3β, CK1, and Axin1, can phosphorylate β-catenin, eventually leading to β-catenin ubiquitination and proteasomal degradation. When the WNT ligand is present (Wnt on), it binds to the Frizzled-LRP5/6 co-receptor, thereby recruiting Dvl and Axin1, which can disrupt the destruction complex and promote intracellular accumulation and nuclear translocation of β-catenin. In the nucleus, β-catenin binds to LEF/TCF and activates the transcription of Wnt target genes such as *c-Myc*, *cyclin D1*, and *Axin2*. Abbreviations: APC, adenomatous polyposis coli; CK1, casein kinase 1; Dvl, Dishevelled; GSK3β, glycogen synthase kinase 3β; HDAC, histone deacetylase 3; LEF/TCF, lymphoid enhancer factor/T cell factor; LRP5/6, low density lipoprotein receptor-related protein 5/6

many protein kinases have been reported to modulate the WCS at various levels (Table 1 and Figure 2). Some protein kinases directly involved in WCS regulation target and phosphorylate the key components in WCS, modulating β-catenin degradation, LRP6 signalosome formation, and LEF/TCF/β-catenin transcription complex activity. Other kinases indirectly modulate the WCS, and this modulation varies from kinase to kinase.

4 | GSK3β

GSK3β is an evolutionarily conserved serine/threonine kinase that is ubiquitously expressed in mammalian cells and functions in diverse

cellular processes including proliferation, differentiation, and motility.¹² GSK3β is directly involved in the WCS, acting as a component of the β-catenin destruction complex to promote cytoplasmic β-catenin degradation by sequential phosphorylation in cooperation with CK1.¹³ In addition, GSK3β phosphorylates Axin1 and increases its binding to β-catenin, allowing the N-terminal region of β-catenin to become a more efficient substrate of CK1 and GSK3β.^{14,15} GSK3β also phosphorylates APC and increases its affinity for β-catenin, followed by ubiquitination of phosphorylated β-catenin by β-TRCP.^{16,17} In addition to its inhibitory role, GSK3β positively regulates the WCS by phosphorylation of LRP6. Wnt induces sequential phosphorylation of LRP6 by GSK3β and CK1, and this dual phosphorylation recruits Axin1 away from the β-catenin destruction complex to the LRP6 signalosome.¹⁸ Recently, it has been

TABLE 1 Kinases that modulate the Wnt/ β -catenin signaling

Kinase name	Expression in TCGA HCC compared with normal liver tissue	Mechanisms	Positive/negative modulation of Wnt signal	Reference
GSK3 β	Up	<ol style="list-style-type: none"> 1. GSK3β phosphorylates APC, Axin1 and β-catenin to facilitate the ubiquitination and proteasomal degradation of β-catenin. 2. GSK3β phosphorylates LRP6 and induces the formation of LRP6 signalosome. 3. GSK3β potentially inhibits many Wnt signaling-associated factors by promoting their protein degradation by phosphorylation. 	Both	13-20
CK1	Up	<ol style="list-style-type: none"> 1. CK1 phosphorylates APC and β-catenin to facilitate the ubiquitination and proteasomal degradation of β-catenin. 2. CK1 phosphorylates LRP6 and induces the formation of LRP6 signalosome. 3. CK1 phosphorylates TCF3 and promotes its interaction with β-catenin. 4. CK1 phosphorylates Dvl and promotes the signaling activity of Dvl. In addition, the phosphorylation of Dvl by CK1 also triggers a negative feedback loop to inhibit the Wnt/β-catenin signaling. 5. CK1 phosphorylates p120-catenin and cadherin, resulting in release of β-catenin from the junctional complex. 	Both	24-30
CK2	Up	<ol style="list-style-type: none"> 1. CK2 phosphorylates β-catenin, leading to its proteasome resistance. 2. The phosphorylation of LEF-1 by CK2 significantly enhances its affinity for β-catenin and stimulates the transactivation of β-catenin:LEF-1 complexes. 	Positive	34,35
CDK1	Up	CDK1 could directly phosphorylate BCL9, which would stabilize BCL9, inhibit clathrin binding to the BCL9/LRP6 complex and suppress clathrin-mediated degradation of LRP6 signalosome	Positive	40
CDK14	Up	CDK14/cyclin Y phosphorylates LRP5/6 and activates mitotic Wnt/ β -catenin signaling	Positive	39
Met	Up	Met phosphorylates β -catenin at tyrosine residues, this causes dissociation of β -catenin from Met and nuclear translocation of β -catenin	Positive	44
EGFR	Down	<ol style="list-style-type: none"> 1. EGFR directly phosphorylates β-catenin, which leads to release of β-catenin from junctional complexes and increase of cytoplasmic β-catenin concentration. 2. EGFR activated ERK phosphorylates LRP6 and dramatically increases the cellular response to Wnt ligand. 	Positive	48
NEK2	Up	<ol style="list-style-type: none"> 1. NEK2 could bind to β-catenin to prevent its ubiquitination and degradation. 2. Dvl accumulated at centrosome is phosphorylated by NEK2, this phosphorylation releases Dvl from the centrosome and increases the Wnt/β-catenin signaling activation. 	Positive	51,54
PAK4	Up	PAK4 phosphorylates β -catenin and prevents its degradation from proteasome pathway	Positive	56
Src	Up	<ol style="list-style-type: none"> 1. Src phosphorylates Fz, allowing Fyn recruitment and activation. Fyn phosphorylates β-catenin, releasing β-catenin from the junctional complexes. 2. Src phosphorylates β-catenin, resulting in the accumulation of β-catenin in the nucleus and the promotion of LEF/TCF transcription. 3. The phosphorylation of LRP6 by Src reduces LRP6 levels on the cell surface, disrupts LRP6 signalosome formation. 	Both	60-62
TAK1	No significant difference	The activation of TAK1 promotes NLK activity, NLK could phosphorylate TCF and interfere with the binding of β -catenin-TCF to the TCF target sites	Negative	66
SIK1	Down	SIK1 phosphorylates SMRT at threonine 1391 and promotes its translocation into the nucleus, then phosphorylated SMRT recruits the NCoR/HDAC3 corepressor complex to β -catenin/TCF/LEF and inhibits the transcription of Wnt target genes	Negative	71

(Continues)

TABLE 1 (Continued)

Kinase name	Expression in TCGA HCC compared with normal liver tissue	Mechanisms	Positive/negative modulation of Wnt signal	Reference
FAK	Up	FAK reduces β -catenin degradation and increases the nuclear accumulation of β -catenin	Positive	75
ROR2	Up	ROR2 directly binds Wnt5a, inhibits the Wnt/ β -catenin signaling and activates non-canonical Wnt signaling	Negative	76,77

Abbreviations: APC, adenomatous polyposis coli; CDK1, cyclin-dependent kinase 1; CK1, casein kinase1; Dvl, Dishevelled; EGFR, epidermal growth factor receptor; FAK, focal adhesion kinase; Fz, Frizzled; GSK3 β , glycogen synthase kinase 3 β ; HCC, hepatocellular carcinoma; HDAC3, histone deacetylase3; LEF/TCF, lymphoid enhancer factor/T cell factor; LRP5/6, low density lipoprotein receptor-related protein5/6; Met, mesenchymal-epithelial transition factor; NCoR, nuclear receptor corepressor; NLK, NEMO-like kinase; PAK4, P21 activated kinase 4; ROR2, tyrosine kinase-like orphan receptor 2; SIK1, salt-inducible kinase 1; SMRT, silencing mediators of retinoic acid and thyroid hormone receptor; TAK1, transforming growth factor- β (TGF- β) activated kinase 1; TCF3, transcription factor 3.

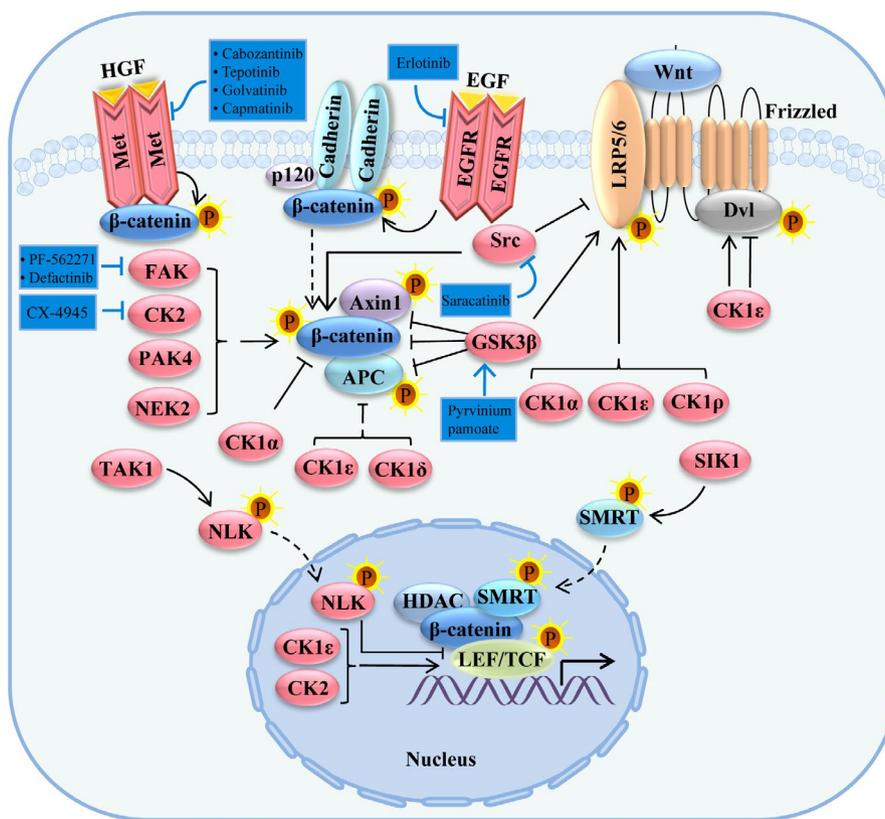


FIGURE 2 Kinases that modulate the Wnt/ β -catenin signaling. Various protein kinases have been reported to modulate the Wnt/ β -catenin signaling in HCC, with some directly phosphorylating the components of this pathway. These protein kinases generally regulate through 3 mechanisms. First, they target β -catenin, Axin1, APC, and so on to regulate the stability, accumulation, and location of β -catenin, such as GSK3 β , CK1, CK2, NEK2, FAK, PAK4, Met, and EGFR. Second, they phosphorylate LRP6 and affect the formation of the LRP6 signalosome, such as CK1 and Src. Finally, they target β -catenin and LEF/TCF and modulate the LEF/TCF/ β -catenin transcription complex, such as CK1 and CK2. In addition, other kinases indirectly modulate the Wnt/ β -catenin signaling, and this modulation varies from kinase to kinase, such as SIK1. The small-molecule inhibitors of the kinases which may be used in combination therapy are indicated in this figure in blue boxes. Abbreviations: APC, adenomatous polyposis coli; CK1, casein kinase 1; CK2, casein kinase 2; Dvl, Dishevelled; EGFR, epidermal growth factor receptor; FAK, Focal adhesion kinase; GSK3 β , glycogen synthase kinase 3 β ; HCC, hepatocellular carcinoma; HGF, hepatocyte growth factor; LEF/TCF, lymphoid enhancer factor/T cell factor; LRP6, low density lipoprotein receptor-related protein 6; Met, mesenchymal-epithelial transition factor; NEK2, NIMA-related kinase 2; PAK4, P21 activated kinase 4; SIK1, Salt-inducible kinase 1

indicated that the Wnt ligand triggers the sequestration of GSK3 β from the cytosol into multivesicular bodies (MVBs) to promote cytoplasmic β -catenin accumulation and, ultimately, activation of the WCS.¹⁹ Moreover, GSK3 β potentially inhibits many Wnt signaling-associated

proteins by promoting their degradation by phosphorylation. However, the WCS stabilizes the GSK3 β targeting proteins, especially in G2/M, indicating that this Wnt-dependent stabilization of proteins may be an alternative mode of the WCS, especially in mitosis.²⁰ The roles of

GSK3 β in HCC remain controversial,²¹ it positively and negatively modulates the WCS. Therefore, whether these modulations contribute to the occurrence and development of HCC remains unclear.

5 | CK1 AND CK2

CK1 and CK2 are serine/threonine kinases, ubiquitously expressed from yeast to humans. Mammalian CK1 possesses 7 family members: α , β , $\gamma 1$, $\gamma 2$, $\gamma 3$, δ , and ϵ , while CK2 is a tetramer consisting of 2 catalytic subunits: CK2 α and CK2 α' , and 2 regulatory subunits: CK2 β and CK2 β' .^{22,23} CK1 regulates diverse cellular processes, such as circadian rhythms, Wnt signaling, membrane trafficking, and cytoskeleton maintenance.²³ CK2 is involved in key cellular processes, such as inhibition of apoptosis, DNA damage response, and cell cycle regulation.²²

Almost all members of the CK1 family have been indicated in WCS regulation, with particular roles. Interestingly, CK1 isoforms have been identified as both positive and negative regulators of the WCS. Similar to GSK3 β , CK1 activates the WCS through the phosphorylation of LRP6 (mostly by CK1 α , CK1 ϵ/δ and CK1 γ in this case)^{24,25} and inhibits it through the phosphorylation of β -catenin (mainly by CK1 α)²⁶ and APC (by CK1 ϵ and CK1 δ).²⁷ Transcription Factor 3 (TCF3) phosphorylation by CK1 ϵ promotes its interaction with β -catenin and activates the WCS.²⁸ Dvl phosphorylation by CK1 ϵ has been well studied, this phosphorylation promotes Dvl signaling and, therefore, the WCS.²⁹ In addition to this activating function, CK1 ϵ triggers a negative feedback loop to inhibit the WCS. Dvl has the capability to multimerize and cluster Wnt-receptor complexes into LRP6 signalosomes. The phosphorylation of Dvl via CK1 ϵ promotes the Huwe1-dependent ubiquitination of Dvl, thereby inhibiting Dvl multimerization and, therefore, inhibiting the WCS.³⁰ Low cytoplasmic CK1 ϵ expression is correlated with a low survival rate and a high probability of tumor vascular invasion in HCC patients, indicating that CK1 ϵ may function as a tumor suppressor in HCC.³¹

β -Catenin is a multifunctional protein that can associate with the transcription complex and junctional complex at the cell membrane where β -catenin interacts with proteins such as α -catenin, p120-catenin and cadherin to mediate cell-cell adhesion.³² The Wnt-induced sequestration of GSK3 β into MVBs is regulated by the junctional complex and CK1 α . In Wnt-stimulated cells, the junctional complex binds the LRP6 signalosome, and CK1 α phosphorylates p120-catenin and cadherin, resulting in the separation of the 2 complexes and the release of β -catenin from the junctional complex, finally activating the WCS. Separated LRP6 signalosome, including GSK3 β , are internalized to MVBs.³³

CK2 appears to be capable of affecting the WCS at multiple levels. CK2 phosphorylates β -catenin at threonine 393, leading to proteasome resistance, and ultimately potentiating the WCS.³⁴ Furthermore, the phosphorylation of nuclear LEF1 via CK2 significantly enhances its affinity for β -catenin and stimulates transactivation of the β -catenin/LEF1 complex.³⁵ CK2 α is aberrantly

overexpressed in HCC,³⁶ and knockdown of CK2 α results in significant apoptosis and inhibition of the migration and invasion of HCC cells,³⁷ confirming that CK2 α could be a marker of poor prognosis for HCC. Based on these findings, it can be concluded that overexpressed CK2 α positively modulates the WCS and promotes HCC progression.

6 | CYCLIN-DEPENDENT KINASE 1 (CDK1) AND CDK14

CDK1 and CDK14 are serine/threonine kinases with reported roles in the regulation of the cell cycle by phosphorylating protein substrates-associated with specific cyclin subunits.³⁸ Mitotic WCS has been discovered to be constitutively activated during mitosis due to phosphorylation of LRP5/6 by CDK14/cyclin Y, and the phosphorylation site has been reported to be the same as that used by GSK3 β .³⁹ Recently, we found that CDK1 could directly and markedly phosphorylate mitotic BCL9 on its N-terminus, especially at threonine 172. This can stabilize BCL9 and inhibit binding of the clathrin complex to the BCL9/LRP6 signalosome to suppress both clathrin-mediated endocytosis and the subsequent degradation of the LRP6 signalosome, and also modulate the WCS dynamically and precisely during mitosis.⁴⁰ This CDK1/BCL9/mitotic Wnt signaling pathway is dynamically controlled by CDK1 activity during mitosis, undergoing higher activity in prophase and metaphase, and a relatively decreased activity in anaphase and telophase. These processes confirmed that CDK1 is directly involved in regulating the WCS, at least by phosphorylating BCL9 in mitosis. More Wnt-related substrates of CDK1 could be revealed in the future to further link CDKs with the WCS in biological and physiological stages or disease processes. CDK1 and CDK14 are upregulated in HCC in accordance with TCGA data, indicating that their positive modulations in WCS may contribute to HCC progression.

7 | MET

RTKs are a subclass of tyrosine kinases that have emerged as key regulators of a wide range of complex biological functions, including cell growth, motility, differentiation, and metabolism.⁴¹ In humans, there are 58 known RTKs, and these have been categorized into 20 subfamilies including EGFR, mesenchymal-epithelial transition factor (Met), fibroblast growth factor receptors (FGFR), and ROR. The Met RTK family contains 3 members: Met, Ron, and c-Sea. Met is the receptor for HGF, and the HGF/Met axis modulates the multiple intracellular signaling pathways involved in muscle and liver formation, cell proliferation, morphogenesis and motility, and epithelial-mesenchymal transition.⁴² Tumorigenic Met mutants (M1268T) contribute to the cellular accumulation of β -catenin and constitutive activation of the WCS, which is partially due to β -catenin tyrosine phosphorylation by mutated Met.⁴³ Further investigation has revealed that Met is associated with β -catenin at the cell membrane

and, upon HGF stimulation, activated Met phosphorylates β -catenin at tyrosine residues. This causes dissociation of β -catenin from Met and the nuclear translocation of β -catenin, resulting in the expression of Wnt target genes.⁴⁴ Aberrant activation of Met promotes the initiation, proliferation, invasion, and metastasis of HCC. HCC patients with a high expression of Met have significantly lower survival rates than the patients with a low or no expression of Met.⁴⁵ Based on these findings, it can be concluded that upregulated Met positively modulates the WCS and promotes HCC progression. Moreover, Met plays a critical role in drug resistance, therefore targeting the HGF/Met axis has been one of the most promising therapies for HCC.⁴⁶

8 | EGFR AND FGFR

Upon ligand binding, activated EGFR and FGFRs turn on downstream signaling pathways such as the Ras-Raf-MEK-ERK1/2 pathway, STAT pathways, and the phosphatidylinositol 3-kinase (PI3K)/Akt pathway, to control cell proliferation, survival, and differentiation.⁴⁷ Intriguingly, FGFR2, FGFR3, and EGFR also significantly activate the WCS through 2 different mechanisms. First, FGFR2, FGFR3, and EGFR directly phosphorylate β -catenin at tyrosine 142, which leads to the release of β -catenin from membrane junctions and an increase of cytoplasmic β -catenin. Second, ERK phosphorylates LRP6 at serine 1490 and threonine 1572 during its Golgi network-based maturation process, and this phosphorylation dramatically promotes the cellular response to the Wnt ligand.⁴⁸ Notably, EGFR is frequently overexpressed in human HCC, and its upregulation is correlated with aggressive tumors, metastasis, and poor patient survival.⁴⁷ According to this research, EGFR may act as an oncogene, partially by enhancing the WCS in HCC. However, EGFR inhibitors have achieved only modest results in HCC clinical trials.⁴⁹

9 | NIMA-RELATED KINASE 2 (NEK2)

NEK2 is a chromosomal instability associated gene that encodes a serine/threonine kinase and belongs to the never in mitosis A (NIMA)-related family of kinases. NEK2 plays a key role in regulating mitotic processes, including centrosome duplication and separation, microtubule stability, kinetochore and microtubule attachment and mitotic spindle formation.⁵⁰ In G2/M phase, Dvl accumulates at the centrosomes and is phosphorylated by NEK2 at several residues, this increases its affinity toward linker proteins at the centrosomes. Dvl then acts as a scaffold to form a complex with the linker proteins and facilitates the release of the complex from the centrosomes. The Dvl released from the centrosome is available for, and increases, WCS activation.⁵¹ Several studies have confirmed that NEK2 is aberrantly overexpressed in human HCC tissues and cell lines, and its expression is significantly correlated with the progression of HCC patients.^{52,53} Moreover, NEK2 induces sorafenib resistance, a first-line treatment for advanced-stage HCC, via WCS activation. In sorafenib-treated HCC cell lines, NEK2 binds and stabilizes β -catenin, promoting its

translocation to the nucleus, which consequently activates the transcription of Wnt target genes.⁵⁴ Therefore, NEK2 acts as a potential therapeutic target to improve the response of HCC patient to sorafenib treatment, especially those resistant to this agent.

10 | P21 ACTIVATED KINASE 4 (PAK4)

PAK4 is a serine/threonine kinase that was originally identified as a downstream effector of the small Rho GTPases, such as Rac1 and Cdc42. It plays vital roles in many biological functions such as cell growth, cell survival, cytoskeletal organization, cell migration, and morphology.⁵⁵ PAK4 has known links to the WCS, and it can shuttle between the cytoplasm and nucleus to positively modulate this pathway. In the cytoplasm, PAK4 phosphorylates β -catenin and prevents its degradation from proteasome pathway. Nuclear accumulation of PAK4 enhances the nuclear import of β -catenin, promotes Wnt target gene transcription and upregulates β -catenin expression.⁵⁶ Interestingly, the methylation of PAK4 has also been connected to the WCS. SETD6, a protein lysine methyltransferase, binds and methylates PAK4 on chromatin, which enhances the interaction between PAK4 and β -catenin, promotes the formation of an active β -catenin/TCF complex, and results in the activation of the WCS.⁵⁷ Importantly, PAK4 is aberrantly upregulated in HCC and contributes to cancer metastasis,⁵⁸ indicating that it may contribute to HCC progression by enhancing the WCS.

11 | SRC TYROSINE KINASES

Src family kinases are nonreceptor tyrosine kinases that act downstream of RTKs and integrins to regulate cell proliferation, adhesion, morphology, and movement.⁵⁹ Src family kinases include isoforms such as Src, Blk (B-lymphoid tyrosine kinase), Fyn, and Lck (Lymphocyte specific kinase). Src is activated by phosphorylation at the key residue tyrosine 416 and dephosphorylation at tyrosine 527. Src acts as either a positive or negative regulator of the WCS. Upon Wnt3a stimulation, Src binds to, and is activated by Dvl, and then Src phosphorylates Dvl and β -catenin, resulting in the accumulation of β -catenin in the nucleus, promoting LEF/TCF-mediated transcription of Wnt target genes.⁶⁰ Src is also associated with and phosphorylates LRP6 directly, which reduces LRP6 expression levels on the cell surface, disrupts LRP6 signalosome formation, and negatively regulates the WCS.⁶¹ Intriguingly, Src and Fyn modulate a new signaling cascade that is different from the WCS. Upon Wnt3a stimulation, activated Src phosphorylates Fz, allowing Fyn recruitment and activation. Fyn phosphorylates β -catenin, releasing β -catenin from the junctional complex to stimulate Wnt target gene expression.⁶² Notably, the protein expression of Src and tyrosine 416 phosphorylated Src (p-Y416Src) are significantly higher in HCC tissues compared with adjacent normal tissues.^{63,64} Increased expression of p-Y416Src is associated with poor patient survival, suggesting that p-Y416Src may serve as an independent prognostic marker for patient survival

in HCC. Although *Src* is an oncogene in HCC, it has dual functions in regulating the WCS. Further investigations are needed to clarify whether these regulations contribute to HCC progression.

12 | TRANSFORMING GROWTH FACTOR- β (TGF- β) ACTIVATED KINASE 1 (TAK1)

TAK1 is a member of the mitogen-activated protein kinase kinase (MAP3K) superfamily, it is activated by TGF- β and regulates both nuclear factor- κ B (NF- κ B) and MAPK signaling pathways, which play key roles in embryogenesis, development, inflammation, the immune response, and metabolism.⁶⁵ TAK1 has been reported to be involved in the WCS by stimulating NEMO-like kinase (NLK) activity and inhibiting the transcriptional activation. TAK1 activation promotes the activity of NLK, which can phosphorylate TCF and then interfere with the binding of β -catenin-TCF to the TCF binding element, thereby negatively regulating the WCS.⁶⁶ More recent studies have revealed that Wnt1 can directly activate the TAK1-NLK cascade, resulting in the phosphorylation of TCF.⁶⁷ Therefore, Wnt signal transduction through the WCS activates β -catenin/TCF whereas, through the TAK1-NLK pathway, it phosphorylates and inhibits TCF, which might function as a feedback mechanism. Hepatocyte-specific deletion of TAK1 in mice results in spontaneous hepatocyte dysplasia, liver inflammation, and the development of HCC, indicating that it acts as a tumor suppressor.⁶⁸ TAK1 may exert anti-tumor effects by inhibiting the WCS in HCC.

13 | SALT-INDUCIBLE KINASE 1 (SIK1)

SIK1 is a serine/threonine protein kinase belonging to the AMP-activated protein kinase (AMPK) family. SIK1 is activated by liver kinase B1 phosphorylation and plays crucial roles in a series of cellular processes, including cell proliferation and apoptosis.⁶⁹ The expression of SIK1 is significantly downregulated in HCC, and the regulation of SIK1 occurs at both the transcriptional and post-transcriptional levels.⁷⁰ Recently, it has been reported that the loss of SIK1 accelerates HCC growth and invasion through activation of WCS. Mechanistically, SIK1 phosphorylates the silencing mediators of retinoic acid and thyroid hormone receptor (SMRT) at threonine 1391 and promotes its translocation into the nucleus. Then, phosphorylated SMRT recruits the nuclear receptor corepressor (NCoR)/histone deacetylase 3 (HDAC3) corepressor complex to β -catenin/TCF and inhibits the transcription of Wnt target genes. Loss of SIK1 leads to the dephosphorylation of SMRT and its export from the nucleus, therefore activating the WCS.⁷¹

14 | FOCAL ADHESION KINASE (FAK)

FAK is a highly conserved non-RTK encoded by the protein tyrosine kinase 2 gene in humans. FAK acts at the intersection of various

signaling pathways, including PI3K/Akt signaling and JNK signaling.⁷² When activated, FAK controls cell adhesion, proliferation, migration, and cancer stem cell self-renewal through both kinase-dependent and kinase-independent mechanisms.⁷² FAK protein and mRNA are over-expressed in HCC compared with corresponding normal liver tissues and are positively correlated with tumor stage, vascular invasion, and intrahepatic metastasis.^{73,74} Recently, FAK has been reported to stimulate the WCS by inhibiting β -catenin degradation and promoting its nuclear accumulation in HCC. In this manner, FAK promotes a cancer stem cell-like phenotype and enhances tumorigenicity, leading to HCC recurrence and sorafenib resistance.⁷⁵ However, the precise molecular mechanism by which FAK regulates β -catenin in HCC remains unclear.

15 | TYROSINE KINASE-LIKE ORPHAN RECEPTOR 2 (ROR2)

ROR2 is a member of the ROR RTK family, with functional extracellular Wnt-binding domains and is implicated in Wnt signal transduction. ROR2 was first found to bind directly to the non-canonical Wnt ligand Wnt5a, to inhibit the WCS, with no effect on the regulation of cellular calcium and β -catenin levels.⁷⁶ In addition, ROR2 functions as a co-receptor of Wnt5a and activates non-canonical Wnt signaling. After Wnt5a binds to Fz, it interacts with ROR2 and recruits GSK3 β to phosphorylate ROR2 at serine 864, which is required for the activation of ROR2 function. Interestingly, Wnt5a and the canonical Wnt ligand Wnt3a compete for binding to Fz at the cell surface. The identity of the Wnt ligand can determine whether the Fz co-receptor would be LRP5/6 or ROR2, therefore dictating whether Wnt/ β -catenin-dependent or -independent signaling would be activated, respectively.⁷⁷ However, the details of how activated ROR2 modulates Wnt signaling is completely unknown, and elucidation of more downstream components of ROR2 would provide a more detailed mechanisms of Wnt/ROR2 signal transduction. TCGA data revealed that ROR2 is upregulated in HCC, however, it negatively modulates the WCS. Therefore, it may not promote HCC progression by enhancing the WCS.

16 | TARGETING THE KINASES RELATED WITH WCS IN HCC

To date, increasing numbers of studies on the molecular mechanisms of tumorigenesis have shown that protein kinases play pivotal roles in various types of cancer. Because of mutation or overexpression, some constitutively active protein kinases promote cancer cell proliferation and survival, therefore they are considered oncogenic.⁷⁸ Other kinases expressed in the tumor or surrounding tissues are also required for tumor maintenance such as the VEGFRs, which are important for inducing angiogenesis.⁷⁹ Surprisingly, treating cells with kinase inhibitors does not cause excessive damage to normal cells, therefore these inhibitors can be used to selectively kill tumor cells.⁴ For the above reasons, kinases have become important targets in

TABLE 2 Kinase inhibitors or activators modulating Wnt/ β -catenin signaling potentially in HCC

Target	Molecule	Product description	Reference
GSK3 β	Pyriminium pamoate	It is an FDA-approved anti-parasite drug, it activates GSK3 β to phosphorylate β -catenin for degradation and ultimately inhibits Wnt/ β -catenin signaling	85
CDK1	Roniciclib	This drug targets CDK1 with IC ₅₀ of 7 nM. It has entered phase II clinical trials for the treatment of solid tumor	86
	Dinaciclib	This drug targets CDK1 with IC ₅₀ of 3 nM. It has entered phase III clinical trials for the treatment of chronic lymphocytic leukemia	87
CK2	CX-4945	This drug targets CK2 with IC ₅₀ of 1.5 nM. It has entered phase II clinical trials for the treatment of multiple myeloma, brain, and gastrointestinal cancer	88
FAK	PF-562271	This drug targets FAK with IC ₅₀ of 1.5 nM. Its phase I clinical trials for the treatment of advanced solid cancers has completed	89
	Defactinib	This drug targets FAK. It has entered phase II clinical trials for the treatment of advanced solid cancers	90
Src	Saracatinib	This drug targets Src, Lyn, Fyn, Blk, EGFR with IC ₅₀ of 2.7, 5, 10, 11, 66 nM, respectively. Its phase II clinical trials for the treatment of advanced solid cancers has completed	91
Met	Cabozantinib	This drug targets Met and VEGFR2 with IC ₅₀ of 1.3 and 0.035 nM, respectively. It is in phase IV clinical trials for the treatment of HCC	92
	Tepotinib	This drug targets Met with IC ₅₀ of 4 nM. It is in phase II clinical trials for the treatment of HCC	93
	Golvatinib	This drug targets Met and VEGFR2 with IC ₅₀ of 14 and 16 nM, respectively. It is in phase II clinical trials for the treatment of advanced solid tumors	94
	Capmatinib	This drug targets Met with IC ₅₀ of 0.13 nM. It is in phase II clinical trials for the treatment of advanced HCC	95

(Continues)

TABLE 2 (Continued)

Target	Molecule	Product description	Reference
EGFR	Erlotinib	This drug targets EGFR with IC ₅₀ of 2 nM. Its phase III clinical trials for the treatment of HCC was unsuccessful	96

Abbreviations: Blk, B-lymphoid tyrosine kinase; CDK1, cyclin-dependent kinase 1; CK2, casein kinase2; EGFR, epidermal growth factor receptor; FAK, focal adhesion kinase; GSK3 β , glycogen synthase kinase 3 β ; HCC, hepatocellular carcinoma; Met, mesenchymal-epithelial transition factor; VEGFR2, vascular endothelial growth factor receptor 2.

cancer treatment. For instance, sorafenib and lenvatinib are small-molecule multikinase inhibitors, both of which are US FDA approved for first-line systemic treatment of advanced-stage HCC patients and have been shown to prolong survival.⁸⁰

Met is emerging as a biologically rational target in HCC and it positively modulates the WCS. Met inhibitor crizotinib has been reported to inhibit the WCS.⁸¹ A phase I study of Met inhibitors combined with sorafenib has illustrated the safety of the combination strategy,^{80,82} therefore the combination of Met inhibitors with WCS inhibitors may synergistically enhance their anti-tumor effect and may be a safe and promising treatment approach. Pyriminium pamoate is an FDA-approved anti-parasite drug. A previous study revealed that it selectively activates CK1 α to suppress the WCS,⁸³ however a further study supported that it did not activate CK1 α , but alternatively activated GSK3 β to phosphorylate β -catenin for degradation and ultimately inhibited the WCS.^{84,85} Pyriminium pamoate dose dependently inhibited cancer stem cell regeneration and proliferation in various breast cancer cell lines, indicating that it is a feasible agent for HCC therapy. Some small molecules targeting the kinases mentioned above are selectively listed (Table 2) to offer new perspectives for preclinical and clinical HCC studies.

17 | CONCLUSION AND PERSPECTIVE

The initiation and development of HCC is a complex process with many factors and stages.⁹⁷ The WCS is frequently hyperactivated in HCC, and a substantial proportion of these patients with HCC have β -catenin mutations. Targeting the WCS represents a new opportunity for HCC treatment that is currently under clinical investigation.⁸³ Protein phosphorylation modulates almost every aspect of cellular processes, including the WCS, and protein kinase dysfunction is the cause of many diseases, especially cancer. Consequently, protein kinases are emerging as the second largest group of drug targets after G protein coupled receptors.⁴ In this review, we focus on protein kinases that modulated the WCS in HCC. CDK1, CDK14, CK2, NEK2, FAK, PAK4, Met, and EGFR activate the WCS, while TAK1, SIK1 and ROR2 inhibit it, and GSK3 β , CK1, and Src have dual functions. Most of these kinases phosphorylate WCS components and directly regulate this pathway through different mechanisms.

Other kinases target non-WCS components, indirectly regulating the pathway in various ways. Some regulation of WCS by protein kinases has been linked to HCC, for example downregulated SIK1 phosphorylates SMRT, promotes its translocation into the nucleus and recruits the NCoR/HDAC3 corepressor complex to β -catenin/TCF/LEF, inhibiting the transcription of Wnt target genes and ultimately contributing to HCC.⁷¹ However, the relationship between some other regulations and HCC remains unclear, for example GSK3 β regulation, and needs to be explored in the future. Given the abundance of kinase substrates and the pivotal role of the WCS in HCC, an increasing number of kinases will be discovered to modulate this pathway directly or indirectly in prospective studies.

With the advent of personalized precision medicine, it is now clear that each HCC patient is unique and that the mechanisms of HCC occurrence and progression may be diverse in different patients.⁸² Therefore, single target inhibitors aimed at one molecule or pathway are not universally effective and have the problem of drug resistance resulting from target gene mutations or upregulation of alternative signaling pathways.⁴⁶ Strategies for combinations of multiple drugs targeting multiple targets are urgently needed.⁴ Furthering our understanding of the crosstalk between the WCS and its regulatory kinases will provide a theoretical basis for better combination therapy. Some pioneering studies have suggested that targeting the WCS and its regulatory kinases will be possible and is a promising research direction to be explored in the near future.^{98,99}

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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