Molecular Pathways

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# Molecular Pathways: Epigenetic Modulation of Wnt–Glycogen Synthase Kinase-3 Signaling to Target Human Cancer Stem Cells

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## Abstract

Aberrant regulation of the canonical Wnt signaling pathway (Wnt–β-catenin–GSK3 axis) has been a prevalent theme in cancer biology since earlier observations until recent genetic discoveries gleaned from tumor genome sequencing. During the last few decades, a large body of work demonstrated the involvement of the Wnt–β-catenin–GSK3 signaling axis in the formation and maintenance of cancer stem cells (CSC) responsible for tumor growth in several types of human malignancies. Recent studies have elucidated epigenetic mechanisms that control pluripotency and stemness, and allow a first assessment on how embryonic and normal tissue stem cells are dysregulated in cancer to give rise to CSCs, and how canonical Wnt signaling might be involved. Here, we review emerging concepts highlighting the critical role of epigenetics in CSC development through abnormal canonical Wnt signaling. Finally, we refer to the characterization of novel and powerful inhibitors of chromatin organization machinery that, in turn, restore the Wnt–β-catenin–GSK3 signaling axis in malignant cells, and describe attempts/relevance to bring these compounds into preclinical and clinical studies. *Clin Cancer Res; 20(21); 5372–8. ©2014 AACR.*

## Background

The Wnt family of secreted glycoproteins act as ligands to activate multiple signal transduction pathways (1). Upon activation, Wnt signaling promotes mainly β-catenin nuclear translocation to regulate expression of target genes via T-cell factor/lymphoid enhancer factor (TCF/LEF) transcription factors (2). The Wnt–β-catenin pathway acts in a context-dependent manner to regulate cell proliferation and differentiation in both embryonic development and adults (2). Perturbations in the levels of Wnt–β-catenin signaling are linked to many disease processes, including solid tumors and leukemia (3, 4). The activity of the Wnt–β-catenin signaling pathway depends primarily on the activity of glycogen synthase kinase-3 (GSK3), which plays a key role in controlling β-catenin stability/degradation. GSK3-dependent phosphorylation of β-catenin restricts its nuclear translocation by inducing proteasome-dependent proteolysis (5). Active β-catenin complexes recruit transcriptional coactivator cAMP responsive element binding protein (CREB)–binding protein (CBP) or its closely related homolog p300 (6) to potentiate the expression of downstream Wnt target genes (Fig. 1A). Consequently, GSK3 acts as a tumor suppressor by curbing canonical Wnt–β-catenin signaling.

Recent advances in cancer genomics identified the Wnt–GSK3–β-catenin pathway as one of the most prevalent signaling mechanisms studied in cancer biology since multiple genetic alterations of its components were recurrently associated with human tumorigenesis, including medulloblastoma, hepatocellular cancer, colorectal cancers, and leukemia (7–10). To date, several reports also highlighted the importance of Wnt–GSK3–β-catenin signaling on self-renewal in both normal and cancer stem cells (CSC). Specifically, CSCs were identified as rare populations of cancer cells within a hierarchical model of tumorigenesis, displaying the ability to sustain long-term neoplastic dissemination in both leukemia and solid cancers (11, 12). Considering their malignant and metastatic properties that might cause relapse, the CSC represents, to date, the major clinical obstacle for effective cancer eradication by conventional therapeutic measures (13). Examples of the participation of Wnt–GSK3 signaling in CSC development include cases of BCR-ABL chronic myeloid leukemia (CML; refs. 14, 15) presenting an aberrant nonfunctional form of GSK3 showing neoplastic progression toward an aggressive stage of the disease, marked by the progressive accumulation of nuclear active β-catenin within BCR-ABL CSCs (10).

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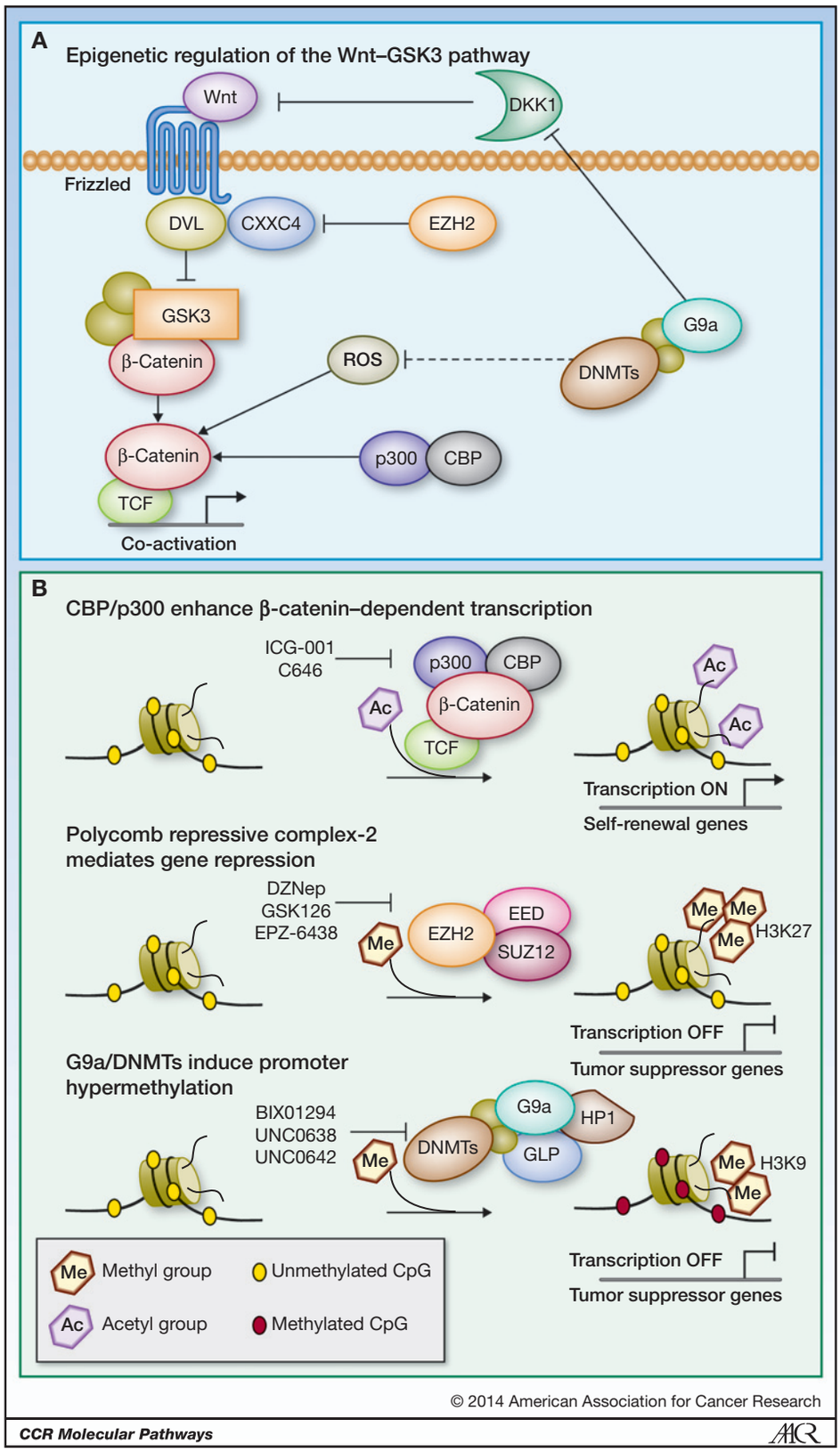




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**Figure 1.** Epigenetic modulation of the Wnt–GSK3 pathway in human CSCs. A, Wnt–GSK3 signaling is cell context–dependent (hyperstimulated in CSCs), and the activity of such a pathway relies on multiple epigenetic factors. B, pharmacologic targeting of histone acetyltransferase and methyltransferase-potentiating Wnt–GSK3 activity represents a new therapeutic strategy to eradicate CSCs.

Moreover, elevated β-catenin levels observed in acute myeloid leukemia (AML) were related to Wnt–GSK3 pathway deregulation, enhanced self-renewal, and CSC development associated with high relapse rates and poor survival outcomes (16).

Considering the essential role of the Wnt–GSK3–β-catenin axis in differentiation of normal progenitors, drug targeting of neoplastic-specific upstream and/or downstream events affecting this pathway may represent a particularly powerful approach, in the future, to restore normal signaling in CSCs that warrant further molecular exploration. Interestingly, Wnt–GSK3–β-catenin signaling has been associated with the deregulation of epigenetic modulators. Cancer-specific changes in histone acetylation have been associated with enhanced activity of CBP–p300 complexes (17, 18), whereas alterations in DNA and/or histone tail methylation processes involve activating or loss-of-function mutations, hyperstimulation, or overexpression of chromatin writers, such as DNMT3A/B, EZH2, or G9a (19–21), which collectively converge on pivotal self-renewal pathways, such as the Wnt–GSK3–β-catenin pathway (Fig. 1A).

Advances in cancer epigenetics have provided fundamental insights into the participation of the aforementioned chromatin remodelers during cancer initiation and CSC development (22, 23). Multiple lines of evidence support the hypothesis that CSCs are functionally dependent upon the maintenance of their epigenetic state, and that this could represent a valuable therapeutic opportunity. Lineage-mapping studies have identified epigenetic dysregulation as a critical early event in human tumorigenesis (24), supporting the concept of an epigenetic progenitor origin of cancer (22). Furthermore, cells with CSC features appear molecularly dissociable from non-CSCs (25–29), and animal models have demonstrated that epigenetic interventions can reduce CSC frequencies and attenuate tumorigenesis (30, 31). For instance, microRNAs suppressing the expression of members of the polycomb repressive complex-2 (PRC2), including Let-7 and the miR200 family, are downregulated in CSC-containing fractions of breast and prostate cancer whereas increased levels were observed in non-stem cancer cells (25, 28, 29). Accordingly, the expression of PRC2 members SUZ12 and EZH2 was shown to be upregulated in CSC-enriched fractions of breast and prostate tumors (25, 29). Taken together, these observations suggest the existence of epigenetic patterns acting as early key prooncogenic events in CSC development, and distinct from normal undifferentiated cells of the same tissues (19, 32, 33), which, subsequently, could affect the onset of tumorigenic signaling cascades. Ultimately, the identification of epigenetic marks that influence CSC self-renewal pathways, such as Wnt–GSK3, is of particular interest because the correction of aberrant epigenetic pathways could represent a powerful strategy to restore normal stem cell phenotypes from CSCs in a process of "cancer reprogramming." Thus, using selective small molecules to suppress aberrant chromatin modifiers activity affecting Wnt–GSK3 signaling is sought to effectively restore normal chromatin organization by targeting the problem at its very source, and restore normal pathway activity (Fig. 1B; ref. 34).

## Clinical–Translational Advances

Multiple studies recently proposed that disruption of epigenetic regulatory mechanisms represents a promising pharmacotherapeutical strategy in the context of several human malignancies (Fig. 1B; reviewed by Helin and Dhanak; ref. 34). Thus, an attractive therapeutic strategy to eradicate CSCs while sparing normal stem cells could consist of targeting CSC-specific epigenetic features that contribute to hyperactivation of oncogenic signaling pathways. As stated through several reports (see the following subsections for references), the activity of the Wnt–GSK3 pathway has the potential to be modulated epigenetically on several fronts (Fig. 1A). The development of drugs altering a mechanism on which CSCs rely (epigenetic in this case), although no such dependency exists in normal stem cells or progenitors, represents a major challenge in actual cancer pharmacology. Examples of CSC-targeting small molecules affecting chromatin organization were recently reported in preclinical studies, including PTC-209, which inhibits BMI-1 activity, a member of the PRC1 and dose dependently compromises colorectal tumor formation in xenograft models (35). Moreover, the G9a inhibitor UNC-0638 was shown to suppress self-renewal in AML CSCs by triggering differentiation programs as evidenced by the acquisition of mature cell morphology, whereas only minor effects were observed on long-term hematopoiesis (31, 35). However, the efficacy of these compounds remains to be tested in clinical trials. The following sections describe chromatin-linked regulatory modes for Wnt–GSK3 pathway modulation, including histone acetyltransferases, PRC2 and G9a, along with the associated therapeutic utility of small molecules related to these nodes of epigenetic activity (Fig.1B).

### Histone acetyltransferase complex CBP–p300 is critical to Wnt–GSK3 target genes transcriptional regulation

CBP and p300 are histone acetyltransferases acting as transcriptional coactivators of Wnt–GSK3 target genes in normal and cancer tissues (36, 37). Chromatin-bound TCF–β-catenin complexes recruit coactivator CBP to potentiate the transactivation of Wnt–GSK3 target genes, stimulating self-renewal programs in CSCs. Inversely, β-catenin–p300 interactions have also been suggested to influence physiologic prodifferentiation transcriptional programs (38). Conversely, other studies have also described chromosomal aberrations resulting in p300 fusion products in human AML, stimulating acetyltransferase activity characterized by important epigenetic dysfunctions (17, 39). Thus, using small molecules to inhibit the recruitment of CBP–p300 coactivators to TCF–β-catenin target genes represents an interesting therapeutic axis to restrict Wnt–GSK3 signaling in CSCs. Compounds such as C646 and ICG-001, which preferentially target p300 and CBP respectively, were developed and tested on human cell lines or

*in vivo* preclinical models. ICG-001 effectively suppressed the tumor growth by over 80% in colon carcinoma xenograft models (40), and extended the survival of mice xenografted with human lymphoblastic leukemia when applied in combination with chemotherapy (41). Mechanistically, C646 was shown to inhibit p300 acetyltransferase activity, which plays a key role in β-catenin (K345ac)–dependent transactivation (42). On the other hand, ICG-001 selectively binds to the CBP nuclear receptor interaction domain to restrict physical interactions with β-catenin (Fig. 1B; refs. 40, 41). Collectively, the studies involving these small molecules clearly highlight the potential therapeutic utility for such epigenetic inhibitors to mediate Wnt–GSK3–β-catenin axis activity at the expense of CSC self-renewal and survival (40, 43).

Although the existing CBP–p300 inhibiting molecules may show a certain degree of selectivity for transformed cells over normal cells in some studies (40, 43), it is still expected that disrupting the TCF–β-catenin–coactivator axis will affect normal stem cell development. One of the current major challenges is to identify cell context–specific compounds that could selectively affect CBP–p300 only in CSCs, which may include yet uncharacterized players that regulate this complex to therapeutically alter gene expression and, in turn, human CSC behavior.

## PRC2 alters GSK3 activity in human cancers

Polycomb group proteins (PcG) are epigenetic transcriptional repressors acting as multiprotein complexes (PRC1/2) catalyzing covalent addition of posttranslational modifications on histone tails. Polycomb groups are divided into two main transcriptional repressive complexes, PRC1 and PRC2 (44). Specifically, PRC2 is responsible for trimethylation of lysine 27 of histone H3 (H3K27me3) via its *Enhancer of Zest* subunit (EZH), for which enhanced histone methyltransferase activity was extensively described in cancer (mostly EZH2; ref. 45). EZH2 activity was also shown as essential for the maintenance of xenograft tumor growth in glioblastoma (46). Aberrant EZH2 activity plays a significant role in the epigenetic repression of differentiation and proapoptotic genes in a plethora of human cancers, including solid tumors as well as in leukemia (45–48), and such a role is potentially related to its ability to promote Wnt–GSK3 activity (Fig. 1A). Accordingly, a number of EZH2 targets have been associated with increased nuclear accumulation of β-catenin, ultimately contributing to CSC self-renewal. For instance, CXXC4 was identified as a target of EZH2, and is known to stabilize the β-catenin degradation complex by inhibiting Dishevelled (Dvl; Fig. 1A; ref. 49). Low levels of CXXC4 were observed in gastric and renal carcinomas and were associated with β-catenin nuclear translocation, metastasis formation, and poor prognosis (50). Furthermore, elevated EZH2 expression in breast CSCs ($CD44^{+}/CD24^{low}$) causes epigenetic silencing of the DNA repair factor RAD51, which in turn stimulates RAF1–ERK activity. In such a context, enhanced p-ERK levels promote functional β-catenin stabilization (21).

The utility of pharmacologic EZH2 targeting to restore proper Wnt–GSK3 activity was further reinforced by the use of an indirect EZH2 inhibitor, 3-deazaneplanocin A (DZNep), against colorectal cancer cells (Fig. 1B; ref. 51). When applied in combination with a histone deacetylase (HDAC) inhibitor, DZNep caused massive apoptosis induction upon the restoration of DACT3 expression, ultimately allowing Dishevelled activation. DZNep has been initially characterized as an S-adenosylhomocysteine hydrolase inhibitor, indirectly causing histone methyltransferase inhibition (52–54). Although DZNep has limited clinical potential due to its untargeted, global methyltransferase inhibitory effects (54), its uses in fundamental studies on cancer epigenetics paved the road for the development of other potent EZH2 inhibitors (46–48, 53). Pharmaceutical companies have recently developed new direct and specific EZH2-targeting small molecules, giving exciting perspectives for the future of epigenetic therapies. Specifically, Epizyme and GlaxoSmithKline have developed EPZ-6438 and GSK126, respectively, which both target hyperactive EZH2 mutants (Y641 and A677) with high specificity (Fig. 1B; refs. 55, 56). At the moment, only EPZ-6438 is being tested in phase I/II clinical trials on patients with advanced solid tumors or with B-cell lymphomas (NCT01897571). Interestingly, these point mutations within the catalytic SET domain of EZH2 were demonstrated to favor the formation of trimethylated H3K27, leading to important changes of the epigenetic landscape (55, 56). These mutations have recently been reported as frequent events in diffuse large B-cell lymphoma, causing transcriptional silencing of cell-cycle checkpoints and differentiation factors (57). Considering the existence of oncogenic EZH2 variants, the emergence of such highly selective small molecules could represent a powerful approach to specifically and epigenetically target CSCs over healthy stem cells inside a chemotherapeutic regimen, to restore normal Wnt–GSK3 activity (Fig. 1B).

## G9a/GLP histone lysine methyltransferase complexes affect Wnt–GSK3 activity

In addition to EZH2, other histone methyltransferases have also been suggested to play critical roles in the oncogenic regulation of the Wnt–GSK3 pathway. As dopamine receptors and the associated signaling cascade were recently linked to CSCs (58), new insights into the histone methyltransferase G9a suggest a role for such a chromatin writer as a downstream effector of this pathway. Psychoactive drugs are known to have a major impact on neuron epigenetic landscapes and are likely to have similar effects on CSCs (59). Notably, antidepressants, repeated cocaine administration, and dopamine receptor signaling were all linked to G9a deregulation and aberrant H3K9 methylation patterning (60–62). G9a is also closely related to malignancy and Wnt–GSK3 hyperactivation by (i) decreasing reactive oxygen species (ROS) via FBP1 epigenetic silencing (H3K9me2/DNA methylation), which in turn enhances TCF–β-catenin interactions, and (ii) by directly repressing Dickkopf-1, 2, and 3 promoters (Fig. 1A; refs. 20, 63, 64). Moreover, it is

now clear that G9a/H3K9me patterning is associated with 5-methyl cytosine deposition catalyzed by *de novo* DNA methyltransferases (DNMT3A/B) that were found to play a pivotal role in the development of preleukemic progenitors (24, 65). Although a persistent G9a expression/activity was described in induced pluripotent stem cells and further associated with sustained epigenetic memory (65, 66), a fascinating parallel can be drawn to the differentiation blockade seen in CSCs (67). Interestingly, leukemic stem cell–driving mutations of IDH1 and 2 were shown to impair H3K9 demethylation, leading to abnormal accumulations of repressive H3K9me2/3 marks, catalyzed by G9a on key loci, which subsequently impairs differentiation (68). Recently, *in vivo* deletion experiments in mouse AML models demonstrated that CSCs depend on G9a histone methyltransferase activity to maintain self-renewal and blockage of differentiation, whereas G9a is not essential for the function of long-term repopulating hematopoietic stem cells (31).

Two potent G9a inhibitors were reported in preclinical literature. Both BIX01294 and UNC-0638 were shown to robustly reduce the abundance of the H3K9me2 mark, with variable toxicity *in vitro* (Fig. 1B; refs. 64, 69, 70). Currently, little is known about the effects of these small molecules on Wnt–GSK3 activity and cancer metabolism (FBP1 expression, serine–glycine synthesis, and ROS levels; refs. 20, 71). However, treatment of human primary AML cells using UNC-0638 has shown clonal growth inhibition and increased differentiation to the mast cell lineage (31). Developing novel, CSC-targeting G9a inhibitory small molecules should be sought to better focus therapies at CSCs versus normal stem cells that remain in the patient. An important milestone has recently been reached with the development of an *in vivo* suitable G9a inhibitor, UNC-0642, which displays enhanced pharmacokinetic properties compared with BIX01294 and UNC-0638 (Fig. 1B; ref. 72). The uses of UNC-0642 in xenograft tumor models will allow further investigation on the impact of G9a inhibition in CSCs versus non–stem cancer cells, which will give insights into the CSC-specific aspect of such a mechanism.

Given the heterogeneous nature and genetic/epigenetic variegation of clonal architecture within tumors and leukemia (73), targeting multiple pathways with unique molecules/drugs is likely required to eradicate CSCs and the resultant disease. Recently, combination therapies using molecules thought to modulate epigenetic regulators are showing promise in clinical trials, and demonstrated alterations of histone and/or DNA methylation status in patient CSCs is now getting clearer (24). However, the variability in CSC cell surface phenotypes (74) remains a challenge toward prospective isolation of pure CSC populations for detailed downstream epigenetic characterization. Deliberate functional studies will become necessary to determine to what extent epigenetic-modifying agents target CSCs in dissociable ways relative to non-CSCs in a tumor, and to carefully resolve differential effects relative to normal stem cells that may still share overlapping genetic and epigenetic properties. This further highlights the importance of future efforts in dissecting the molecular pathways that are deregulated in CSCs specific to disease pathogenesis and evolution for designing effective cancer therapies in patients.

## Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

## Authors' Contributions

**Conception and design:** Y.D. Benoit, B. Guezguez, M. Bhatia
**Writing, review, and/or revision of the manuscript:** Y.D. Benoit, B. Guezguez, A.L. Boyd, M. Bhatia

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