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Autocrine signaling by Wnt-5a deregulates chemotaxis of leukemic cells and predicts clinical outcome in chronic lymphocytic leukemia

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Abstract

Background—ROR1, a receptor in the non-canonical Wnt/planar cell polarity (PCP) pathway, is up-regulated in malignant B cells of chronic lymphocytic leukemia (CLL) patients. It has been shown that Wnt/PCP pathway drives pathogenesis of CLL, but which factors activate ROR1 and PCP pathway in CLL cells remains unclear.

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Authorship Contributions

P.J., L.P., K.P., H.P., M.Be., M.K., M.H., M.Bo, O.S. and S.Pa. designed and performed the research, collected, analyzed and interpreted the data; S.B. contributed vital reagents (anti-ROR1 mABs); Y.B., M.D and IS collected samples from patients and provided them for analysis; P.O. performed statistical analysis; S.Po, A.K. and V.B. designed and supervised the research and interpreted the data; P.J., L.P., K.P., S.Pa and V.B. wrote the manuscript.

Supplementary information is available.

Aims—To analyze the expression, function and clinical relevance of two putative ROR1 ligands, Wnt-5a and Wnt-5b, produced by CLL cells.

Methods—B lymphocytes from peripheral blood of CLL patients were negatively separated using RosetteSep (StemCell) and gradient density centrifugation. Relative expression of *WNT5A*, *WNT5B* and *ROR1* was assessed by quantitative real-time PCR. Protein levels, protein interaction and downstream signaling were analyzed by immunoprecipitation and western blotting. Migration capacity of primary CLL cells was analyzed by transwell migration assay.

Results—By analyzing the expression in 137 previously untreated CLL patients we demonstrate that *WNT5A* and *WNT5B* genes show dramatically (five orders of magnitude) varying expression in CLL cells. High *WNT5A* and *WNT5B* expression strongly associates with unmutated IGHV and shortened time-to-first-treatment. In addition, *WNT5A* levels associate, independent of IGHV status, with the clinically worst CLL subgroups characterized by dysfunctional p53 and mutated *SF3B1*. We provide functional evidence that *WNT5A*-positive primary CLL cells have increased motility and attenuated chemotaxis towards CXCL12 and CCL19 that can be overcome by inhibitors of the Wnt/PCP signaling.

Conclusion—These observations identify Wnt-5a as the crucial regulator of ROR1 activity in CLL and suggest that autocrine Wnt-5a signaling pathway allows CLL cells to overcome natural microenvironmental regulation.

Keywords

Wnt-5; Chronic lymphocytic leukemia (CLL); non-canonical Wnt pathway; cell migration; microenvironment

Introduction

ROR1, a transmembrane receptor tyrosine-protein kinase, is upregulated in chronic lymphocytic leukemia (CLL). It is uniformly expressed on the surface of CLL cells (1–3), whereas it is almost absent in healthy B cell subsets, with the notable exception of hematogones (4). Signaling through the ROR1 receptor is far from being completely understood. It is, however, widely accepted that ROR1 and closely related ROR2 act as receptors for Wnt ligands, mainly from the Wnt-5 family, and mediate the activation of the non-canonical Wnt pathway referred to as the planar cell polarity (PCP) pathway. Wnt/PCP controls various aspects of cell polarity and migration with the most prominent role in embryonic development (5).

There is increasing evidence that ROR1 plays an important role in the pathogenesis of CLL. First, siRNA-mediated silencing of *ROR1* (6) as well as treatment by specific anti-ROR1 antibodies can induce apoptosis of CLL cells (7). Second, we have recently shown that PCP pathway – driven by ROR1 – controls migratory properties of CLL cells in the chemokine gradient and that several PCP genes are expressed differently between IGHV-mutated (M-CLL) and unmutated (U-CLL) CLL subgroups (8). Third, it was shown that *ROR1* overexpression can enhance leukemogenesis in the Eμ--TCL1 mouse model (9).

Strikingly, *ROR1* expression in CLL patients is remarkably uniform. The amount of ROR1 does not correlate with markers of disease aggressiveness and does not predict clinical outcome (1, 2, 8). This is in distinct contrast with the fact that ROR1-controlled non-canonical Wnt pathway has an impact on CLL progression (8, 9). These observations suggest that ROR1 activity and its downstream signaling in CLL are controlled by unknown factors.

We have shown earlier that acute stimulation by exogenous Wnt-5a, a putative ROR1 ligand, can modulate the chemotactic response of CLL cells (8). However, nothing is known if and how this is relevant for CLL biology. Here we hypothesized that ROR1 ligands from the Wnt-5 family (Wnt-5a and Wnt-5b) control ROR1 activity in CLL cells and may help to explain the functional variability despite uniform *ROR1* expression. We demonstrate that Wnt-5 ligands show dramatically varying expression among CLL patients and also among individual populations of healthy B cells, which implicate their role in the interaction of B cells with the secondary lymphoid tissue microenvironment. Furthermore, we provide evidence that autocrine Wnt-5a is a key factor, which contributes to the deregulated motility and chemotaxis of primary CLL cells. These observations identify Wnt-5a as the crucial regulator of ROR1 activity in CLL and further support the importance of the Wnt-5a-ROR1 axis for CLL pathogenesis.

Methods

Patient and control samples

CLL patients were monitored and treated at the Dept. of Internal Medicine – Hematology and Oncology, University Hospital Brno according to international criteria (10). All samples including non-malignant controls were taken after written informed consent in accordance with the Declaration of Helsinki under protocols approved by the Ethical Committee of the University Hospital Brno. B lymphocytes from peripheral blood of CLL patients and healthy volunteers and from non-malignant tonsilar tissues were separated using non-B cell depletion techniques (RosetteSep kits, StemCell, or magnetic separation - B-cell isolation kit II, Miltenyi Biotec) (8). The separation efficiency was assessed by flow-cytometry and all tested samples contained ≥98% B cells. Individual tonsilar B cell populations were separated by FACS sorting.

The CLL cohort selected for expression analyses represent patients monitored at the University Hospital where more aggressive cases with inferior prognosis are referred. The patients were examined for presence of mutations in *TP53* and *SF3B1* genes, for recurrent cytogenetic abnormalities, and for somatic hypermutations in IGHV gene. To validate correlation of *WNT5* expression with less frequent abnormalities (trisomy 12, and *SF3B1)*, basic cohort was enriched for patients carrying these aberrations. Serial samples of 36 patients were obtained to measure expression changes in time. For cohort characterization and further details see Supplementary Information.

Gene expression analysis by real-time qPCR

Relative gene expression of *WNT5A*, *WNT5B* and *ROR1* was assessed by quantitative real-time PCR. Briefly, cells were lysed in TriReagent (MRC) and total RNA was isolated by isopropanol precipitation. mRNA was reverse-transcribed using oligo(dT) (Fermentas) and SuperScriptII (Invitrogen). *ROR1* expression was measured using Power SYBR Green Master Mix (Applied Biosystems) and primers described previously (8). *WNT5A/B* genes were quantified using TaqMan Gene Expression Assays Hs00998537_m1 Wnt-5a and Hs01086864_m1 Wnt-5b (Life Technologies). The relative expression was calculated by the $2^{-ddCt}\times100\%$ method using *HPRT1* and *TBP* genes as endogenous controls.

Western Blotting and immunoprecipitation

Immunoprecipitation and Western blotting were performed as previously described (11). For detection, Immobilon Western Chemiluminescent HRP Substrate (Millipore) was used. For more details see Supplementary Information.

Functional assays of primary B cells

The fresh primary B cells were collected as described above and cultivated overnight in RPMI1640 (Life Technologies) supplemented with 1% Fetal Bovine Serum (FBS, Gibco) and antibiotics (penicillin/streptomycin, TPP) at 37 °C and 5% CO_2. The migration assay was performed in HTS Transwell®-96 well plates (Corning Incorporated) with 5.0 μm pore size polycarbonate membranes following the manufacturer's instructions. After treatment (for details of individual treatments see Supplementary Information) 0.5×10^6 cells were seeded in the Transwell upper insert and incubated for 6 hours at 37 °C and 5% CO_2. Cell number in the bottom chamber was counted by Accuri C6 Flow Cytometer (BD Biosciences). The basal migration was defined as the percentage of transmigrated cells out of the total. Directed chemotaxis towards medium containing 200 ng/ml of CXCL12 or CCL19 (R&D Systems, 350-NS-010 and 361-MI-025) chemokines was defined as migration index (MI) calculated as ratio of migrated cells in the presence and absence of chemokine. The apoptosis was assessed in parallel by TMRE staining (2 μM TMRE, 15 min at room temperature, T-66915, Invitrogen).

Statistics

The distribution normality was tested by Kolmogorov-Smirnov, Shapiro-Wilk or D'Agostino & Pearson normality test. Parametric or non-parametric tests were used accordingly to assess the difference between two variables (unpaired t-test and Mann-Whitney test), the difference in the paired samples (Wilcoxon test) and the correlation between two variables (Pearson and Spearman test). Fisher's exact test was used for categorical datasets. Differences in survival were analyzed using the Log-rank test. The standard level of statistical significance was $P\leq0.05$. All statistical tests were performed as two-sided using GraphPad Prism 5 (GraphPad Software Inc). Cut-offs stratifying patients into subgroups with short/long time-to-first-treatment were determined using CutOff Finder web application (12).

Results

Wnt-5a ligand signals through ROR1 receptor in primary CLL cells

As the first step we examined the *ROR1* gene expression and observed that CLL B cells (n=93), regardless of the IGHV mutational status, expressed uniformly high levels of *ROR1* mRNA compared to B cells isolated from tonsils and peripheral blood (PB) from healthy volunteers (Fig. 1A). This correlated very well with the ROR1 protein expression on the cell surface, P=0.0038 (Suppl. Fig. 1A, Pearson correlation) defined by flow cytometry. These findings are in agreement with the previous reports (1–3).

Although both Wnt-5a and Wnt-5b have been proposed to be ROR1 ligands, bona fide evidence of interaction was presented only for Wnt-5a (3). However, our co-immunoprecipitation analysis suggested both Wnt-5a and Wnt-5b ligands can be found in complex with ROR1 (Fig. 1B). In order to test whether indeed these two ligands signal in primary CLL cells via ROR1 we stimulated primary CLL cells with recombinant Wnt-5a and Wnt-5b. As a control we used Wnt-3a, a ligand known to signal exclusively via Wnt/beta-catenin pathway (13), which is independent of ROR receptors (14). Ligands from both families could induce phosphorylation of Dishevelled (DVL) family of proteins, which can be subsequently used as a universal downstream readout (15). Out of the tested Wnt ligands, only Wnt-5a and Wnt-3a were able to trigger DVL3 phosphorylation in primary CLL cells (Fig. 1C, lanes 1–4). To prove that this effect is dependent on the ROR1 receptor, we pre-treated cells with the blocking mouse monoclonal anti-ROR1 (2A2) antibody (16). Despite the fact that both Wnt-5a and Wnt-3a promote DVL3 phosphorylation in primary CLL cells, only the Wnt-5a effect is dependent on ROR1 receptor as it was blocked by prior treatment with the anti-ROR1 antibody (Fig. 1C, lanes 5–8). Effect of Wnt-3a is ROR1-independent and not affected by anti-ROR1 antibody. Recombinant Wnt-5b does not promote DVL3 phosphorylation in these cells, however we cannot exclude that it acts via other pathway. Out of a panel of four anti-ROR1 antibodies targeting different epitopes in the ROR1 extracellular domain (Fig. 1D), only anti-ROR1 2A2 antibody was able to completely block Wnt-5a-induced DVL3 shift (Fig. 1D, Suppl. Fig. 1B/C) pinpointing the critical role of N-terminal immunoglobulin-like domain of ROR1 in the downstream signaling triggered by Wnt-5a. In summary, these data demonstrate that both Wnt-5a and Wnt-5b can interact with ROR1 and ROR1 is required for Wnt-5a-induced signaling towards DVL in CLL cells.

WNT5A and *WNT5B* mRNA expression strongly correlates with IGHV mutational status

In order to understand the Wnt-5 role in CLL, mRNA expression of two genes encoding Wnt-5 ligands – *WNT5A* and *WNT5B* – was assessed in 137 previously untreated CLL patients (34% M-CLL, 66% U-CLL) and control non-malignant samples from peripheral blood (N=6) and tonsils (naïve, N=8; centrocyte, N=4; centroblast, N=4; memory, N=8; for details on separation/phenotyping see Supplementary Information). The levels of both *WNT5*s are homogeneously low in control peripheral blood B cells and naïve tonsilar B cells but more than 10-times higher in tonsilar centroblasts and centrocytes (Fig. 2A, B). The *WNT5* levels drop again in tonsilar memory B cells with the more profound decrease observed for *WNT5A* (Fig. 2A, B). CLL samples show highly variable expression of both *WNT5* genes ranging from levels resembling healthy peripheral B cells or even lower to

values exceeding median expression in germinal center B cells by more than 40–70-times in the case of *WNT5A* (Fig. 2A) and 6–7-times in the case of *WNT5B* (Fig. 2B). Interestingly, *WNT5A* transcript was undetectable in a significant proportion of CLL patients (85/137 ≈ 62 %; referred as *WNT5A*-negative) whereas it was present, albeit at low levels, in most healthy (5/6) control peripheral B cells. The differences at the mRNA level translate into the increased protein amount (Fig. 2C), which suggests that cells high in *WNT5A* produce also functional Wnt-5a.

In U-CLL the proportion of *WNT5A*-negative samples was significantly lower (50 % vs 85 %, P<0.0001, Fisher's exact test) and the expression of *WNT5B* was remarkably higher (P<0.0001, Mann-Whitney test) (Fig. 2A, B). Although the association of the *WNT5A* and *WNT5B* expression with IGHV mutational status is comparably strong, we did not observe a direct correlation between the two genes (Fig. 2D). Further, *WNT5A* and *WNT5B* expression does not correlate with the expression of *ROR1* receptor (Suppl. Fig. 2A–D), which opens the possibility that ROR1 signaling might be regulated by the Wnt-5 ligands produced by CLL cells.

Higher *WNT5A/B* expression levels in a prognostically worse U-CLL subgroup raised the question whether the *WNT5* genes could play a role in disease progression. To answer this issue, we measured their expression in paired samples from 36 patients. Median time between samplings was 36.2 months (range 9.3–92.3). 17 patients were examined in relapse after therapy administered between the samplings. Out of the remaining 19 patients, remarkable progression of the disease was observed in 8 patients, whereas 11 patients remained stable. We did not observe significant difference in *WNT5* expression between the initial and subsequent samples (Wilcoxon paired t-test, *n.s.*) (Suppl. Fig. 2E, F). The fact that there are no remarkable changes in *WNT5* expression comparable to the expression range within the studied cohort suggests that *WNT5* expression is determined in the early disease state and is not, in most cases, affected by disease evolution.

Increased levels of *WNT5A* and *WNT5B* correlate with CLL aggressiveness

Initial characterization of *WNT5A* and *WNT5B* expression (Fig. 2) uncovered significant differences between U-CLL and M-CLL, two major CLL subsets with a different clinical outcome. Subsequently we retrospectively analyzed time-to-first-treatment (TTFT) in patient cohorts divided according to the *WNT5A* (negative vs. positive cases, Fig. 2A) and *WNT5B* expression levels (high vs. low; Fig. 2B). As we show in Fig. 3A (*WNT5A*) and 3B (*WNT5B*), the subset of patients with lower *WNT5A* and *WNT5B* expression levels had a significantly longer TTFT (median 43.3 months for *WNT5A*-negative vs. 16.5 months for *WNT5A*-positive, P<0.0001; median 56.8 months for *WNT5B* low vs. 22.2 months for *WNT5B* high, P<0.0001).

Interestingly, increased *WNT5A* (Fig. 3C; positive vs. negative *WNT5A* expression: P=0.0046, Log-rank test) but not increased *WNT5B* (Suppl. Fig. 3A) expression also had prognostic value within M-CLL and identified a subgroup of M-CLL patients with shorter TTFT. *WNT5A* expression was dramatically higher in M-CLL patients with borderline mutated IGHV (97–98% of germline identity) (Fig. 3D: P<0.0001; Spearman correlation). Similar but weaker association was also seen for *WNT5B* (Supp. Fig. 3D; P=0.0047,

Spearman correlation). M-CLL patients with IGHV identity between 97–98% had shorter TTFT in our cohort (see Fig. 3C; TTFT in M-CLL below and above 97%; P=0.0062, Log-rank test), which is in agreement with the previously reported observation (17). Thus, M-CLL patients positive for *WNT5A* have more progressive disease and often carry less hypermutated IGHV gene in comparison to *WNT5A*-negative M-CLL patients. This correlation, however, did not hold true within U-CLL (Suppl. Fig. 3B, 3C).

WNT5A expression correlates with other negative prognostic factors independently of IGHV mutational status

In order to gain further insight into the role of *WNT5* in CLL, we examined the correlation of *WNT5A/B* expression with other clinical parameters both in the whole cohort and in U-CLL and M-CLL separately (summed up in Supplementary Table 1). High expression of *WNT5A* and/or *5B* expression in the whole cohort significantly positively correlated with numerous parameters associated with unmutated IGHV – leukocytosis, clinical stage at diagnosis, *TP53* mutation/17p deletion, and 11q deletion. However, *WNT5A* and/or *WNT5B* gene expression levels were also significantly associated with several parameters even within U-CLL and/or M-CLL subgroups. Most strikingly, within U-CLL cohort the *WNT5A* expression was increased in the most aggressive CLL defined by 17p-deletion (P=0.0071; Mann-Whitney test) (Fig. 3E). Significantly higher *WNT5A* expression was also found in patients with aggressive CLL carrying a mutation in *TP53* (P=0.02; Mann-Whitney test) (Fig. 3F) or in *SF3B1* (Fig. 3G; *SF3B1*-mutated samples added to validate significance; P=0.0218; Mann-Whitney test). Of note these mutations are often mutually exclusive (18, 19), which suggests that high *WNT5A* is a hallmark of an aggressive disease. For *WNT5B* data see Supplementary Fig. 4A/B. For complete data describing the *WNT5* gene expression in patients stratified according to the FISH hierarchical risk model (20) see Suppl. Fig. 4C/D.

WNT5A-high primary CLL cells show higher basal motility and impaired chemotaxis

The association of *WNT5A* and *WNT5B* mRNA levels with markers of unfavorable prognosis observed here and the previously reported link between Wnt/PCP pathway signaling and CLL pathogenesis (8) prompted us to investigate the functional role of Wnt-5 ligands in the behavior of CLL cells. We analyzed 27 primary CLL samples (M-CLL, N=12; U-CLL, N=15) with variable *WNT5A* (i) and *WNT5B* expression (ii) (Fig. 4A) for their ability to migrate in vitro in the absence or presence of chemokines using Transwell system (8). Based on the *WNT5A* and *WNT5B* expression, respectively, the samples were divided into two groups using the same criteria as in Fig. 2.

The basal migration capacity – defined as the proportion of CLL cells migrating in the absence of chemokine – varied strongly among individual samples, ranging from 0.02 % to 7.74 % (range from 10^2 to 10^4 cells) of migrated cells (median 0.3 %, N = 27) (Fig. 4B). Following stratification based on (i) IGHV mutational status, (ii) expression of *WNT5A* and (iii) *WNT5B*, only high *WNT5A* expression could distinguish cells with a significantly higher migration capacity (median 1.08 % vs. 0.23 %, P = 0.0042, Mann-Whitney test) (Fig. 4B). Importantly, high *WNT5A* expression also defined more motile cells within U-CLL cohort (Fig. 4B, panel iv; P=0.0128, Mann-Whitney test, N=27), which suggests that *WNT5A*, and

not unmutated IGHV, is the primary parameter from those tested associated with the higher CLL cell migratory capacity.

Next, we studied the CLL cell ability to respond to chemokines CXCL12 and CCL19 known to stimulate via the receptors CXCR4 and CCR7, respectively (21). The chemotaxis was depicted as a migration index (MI) defined as the ratio of cells migrated in the presence vs absence of a chemokine. The MI varied ranging from 0.4 to 75.5 for CXCL12 (N = 25) and from 0.35 to 121 for CCL19 (N = 25) (Fig. 4C, D) and cannot simply be explained by the expression levels of relevant receptors (ROR1 receptor and chemokine receptors CXCR4 and CCR7) since the surface levels of individual receptors, defined by flow-cytometry, did not correlate significantly with migratory parameters (Suppl. Fig. 5A–C). In contrast, following patient stratification, we found significant differences between *WNT5A*-positive and *WNT5A*-negative groups (Fig. 4C, 4D) and in case of CCL19 also between *WNT5B*-high and *WNT5B*-low groups. Higher *WNT5A* expression defined the CLL cells generally less able to respond to the chemokine stimuli compared to *WNT5A*-negative group (MI CXCL12: P=0.0143; MI CCL19: P=0.0202; Mann-Whitney test). Similar to the basal migration, *WNT5A*-positive CLL samples showed a tendency towards a more impaired response to chemokines within the U-CLL patients, although in our sample, this trend was not significant. In summary, our functional data demonstrate that *WNT5A*-high CLL cells show deregulated migratory properties defined by higher basal motility and decreased response to chemokines.

Aberrant migration of *WNT5A*-positive cells is rescued by inhibition of Wnt/PCP pathway

The finding that *WNT5A*-positive CLL cells exhibited higher basal migration suggested a causative connection between Wnt-5 signaling and cell motility. To support this possibility we first tested the effect of recombinant Wnt-5a on CLL cell migration. We found that recombinant Wnt-5a has a significant positive effect on the migration of cells lacking endogenous *WNT5A* expression in comparison with cells expressing *WNT5A* (P=0.0223; N=15) (Fig. 5A). The differential response of CLL cells to *WNT5A* cannot be explained by the IGHV mutational status (Fig. 5A). In addition, overnight stimulation of *WNT5A*-negative CLL cells (N=5) with Wnt-5a was able to decrease migratory response to CXCL12, which suggests that chronic Wnt-5a stimulation can desensitize CLL cells for chemotactic stimuli (Fig. 5B).

Next, we inhibited the Wnt/PCP pathway by (i) Rho/ROCK kinase inhibitor (100 μM Y-27632), (ii) porcupine inhibitor IWP2 (10 μM), which specifically blocks secretion of Wnt ligands, (iii) soluble Frizzled-related protein (sFRP1, 0.3 μg/ml), a natural decoy receptor, which prevent Wnt ligands binding to their receptors; and (iv) Rac1 inhibitor (10 μM NSC23766). Out of these drugs sFRP1 and IWP2 are specific for Wnt signal transduction, whereas inhibitors of Rho/Rac have more general effect and may interfere with other signaling pathways regulating cytoskeleton. See Fig. 5C for a schematized mode of action for each drug. As expected, all these inhibitors with the exception of Rac1 inhibitor were able to decrease CLL cell migration in the 6h Transwell assay (Fig. 5D, N=16 for Y-27632, N=19 for IWP2, N=21 for sFRP1, N=10 for NSC23766). Cell viability was not significantly affected by any of these treatments (Fig. 5D).

Patient stratification (see Fig. 4) showed that only CLL cells positive for *WNT5A* but not unmutated IGHV (Fig. 5 E, F, G) or high *WNT5B* expression (Suppl. Fig. 6A–C) are significantly more prone to inhibition by the mechanistically unrelated Wnt/PCP pathway inhibitors Y-27632 (Fig. 5E, P=0.0247, Unpaired t-test), IWP2 (Fig. 5F, P=0.0429, Unpaired t-test) and sFRP1 (Fig. 5G, P=0.0305, Unpaired t-test). We can exclude the possibility that the response associated primarily with U-CLL, because *WNT5A*-positive cells responded significantly more to both Rho/ROCK inhibitor (Fig. 5E, panel iv, P=0.0391) and porcupine inhibitor IWP2 (Fig. 5F, panel iv, P=0.0214) also within U-CLL cohort. Interestingly, Wnt/PCP pathway inhibition can rescue also the chemotactic response and increase the MI index for both CXCL12 (Fig. 5H–i) and CCL19 (Fig. 5H–ii) (Wilcoxon paired test of raw data shown in Suppl. Fig. 6D–F). The MI median was increased up to 5.83-fold in case of sFRP1 and CXCL12 and it was significant for all combinations with the exception of 1.74-fold increase of MI CXCL12 by Rho/ROCK inhibitor Y-27632.

In summary, these data suggest that inhibiting the Wnt/PCP rescues migratory defects, namely increased basal migration and decreased chemotaxis, associated with *WNT5A*-positive cells and support causative connection between high Wnt-5 levels and deregulated migration.

Discussion

In the present study, we have investigated the role of Wnt-5a and Wnt-5b, the two known ROR1 physiological ligands, in CLL biology. Despite many similarities between Wnt-5a and Wnt-5b, several important distinctive features were observed. First, only Wnt-5a but not Wnt-5b can induce downstream DVL phosphorylation via ROR1, an event associated with the activation of the non-canonical WNT pathway (15). Second, only high *WNT5A* but not *WNT5B* expression correlated with the most aggressive form of CLL characterized by dysfunctional p53. Third, only patient stratification based on *WNT5A* but not *WNT5B* expression could explain the differences in the CLL migratory potential and their response to the inhibitors of the Wnt/PCP pathway. The typical features of WNT5A-positive cells are schematized in Fig. 6.

In agreement with previous reports, our study shows that WNT/PCP pathway is deregulated in CLL at multiple levels. First, its activity can be controlled by the availability of the receptor, in CLL primarily by ROR1. It was shown that cell surface levels of ROR1 vary in CLL cells depending on its glycosylation (22, 23). Second, this work demonstrates that the *WNT5* ligand expression differs dramatically among individual CLL patients, which suggests that the availability of ligands is another important control step in the pathway activation. Third, Wnt-5 ligand activity can be eliminated by several soluble pathway inhibitors, mainly by the soluble FZD decoy receptors from the sFRP family. Interestingly, sFRP1, the most efficient inhibitor of Wnt-5a induced signaling (24), and other proteins from the sFRP family were found to be epigenetically silenced in CLL but not in healthy CD19+ B cells (25).

We provide evidence that the high *WNT5A* expression and high Wnt-5a autocrine signaling define the subgroups of patients with poor prognosis whose CLL cells often have higher

basal motility and attenuated response to chemokines. In general, it has been repeatedly shown that (i) circulating CLL lymphocytes display changed migratory and chemotactic capacity in comparison to normal B cells (26, 27) and (ii) the chemotactic potential differs dramatically among individual CLL samples (28–30). There is, however, no consensus how attenuated chemotaxis contributes to CLL pathogenesis and how it correlates with prognosis. Some studies argue that robust chemotaxis (high MI) associates with high CD38 and ZAP-70 (28, 29), which are generally markers of unfavorable prognosis (31, 32). Others, however, demonstrated that low CXCR4 defines patients with shorter overall survival (33). Moreover, it has been shown that B cell receptor (BCR) stimulation attenuates (probably via decreased expression of CXCR4) chemotaxis towards CXCL12 and CLL with the strongest BCR-triggered downregulation of CXCR4 were associated with the worst progression free survival (34). The reasons for these discrepancies are unclear but may reflect differences between *in vivo* and *in vitro* situation and/or between chronic and acute stimulation. For example, we have shown previously that acute treatment by Wnt-5a can promote chemotaxis of CLL cells (8) whereas CLL cells with high *WNT5A* expression respond to Wnt-5a worse despite high migration when unstimulated (as shown in this study). Herein presented ability of WNT5A-low but not WNT5A-high cells to migrate better in response to Wnt-5a suggests the existence of negative feedback loops, which affect the final outcome.

The differences in chemotaxis cannot simply be explained by differences in chemokine receptor expression (27, 28). These studies implicate that other factors significantly modulate the response and we propose that autocrine Wnt-5a signaling via ROR1 is one of the key mechanisms in this process. Currently, it is not clear if CLL cells can experience Wnt-5a produced by other cell types in the body in a paracrine fashion. *WNT5A* is, however, highly expressed by several immune cell types (http://www.immgen.org/) and germinal canter B cells functionally depend on Wnt-5a produced by dendritic cells (35). We can speculate that autocrine Wnt-5a protein production in *WNT5A*-high CLL cells can bypass the requirement for the ligand, which can be under physiological conditions provided in a tightly regulated manner by supportive cells from the microenvironment.

In summary, our data demonstrate that varying levels of *WNT5A* (and to a lower extent also *WNT5B*) help to explain CLL behavior diversity and are useful markers of disease aggressiveness. Functionally, high autocrine Wnt-5a signaling leads to the impaired CLL cell migration. This opens the interesting possibility that permanent autocrine Wnt-5a signaling allows CLL cells to escape normal regulatory mechanisms controlling B cell trafficking.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Statement of Translational Relevance

Chronic lymphocytic leukemia (CLL) is the most common leukemia diagnosed in the Western world. Despite increased understanding of the disease biology and better risk stratification, the disease is still considered incurable. In the current study, we provide evidence that CLL cells with positive expression of *WNT5A*, encoding ligand for the surface receptor ROR1 uniformly upregulated in CLL, define the subgroup of patients with poor prognosis. The most aggressive CLL cases with dysfunctional p53 and mutated *SF3B1* are enriched with *WNT5A*-positive cells, independently of their IGHV mutational status. Functional data show that *WNT5A*-positive CLL cells have increased motility and deregulated chemotactic responses due to Wnt-5a autocrine signaling. In summary, our data identify *WNT5A* expression as a new, stable and strong marker of time-to-first-treatment. Moreover, altered migration properties of *WNT5A*positive patient cohort further substantiate the clinical importance of the interaction of CLL cells with their microenvironment.

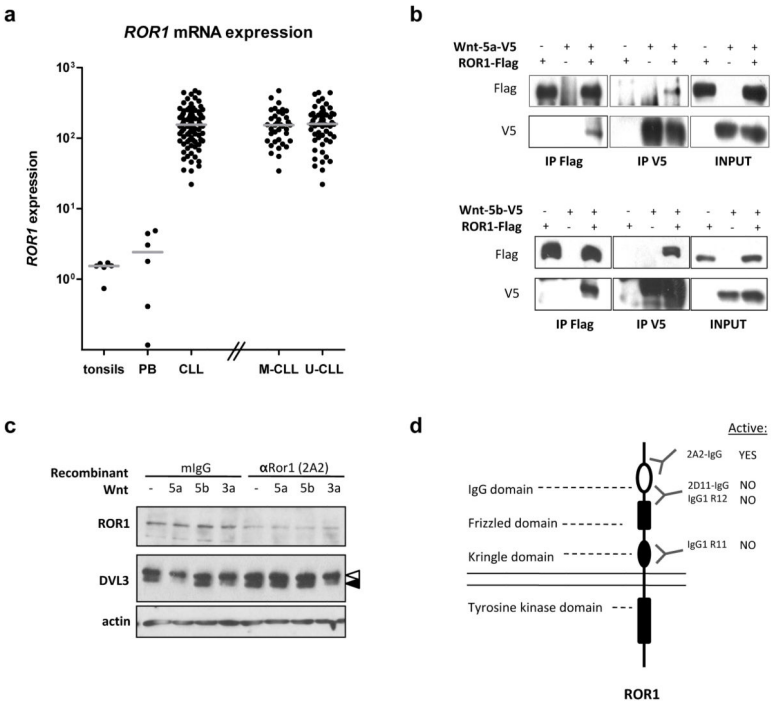

Fig. 1. Wnt-5a signals in CLL cells via ROR1

A) ROR1 mRNA is uniformly overexpressed in CLL cells. qPCR analysis of ROR1 mRNA expression confirmed that in comparison to healthy peripheral (PB) and tonsilar B cells it is uniformly overexpressed in CLL regardless of its IGHV mutational status (N=93). **B**) Immunoprecipitation of overexpressed ROR1-flag and Wnt-5a-V5 or Wnt-5b-V5 in HEK293 cells demonstrates the interaction of the Wnt-5 ligands and ROR1 receptor. **C)** Wnt-5a signals in CLL via ROR1. Primary CLL cells were stimulated by recombinant Wnt proteins (200 ng/ml) and downstream activation was assessed by formation of phosphorylated and shifted DVL3 (PS-DVL) on Western blotting. Position of individual bands is indicated by arrowheads (DVL3, full; PS-DVL3, open). The shift is detectable after Wnt-5a and Wnt-3a treatment but only Wnt-5a triggered phosphorylation can be blocked by monoclonal mouse anti-ROR1 (2A2) antibody (3 μg/ml, overnight treatment). **D)** Epitopes targeted by monoclonal mouse and rabbit anti-ROR1 antibodies and their requirement for downstream signaling (see Suppl. Fig. 1B and C for details). Only the anti-ROR1 2A2 antibody with the epitope in the very N-terminal Ig-like domain effectively blocks a Wnt-5a triggered DVL3 shift. The other antibodies lack this ability. Representative examples from at least 3 independent experiments are shown in B, C.

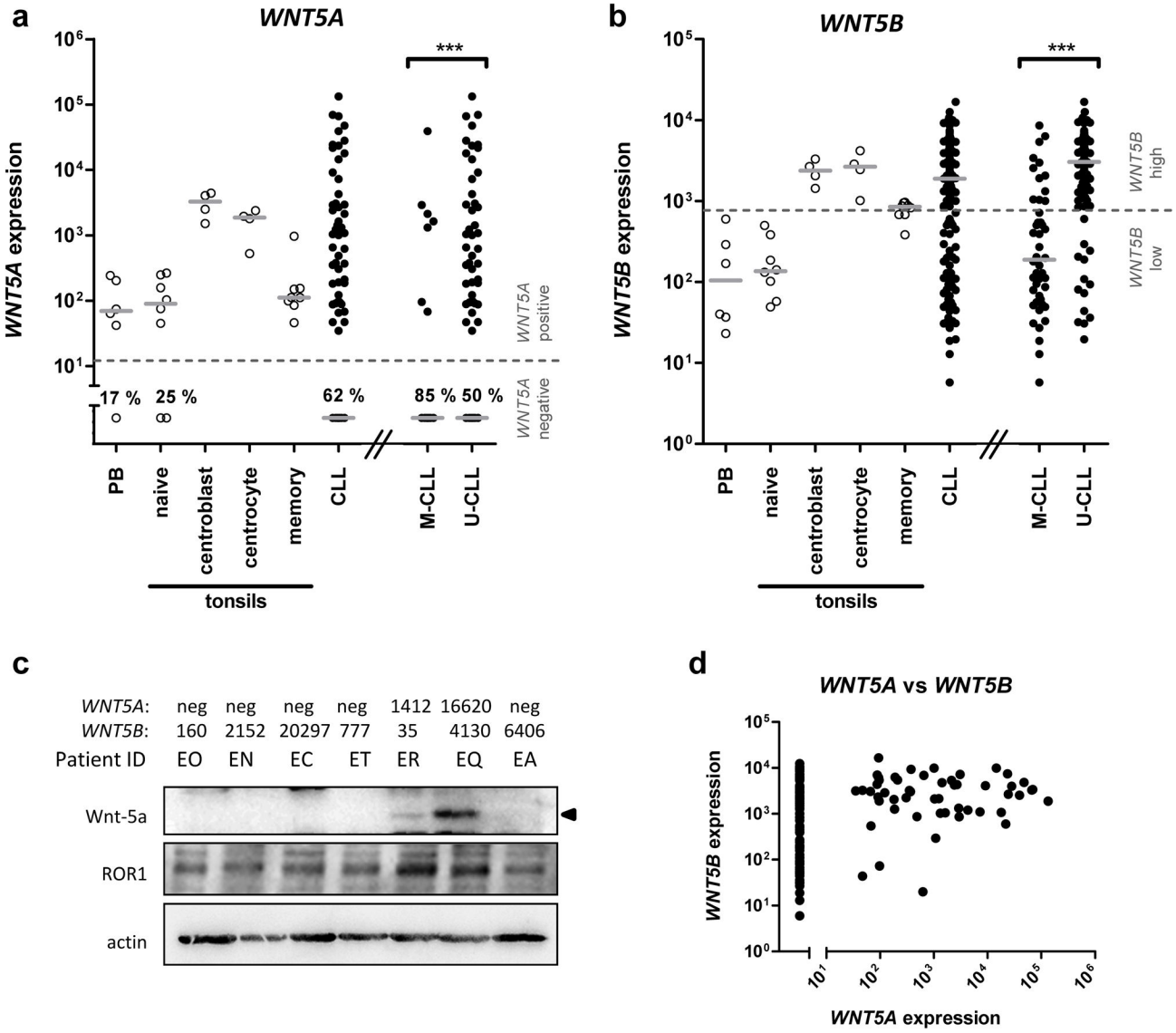

Fig. 2. High expression of *WNT5A* and *WNT5B* associates with unmutated IGHV

A) – B) Relative expression of *WNT5A* **(A)** and *WNT5B* **(B)** mRNA in previously untreated CLL samples (N=137) and in non-malignant B cells isolated from tonsils (N=8 for naïve and memory, N=4 for centrocytes and centroblasts) and from peripheral blood (PB; N=6) assessed by qPCR analysis. On the right side of each graph, CLL samples are divided based on their IGHV mutational status (mutated IGHV – M-CLL, unmutated IGHV – U-CLL). Please note that a significant proportion of CLL samples shows undetectable expression of *WNT5A* (neg). Line indicates median. P<0.001, Mann-Whitney test, Fisher's exact test. **C)** Western blotting analysis of Wnt-5a levels in peripheral B cells of CLL patients. Corresponding mRNA expression for *WNT5A* and *WNT5B* and patient ID is indicated. Actin was used as a loading control. **D)** Expression of *WNT5A* and *WNT5B* in CLL samples from A/B plotted in one graph. Note the absence of *WNT5B*-low/*WNT5A*-high patients.

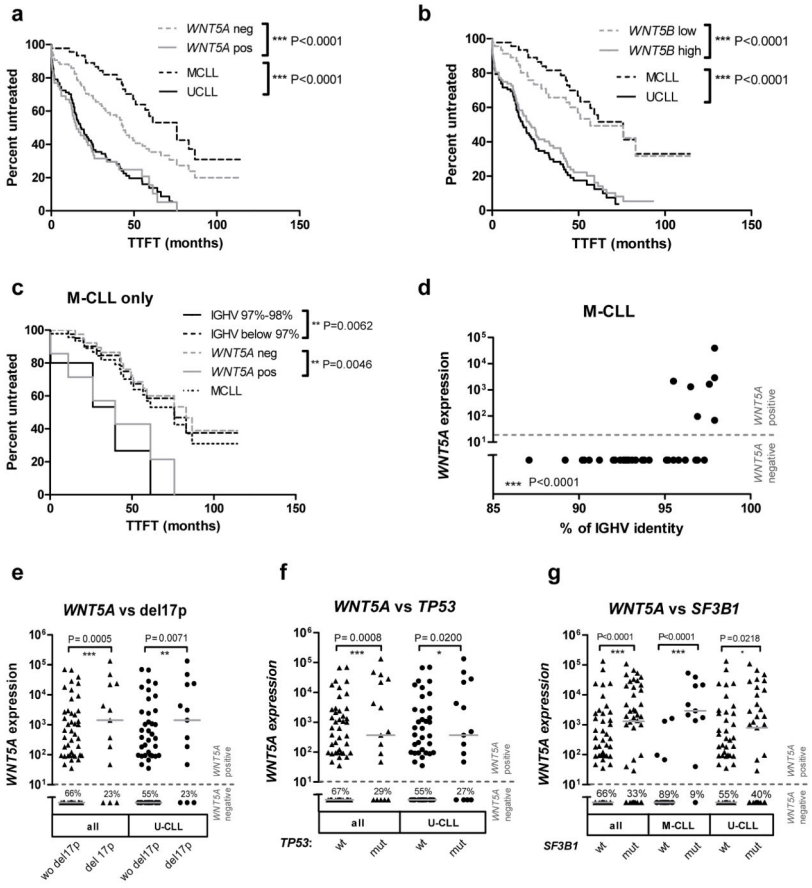

Fig. 3. Clinical characteristics of patients high in *WNT5A* and *WNT5B*

A) – C) Patients were separated into two groups based on their expression of *WNT5A* and *WNT5B*. Time-to-first-treatment (TTFT) in individual groups was plotted using Kaplan-Meier survival curves for *WNT5A*-negative/*WNT5A*-positive **(A)** and *WNT5B*-low/*WNT5B*-high **(B)** patients. TTFT in the same patient cohort separated by their IGHV status (M-CLL and U-CLL) is plotted in both graphs for comparison. **C)** Patients with mutated IGHV (M-CLL) were divided by IGHV identity, below 97 % vs. 97–98 %, and by *WNT5A* (neg vs. pos). (A–C) Statistical significance (P) was analyzed by Log-rank test. **D)** *WNT5A* levels in M-CLL correlate with higher % of IGHV identity. **E–F)** Expression of *WNT5A* mRNA in patients with **(E)** del(17p) and **(F)** *TP53* mutation in the whole cohort (left) and in U-CLL only (right) **G)** Expression of *WNT5A* mRNA in patients with mutated *SF3B1* in the whole patient cohort enriched for *SF3B1*-mutated samples (left), in M-CLL only (middle) and in U-CLL only (right). Statistical significance was assessed by Mann-Whitney test (E, F, G).

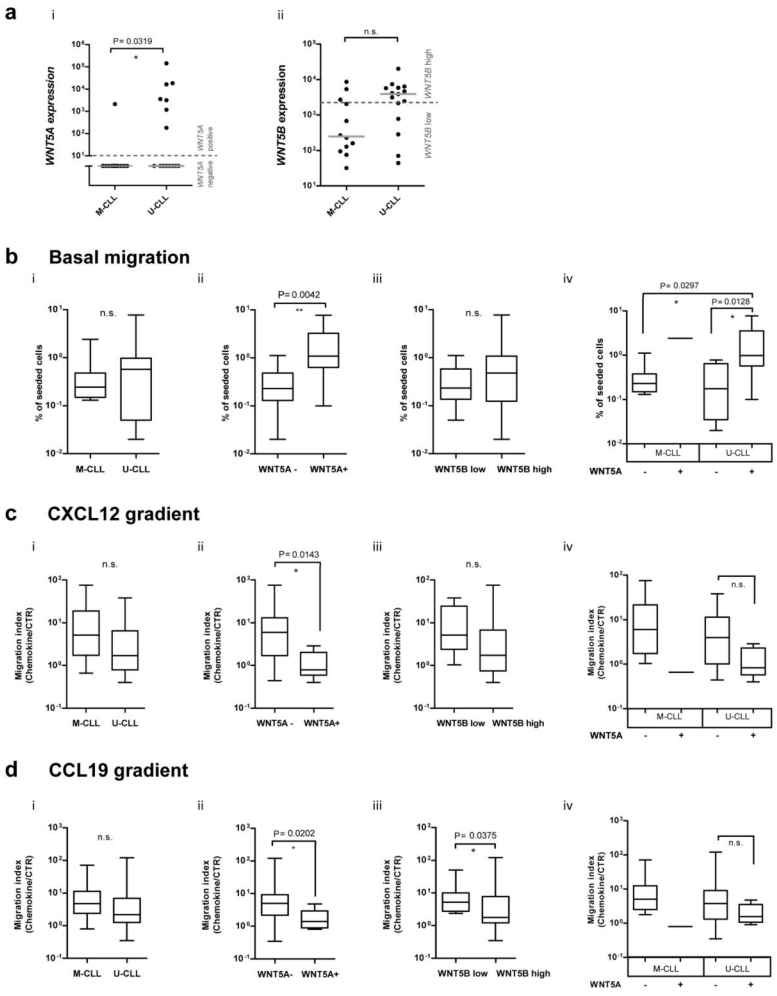

Fig. 4. CLL cells positive for *WNT5A* have increased motility and deregulated chemotaxis

Migration of freshly isolated CLL cells was analyzed using Transwell assays. **A)** Expression of *WNT5A* and *WNT5B* mRNA in primary CLL samples used for Transwell assays. The samples were stratified according to patients' IGHV status (mutated, M-CLL, N=12; unmutated, U-CLL N=15), *WNT5A* expression (positive vs. negative) and *WNT5B* expression (high or low, cut off as Fig. 2). U-CLL cells display significantly higher *WNT5A* expression than M-CLL cells (Mann-Whitney test, P=0.0319). **B–D)** Migratory properties – basal migration **(B)**, chemotaxis towards CXCL12 **(C)** and CCL19 **(D)** of CLL cells in the individual patient subgroups defined by (i) IGHV status, (ii) *WNT5A* expression, (iii) *WNT5B* expression and (iv) combination of IGHV status and *WNT5A* expression. Stratification based on the expression of *WNT5A* was able to define CLL cells with significantly higher basal migration (B) and deregulated chemotaxis (C, D). P – statistical significance (Mann-Whitney test), n.s. – not significant.

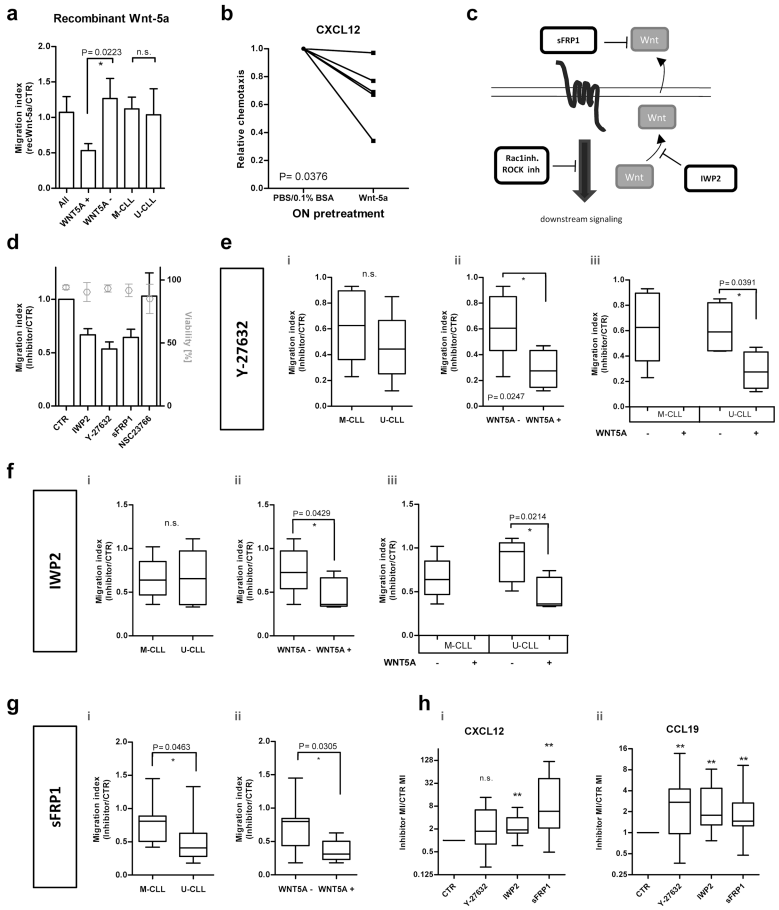

Fig. 5. Inhibition of the Wnt/PCP pathways restores migratory properties of *WNT5A*-positive cells

Freshly isolated CLL cells were treated as indicated and their migration was analyzed using the Transwell assays. Stratification into individual groups was the same as described in Fig. 4. **A)** Recombinant Wnt-5a protein increases basal migration of CLL cells negative for *WNT5A*. Response in *WNT5A*-negative cells is significantly better than in the *WNT5A*-positive group (Mann-Whitney test, P=0.0223). **B)** CLL cells from five *WNT5A*-negative patients were treated with recombinant Wnt-5a (or 0.1% BSA/PBS) overnight and analyzed for their chemotaxis towards CXCL12. Graph shows relative difference in the number of trans-migrated cells (normalized to 0.1% BSA/PBS for each patient). P – statistical significance (One sample t-test, P=0.0376). **C)** Scheme depicting the mechanism of action of Wnt/PCP pathway inhibitors used in D–H. IWP2 blocks Wnt secretion by inhibiting porcupine, sFRP1 binds soluble Wnts, Rac1 and Rho/ROCK kinase inhibitors block downstream signaling. **D)** The effect of the inhibitors on basal migration (left axis) and viability (right axis) of primary CLL cells. Treatment by 0.3 μg/mL sFRP1, 10 μM IWP2 and 100 μM Rho/ROCK inhibitor (Y-27632) inhibited the migration, whereas 10 μM Rac1 inhibitor (NSC23766) did not affect CLL cells. **E) – G)** Graphs show the effect of Rho/ROCK inhibitor (Y-27632), IWP2 and sFRP1 pretreatment on basal migration of CLL cells stratified subgroups according to their IGHV mutational status (i), *WNT5A* status (ii), or both *WNT5A* and IGHV (iii). Stratification according to *WNT5A* expression distinguishes

the best responding cells, even within U-CLL only. P – statistical significance (Unpaired t-test, n.s. not significant). **H**) Changes in the migration index (MI) of primary CLL cells following Rho/ROCK inhibitor (Y-27632), IWP2 and sFRP1 treatment in CXCL12 (i) and CCL19 (ii) gradient. Normalized data represent ratio between MI affected by inhibitor and control condition. Significance values come from paired Wilcoxon rank test of raw data (see Suppl. Fig. 6).

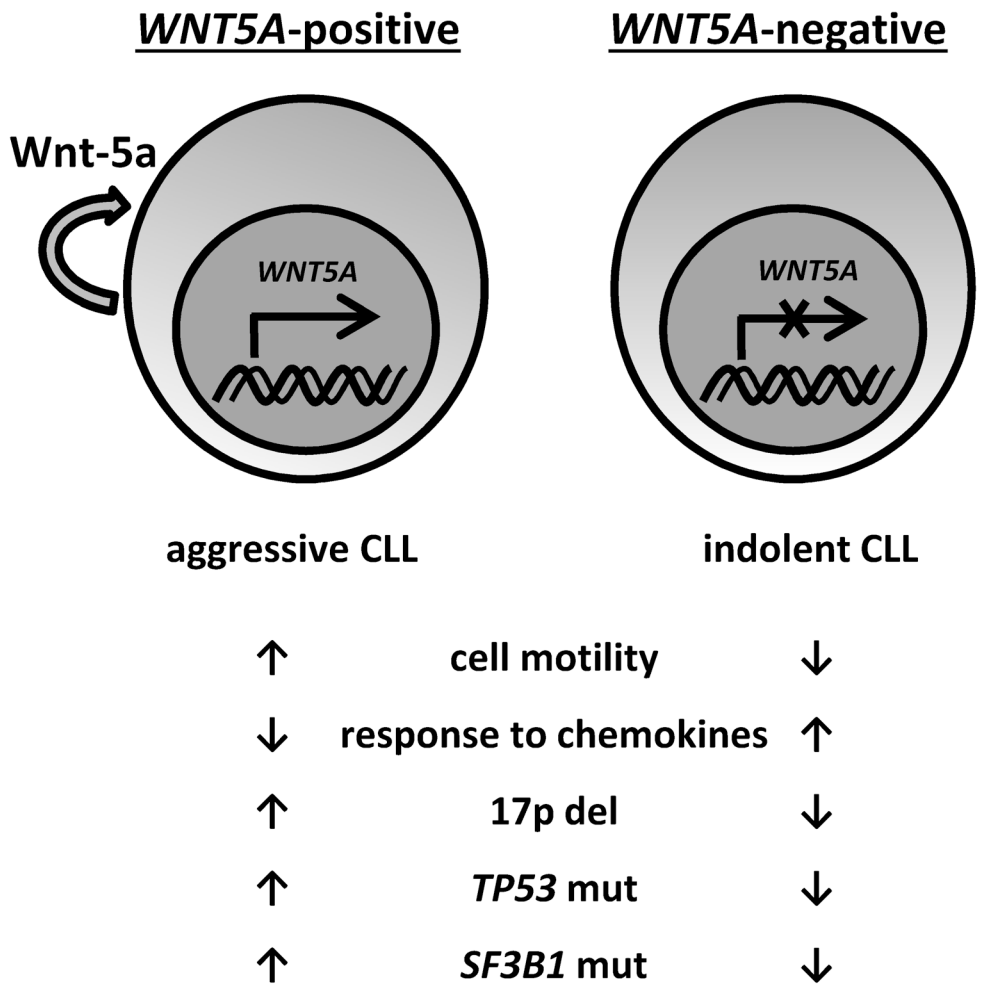

Fig. 6. Schematized differences between *WNT5A*-positive and negative CLL cells

Summary of molecular and functional characteristics of *WNT5A*-positive and *WNT5A*-negative CLL cells presented in Figures 1–5. The causality remains to be determined.