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Epigenetic regulation of WNT signaling in chronic lymphocytic leukemia

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Abstract

Certain WNT and WNT network target genes are expressed at higher or lower levels in chronic lymphocytic leukemia compared with normal B-cells. This includes upregulation of nuclear complex genes, as well as genes for cytoplasmic proteins and WNT ligands and their cognate receptors. In addition, epigenetic silencing of several negative regulators of the WNT pathway have been identified. The balance between epigenetic downregulation of negative effector genes and increased expression of positive effector genes demonstrate that the epigenetic downregulation of WNT antagonists is one mechanism, perhaps the main mechanism, that is permissive to active WNT signaling in chronic lymphocytic leukemia. Moreover, constitutive activation of the WNT network and target genes is likely to impact on additional interacting signaling pathways. Based on published studies, we propose a model of WNT signaling that involves mainly permissive expression, and sometimes overexpression, of positive effectors and downregulation of negative regulators in the network. In this model, DNA methylation, histone modifications and altered expression of microRNA molecules interact to allow continuous WNT signaling.

Keywords

chronic lymphocytic leukemia; DNA methylation; epigenetics; histone modification; microRNAs; WNT pathway

Chronic lymphocytic leukemia

B-cell chronic lymphocytic leukemia (CLL) has been considered a single disease that occurred mainly in the elderly and underwent a variable but generally indolent clinical course (reviewed in [1,2]). It is now clear that CLL is a more heterogeneous disease with at least two major subtypes in terms of cellular proliferation, clinical aggressiveness and prognosis. Rising cell counts in patients with CLL could be the result of increased production, decreased cell death or both events to some degree. Circulating peripheral blood CLL cells comprise what is sometimes referred to as the accumulation compartment. However, there is also a proliferation compartment comprised of prolymphocytes and paraimmunoblasts that together form pseudofollicular proliferation centers (PCs) in the bone marrow (BM) and to a lesser extent in lymph nodes. These are a hallmark of CLL as other lymphoid malignancies generally do not contain PCs. The size and number of these correlates with the lymphocyte doubling time, a

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surrogate of clinical aggressiveness. Even though CLL cells proliferate at a slower rate than normal B cells [3], they do still proliferate in PCs and this then contributes to the accumulation compartment. There is considerable intra- and inter-clonal heterogeneity in the proliferative rate of CLL cells, with newly-produced CD38⁺ cells dividing about twice as rapidly as CD38⁻ cells [4]. While CLL cells accumulate *in vivo*, they rapidly undergo spontaneous apoptosis *in vitro*, implying that their accumulation, and perhaps apoptotic resistance, is likely to depend upon external signals, as supported by multiple studies [2,5]. This is not surprising considering that development of normal B-cells is also dependent upon microenvironmental signals. Most or all cases of CLL seem to arise from antigen-experienced and activated B-cells and have gene-expression profiles similar to memory B-cells [2]. It is still not entirely known why CLL proliferates more or less rapidly among patients, although recently described *in vitro* model systems may provide tools to investigate this issue [6,7].

The biological behavior of CLL is also affected by other cell types and the microenvironment. Within the PCs, CLL cells are supported and their growth is partly controlled by signals from interspersed T-cells and stromal cells. These stromal cells are thought to contribute to the long-term support of CLL cells at least in part through interactions of WNT proteins (and others) produced by both the stromal and CLL cells [8,9]. Recent evidence points to the fact that interactions between CLL and marrow stromal cells is bidirectional [7]. There is mounting evidence that a functional and delicately balanced WNT signaling network may dictate whether cells proliferate or instead undergo apoptosis. Therefore, this pathway may be quite important in CLL, but other signaling pathways undoubtedly also contribute to aberrant cellular behaviors.

Homing and/or retention of CLL to the BM is mediated by the chemokine receptor CXCR4, which interacts with CXCL12-expressing stromal cells that also produce ligands for additional adhesion molecules and WNT-related proteins [10]. Activation of CXCR4 prolongs CLL cell survival, indicating that chemokine receptor activation not only attracts CLL cells, but along with secretion of additional molecules, directly affects their biological fate.

Chromosomal abnormalities and genetic alterations are common in CLL, but thus far it is not clear if they might be the cause rather than an effect of CLL development. There is now strong evidence that genetic and also epigenetic mechanisms contribute prominently to this disease [11–14]. For instance, certain microRNA (miR) genes reside in some of these regions of chromosomal genetic alterations and their functional status also affects CLL biology [15–19].

Many of the earlier research publications have, out of necessity, discussed genetic and epigenetic alterations at a single gene or single modification level. However, the international research community is now poised to explore the genome and epigenome at a systems level whereby many or all of the currently known biological modifications in CLL are brought together to model an entire virtual cell. This has been well addressed in recent publications [16,20–26] and has become a major Roadmap Initiative at the US NIH. Systems approaches have the capacity to explore individual or sets of signaling pathways, chromosomal regions, or the entire epigenome using modern technologies. This review will focus on epigenetic alterations involving the dysregulated WNT signaling network in normal and malignant cells as it pertains to CLL and potential crosstalk with certain other important pathways in this disease.

WNT signaling network

The WNT proteins can activate at least three main branches of this network of signaling molecules: the noncanonical planar cell pathway, which regulates cytoskeletal interactions and cellular polarity, a second noncanonical pathway, the WNT/Ca⁺⁺ pathway, which leads to activation of protein kinase C and calcium-calmodulin dependent protein kinase II, and finally

the canonical WNT/ β -catenin branch. Activation of the noncanonical pathways is β -catenin independent, while the canonical pathway is mediated by β -catenin, which is encoded by *CTNNB1*.

In the canonical WNT/ β -catenin pathway, the proto-oncogene β -catenin can switch between at least two different intracellular pools. In the presence of certain WNT proteins, a series of intracellular events leads to the stabilization of β -catenin and its translocation into the nucleus where it forms complexes with a number of partner proteins ultimately leading to transcriptional activation of many target genes. These WNT target genes are likely to be cell-type and context specific [27]. In general, pathway activation is thought to begin with binding of WNT glycoproteins to frizzled (FZD) transmembrane receptors that subsequently heterodimerize at the cell surface with the low-density lipoprotein receptor-related proteins 5 and 6 (LRP5/6). This then leads to inhibition of GSK3 β and stabilization of β -catenin, which can then translocate into the nucleus through actions of PYGO and BCL9, as well as other binding partners [28]. Here, it participates in a complex including the transcriptional factors lymphoid enhancing factor 1 (LEF1)/T-cell factor (TCF) to facilitate activation of many WNT target genes that can then also affect other signaling pathways [29]. To add to this complexity, isoforms of LEF1 may regulate different sets of target genes [30]. WNT signaling through β -catenin mediates certain of its transcriptional effects through interactions with chromatin-modifying enzymes and this process is largely responsible for target gene activation (reviewed in [31]).

Conversely, in the absence of WNT ligands, β -catenin forms a stable complex with E-cadherin (*CDH1*) and excess β -catenin is also bound by a destruction complex or supercomplex comprised of the COP9 signalosome [32,33]. The destruction complex contains GSK3 β , AXIN, protein phosphatase 2A activator, regulatory subunit 4 (*PPP2R4*; formerly known as PP2A), casein kinase 1, α 1 (*CSNK1A1*; formerly known as CK1 α) and adenomatosis polyposis coli (*APC*), which work together to ensure that β -catenin becomes phosphorylated and ubiquitinated by β -transducin repeat containing (BTRC; formerly known as β -TrCP) and subsequently targeted for proteasome-mediated degradation (reviewed in [33]). The COP9 signalosome has at least two important functions in the regulation of WNT signaling; balancing β -catenin degradation by deneddylation and protection of APC from ubiquitin-specific protease-mediated degradation [32]. The balance between β -catenin and APC seems critical to avoid malignant transformation.

In the noncanonical planar cell pathway, certain transmembrane proteins including receptor tyrosine kinase-like orphan receptor 1 (ROR1), or 2 (ROR2), and RYK receptor tyrosine kinase can also bind certain WNT ligands and other molecules [34,35], and DVL via disheveled-associated activator of morphogenesis 1 (DAAM1), is connected to downstream effectors including the small GTPase *RHOA* and JUN kinase (*JNK*) to control cell polarity and movement. Activation requires DVL–*RHOA* complex formation, and assembly mediated by DAAM1, which functions as a scaffolding protein. The WNT/ Ca^{++} pathway participates in the release of intracellular Ca^{++} and activation of phospholipase C (PLC) and protein kinase C (PKC). Then, Ca^{++} can also activate calmodulin-dependent protein kinase C (CaMKII), and Nemo-like kinase (NLK), which can ultimately act to suppress canonical WNT signaling.

There are more than 100 genes involved in WNT signaling, and there is considerable recent evidence that all these genes and pathways may be active and interact with each other in a ‘WNT signaling network’ (reviewed in [36,37]). Thus, there is a move away from the concept of linear pathways to that of feedback loops and signaling cascades that involve more than a single pathway [36]. However, even by examining a few target genes, we can also gain some insights into other pathways that may be affected beyond WNT.

Pathway crosstalk

The concept of a sequential linear cassette of genes comprising a signaling pathway has been challenged by evidence that signaling networks are comprised of multiple interacting regulatory pathways, several of which involve the WNT network [37–39]. As described in an excellent review [39], GSK3 β is one central regulator that resides at a junction between the PI3K/AKT and WNT pathways and also interacts directly with certain nuclear receptor proteins, such as the androgen receptor (AR) and glucocorticoid receptor (GR), which are both active transcription factors in many additional pathways. The AKT pathway is activated in CLL and delivers pro-survival signals [40]. Both GSK3 β and CSNK1A1 have also been demonstrated to be active in NF- κ B activation [41] and activation of the sonic hedgehog (SHH) pathway (reviewed in [38]). Conversely, many nuclear receptor proteins and their agonists can promote transrepression of WNT signaling [39].

The B-cell receptor (BCR) signaling system is a key component of all B-cells, but is dysregulated in CLL, and is likely to contribute to the underlying biology, in part through interaction with downstream pathways that are also used by WNT signaling pathways [42]. BCR signaling affects production of β -catenin via PKC-mediated inhibition of GSK3 β , thus linking these important pathways through this central molecule [43]. Beyond BCR signaling, an increasing body of knowledge suggests that the apoptotic block in CLL may be related to additional activated pathways, such as the NF- κ B pathway and the NOTCH pathway, which then effect expression of anti-apoptotic proteins such as BCL-2 and XIAP [44,45]. One link between these and other dysregulated pathways is through GSK3 β [46,47,38].

Crosstalk between WNT and TGF β pathways has been extensively studied [48]. WNT and TGF β proteins can reciprocally regulate production of their reciprocal ligands that then creates a level of interdependence. In the nucleus, the SMAD/ β -catenin/LEF1 protein complex regulates a host of shared target genes.

This multilevel paradigm also holds true for the crosstalk between other pathways such as WNT, SHH and NOTCH in many types of cancer, including CLL [49–52]. Other than WNT, both the SHH and NOTCH pathways are constitutively active in CLL [45,51,53]. For example, the transducin-like enhancer co-repressor proteins are involved in many pathways and interact with transcription partners including TCF/LEF1 in the WNT pathway and Hairy/Enhancer of Split 1 (HES1) in the NOTCH pathway. In CLL, NOTCH1 and NOTCH2, along with their ligands (JAGGED1 and JAGGED2) are overexpressed in a manner that suggests autocrine/paracrine mechanisms [45]. Gene-expression profiling of CLL cells indicates that the expression of SHH signaling molecules, such as GLI1, GLI2, SUFU and BCL2, is significantly increased and correlates with disease progression and clinical outcome [53]. Further, expression of WNT2B and WNT5A may be regulated through SHH and NOTCH signals [52,54]. Thus, in cases where lymphoid malignancies demonstrate evidence of epigenetic repression of *TLE* genes through DNA methylation [55], there may be effects involving both the WNT and NOTCH pathways.

Lymphopoiesis in the bone marrow

The BM microenvironment is a complex arrangement of lymphohematopoietic and accessory cells that provide cell–cell contacts and production of secretory molecules that provide structural and functional support for developing cells [56]. WNT proteins are produced by BM stromal cells, also known as BM derived mesenchymal stem cells (MSC) that can differentiate into multiple cell types. Expression of MSC mRNA for various *WNT* genes has been reported by multiple groups with somewhat variable results [8,9,57,58]. One consistent finding was expression of WNT5A, and absence of WNT8A, 8B and 9B. A recent investigation of 19 WNT genes in human BM stromal cells provided insights into potential age- and gender-related

difference that may have biological importance into the reasons for gender bias in certain disease states [9]. Primary stromal cells from low-passage cultures demonstrated expression of 12 of the 19 *WNT* genes studied (*WNT2*, 3, 4, 5A, 5B, 6, 7B, 10B, 11, 13, 14 and 16) with absence of *WNT1*, 3A, 7A, 8A, 8B, 10A and 15 across all samples tested. There also appeared to be a few age- and gender-related differences in expression. Dosen *et al.*, reported stromal cell expression of *WNT2B*, 5A, 5B, 8B and 9B, as well as *FZD3*, 4 and 6, *DKK1*, and *SFRP2* and 3 [8]. Whether any of these studies in primary stromal cell cultures truly recapitulates the *in situ* representation in the BM microenvironment awaits further investigations. A recent study describes interactions between MSC and CLL cells and demonstrates that cellular signaling is interdependent [7]. All these studies reinforce the prominent role of stromal cells in the microenvironment and in WNT signaling within the BM.

In the context of human B-cell lymphopoiesis, the WNT pathway(s) take on an important role [8]. *WNT3A* does not affect apoptosis, but does inhibit stromal cell-dependent initial divisions of B progenitor cells *in vitro* thus suggesting some control over the early B-cell compartment in the BM. In a study of CD10⁺ BM B-cells involving five stages of development, there were clear patterns of gain and loss of certain WNT proteins at each stage of maturation on the B-cells themselves, as well as on normal BM stromal cells. This points to a regulatory network in early differentiation that is as yet not entirely understood. The most mature cell type studied was just prior to the so-called naive B-cell that co-expresses IgD and IgM and that some believe is a candidate normal counterpart from which CLL derives [59].

Another B-cell WNT-related function involves *WNT5A* signaling through noncanonical pathways, but this is thought to negatively regulate B-cell proliferation, possibly through direct downregulation of *CCND1* [60]. Deletion of *WNT5A* results in B-cell lymphomas in both mice and humans, thus suggesting its function as a tumor suppressor gene. Interestingly, *WNT5A* can also inhibit *WNT3A* function in a GSK3 β - and β -catenin-independent manner [61]. Recently, the receptor tyrosine kinase-like orphan receptor 1 (ROR1), a transmembrane receptor aberrantly expressed in CLL, has been demonstrated to bind *WNT5A*, and possibly additional ligands to stimulate WNT signaling [34,35]. Upon LRP5/LRP6-independent binding of *WNT5A* by ROR1, NF- κ B signaling is induced in a noncanonical pathway that apparently fails to activate reporters for LEF/TCF, nuclear factor of activated T-cells (NFAT), or activator protein-1 (AP-1) [35]. While the regulation of ROR1 is currently not understood (and may not be epigenetic), its overexpression is characteristic of CLL malignancy and it is likely to have multiple effects with respect to WNT signaling. These complex interactions require further study to understand not only WNT-related normal lymphopoiesis, but also neoplastic lymphoid development.

Illegitimate WNT signaling in lymphoid malignancies

The WNT signaling network is dysregulated in many types of cancer, including CLL [62]. Owing to the large number of genes involved, and also a level of functional redundancy, there is a myriad of interactions that can occur (reviewed in [63]). Therefore, the precise role of WNT/ β -catenin signals or of noncanonical signaling, in regulation of CLL growth is not yet entirely understood. Nevertheless, certain proteins are considered WNT signaling agonists, while others function mainly as antagonists, and it is the overall balanced inputs of these proteins that comprise the functional state of the signaling network. In addition, certain of the agonistic WNT proteins were previously thought to act either in the canonical or noncanonical pathways, but it is more likely that all WNT pathways can be activated by multiple WNT proteins and can interact with each other.

Early agonistic events are negatively regulated by antagonist proteins, including WNT inhibitory factor (WIF)1, as well as members of the DICKKOPF (DKK) and soluble frizzled

protein (SFRP) families. The DKK proteins antagonize signaling through binding to LRP5/LRP6, whereas WNT inhibitory factor 1 (*WIF1*) and SFRPs interact directly with WNT proteins to buffer their activity and binding to FZD receptors. Individual SFRPs are expressed in a partially overlapping manner that might be complementary to the expression of certain WNT proteins, suggesting some functional redundancy in their activity as inhibitors [64].

Another group of potential negative regulators is the SOX family [65]. Sinner *et al.* reported a novel mechanism by which this family of transcription factors regulates WNT signaling [66]. Some SOX proteins antagonize, while others enhance, β -catenin/TCF activity. While SOX17 promotes degradation of both β -catenin and TCF proteins through a GSK3 β -independent mechanism, SOX4 may function to stabilize β -catenin protein. Thus, SOX proteins are likely to act as both antagonists and agonists of β -catenin/TCF activity, but these responses may depend on cellular context. A recent study concluded that SOX9 inhibited β -catenin/TCF-dependent transcription and promoted β -catenin degradation by two separate mechanisms involving different domains of SOX9 [67]. The N-terminus and high-mobility group domain were required for β -catenin degradation, whereas the C-terminus was required to repress β -catenin-stimulated transcription. SOX9 associated with components of the cytoplasmic destruction complex, but then also translocated the complex into the nucleus where it phosphorylated and degraded nuclear β -catenin, a process normally thought to occur in the cytoplasm. The transducin-like enhancers of split proteins function as transcriptional co-repressors of LEF1 and this function requires histone deacetylase (HDAC) activity [68]. Depending on the isoform of LEF1 that is expressed, different sets of WNT target genes may be activated [30]. Regulation of these isoforms depends, at least in part, on interactions with β -catenin and *PITX2*, a gene commonly affected by DNA methylation in multiple tumor types [69].

In one study, mRNA from CLL revealed that six of the 19 WNT genes examined (*WNT3*, *WNT5b*, *WNT6*, *WNT10a*, *WNT14* and *WNT16*), as well as the WNT receptor *FZD3* and the coreceptors *LRP5* and *LRP6*, were all highly expressed in human CLL when compared with normal B-cells [62]. The increased expression of *WNT3* was also reported as a signature gene in an earlier microarray-based study [70]. The mRNA of the transcription factor *LEF-1* and its downstream target *CCND1* were also overexpressed. Interestingly, *WNT3*, *WNT5B* and *WNT14* mRNA levels were higher in the subtype of CLL demonstrating unmutated IgVH. These results indicate that some agonistic WNT signaling genes are overexpressed in CLL cells, but did not address the role(s) of agonistic WNT proteins produced by BM stromal cells. It remains to be seen if the protein levels are also increased and/or functional.

The D-type cyclins (*CCND1*, *CCND2* and *CCND3*) are targets of WNT signaling and are also highly expressed in CLL, the magnitude of expression was *CCND3* higher than *CCND2*, which, in turn, was higher than *CCND1* [71]. The mean *CCND1* and *CCND3* mRNA levels were reported to be four- to six-fold higher in CLL cells than in normal B-cells, but this has been contradicted by others [72]. Both *CCND1* and *CCND3* were overexpressed in CLL cells of patients; higher levels are more common in low-stage disease with prolonged survival [71].

Using WNT signaling quantitative RT-PCR arrays (the human WNT signaling pathway RT2 profiler™ PCR array [SABiosciences, MD, USA]), which measure mRNA levels of 84 genes involved in WNT signaling or targets of the WNT pathway, our laboratory has studied expression in normal B-cells and CLL cells. We confirmed the results above, but also found that in general, most of the positive effectors (agonists) that are expressed at measurable levels in normal B-cells and several target genes were expressed in both cell types or upregulated in CLL. Conversely, many genes that have negative effector functions were downregulated

[RAHMATPANAH FB ET AL., MANUSCRIPT SUBMITTED].

In a less studied area, one noncanonical pathway that might use the overexpressed ROR1 WNT receptor [73], PI3K signaling in CLL clearly demonstrates constitutive activation of AKT signaling and decreased activation of GSK3 β through phosphorylation [40]. Within the same pathway, certain isoforms of PKC are overexpressed and support cellular survival [74]. It is also known that the constitutively activated BCR signaling in CLL uses some of these same downstream signaling pathways, and therefore any defects could affect multiple signaling processes.

Additional noncanonical WNT signaling can result in RAC or RHO activation, dependent on DVL activity. These play fundamental roles in numerous cellular processes initiated by extracellular stimuli working through G-protein coupled receptors (or certain WNTs) and is involved in control of cytoskeletal organization. Generally, RAC activation leads to MAPK and JNK signaling downstream and cell survival, while RHO signaling results in activation of ROCK and cytoskeletal reorganization. Within the RHO pathway, RHOA is kept in an inactive or active state depending on the balance between RHO guanine nucleotide exchange factor (ARHGEF) proteins and RHO GTPase activating proteins (ARHGAP) that determine the balance between GDP and GTP. *ARHGEF5* is one member of the ARHGEF family that we have previously found to be methylated in CLL [12]. Related proteins include the ARHGAP group that counterbalance ARHGEF proteins. *ARHGAP7*, now known as deleted in liver cancer 1 (*DLC-1*), helps maintain an inactive state of RHOA (and related members) proteins through converting GTP to GDP and contains a GAP domain specific for the small GTPases RHOA, RHOB, RHOC and to a lesser extent CDC42 [75]. We have previously reported DNA methylation involving *DLC-1* [12,76]. *DLC1* is thought to be involved in the remodeling of the actin cytoskeleton by local suppression of active Rho proteins and controls directed cell migration through a Dial-dependent pathway [77]. Therefore, the methylation of this gene, as well as an *ARHGEF* gene, may have functional effects on cell migration and cytoskeletal organization in CLL. This, and the WNT/Ca⁺⁺ pathway, may also be affected via binding of lysophosphatidic acid (LPA) to cognate G-coupled receptors (LPA₁₋₅). This produces cell proliferation and increased intracellular calcium in B-cells and leads to activation of the MAPK and JNK pathways and transcription factors such as NF- κ B and AP-1 and promotes cell survival through inhibition of apoptosis in CLL cells [78,79]. CLL cells are also known to have aberrantly increased expression of the LPA₁ receptor [78]. One mechanism for this is through LPA induction of HDAC1 and reduction in histone acetyltransferase activity that in combination lead to reduced histone acetylation [80]. Thus, stimulatory signaling through a noncanonical WNT pathway can also have additional epigenetic manifestations through alterations of histone acetylation.

DNA methylation

Studies of DNA methylation in CLL have demonstrated that many genes do demonstrate aberrant global hypomethylation and regulatory region hypermethylation. Few large-scale discovery studies have been reported [11,14,76,81], but these have been useful to identify genes that contain DNA methylation in regulatory regions, particularly those that have been confirmed by independent methods. However, when compared with other lymphomas, CLL demonstrates remarkably fewer methylated genes than follicular lymphoma, another low-grade lymphoma [11,76,81,82]. This may well be a reflection of the microenvironment where follicular lymphoma arises in germinal centers of lymph nodes, an area known to undergo rapid DNA strand breaks and attempted repairs, although not all are faithfully repaired. This then may lead to accumulation of abasic sites, uracil misincorporation or other aberrant attempts at repair. All these are candidate sites for increased DNA methylation. Nevertheless, aberrant DNA methylation of a small number of WNT-related genes has been reported in CLL [13, 83–86], and our group recently reported results from DNA methylation microarray experiments

that demonstrated an over-representation of hypermethylated WNT pathway genes that are mainly negative effectors of WNT signaling [12].

Methylation of DNA in humans is generally thought to take place via some combination of three DNA methyltransferase enzymes (DNMT1, 3A and 3B). Whereas DNMT1 has been mainly considered responsible for maintenance of normal methylation patterns, DNMT3A and 3B are mainly involved in *de novo* methylation. Large-scale studies of DNA methylation across the genome have demonstrated that many genes demonstrate evidence of DNA methylation within regulatory regions of genes in a tissue-specific manner. However, there is also strong evidence for aberrant DNA methylation in virtually every tumor type studied, but in a generally nonrandom manner that differs between tumor types. Just how the methylation is directed and controlled is still a largely unanswered question. Polycomb group proteins are likely to be key in marking certain genes for DNA methylation and formation of repressive chromatin by altering histone methylation, but the mechanisms are not completely elucidated [87–92]. These proteins also function jointly with noncoding RNAs to control certain genes [93]. For instance, in bladder cancer, miR-101 represses the polycomb group protein EZH2, which acts through trimethylation of histone H27 to create repressive chromatin [94]. As discussed in more detail below, a class of noncoding RNAs, the miRNAs, are also likely modulators of DNA methylation. For instance, miR-143 has been demonstrated to control DNMT3A in colorectal cancer [95], the miR-29 family regulates DNMT3A and 3B in lung cancer [96], and miR-29b has also recently been demonstrated to control all three DNA methyltransferase genes either directly or indirectly in acute myeloid leukemia [97]. The miRs-29a and -29c are reportedly increased in CLL, but miR-29c is also reportedly downregulated in cases of CLL with TP53 abnormalities [19,98]. Therefore, this is an area in need of further exploration.

Within the group of WNT pathway transmembrane signaling genes, none of the agonistic *WNT* (except *WNT7A*) or *FZD* genes demonstrated any direct evidence of DNA methylation [12]. However, the *SFRP* family is generally thought to antagonize WNT/FZD interactions and earlier studies, as well as our own, have reported DNA methylation involving *SFRP1*, *SFRP2*, *SFRP4* and *SFRP5* in CLL; *SFRP3* has no CpG island and was not tested [11,13,86]. Another inhibitory gene, *WIF1*, was methylated in only approximately 12% of CLL patients [83].

The APC protein serves a critical function in the destruction complex, and since our microarrays suggested the presence of DNA methylation, we examined DNA fragments of APC2 by combined bisulfite restriction analysis (COBRA) in a CpG island located toward the 3' end and one located in the 5' coding region [12]. DNA methylation of the 3' CG was present in 25 out of 36 CLL patients, as well as CLL cell lines. The coding region CpG island demonstrated a consistent pattern of methylation across all the cases examined. Thus, the role of DNA methylation on APC/APC2 expression needs further examination.

With respect to WNT activity in the nucleus, SOX–WNT interactions can regulate a variety of processes [99]. Moreover the same SOX protein, such as SOX9 appears to employ different mechanisms in different contexts in the nucleus and the cytoplasm [67]. Whether these reflect distinct cell-specific functions or different aspects of a common underlying mechanism is not yet known. However, *SOX* gene expression is dysregulated in a wide variety of human cancers, and there is evidence that SOX factors impact tumorigenesis by modulating β -catenin/TCF activity and the expression of oncogenic WNT target genes such as *CCND1* and *MYC* [65, 99].

While we have not yet examined all 20 genes from this family, validation of 6 *SOX* members using COBRA revealed methylation of *SOX1* in 65%, *SOX3* in 100%, *SOX4* in 77%, *SOX9* in 21%, *SOX11* in 100% and *SOX17* in 50% of the 48 primary CLL samples [12]. Given this high

rate of methylation and the complexity of SOX functions, more investigation is required in order to fully understand the overall impact of these epigenetic alterations. It is possible that with such a large family, there may be compensatory processes that will blunt these observed methylation changes.

Recent unpublished experiments using the COBRA method in our laboratory have also demonstrated methylation within CpG islands of *CDH1* and *NLK*. *CDH1* is a negative regulator that functions by sequestering β -catenin at the cell membrane and has been reportedly methylated by others [86], and mutations of the gene that result in alternative transcripts have also been reported [100]. *NLK* provides feedback to negatively regulate the canonical pathway via the Ca^{++} /WNT arm and therefore also functions as a negative regulator of WNT signaling. Additionally, as described above, we have found DNA methylation of two additional noncanonical WNT-related genes that affect RHOA activation; *ARHGEF5* and *DLC-1* [12, 76].

The human *TERT* encodes the telomerase enzyme that is elevated in some cases of CLL, and this correlates with clinical outcomes [101]. It has also been reported that *TERT* is methylated in CLL and this leads to downregulation [102]. Therefore, it is likely that differential expression of this protein has some impact on underlying cell biology in CLL. Telomerase has recently been demonstrated to play an important role in modulating WNT signaling through associations with downstream target gene chromatin [103].

Overall, it appears that in CLL, most of the WNT-related genes that have demonstrated evidence of DNA methylation are those involved in negative regulation rather than positive effector functions, thus suggesting an effect on creating a permissive signaling environment that perhaps helps explain a role for DNA methylation in the observed constitutive activation of the network. This has also been observed in many, but not all, other cancers (reviewed in [104]).

Histone modifications in chronic lymphocytic leukemia

In normal cells, histone acetyltransferases (HATs) add acetyl groups to residues on histone tails that leads to a relaxation of the chromatin structure, allowing gene transcription to occur. HAT activity is counteracted by HDACs, enzymes that remove the acetyl groups leading to compaction of nucleosomes and transcriptional downregulation. Inhibitors of HDACs (HDACi) are thought to maintain chromatin in an open structure and are therefore potential pharmacologic agents because many tumors overexpress HDACs and this results in suppression of gene transcription (reviewed in [105]). HDACi also have effects that are independent of histone deacetylation, including acetylation of nonhistone proteins such as p53, Rb and hsp90, induction of p21 with consequent cell-cycle arrest, and generation of oxidative stress. Several different HDACs have been identified, and HDACi generally target one or more of these molecules, depending upon their structure.

The modifications that can occur in histone proteins are many [16,106], and a number of genome-wide studies have identified combinatorial patterns of histone modifications that may correlate with gene structures and functions [107–110]. These studies have mainly addressed the processes of acetylation or methylation at lysine (K) or arginine (R) residues in histone protein tails. Generally, trimethylation of histone H3K4 is associated with active transcription, while that of H3K27 is most commonly associated with downregulation, although both marks can appear simultaneously on bivalent gene promoters that may be poised for rapid expression or downregulation.

The mechanism of apoptotic resistance in CLL is complex and involves expression of anti-apoptotic proteins including GSK3 β that positively regulates NF- κ B-mediated gene

transcription and cell survival, as well as alterations that induce downregulation of normally apoptotic effects through p73. In CLL, both GSK3 β and NF- κ B accumulate in the nucleus, and pharmacologic inhibition of GSK3 β results in decreased expression of the NF- κ B target genes *BCL-2* and *XIAP* by abrogating NF- κ B binding to target gene promoters, in part through epigenetic modification of histones [44]. A substantial increase in methylation of H3K9, H3K27 and H4K20, all of which are repressive modifications, was found in these target gene promoters. Through another mechanism, the promoter region of a miR gene (miR-106b) is repressed via histone deacetylation, which results in decreased expression of the proapoptotic protein p73, but this can be pharmacologically reversed and ultimately induce apoptosis in CLL cells [111].

It is now well accepted that DNA methylation and histone modifications work in concert to affect gene transcription. In a study of WNT target genes and promoter occupancy by TCF family members in various cell types [112], WNT-responsive promoters were bound by specific subsets of TCFs, whereas the unresponsive promoters were not, and this was independent of the cell type. The TCF-bound WNT-responsive promoters demonstrated hypomethylation of DNA, hyperacetylation of histone H3 and trimethylation of histone H3K4, all of which are associated with active transcription. The inactive promoters demonstrated the opposite pattern. Interestingly, the actual chromatin state was independent of WNT pathway activity.

Another important mechanism within the nucleus involves PYGO family members and the adaptor protein BCL9 that function jointly to promote the transcriptional activity of β -catenin in normal and malignant cells. Human PYGO PHD fingers associate with HD1 domains in BCL9 to specifically bind histone H3 methylated at lysine 4 (H3K4me) [113]. This involves efficient histone binding via the HD1 association, wherein the PHD–HD1 complex binds preferentially to H3K4me2 while displaying insensitivity to methylation of H3R2. This is a prime example of histone tail binding by a PHD finger (of PYGO) being modulated by a cofactor (BCL9). In mammary epithelial cells, PYGO2 promotes WNT signaling by recognition of the methylated histones and the recruitment of β -catenin-BCL9 complexes to the promoters of β -catenin target genes [114]. This mechanism has not been reported in CLL, but is surely one worthy of further investigation.

Certain epigenetic modifications of chromatin by protein arginine methyltransferases (PRMTs) are also crucial for normal cell growth and differentiation. The mechanisms controlling this epigenetic process are not as well understood compared with lysine methylation. The SWI/SNF-associated protein PRMT5 transcriptionally represses its target genes by methylating histone 3 at arginine 8 (H3R8) and histone 4 at arginine 3 (H4R3) [115]. PRMT5 protein levels are increased in CLL cell lines secondary to the altered expression of PRMT5-specific miRs 19a, 25, 32, 92, 92b and 96 (discussed below) that results in an increase of global symmetric methylation of H3R8 and H4R3. An evaluation of both epigenetic marks at PRMT5 target genes such as cell cycle pocket proteins RB1 (p105), RBL1 (p107) and RBL2 (p130) demonstrated that at their promoters H3R8 and H4R3 are hypermethylated, which then leads to transcriptional repression. These proteins also interact with the WNT network via cell-cycle control. Thus, miR-induced PRMT5 overexpression epigenetically alters the transcription of key target genes and suggests a causal role of the elevated symmetric methylation of H3R8 and H4R3 at target gene promoters in CLL. Although not reported in studies of CLL, using the human β -globin locus as a model, symmetric dimethylation of H4R3me2 by PRMT5 is required for subsequent DNA methylation [115]. H4R3me2 is a direct binding target for DNMT3A, which interacts through the ADD domain containing the PHD motif. Loss of the H4R3me2 mark leads to reduced DNMT3A binding, loss of DNA methylation and gene activation. Thus, it was proposed that DNMT3A functions as both a reader and a writer of

repressive epigenetic marks at this locus, thereby directly linking histone and DNA methylation in gene silencing.

MicroRNA in chronic lymphocytic leukemia

Small RNA molecules, referred to as miR, act as translational repressors of many mRNAs and each may affect hundreds of genes [116]. MiRs are processed after transcription by Drosha and Dicer enzymes and ultimately associate with an RNA-induced silencing complex (RISC) that binds target mRNAs through partially complementary sequences that then reduce their translation or stability. Excellent reviews are available that discuss the multiple aspects and interacting networks of miRs and their multitude of putative targets [117,118] and much more information can be gleaned from the public miRBase database [201], as well as from others including TarBase and DIANA-mirPath [118,119].

Global underexpression, but some specific overexpression, of miRs is a characteristic of CLL [120]. This is a rapidly expanding research area where new functional aspects of miR dysregulation in cancers is continually being reported. Thus, we offer up only a few examples pertinent to CLL and WNT signaling. Recently, miR-8 has been reported to negatively regulate the WNT network at multiple levels, but investigations of this miR in CLL have not been reported [121]. By contrast, a functional screen identified miR-315 as a potent activator of WNT signaling [122]. These findings should stimulate similar studies in mammalian systems and in cancers such as CLL that demonstrate dysregulation of the WNT network.

Expression profiling has identified significant differences in the patterns of miR expression between normal B-cells and CLL cells; some specifically target WNT network or WNT target genes, while others target other gene sets. MiR profiling of naive, germinal center and memory subsets of B-cells from lymphoid tissues revealed that different sets of miRs are preferentially involved at different stages of cellular differentiation and found some similarities with CLL [123]. For instance, miR-150 is expressed at a high level in CLL cells, but not in their proliferation centers. This is consistent with the patterns in normal tissues whereby miR-150 is high in naive and memory B-cells, but low in germinal center cells. It is not currently known if miR-150 is an active component of WNT network regulation, either directly or indirectly.

Human miR genes are distributed throughout the genome in a nonrandom manner and are frequently located in chromosomal fragile sites and cancer-associated genomic regions where they may act as molecular switches (reviewed in [124,125]). As with other coding genes, miR genes have specific functions, and are also susceptible to epigenetic modifications such as DNA hyper- and hypomethylation [16,126]. Some miRs play a fairly direct role in chromatin modification by targeting post-transcriptional regulation of key enzymes. For instance, the miR-29 family targets the three main DNA methyltransferases of humans either directly or indirectly in lung cancer and acute myeloid leukemias [97,96]. These miRs directly target the 3' UTR of DNMT3A and DNMT3B, but affect DNMT1 indirectly by targeting SP1, which functions as a transactivator of this enzyme gene. Whether this is also true in CLL remains to be examined, but miR29a, b and c are reportedly increased in CLL compared with normal B-cells [98]. The level of DNMT1 in CLL is similar to that of normal B-cells, but DNMT3B is decreased [127]. These enzymes are clearly important for DNA methylation in all cancers including CLL, and of the WNT network genes. One of the hallmarks of CLL is global genomic hypomethylation, which then suggests further research regarding miR-29 in CLL [128].

Following the initial description of altered miR-15a and miR-16-1 in CLL by Calin *et al.* [129], further studies by this group defined a signature composed of 13 miRs that could discriminate between different prognostic groups with unmutated and mutated *IgVH* gene cases [130]. It is also clear that different subtypes and different karyotypes of CLL demonstrate characteristic patterns of miR expression [131]. As relates to WNT signaling, miR-15a and

miR-16-1 function by targeting *WNT3A*, a target gene *CCND1*, and multiple oncogenes, including *BCL-2* and *MCL1* [15]. Downregulation of these miRs has been reported in CLL as well as prostate carcinoma where additional WNT-related targets included *SMAD7*, *SOX5*, *WNT2B* and *FOSL1* [132]. It now seems that miR-15a and miR-16-1 are hosted in a gene, *DLEU2*, located in chromosomal band 13q14 [18]. This region comprises the most common chromosomal deletion in CLL (~50%) and is associated with less aggressive disease. In addition, mutations in this region have also been reported in CLL [130]. Mature miR-15a and miR-16-1 are produced in a Drosha-dependent manner from *DLEU2* and binding of MYC to two alternative *DLEU2* promoters represses expression of both the host gene and both miRs. *DLEU2* also negatively regulates a WNT target gene, *CCND1* and *CCNE1*, through these miRs. Thus, in those cases of CLL where *DLEU2* is genetically deleted along with miR-15a and miR-16-1, cellular proliferation ensues and this is at least partially affected via WNT signaling.

In addition, miRs-21, -92, -106b, -150, -155, and -222 are deregulated in CLL [111,133,134]. One of the reported targets of miR-21 is WNT1 [17]. MiR-92-1 (formerly miR-92a) is elevated in CLL and promotes the HIF/VEGF signaling axis through regulation of pVHL and this is likely at least partly responsible for autocrine VEGF secretion from CLL cells [135]. Although its role in CLL might be a bit different, miR-181 directly targets transcriptional regulators including GATA binding protein 6 (*GATA6*) and an inhibitor of WNT signaling (*NLK*) in hepatic cancer stem cells. Forced expression of miR-181 resulted in upregulation of the WNT target *CCND1* [136]. In primary pigmented nodular adrenocortical disease miR-449 targets *WISP2* (WNT1 inducible signaling pathway protein 2), as well as *CTNNB1* and *GSK3 β* [137]. Whether these same mechanisms are also found in CLL requires further investigations, but reinforces the multiplicity of mechanisms by which WNT and WNT targets can be dysregulated. A very interesting epigenetic process involving HDAC suppression of miR-106b and its host gene *MCM7* has been reported in CLL [111]. When miR-106b is downregulated, a ubiquitin E3 ligase protein, ITCH, is increased and this supports an anti-apoptotic degradation of p73 protein. Following HDACi, reacylation of H3K9 occurred in the *MCM7/miR-106b* promoter, followed by binding of E2F1 and MYC transcription factors and re-expression of miR-106b that in turn downregulated ITCH and led to induction of PUMA, caspases and apoptosis. This is another example of how normally antagonistic processes may be epigenetically dysregulated. Future investigations will surely provide important insights into these pathway and gene interactions and the overall output on clinical behavior of CLL cells.

Pharmacologic targeting the WNT pathway

Epigenetic alterations are reversible through pharmacological manipulation of enzymes that are responsible for chromatin modifications. These include HDACi and DNA demethylating agents, and some are currently on the market that can induce proliferative arrest and cell death in hematologic malignancies [138,139]. However, there are likely to be additional effects on cellular function beyond the intended HDAC and methylated DNA targets. So far, epigenetic therapies have demonstrated considerable activity in hematologic malignancies, but their success in the treatment of solid tumors remains much more uncertain [140]. There is an increasing need for clinical trials driven by pharmacodynamic biomarkers aimed at determining the optimum dose rather than the maximum tolerated dose, and also evaluating their use in combination with cytotoxic chemotherapies, perhaps as chemosensitizers. Such trials already suggest that improved tumor delivery, with decreased normal tissue toxicity, will be required to take full advantage of this class of agents. Current studies are revealing a high level of complexity in the mechanisms controlling the epigenome, and the need for combination therapies targeting different chromatin modifiers to reach an effective reversal of epigenetic alterations [141].

While several clinical trials have been reported or are in progress in hematologic malignancies, only a few have been reported using epigenetic modifiers in CLL (reviewed in [138]). A Phase I trial with an HDACi, depsipeptide (FK228), demonstrated significant histone acetylation and several patients had a positive treatment effect, but there were also unacceptable treatment-associated toxicities that were also subsequently observed in patients with acute myeloid leukemia [140,142]. For cancer treatment in general, several structurally distinct HDACi have been described beyond depsipeptide, including MGCD0103, the hydroxamic acid-based HDACi (Vorinostat, LAQ824 and PXD101), and aminobenzamide-based HDACi (CI-994 and MS-275). These have been demonstrated to have potent *in vitro* anti-tumor activities in preclinical studies. A recent, second-generation HDACi compound (JNJ-26481585) has been found to be a potent pan-HDACi with activity in myeloid leukemias [143]. Certainly, we are at an early point in optimizing HDACi treatments, and it is possible that as single agents they may not be able to deliver the responses needed.

In a recent study, a GSK3 β -specific inhibitor (AR-AO14418) was used to treat CLL cells [44,144]. In this case, abnormal nuclear localization of GSK3 β in CLL cells was reversed along with decreased binding of the NF- κ B p65/p50 subunit at the promoters of the target genes *BCL-2* and *XIAP*. This treatment was also accompanied by increased methylation of histones H3K9, H3K20 and H3K27 in the same promoter regions, thus suggesting an epigenetic underpinning to NF- κ B binding at the promoters of these target genes.

In the context of DNA methylation, a Phase II study of 5-azacytidine (azacytidine; Vidaza[®]; Celgene Corporation, NJ, USA) in CLL is currently in progress [202]. One important point is that the *in vivo* mechanisms of action of this, and other, demethylating agents is not entirely clear, even though clinical trials in many types of cancer are ongoing (reviewed in [145]). Of interest, the demethylating effects of three common demethylating agents have been found to be quite different from each other, thus arguing that they may need to be used in some rational combination for a full effect [146]. Therefore, we do not yet understand the optimal manner to use these agents or in what combinations clinically. Further clinical research will be needed to answer these important questions.

In our current state of knowledge, epigenetic therapeutics may be wide-ranging in their effects [141]. Since the WNT network plays such a key role in many developmental and disease processes, it is not surprising that drug development to target this key pathway is of great importance. Much of our recent knowledge derives from functional genomics screening that identifies target genes of WNT network proteins. The applications of RNAi to study the WNT network, as well as advantages, disadvantages, and additional technologies have been detailed in an excellent review by DasGupta [38].

As one example, a novel class of small molecule inhibitors of WNT signaling has been described [147]. Two new classes of small molecules that disrupt WNT signaling were discovered from screening a large synthetic chemical library. One class, or inhibitors of WNT production (IWP) inhibits the activity of porcupine, a membrane-bound acyltransferase that is essential to the production of WNT proteins. The other class, inhibitors of WNT response (IWR) inhibits destruction of AXIN proteins, which are part of the destruction complex and cooperatively acts to suppress WNT signaling. Targeted agents such as these may become quite useful in further studies and possible clinical trials in the future. In addition, new chemical inhibitors of GSK3 β have been described [148], and at least one of these affects *TERT* expression via a group of transcription factors that are also important in WNT signaling [149].

Several plant-derived phytochemicals have also been demonstrated to have intriguing signs of effect on WNT signaling and potential therapeutic benefits (reviewed in [150]). For instance,

curcumin, resveratrol and epigallocatechin-3-gallate all seem to affect WNT signaling. A recently reported Phase I trial using green tea extract in early stage CLL was reported by Shanafelt *et al.* [151]. Polyphenon E with a standardized dose of epigallocatechin-3-gallate was used in a standard Phase I design with oral dose levels ranging from 400 to 2000 mg twice a day. This treatment was well tolerated by CLL patients and the majority had declines in the absolute lymphocyte count and/or lymphadenopathy. A Phase II trial to evaluate efficacy began in November 2007. Studies such as this that can use epigenetic modifiers with minimal toxicity to achieve positive results that may improve the quality of life, achieve cytostasis or move toward curative intent, are exciting. Nitric oxide has also taken on renewed enthusiasm with respect to modulating chromatin and chromosome structure, thereby also modulating epigenetic functions [152].

Future perspective

In cancers, three major types of relationships between tumor cells and their microenvironment have been proposed; the loss of interconnection with the microenvironmental network, a dysfunctional environment, or a friendly, regulated coexistence between the tumor and the microenvironment [5]. There is evidence to support this latter type in CLL where interactions largely recapitulate those of normal B-cell/microenvironment interactions. Thus, the proliferative drive for the CLL cells might derive largely from continued external microenvironmental signals such as antigens, cell–cell interactions, growth factors and signaling molecules such as WNT proteins. It seems likely that either too much or too little WNT signaling may lead to apoptosis, while an intermediate level may be permissive to cellular proliferation at some level. Therefore, it is quite plausible that disruption of this delicate balance may lead to any of a spectrum of observations ranging from apoptosis to proliferation, or perhaps an intermediate phase resulting in decreased proliferation but not apoptosis.

From the reported literature and our own investigations, we suggest a model whereby epigenetic dysregulation affects mainly those genes that normally act as inhibitors of WNT signaling (FIGURES 1 & 2). These epigenetic alterations include a complex interaction between DNA methylation, histone modifications and miRs that have the aggregate effect of creating a permissive environment where WNT signals from BM stromal cells, as well as perhaps autocrine production of certain WNT proteins by CLL cells, then allows what appears to be constitutive activation of this key cellular network. Further research will surely provide insights into exactly how each type of malignancy is affected by WNT dysregulation, additional pathways that may be affected by this, and how the underlying cellular biology can be manipulated either through targeting the WNT network directly, or perhaps through key partners that are yet to be identified.

Executive summary

Chronic lymphocytic leukemia

- This is a very heterogeneous disease, mainly affecting older individuals, and although treatable, it is not currently considered curable.

WNT network

- The historical perspective is that canonical and noncanonical WNT pathways are functional under different conditions and in different cellular contexts.
- The ever-expanding candidates involved in noncanonical signaling are shedding new insights into biological functions and pathway crosstalk.
- It is likely that all these putative pathways function as one large WNT signaling network.

Pathway crosstalk

- Several key signaling pathways in normal B-cell development are constitutively activated in chronic lymphocytic leukemia (CLL).
- Crosstalk involves multiple pathways that share downstream gene cassettes as well as cross-regulation.
- One key mediator, GSK3 β , is involved in many pathways, and can modulate their functions and conversely can be modulated by other pathways.

Bone marrow lymphopoiesis

- B-cells are produced in the bone marrow and undergo several serial steps in maturation under the influence of stromal cells and secreted proteins such as WNT ligands and LPA.
- CLL is a disease based mainly in the bone marrow and is likely to be affected by the marrow microenvironment in a bidirectional way.

Illegitimate WNT signaling & epigenetic mechanisms

- The WNT network and downstream target genes are constitutively active in CLL.
- Most of the reported epigenetic dysregulation of WNT in CLL seems to affect negative regulators of the network to allow permissive signaling.
- DNA methylation seems to target mainly genes involved in antagonism of the signaling network.
- Histone modifications contribute to this dysregulation through contributions to repressive or active chromatin.
- MicroRNAs are frequently deleted or dysregulated in ways that further contribute to the overall epigenetic dysregulation of WNT signaling.
- Frequently, there is a multifaceted epigenetic process that involves all these major mechanisms that affect WNT signaling, but also many other biological processes.
- Overall, the epigenetic alterations create a permissive environment for WNT signaling in CLL.

Therapeutic opportunities

- Many opportunities currently exist to target WNT network genes and underlying epigenetic alterations with the ultimate goal of curative intent.
- Even without curative intent, there are many opportunities to manage CLL as a chronic, but not fatal disease, particularly as milder forms of effective therapies are discovered.

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Figure 1. Selected gene interactions and epigenetic dysregulation of the canonical WNT pathway
 The genes shown in solid black have previously been reported to be dysregulated through DNA methylation, histone modifications, miRNA expression and combinations of these mechanisms. How they affect the overall signaling pathway is not yet known.
 LEF: Lymphoid enhancing factor; SFRP: Soluble frizzled protein.

Figure 2. Selected gene interactions and epigenetic dysregulation of selected noncanonical WNT pathways

The genes shown in solid black have previously been reported to be dysregulated through DNA methylation, histone modifications, miRNA expression and combinations of these mechanisms. Other mediators such as ROR1 overexpression and LPA overexpression are also indicated. How these alterations affect the overall signaling pathway(s) is not yet known.

LPA: Lysophosphatidic acid; ROR1: Receptor tyrosine kinase-like orphan receptor 1.