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Expression of *COBLL1* encoding novel ROR1 binding partner is robust predictor of survival in chronic lymphocytic leukemia

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ABSTRACT

Chronic lymphocytic leukemia is a disease with up-regulated expression of the transmembrane tyrosine-protein kinase ROR1, a member of the Wnt/planar cell polarity pathway. In this study, we identified COBLL1 as a novel interaction partner of ROR1. *COBLL1* shows clear bimodal expression with high levels in chronic lymphocytic leukemia patients with mutated IGHV and approximately 30% of chronic lymphocytic leukemia patients with unmutated IGHV. In the remaining 70% of chronic lymphocytic leukemia patients with unmutated IGHV, *COBLL1* expression is low. Importantly, chronic lymphocytic leukemia patients with unmutated IGHV and high *COBLL1* have an unfavorable disease course with short overall survival and time to second treatment. *COBLL1* serves as an independent molecular marker for overall survival in chronic lymphocytic leukemia patients with unmutated IGHV. In addition, chronic lymphocytic leukemia patients with unmutated IGHV and high *COBLL1* show impaired motility and chemotaxis towards CCL19 and CXCL12 as well as enhanced B-cell receptor signaling pathway activation demonstrated by increased PLCγ2 and SYK phosphorylation after IgM stimulation. *COBLL1* expression also changes during B-cell maturation in non-malignant secondary lymphoid tissue with a higher expression in germinal center B cells than naïve and memory B cells. Our data thus suggest *COBLL1* involvement not only in chronic lymphocytic leukemia but also in B-cell development. In summary, we show that expression of *COBLL1*, encoding novel ROR1-binding partner, defines chronic lymphocytic leukemia subgroups with a distinct response to microenvironmental stimuli, and independently predicts survival of chronic lymphocytic leukemia with unmutated IGHV.

Introduction

Upregulation of transmembrane receptor tyrosine kinase-like orphan receptor 1 (ROR1) in chronic lymphocytic leukemia (CLL) cells was revealed as one of the most stable CLL markers.[1,2] ROR1 is expressed on the cell surface of patients with mutated (M-CLL) as well as unmutated (U-CLL) IGHV. ROR1 is highly expressed during embryonal development but largely undetectable in the adult organism.[3,4] Negligible ROR1 expression on healthy B cells[4,5] makes it a suitable candidate for monitoring CLL remission[6] and a candidate target for therapy with monoclonal antibodies[7] or T cells with ROR1-specific chimeric antigen receptor.[8,9] Although ROR1 is up-regulated in CLL patients, its activity may vary depending on its post-translational modification[10] and on the availability of its dedicated ligands.[11]

ROR1 is a member of the Wnt/PCP (planar cell polarity) signaling pathway,[4] which regulates various processes during embryonic development, mainly linked to cell polarity, survival and migration. We have previously reported, in accordance

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with others, that Wnt/PCP components are expressed differently in CLL subgroups defined by IGHV mutational status and CLL aggressiveness.[11-13] It has also been well described that deregulated ROR1 and Wnt/PCP pathway affect CLL cell migration, survival and chemotaxis.[11,13,14] Despite the generally accepted importance of ROR1/PCP signaling in CLL, surprisingly little is known about downstream effectors and links to other signaling pathways critical for CLL pathogenesis.

In this study, we focused on the analysis of ROR1 downstream signaling in CLL. We took advantage of the proteomic approach and analyzed the protein composition of endogenous ROR1 complexes from primary CLL cells. This unbiased approach allowed us to identify a poorly known protein, Cordon-blue protein-like 1 (COBLL1), as a novel ROR1 binding partner. Examining *COBLL1* expression in CLL cells showed that *COBLL1* expression can serve as an independent molecular marker in U-CLL: U-CLL *COBLL1*-high patients had a deregulated response to microenvironmental stimuli and significantly worse prognosis, resulting in shorter overall survival (OS) and time to second treatment (TTST). These data further pinpoint the importance of the ROR1/PCP signaling axis in CLL and identify COBLL1 as an important and clinically relevant regulator of this process.

Methods

Patients and samples

All samples were taken after informed consent in accordance with the Declaration of Helsinki, under protocols approved by the Ethical Committee of the University Hospital Brno, Czech Republic.

Peripheral blood (PB) B cells from CLL patients or healthy volunteers and non-malignant tonsillar tissue were separated by non-B-cell depletion (RosetteSep CD3$^+$ Cell Depletion Cocktail, RosetteSep B Cell Enrichment Cocktail, StemCell Technologies or magnetic B-cell isolation kit II, Miltenyi Biotec). Isolated B-cell purity was assessed by flow cytometry and exceeded 98%. Tonsillar B cells were stained and sorted as described previously.[11] RNA was extracted with TriReagent (Molecular Research Center). For information on how IGHV mutation status[15] and genetic aberrations[16-20] were determined and HEK293 and MAVER-1 cells cultured, see the *Online Supplementary Appendix*.

Mass spectrometry, transfection, immunoprecipitation, immunofluorescence and western blotting

To identify and confirm potential ROR1 binding partners, immunoprecipitation of ROR1 from primary CLL cells coupled to mass-spectrometry,[21,22] transfection of HEK293 cells,[10,23] immunoprecipitation of MAVER-1 and transfected HEK293 cells,[11] immunofluorescence of transfected HEK293 cells[24] and western blotting[25] were performed as previously described. For details, see the *Online Supplementary Appendix*.

Gene expression analysis

COBLL1 and *ROR1* mRNA expression was assessed using qRT-PCR. Three *COBLL1* expression datasets were obtained; for details see the *Online Supplementary Appendix*. Since all datasets showed a similar bimodal distribution (*Online Supplementary Figure S1*), dCt values ($dCt = Ct_{COBLL1} - Ct_{mean\ of\ reference\ genes}$) were normalized using the mean expression and standard deviation of the U-CLL samples and subsequently merged into one dataset. *ROR1* mRNA expression was examined as previously described.[13] The expression was further calculated from dCt values (*ROR1*) and normalized dCt values (dCtN, *COBLL1*) by the 2^{-dCt} x100% and 2^{-dCtN} x100% method, respectively.

Transwell assay

Cell migration in RPMI supplemented with 1% FBS and antibiotics towards chemokines CCL19 or CXCL12 (200 ng/mL; 350-NS-010, 361-MI-025, R&D Systems) or chemokine-free media was analyzed as described previously.[13] Migrated cells were counted using Accuri C6 flow cytometer (BD Biosciences).

BCR stimulation

The protocol previously described by Palomba *et al.* was adopted.[26] For response quantification, phosphorylation increase was assessed and calculated as a ratio of positive cells in a stimulated and unstimulated sample. For details and western blot analysis, see the *Online Supplementary Appendix*.

Table 1. Results of the proteomic analysis of proteins co-immunoprecipitated with ROR1 from chronic lymphocytic leukemia samples.

Accession n.	Protein name	#1	#2	#3	#4	#5
ROR1_HUMAN	Tyrosine protein kinase transmembrane receptor ROR1 OS Homo sapiens GN ROR1 PE 2 SV 2	x	x	x	x	x
O75805_HUMAN	HOXA 9A OS Homo sapiens GN HOXA 9 PE 2 SV 1	x			x	
CENPE_HUMAN	Centromere associated protein E OS Homo sapiens GN CENPE PE 1 SV 2		x			x
COBL1_HUMAN	Cordon bleu protein like 1 OS Homo sapiens GN COBLL1 PE 1 SV 2	x	x			
S17A4_HUMAN	Putative small intestine sodium dependent phosphate transport protein OS Homo sapiens GN SLC17A4 PE	x			x	
APC2_HUMAN	Adenomatous polyposis coli protein 2 OS Homo sapiens GN APC2 PE 1 SV 1		x			x
KINH_HUMAN	Kinesin 1 heavy chain OS Homo sapiens GN KIF5B PE 1 SV 1	x				x

Statistical analysis and data visualization

For statistical analysis, GraphPad Prism 5 (GraphPad Software), Statistica 10 (StatSoft) and R v.3.1.2.27 supplemented with a KEGG profile package[28] were used. Genomic aberrations were visualized as Circos plots.[29] *COBLL1*-linked signaling pathways were analyzed using CLLE-ES dataset (*www.icgc.org*).[30] The cut off dividing patients into *COBLL1*-low and *COBLL1*-high subgroups was determined according to their OS. Kaplan-Meier curve dichotomization was accessed for each $dCtN_{COBLL1}$ and the value with the strongest difference was further used as cut off. For details, see the *Online Supplementary Appendix*.

Results

COBLL1 is a novel binding partner of ROR1

In order to investigate how ROR1 modulates CLL biology and pathogenesis, we decided to apply a proteomic approach and looked for novel ROR1 protein interaction partners. We immunoprecipitated endogenous ROR1 molecular complexes from the primary CLL cells of 5 CLL patients using anti-ROR1 specific antibody and analyzed the proteins pulled down with mass spectrometry. The hits that were identified in the ROR1 pulldown in at least

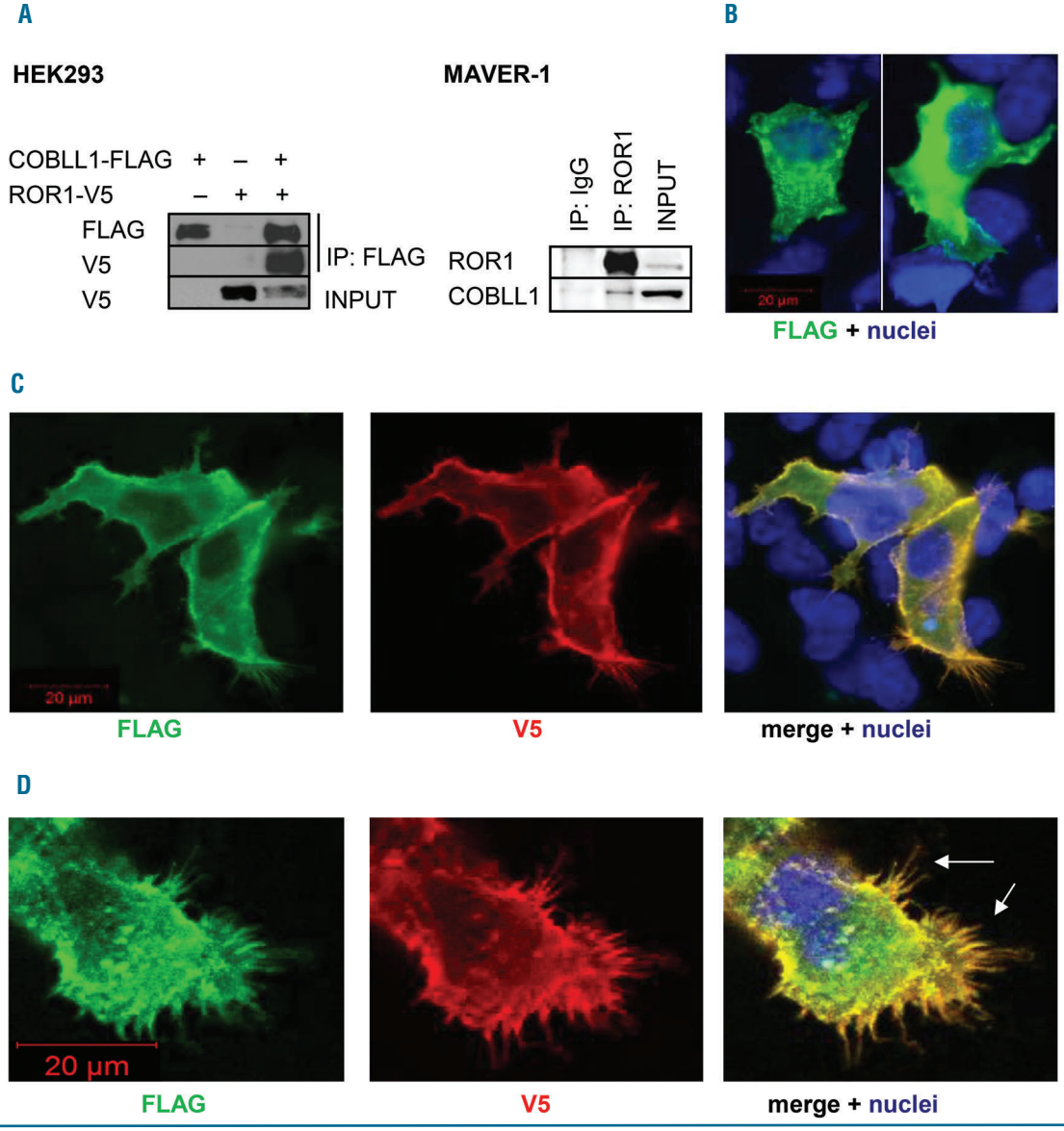


Figure 1. COBLL1 is an ROR1-interaction partner. (A) (Left) COBLL1-ROR1 complex was efficiently immunoprecipitated in HEK293 cells transfected with plasmids encoding FLAG-tagged COBLL1 and V5-tagged ROR1. (Right). Endogenous COBLL1 was pulled down with endogenous ROR1 in MAVER-1 cells; unspecific IgG was used as a negative control. Immunoprecipitation input is loaded on the right. Protein levels were determined using western blotting and anti-FLAG, anti-V5, anti-ROR1 and anti-COBLL1 antibodies. IP: immunoprecipitation. (B-D) Immunofluorescence of HEK293 cells transfected with plasmids encoding FLAG-COBLL1 (B-D) and V5-ROR1 (C and D). COBLL1 over-expressed in HEK293 cells shows mostly cytoplasmic localization (B), but co-localizes with ROR1 in the membrane when ROR1 is co-expressed (C). The most efficient ROR1 and COBLL1 co-localization is observed in filopodia formed as a consequence of ROR1 overexpression (D, indicated by arrows). Protein expression was visualized using anti-FLAG, anti-V5 and corresponding secondary fluorescein-conjugated antibodies. Nuclei were visualized using DAPI staining.

2 patients are shown in Table 1. We compared this list of putative ROR1 interaction partners with the microarray-based dataset of genes differentially expressed in M-CLL *versus* U-CLL samples.[31] This comparison pointed out the cordon blue protein-like 1 (COBLL1) protein as one of the most promising targets. In the next step, we focused on the validation and functional characterization of COBLL1, encoded by the *COBLL1* gene.

First, we aimed to independently confirm that COBLL1 can indeed physically interact with ROR1. We transfected HEK293 cells with plasmids encoding FLAG-tagged COBLL1 and V5-tagged ROR1 plasmids, and immunoprecipitated COBLL1 using anti-FLAG specific antibody. As shown in Figure 1A left, ROR1 can be efficiently co-immunoprecipitated with COBLL1. In order to confirm the interaction on an endogenous level in lymphoid cells, we also co-immunoprecipitated COBLL1 in endogenous ROR1 pulldown using anti-ROR1 antibody from protein lysates of MAVER-1 cells,[32] a mantle cell lymphoma cell line expressing both COBLL1 and ROR1 (Figure 1A right).

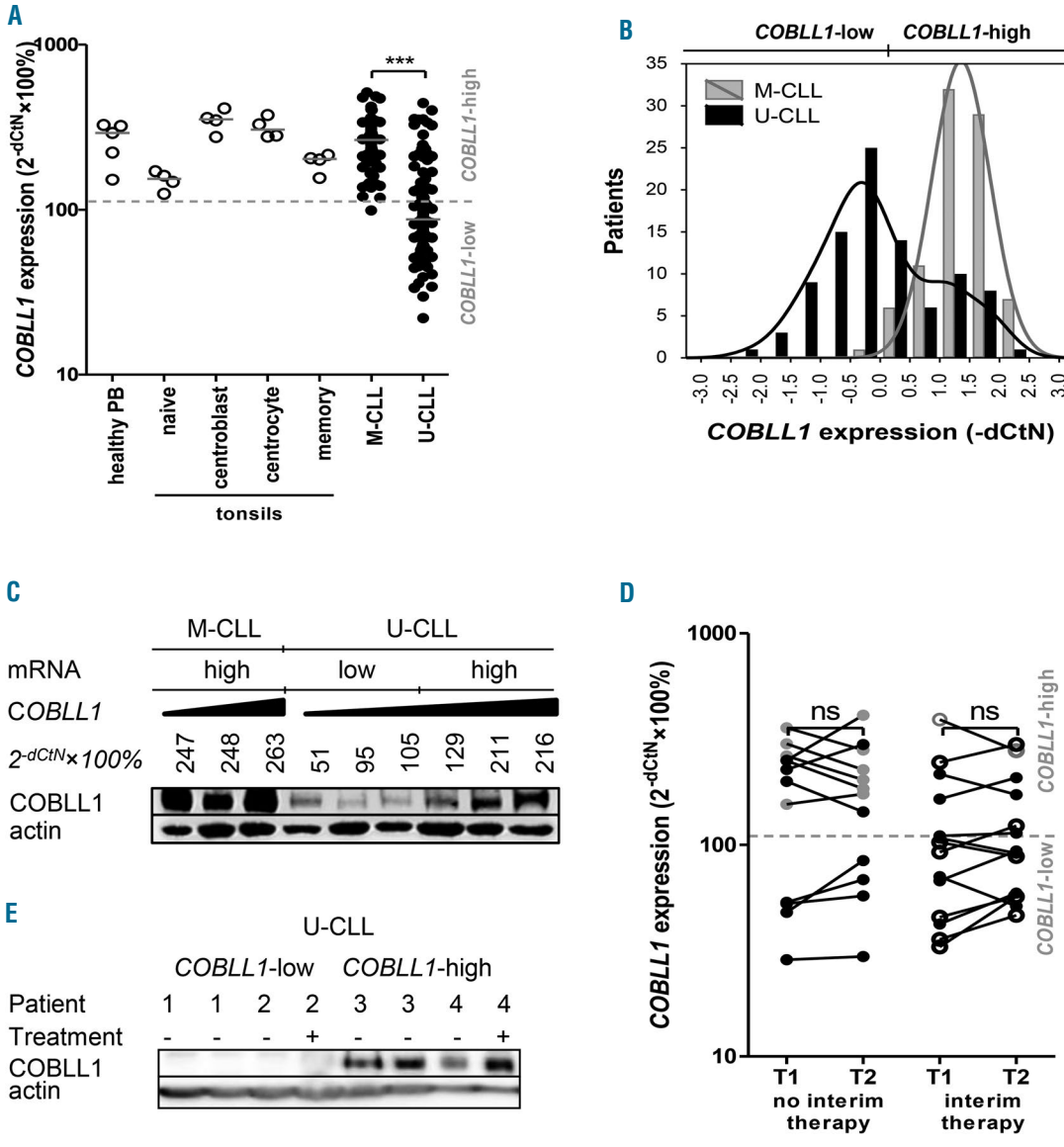


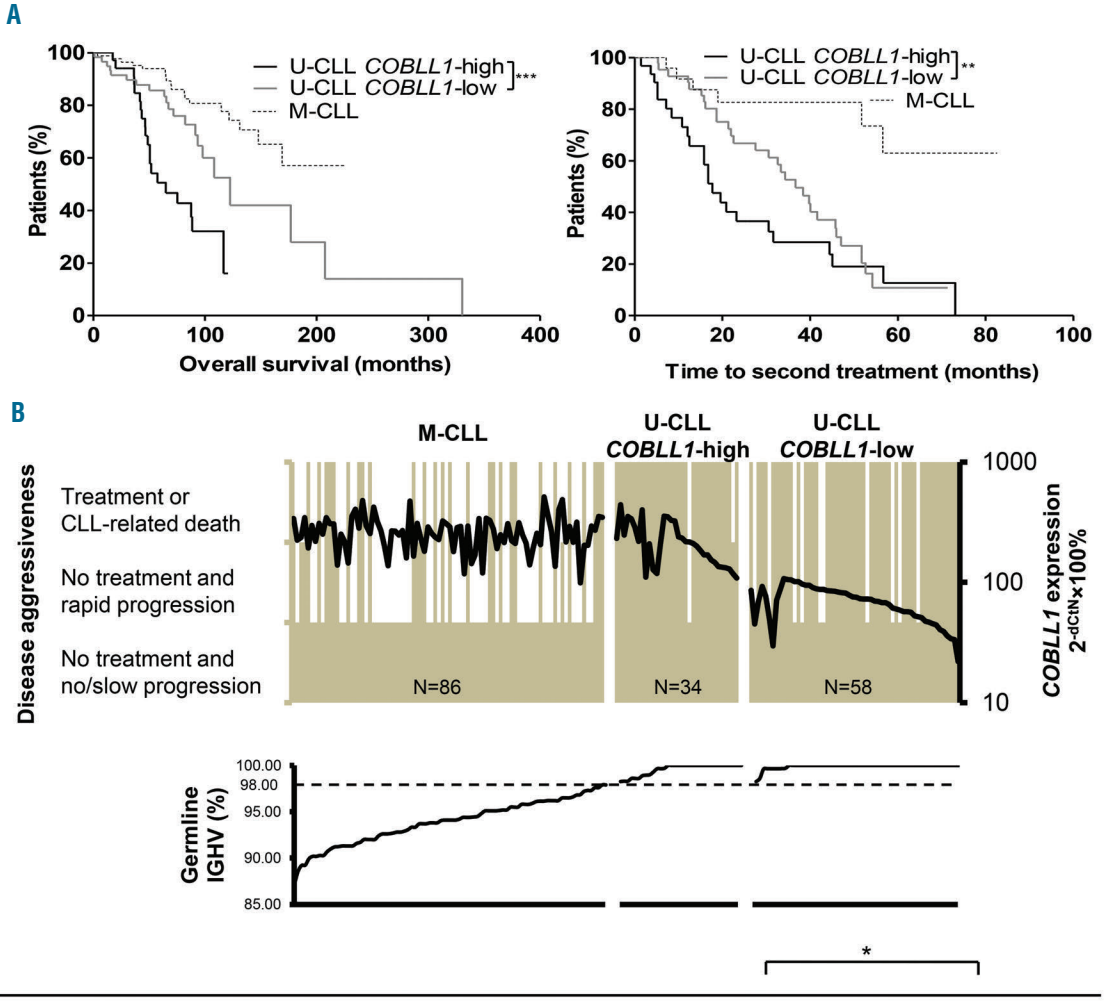
Figure 2. *COBLL1* expression in chronic lymphocytic leukemia (CLL) and non-malignant B cells. (A) *COBLL1* mRNA expression in 86 mutated CLL (M-CLL), 92 unmutated CLL (U-CLL), and healthy B cells isolated from 4 tonsils and 5 peripheral blood (PB) samples. Individual dots represent individual patients. Full lines indicate median. dCtN - dCt value normalized for three independent datasets (see Methods); ***P<0.0001, Mann-Whitney test. (B) *COBLL1* expression histogram follows a bimodal distribution pattern in U-CLL. (C) COBLL1 protein levels correspond very well with *COBLL1* mRNA both in M-CLL and U-CLL cells. COBLL1 protein levels in CLL cells were determined using western blotting and anti-COBLL1 antibody. Actin was used as a loading control. Patient samples are ordered according to their IGHV mutation status and *COBLL1* mRNA expression [in the ascending order; numbers indicate patients' relative *COBLL1* expression determined by qRT-PCR (see A)]. (D) *COBLL1* mRNA expression does not change with time or treatment. *COBLL1* expression was analyzed in each patient at two time points (T1 and T2, connected by line) with (left; 6 M-CLL, 6 U-CLL) or without (right; 1 M-CLL, 13 U-CLL) therapy in the interim. Patients were administrated mainly fludarabine-cyclophosphamide-rituximab (FCR) regimen. ● M-CLL, ● U-CLL: open circle; FCR; full circle: other therapy. Wilcoxon signed rank test. ns: not significant. (E) COBLL1 expression does not change with time or treatment. Protein expression was detected in 2 U-CLL *COBLL1*-low patients (1 and 2) and 2 U-CLL *COBLL1*-high patients (3 and 4) at two time points with or without therapy in the interim. Western blotting and anti-COBLL1 and anti-actin (as a loading control) antibody was used.

These co-immunoprecipitation experiments confirmed that ROR1 can indeed interact with COBLL1 both at the exogenous as well as endogenous level.

ROR1 is a transmembrane receptor, which in most cell types has the capability to induce filopodia formation.[33] Immunofluorescence staining of transfected HEK293 cells showed that, when solely COBLL1 is expressed, it was localized mainly in the cytoplasm (Figure 1B). However, when ROR1 was co-expressed, the COBLL1 signal was detected predominantly in the plasma membrane (Figure 1C) where it co-localized with ROR1. The co-localization was most prominent in the filopodia, which formed as a consequence of ROR1 overexpression (Figure 1D). These data demonstrate that COBLL1 is a true ROR1 binding partner which is recruited to the ROR1 signaling complexes in the membrane.

COBLL1 expression levels vary dramatically among CLL

To evaluate *COBLL1* relevance in CLL, we analyzed its expression in the cohort of 178 CLL untreated patients (86



Patients	M-CLL (%)	U-CLL *COBLL1*-high (%)	U-CLL *COBLL1*-low (%)
Treatment or CLL-related death	31.4	94.1	82.8
No treatment and rapid progression	8.1	2.9	0.0
No treatment and no/slow progression	60.5	2.9	17.2

Figure 3. Unmutated chronic lymphocytic leukemia (U-CLL) *COBLL1*-high patients show significantly shorter survival and progress more often compared to U-CLL *COBLL1*-low patients. (A) U-CLL *COBLL1*-high patients show shorter overall survival (left) and time to second treatment (right). Survival data are presented using Kaplan-Meier plots and tested by Gehan-Breslow-Wilcoxon test. (B) U-CLL *COBLL1*-high patients progress more often than patients in other groups. Progression (left y-axis, gray columns) categorized as 1 - no treatment and no/slow progression (clinical stage Rai 0/I at both diagnosis and sampling); 2 - no treatment but rapid progression (clinical stage Rai 0/I at diagnosis and II/III/IV at sampling); 3 - treatment or CLL-related death (various clinical stages at diagnosis and sampling). Patients are grouped based on their IGHV mutation/*COBLL1* expression status, and ordered according to germline IGHV (in the ascending order, x-axis) and *COBLL1* expression (descending order, full line, right y-axis). (A left and B). N: 86 mutated CLL (M-CLL), 58 U-CLL *COBLL1*-low, 34 U-CLL *COBLL1*-high. (A right). N: 28 M-CLL, 48 U-CLL *COBLL1*-low, 32 U-CLL *COBLL1*-high. *$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$. CLL progression was tested by Fisher's exact test (U-CLL *COBLL1*-high *vs.* U-CLL *COBLL1*-low).

Table 2. Multivariate Cox analysis in unmutated chronic lymphocytic leukemia overall survival.

	HR	95%CI for HR		P
		Lower	Upper	
COBLL1 - high	2.924	1.372	6.232	**0.005**
Age at diagnosis	1.025	0.982	1.070	0.261
Risk according to Rai stage at diagnosis*				
Intermediate	1.514	0.668	3.427	0.320
High	6.029	2.003	18.145	**0.001**
CD38 - positive	3.086	1.087	8.757	**0.034**
Cytogenetic hierarchical model**				
del(17p)	5.049	1.691	15.076	**0.004**
del(11q)	2.503	0.853	7.344	0.095
trisomy 12	0.816	0.192	3.468	0.783
del(13q)	1.232	0.399	3.811	0.717

*Compared to low risk. **Compared to normal karyotype. HR: hazard ratio; CI: confidence interval. Statistically significant *P*-values are highlighted in bold.

M-CLL, 92 U-CLL) and compared it with non-malignant B cells from PB (5 samples) and tonsillar tissue (4 samples). *COBLL1* was highly expressed in normal PB and tonsillar tissue (Figure 2A). The expression in individual tonsillar B-cell subpopulations varied; *COBLL1* expression in centroblasts and centrocytes was increased compared to naïve and memory B cells.

The expression in CLL cells differed significantly according to the IGHV mutation status ($P<0.0001$, Mann-Whitney test). *COBLL1* levels were higher in M-CLL patients with an expression comparable to that of healthy tonsillar and PB B cells. On the contrary, the *COBLL1* expression in U-CLL showed bimodal distribution (Figure 2B). A subgroup of U-CLL patients expressed *COBLL1* at a level comparable with M-CLL patients, but in the majority of U-CLL samples *COBLL1* expression was much lower. Since the *COBLL1* expression had such a clearly bimodal distribution in all three independently analyzed datasets (see Methods section and *Online Supplementary Figure S1*), we set a cut off to distinguish *COBLL1*-high and *COBLL1*-low patients (for details see Methods section). The cut off is set close to the local distribution minimum (Figure 2B). Following this approach, all but one M-CLL patient was classified as *COBLL1*-high. The majority of U-CLL patients (n=58; 63%) were classified as *COBLL1*-low, whereas the remaining U-CLL patients (n=34; 37%) were classified as *COBLL1*-high. Different expression in both cohorts was also confirmed at protein level (Figure 2C).

To analyze the changes in *COBLL1* expression over time and after treatment, we examined 26 patients at two time points (7 M-CLL, 19 U-CLL) (Figure 2D). A part of the cohort was not treated in the interim (6 M-CLL, 6 U-CLL; median 37 months), whereas the remaining patients (1 M-CLL, 13 U-CLL; median 35 months) were administrated a fludarabine-cyclophosphamide-rituximab regimen or another chemoimmunotherapy. The *COBLL1* expression category did not change, with one exception: *COBLL1* expression was slightly increased after treatment in one borderline U-CLL *COBLL1*-low patient. We also examined the changes in expression at protein level in 4 U-CLL patients and obtained similar data (2 U-CLL *COBLL1*-low, 2 U-CLL *COBLL1*-high; 2 with treatment in the interim, 2 without treatment in the interim) (Figure 2E). This suggests that *COBLL1* expression at mRNA as well as protein level does not dramatically change with time or treatment.

Recently, CLL patients with high ROR1 expression were found to suffer from a more aggressive disease.[34] Since COBLL1 and ROR1 form a protein complex, we correlated *COBLL1* and *ROR1* expression (protein levels of *COBLL1* and *ROR1*[11] correspond well with mRNA levels) (Figure 2C) but did not find any correlation (*Online Supplementary Figure S2A*). We were also unable to detect any obvious changes in COBLL1 levels or phosphorylation (detected as phosphorylation-dependent mobility shift) upon activation of ROR1 by its ligand Wnt-5a (*Online Supplementary Figure S2B*). This suggests that COBLL1 rather represents an independently-regulated ROR1 signaling modulator than a bona fide component of ROR1 signaling pathway.

High *COBLL1* expression identifies a subgroup of U-CLL patients with inferior prognosis independent of other prognostic markers

To explore the possible *COBLL1* association with CLL disease course, we analyzed the survival of M-CLL, U-CLL *COBLL1*-low and U-CLL *COBLL1*-high patients. As expected, M-CLL patients showed the best prognosis according to OS and time to second treatment (median OS and TTST not reached; M-CLL *vs.* U-CLL *COBLL1*-low $P_{OS}=0.0389$, $P_{TTST}=0.0104$; M-CLL *vs.* U-CLL *COBLL1*-high $P_{OS}<0.0001$, $P_{TTST}=0.0004$, Gehan-Breslow-Wilcoxon test) (Figure 3A). The survival of U-CLL patients differed according to *COBLL1* expression. U-CLL *COBLL1*-high patients showed a more aggressive disease course (median OS 65 months, TTST 17 months), whereas the U-CLL *COBLL1*-low patients progressed more slowly (median OS 123 months, TTST 37 months; U-CLL *COBLL1*-high *vs.* U-CLL *COBLL1*-low $P_{OS}=0.0086$, $P_{TTST}=0.0116$). There was no significant difference in time to first treatment (TTFT) between U-CLL *COBLL1*-high and low (*Online Supplementary Figure S3*).

To get further insight into the role of COBLL1, we categorized M-CLL, U-CLL *COBLL1*-low and U-CLL *COBLL1*-high patients, based on the aggressiveness of the

disease. This parameter was defined based on the disease behavior between diagnosis and sampling (median time between diagnosis and sampling: 35 months in M-CLL, 11 months in U-CLL *COBLL1*-high, 19 months in U-CLL *COBLL1*-low). Patients were categorized into three groups: 1) no treatment and no/slow progression (clinical stage Rai 0/I at sampling); 2) no treatment but rapid progression (progression into clinical stage Rai II/III/IV at sampling); 3) treatment or CLL-related death. U-CLL *COBLL1*-high progressed more often than U-CLL *COBLL1*-low (P=0.0297, Fisher's exact test) (Figure 3B). U-CLL *COBLL1*-high progressed in almost all cases; only 3% did not progress *versus* 17% in U-CLL *COBLL1*-low patients. In line with this observation, treatment or CLL-related death occurred more often in the U-CLL *COBLL1*-high patients than in U-CLL *COBLL1*-low patients (94% *vs.* 83%).

To further confirm the difference in U-CLL patients

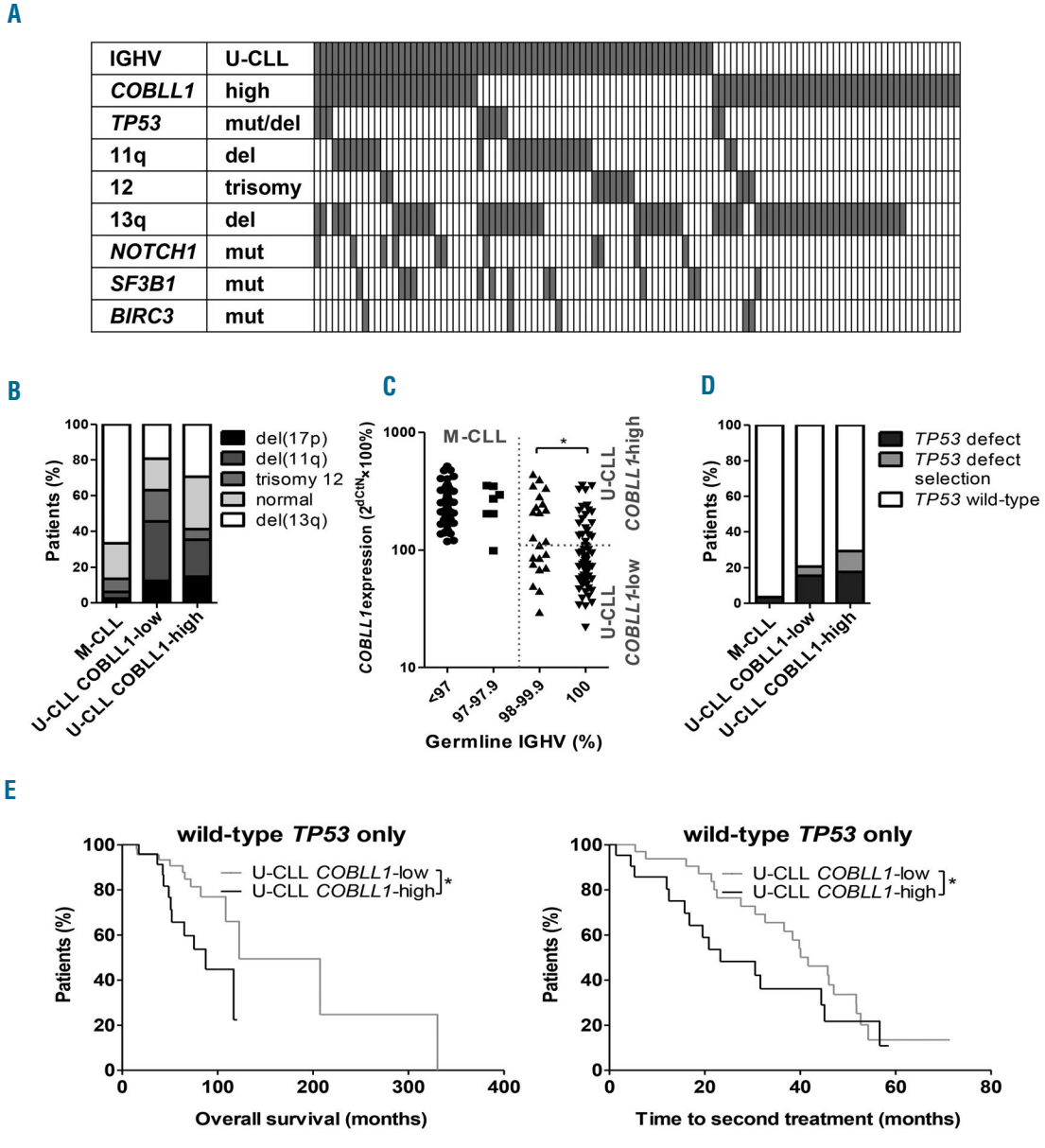
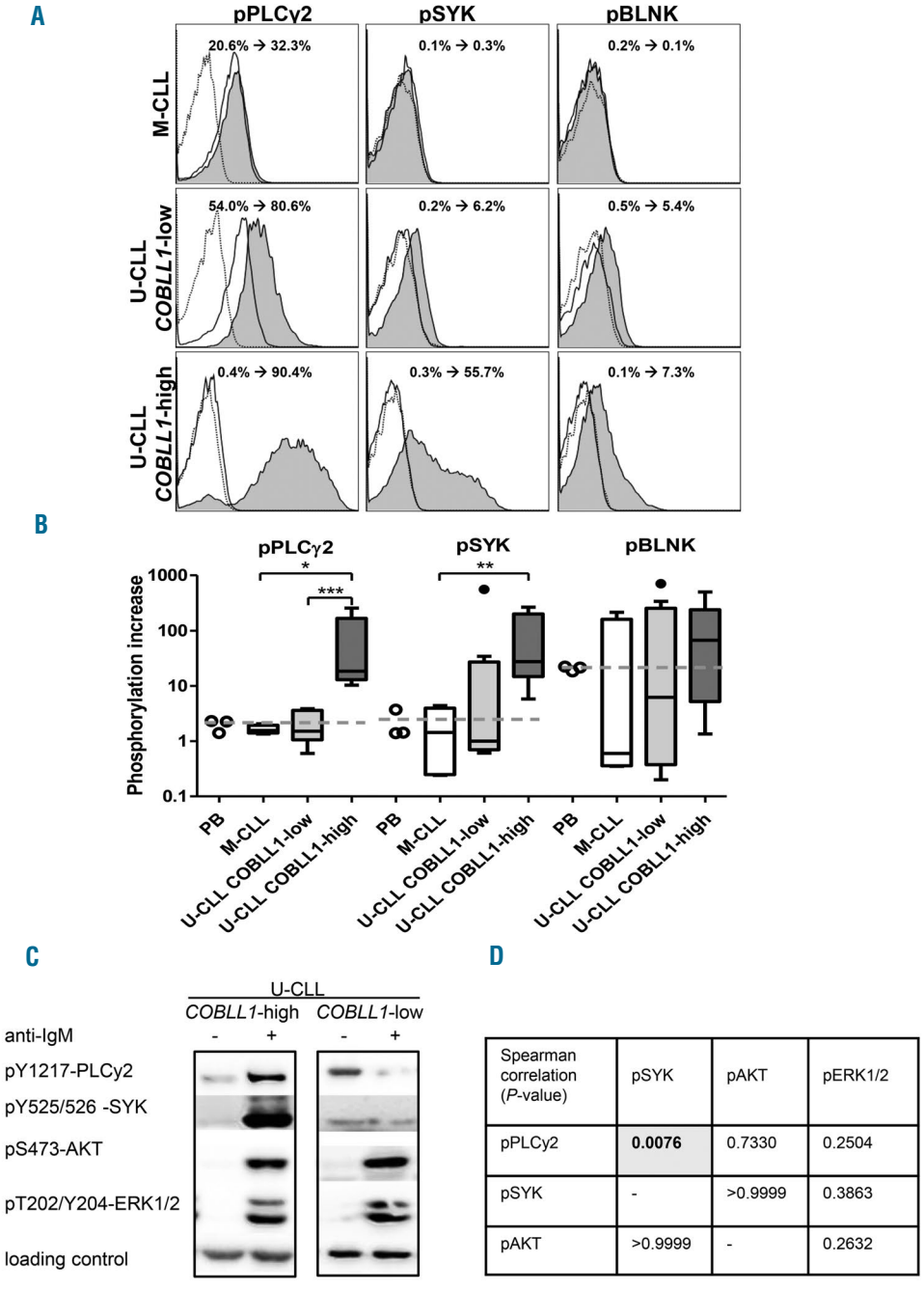


Figure 4. The survival difference between unmutated chronic lymphocytic leukemia (U-CLL) *COBLL1*-high and U-CLL *COBLL1*-low is not caused by recurrent mutations and chromosomal abnormalities. (A-D) Samples were analyzed by I-FISH [del(17p), del(11q), trisomy 12, del(11q)] and sequencing (mutations in IGHV, *TP53, BIRC3, NOTCH1, SF3B1*). *TP53* defect - *TP53* mutation, deletion or both. (A) U-CLL *COBLL1*-low and U-CLL *COBLL1*-high patients do not exhibit any differences in the occurrence of recurrent defects [*TP53* defect, *BIRC3, NOTCH1* and *SF3B1* mutations, del(11q), trisomy 12, del(13q)] or (B) in cytogenetic aberrations evaluated according to the hierarchical model.[36] (C) Expression of *COBLL1* categorized according to the IGHV mutation load. (D) U-CLL *COBLL1*-high patients exhibit non-significantly higher incidence of *TP53* defect at diagnosis or its later selection. (A) 41 mutated CLL (M-CLL), 37 U-CLL *COBLL1*-low, 29 U-CLL *COBLL1*-high. (B-D) 86 M-CLL, 58 U-CLL *COBLL1*-low, 34 U-CLL *COBLL1*-high. (D) Adverse survival of U-CLL *COBLL1*-high patients is retained even in *TP53* wild-type patients. (D) (Left) Overall survival: 46 U-CLL *COBLL1*-low, 24 U-CLL *COBLL1*-high. (D) (Right) Time to second treatment: 37 U-CLL *COBLL1*-low, 22 U-CLL *COBLL1*-high. *P≤0.05, **P≤0.01, ***P≤0.001. Aberrations frequency tested by Fisher's exact test, survival data tested by Gehan-Breslow-Wilcoxon test, germline IGHV tested by Mann-Whitney test.

overall survival, we analyzed our patient cohorts separately (cohort B *vs.* cohort A+C). The U-CLL *COBLL1*-high patients showed a shorter OS than U-CLL *COBLL1*-low in both cases (58 *vs.* 75 months in cohort B and 75 *vs.* 123 months in cohort A+C) (*Online Supplementary Figure S4*) but the difference was significant only in cohort B (P=0.0314, Gehan-Breslow-Wilcoxon test); this is likely due to the relatively small number of patients.

The striking difference in survival of U-CLL *COBLL1*-



D

Spearman correlation (P-value)	pSYK	pAKT	pERK1/2
pPLCγ2	**0.0076**	0.7330	0.2504
pSYK	-	>0.9999	0.3863
pAKT	>0.9999	-	0.2632

Figure 5. U-CLL *COBLL1*-high cells show higher response upon BCR stimulation. (A and B). Chronic lymphocytic leukemia (CLL) cells (4 mutated CLL (M-CLL), 8 U-CLL *COBLL1*-low, 6 U-CLL *COBLL1*-high) were stimulated for 4 minutes with anti-IgM and response to BCR stimulation was analyzed using phospho-specific antibodies targeted against pPLCγ2, pSYK and pBLNK. (A) Representative examples of M-CLL, U-CLL *COBLL1*-low and U-CLL *COBLL1*-high patients. Histograms show a negative control (unstimulated non-stained sample, dotted line), unstimulated sample (full line) and IgM-stimulated sample (full line, gray area). Percentage of positive cells is indicated (unstimulated sample → stimulated sample). (B) Quantification of changes in the pPLCγ2, pSYK and pBLNK. Phosphorylation increase (y-axis) was calculated as a ratio of positive cells in IgM-stimulated *versus* unstimulated samples. Box-and-Whisker plots show quartiles and median. Dashed line indicates phosphorylation increase in non-malignant peripheral blood (PB) B cells (mean), • outliers, *P≤0.05, **P≤0.01, ***P≤0.001. Mann-Whitney test. (C) Western blot analysis of representative U-CLL samples treated with anti-IgM and analyzed for activation of BCR components using phospho-specific antibodies - PLCγ2 (pY1217), pSYK (pY525/526), pAKT (pS473) and pERK1/2 (pT202/Y204). Loading control: β-actin (left), total PLCγ2 (right). (D) Correlation of the response at the level of individual kinases (Spearman correlation). Statistically significant P-values are highlighted in bold with gray background. See *Online Supplementary Figure* S7 for details and raw data.

high and U-CLL *COBLL1*-low patients leads to compare the genetic aberrations which could influence the patients' prognosis in both cohorts. We analyzed cytogenetic aberrations [del(17p), del(11q), trisomy 12, del(13q)] and recurrent mutations (*TP53, NOTCH1, BIRC3, SF3B1*) in 107 patients (41 M-CLL, 37 U-CLL *COBLL1*-low, 29 U-CLL *COBLL1*-high) where all these data were available. We were unable to find any significant difference in U-CLL *COBLL1*-high *versus* *COBLL1*-low categories (see Figure 4A for brick plot, *Online Supplementary Figure S5* for Circos plot, Figure 4B for hierarchically categorized cytogenetic aberrations;[35] Fisher's exact test).

Since U-CLL patients with borderline IGHV mutations have been shown to have a better prognosis than patients with truly unmutated IGHV,[36] we also compared mutation load in U-CLL patients. The worse prognosis of U-CLL *COBLL1*-high patients could not be explained by difference in mutation load; on the contrary, borderline mutated patients (98-99.9%) showed higher expression of *COBLL1* than patients with 100% identity (P=0.0219, Mann-Whitney test) (Figure 4C).

We further assessed the influence of *TP53* aberrations (mutations, deletions or both) present either before treatment or evolving during disease progression. Although U-CLL *COBLL1*-high patients harbored *TP53* aberrations more often and also lost wild-type *TP53* more often during disease evolution, the differences were not significant (P=0.4823; Fisher's exact test) (Figure 4D). Furthermore, when we compared only wild-type *TP53* patient survival, the U-CLL *COBLL1*-high patients still retained a worse OS and TTST (U-CLL *COBLL1*-low: median OS 122 months, median TTST 42 months; U-CLL *COBLL1*-high: median OS 88 months, median TTST 23 months; P_{OS}=0.0276, P_{TTST}=0.0404; Gehan-Breslow-Wilcoxon test) (Figure 4E).

To evaluate *COBLL1* significance in U-CLL survival, we performed univariate and multivariate Cox regression analyses. The univariate analysis revealed *COBLL1* status, age at diagnosis, Rai stage at diagnosis, CD38 expression and cytogenetic aberrations as significant prognostic factors for OS in U-CLL. Multivariate Cox regression analysis confirmed *COBLL1* as an independent molecular marker (Table 2). In multivariate Cox regression analysis for TTST, *COBLL1* did not retain independence.

Moreover, we did not find any difference in the clinical parameters such as leukocytosis, clinical stage, age or sex, explaining the short survival of U-CLL *COBLL1*-high patients. Due to the striking difference in TTST, we also investigated the administered treatment in detail (*Online Supplementary Figure S6*). We did not find any difference in patient treatment response, length or number of received treatment cycles or if categorized as full, reduced, interrupted or reduced therapy. Therefore, we assumed that the aggressive course of U-CLL *COBLL1*-high patients cannot be explained by any common unfavorable clinicobiological disease characteristics.

U-CLL *COBLL1*-high cells show higher phosphorylation upon BCR stimulation

To understand our findings in context, we performed detailed bioinformatics analysis of publicly available RNA sequencing data of 44 U-CLL samples[30] (see Methods section). A subset of 1240 significantly *COBLL1*-correlated genes (P<0.05; Spearman test) (*Online Supplementary Table S1*) was selected for KEGG pathway analyses. Among the transcripts positively correlating with *COBLL1* in U-CLL the genes associated with various cancer-linked signaling pathways and metabolic processes, including the B-cell receptor (BCR) pathway, were enriched (*Online Supplementary Table S2*). Progressive phosphorylation of BCR pathway components promotes cell survival, differentiation and proliferation in CLL (for review see ten Hacken and Burger[37]). Given the crucial biological and clinical importance of BCR signaling in CLL cells, we hypothesized that U-CLL *COBLL1*-high patients might have a deregulated response to BCR stimulation.

To investigate how U-CLL *COBLL1*-high cells respond to BCR stimulation, we adopted a previously described

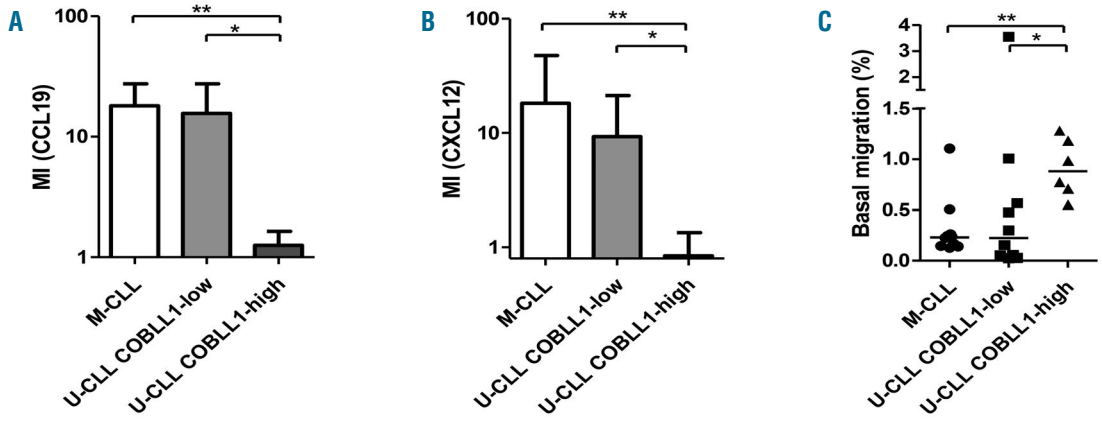


Figure 6. Unmutated chronic lymphocytic leukemia (U-CLL) *COBLL1*-high cells show deregulated chemotaxis and motility. Migratory properties of 10 mutated CLL (M-CLL), 10 U-CLL *COBLL1*-low, and 6 U-CLL *COBLL1*-high samples were assessed using transwell plates. (A) Chemotaxis towards chemokine CCL19 expressed as migration index (MI). (B) Chemotaxis towards chemokine CXCL12 expressed as MI. (C) Basal migration. MI was calculated as the number of cells migrated towards chemokine divided by the number of cells migrated in chemokine-free media. Basal migration was calculated as the percentage of migrated cells from all seeded cells. Each measurement was performed in a technical triplicate. Bars represent mean+Standard Deviation (S.D.) (A and B) Individual dots represent individual patients (C). *P≤0.05, **P≤0.01. (Mann-Whitney test).

protocol,[26] stimulated CLL cells and examined the phosphorylation level of selected BCR signaling pathway components (pPLCγ2, pSYK and pBLNK) *via* flow cytometry. Eighteen CLL samples (4 M-CLL, 8 U-CLL *COBLL1*-low, 6 U-CLL *COBLL1*-high) and 3 peripheral blood (PB) B-cell samples from healthy donors were analyzed. The response to anti-IgM was evaluated as a difference (fold change) in the number of positive cells in stimulated and unstimulated samples.

Not surprisingly, the response to BCR stimulation in CLL cells from individual patients was rather heterogeneous but still showed clear trends in the individual groups (see representative examples in Figure 5A). When quantified (Figure 5B), the number of pPLCγ2-positive cells after BCR stimulation was dramatically increased only in U-CLL *COBLL1*-high [U-CLL *COBLL1*-high *vs.* U-CLL *COBLL1*-low (P=0.0007), U-CLL *COBLL1*-high *vs.* M-CLL (P=0.0007), *vs.* M-CLL (P=0.0139)]. A similar trend could also be seen for pSYK [U-CLL *COBLL1*-high *vs.* U-CLL *COBLL1*-low (P=0.0609), U-CLL *COBLL1*-high *vs.* M-CLL (P=0.0095), Mann-Whitney test] and pBLNK where U-CLL *COBLL1*-high cells responded best, albeit not with statistical significance. The non-malignant PB B-cell controls showed a uniform response, which was very similar to that of M-CLL (Figure 5B).

Interestingly, the western blot analysis confirmed in principle the differences in the activation of upstream BCR signaling components, namely PLCγ2 and SYK, but we were unable to detect any differences between the groups at the activated AKT and ERK1/2 level (Figure 5C). Quantitative analysis of western blot data from a larger cohort of CLL samples (n=10 for pAKT, 11 for all others) showed that pPLCγ2 and pSYK signals correlated strongly with each other (Figure 5D, graphs in *Online Supplementary Figure S7*) but not with the pAKT and pERK1/2 signals that were almost uniformly induced in all patients (*Online Supplementary Figure S7A*). This suggests that regulating the upstream (PLCγ2, SYK) and downstream (ERK1/2, AKT) BCR pathway module can differ. We conclude that U-CLL *COBLL1*-high patients exhibit an enhanced response to BCR stimulation, in particular at the level of upstream components such as pPLCγ2 and pSYK.

U-CLL *COBLL1*-high cells exhibit impaired migration and chemotaxis

COBLL1 can physically interact with ROR1 and thus represents a candidate regulator of the non-canonical Wnt/PCP pathway. Since the Wnt/PCP pathway was shown to be involved in the migration of CLL cells,[11,13] we analyzed their ability to respond to chemokines CCL19 and CXCL12, known to stimulate the cells *via* CCR7 and CXCR4 receptors, respectively.[38] CLL cells were stratified according to the expression of *COBLL1* and IGHV mutational status (10 M-CLL, 10 U-CLL *COBLL1*-low, 6 U-CLL *COBLL1*-high).

Generally, the chemotactic and migratory abilities differed according to the combination of IGHV status and *COBLL1* expression (Figure 6). U-CLL *COBLL1*-high cells showed impaired chemotaxis towards chemokines CCL19 and CXCL12 and increased basal migration compared to U-CLL *COBLL1*-low and M-CLL cells [both CCL19 and CXCL12: U-CLL *COBLL1*-high *vs.* M-CLL (P=0.0017), U-CLL *COBLL1*-high *vs.* U-CLL *COBLL1*-low (P=0.0302); basal migration: U-CLL *COBLL1*-high *vs.* M-CLL (P=0.0030), U-CLL *COBLL1*-high *vs.* U-CLL *COBLL1*-low (P=0.0420) Mann-Whitney test]. U-CLL *COBLL1*-low exhibited an intermediate response to chemokine stimuli. The deregulated migratory abilities of U-CLL *COBLL1*-high cells further point out their altered microenvironmental interactions.

Discussion

In this study, we have identified COBLL1 as a novel binding partner for ROR1 in CLL. COBLL1 is an evolutionary conserved but very little known protein. Its mouse ortholog Cordon blue (Cobl) interacts with Vang-like protein 2 (Vangl2; a component of the Wnt/PCP pathway) and is required for neural tube closure, which is a process typically regulated by the Wnt/PCP pathway.[39] The combination of these results and our findings suggests that COBLL1 can represent a Wnt/PCP pathway regulator in mammals. COBLL1 levels in CLL dramatically vary and do not correlate with ROR1. This opens up the possibility that the way COBLL1 affects ROR1 function may differ depending on the level of COBLL1.

COBLL1 links to human pathological conditions are very limited and restricted to the observation that *COBLL1* upregulation is associated with a better prognosis after surgery in malignant pleural mesothelioma, where it acts as a negative regulator of apoptosis.[40] On the contrary, *COBLL1* upregulation in chronic myeloid leukemia patients was recently associated with a reduction in nilotinib-dependent apoptosis, disease progression and shorter OS.[41] Besides mature B cells, *COBLL1* expression is detectable in various other cell types including other blood elements (such as T cells), although usually at a much lower level.[42] Comparable or higher *COBLL1* levels were detected in mast cells, adipocytes, placenta and esophagus.[42]

Our data show that in M-CLL, *COBLL1* expression is uniformly high, whereas in U-CLL patients it ranges from low to high levels. The U-CLL *COBLL1*-high cohort showed a strikingly worse prognosis than the *COBLL1*-low. Shorter OS and TTST of U-CLL *COBLL1*-high patients remained, even after excluding patients with aberrant *TP53* and the independence of *COBLL1* as a prognostic factor in U-CLL for OS was proven by multivariate analysis. IGHV mutational status and *COBLL1* expression thus represents a novel marker combination, which efficiently identifies patients with short OS and TTST.

We showed bimodal *COBLL1* expression distribution in three independent cohorts. The U-CLL patients can be categorized according to a cut off close to a local distribution minimum which facilitates access to our marker combination by other laboratories if desirable. In addition to qRT-PCR-based assessment of *COBLL1* expression, COBLL1 protein levels can in principle, be analyzed using flow cytometry. However, this would require fixation (COBLL1 is a cytoplasmic protein) and staining with a primary and secondary antibody, since there are currently no well-validated fluorescently-conjugated monoclonal antibodies.

Interestingly, functional analysis of U-CLL *COBLL1*-high CLL cells showed a higher response to BCR stimulation and deregulated chemotaxis in this patient cohort. This is in line with a large body of evidence showing that increased *in vitro* response to BCR stimuli associ-

ates with aggressive CLL.[43,44] *COBLL1*-high CLL cells preferentially responded by activation of BTK, SYK and ERK1/2 whereas *COBLL1*-low U-CLL cells induced only ERK1/2. It has been shown previously that, in healthy B cells, ERK1/2 can be efficiently phosphorylated by Ig-induced BCR crosslinking even in cases when no detectable phosphorylation of BTK or SYK is seen.[45] There is also some evidence that these different modes of BCR activation depend on the stimulus[45] and also differ between healthy and malignant cells.[46] This suggests that COBLL1 can regulate this balance and promote the BCR activation mode that involves the upstream BTK/SYK kinases.

In addition, U-CLL *COBLL1*-high cells exhibit impaired migration towards chemokines CCL19 and CXCL12, a phenotype very similar to aggressive CLL cells expressing ROR1 ligand Wnt-5a.[11] Although we were not able to correlate *WNT5A* and *COBLL1* expression, both studies indicate lower chemotaxis in patients with aggressive CLL and a deregulated Wnt/PCP signaling pathway. Both observations support the generally accepted view that patients with inferior prognosis often exhibit deregulated interaction with the microenvironment and with other cell types. The clear difference in TTST in U-CLL *COBLL1*-high patients suggests that standard therapeutic schemes do indeed have limited efficiency in this cohort. Due to their unmutated IGHV (U-CLL patients have been described as more perceptive to ibrutinib than M-CLL[47]) and high BCR responsiveness, *COBLL1* can thus help to identify patients that will benefit more from the new BCR inhibitor-based therapies.

The role of *COBLL1* in CLL pathogenesis and in B-cell development remains unclear. One striking observation is apparently the difference in importance of high *COBLL1* in M-CLL and U-CLL. We were able to confirm previously reported uniformly high *COBLL1* levels in M-CLL cells.[48,49] Interestingly, M-CLL is generally more indolent than U-CLL, where high *COBLL1* rather counterintuitively defines patients with an inferior prognosis. Upregulation of *COBLL1* in centroblasts and centrocytes compared to naïve and memory cells indicates that *COBLL1* is switched on during B-cell maturation in the germinal center. Together with lower IGHV germline identity in U-CLL *COBLL1*-high patients (compared to U-CLL *COBLL1*-low patients), it suggests that upregulation of *COBLL1* expression may be linked to the process of IGHV mutation. This view is also supported by the gene profiling of monoclonal B-lymphocytosis cells (MBL) with mutated and unmutated IGHV[50] where *COBLL1* expression followed a similar pattern to CLL.[48,49] This suggests that deregulating *COBLL1* expression likely occurs prior to overt CLL, or, alternatively, points to a different origin of a U-CLL subset from a rare B-cell subset with low *COBLL1* expression. This assumption is also supported by the observation that the levels of *COBLL1* in U-CLL *COBLL1*-low samples are lower than any of the healthy B-cell populations analyzed in this study.

In summary, we identified COBLL1 as a component of the ROR1 receptor system in CLL cells. *COBLL1* expression combined with IGHV hypermutation status correlates with CLL prognosis, and identifies the U-CLL *COBLL1*-high patients as those having an adverse disease course. U-CLL *COBLL1*-high cells show an increased response to BCR stimulation and attenuated chemotaxis, which suggests a mutual interplay between Wnt/PCP and BCR pathways in the regulation of CLL response to microenvironmental stimuli.

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