SUPPLEMENTARY APPENDIX

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

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**Supplementary information to:**

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## Supplementary Methods

### Determination of IGHV mutation status and genetic aberrations

*IGHV* mutation status was determined using Sanger sequencing of cDNA and analysis with IMGT/V-QUEST database and tools as described before.[1] Mutated *IGHV* was established as >2% deviation from the germline sequence.

Cytogenetic aberrations del(17p13), del(11q23), del(13q14) and trisomy 12 were analyzed using Metasystems or Abott Vysis I-FISH probes.[2,3] *TP53* mutations were examined by yeast functional analysis (FASAY) and Sanger sequencing.[4,5] Mutations in *SF3B1* (exons 14-16), *NOTCH1* (exon 34) and *BIRC3* (exon 7-10) were identified by Sanger sequencing.[6]

### HEK293 and MAVER-1 cultivation

HEK293 cells were obtained from ATCC and cultured in DMEM supplemented with 10% FBS and 1% antibiotics (penicillin, streptomycin). MAVER-1 cells were obtained from DSMZ and cultured in RPMI-1640 supplemented with 10% FBS and 1% antibiotics. Cells were cultured at 37°C with 5% $CO_2$.

### Mass spectrometry

Immunoprecipitation was performed using anti-ROR1 antibody (AF 2000; R&D Systems) from the lysates of $10^8$ primary CLL cells. Unspecific IgG (sc-2028, Santa Cruz Biotechnology) was used as a control.

Following the co-immunoprecipitation samples were washed in detergent-free RIPA buffer and subjected to proteomics grade trypsin digest (10 ng/µl, 37°C/12h) (Sigma). LC-MS/MS was performed on a NanoAcquity UPLC (Waters) coupled to an ESI Q-Tof Premier (Waters) Mass spectrometer. Digested peptides were first desalted using ZipTip C18 column (Millipore), diluted in MQ water and loaded onto nanoAcquity UPLC Symmetry trap column

(Waters) packed with 5µm BEH C-18 beads. Peptides were eluted through a nanoAcquity (Waters) analytical column packed with 1.7µm BEH C-18 beads at a flow rate of 400nL/min using a gradient of 3 – 40% acetonitril with 0.1% formic acid for 35 minutes. Effluent was directly fed into the ESI source of the MS instrument. Raw data were acquired in $MS^E$ Identity mode, and later subjected to a search using UniProt and NCBI mouse protein database by the PLGS2.3 software (Waters). Acetyl N-terminal, deamidation N and Q, Carbamidomethyl C and Oxidation M were set as variable modifications. Peptide accuracy and MS/MS fragment mass accuracy was set to less than 20 ppm. Generated hit lists were manually curated to remove common contaminants and hits found in IgG IP samples.

In total samples from five U-CLL patients were analyzed. COBLL1 was identified by multiple independent IP/MS experiments and was thus further considered for validation and testing.

**Immunoprecipitation from HEK293 and MAVER-1 cells**

HEK293 cells were transfected with total amount of 8 µg DNA per a 10cm plate as described above and incubated 24 hours. HEK293 cells were washed twice with PBS, lyzed in 0.5% NP40 buffer and further processed as described previously.[7] Lysate of MAVER-1 cells was precleared with Dynabeads Protein G (Life Technologies) for 1 hour, incubated with antibodies overnight at 4°C, incubated 3 hours with Dynabeads Protein G at 4°C and otherwise processed as described previously.[7] Antibodies anti-V5 (R960-25, Invitrogen), anti-FLAG (clone M2, Sigma Aldrich), anti-ROR1 (AF2000, R&D Systems) and Normal Goat IgG Control (AB-108-C, R&D Systems) were used.

**Transfection**

HEK293 cells were transfected with PEI as described previously.[8] FLAG-COBLL1 vector was generated using Gateway system and LR Clonase II kit (Invitrogen), pcDNA3.1-FLAG destination vector (preparation described previously[9]) and ENTRY COBLL1 vector (U0698, GeneCopoeia). To prepare ROR1-V5 vector, we cloned ROR1 from pCMV6-XL6-ROR1 vector (OriGene Technologies) into pcDNA3.1/V5-His vector (Invitrogen). pcDNA3 empty vector was used to equalize the transfected DNA content.

**Immunofluorescence**

HEK293 cells were seeded onto 0.1% gelatin-coated glass cover slips in 24-well plate, transfected as indicated with total amount of 0.2 μg of DNA per well and further processed as described previously.[10] For protein visualization, primary antibodies (anti-FLAG, clone M2, Sigma Aldrich, anti-V5, R960-25, Invitrogen), secondary antibodies (Alexa Fluor 488, Alexa Fluor 645, Invitrogen) and 1μg/ml DAPI (AppliChem GmbH) were used. Coverslips were mounted using Glycerol-gelatin (Sigma Aldrich). Images were captured on a Zeiss 710 LSM laser-scanning microscope (Carl Zeiss, Inc.).

**Western blotting**

Cells were lysed in 1% SDS buffer supplemented with β-mercaptoethanol and processed as described previously.[11] Lysates were separated on 8% SDS-PAGE gel, transferred onto a PVDF membrane, probed with primary antibodies [anti-COBLL1, ab64465, Abcam; anti-ROR1, AF2000, R&D Systems; anti-V5, R960-25, Invitrogen; anti-FLAG, clone M2, Sigma Aldrich; anti-Actin, clone C-11, Santa Cruz Biotechnology; anti-phospho PLCγ2 (Y1217), cs-3871; anti-phospho SYK (Y525/526), cs-2710; anti-phospho Akt (S473), cs-9271; anti-phospho P44/42 MAPK (Erk1/2; T202/Y204), clone 20G11, cs-4376 and total PLCγ2, cs-

3872 (all Cell Signaling)] and secondary antibodies (anti-goat IgG, whole molecule-peroxidase, A4171; anti-rabbit IgG, whole molecule-F(ab')$_2$ fragment-peroxidase, A6667; anti-mouse IgG, whole molecule-peroxidase, A6782, all Sigma-Aldrich). To visualize signals, Immobilon Western Chemiluminiscent HRP Substrate (Merck) was used.

**Gene expression analysis**

Three datasets of *COBLL1* expression were obtained with the following methods: (i) reverse transcription using SuperScript II (Invitrogen) and subsequent qPCR analysis of *COBLL1* and reference genes (*ACT*, *B2M*) with specific primers (Integrated DNA Technologies) and Power SYBR Green Master Mix (Applied Biosystems) detected at LC480 (F. Hofmann-La Roche Ltd; cohort A); (ii) qRT-PCR of *COBLL1* and reference genes (*B2M, HPRT1, GUSB*) with gb ONCO CLL kit[12] and TaqMan probes (both Generi Biotech) measured at CFX (Bio-Rad Laboratories Inc.; cohort B); (iii) qRT-PCR of *COBLL1* and reference genes (*B2M, HPRT1, GUSB*) with gb ONCO CLL kit[12] and TaqMan probes measured at ABI7900HT (Applied Biosystems; cohort C).

**BCR stimulation**

The cells were incubated for 4 min at 37°C with 10 µg/ml anti-IgM F(ab')$_2$ (µ chain specific, Southern Biotech) and 3.3 mM $H_2O_2$. The cells were stained with anti-pPLCγ2 Alexa Fluor 488, anti-pSYK PE and anti-pBLNK PE, respectively (pY759, clone K86-689.37; pY348, clone I120-722; pY84, clone J117-1278; BD Biosciences), washed with 1% BSA in PBS and analyzed by Accuri C6 Flow Cytometer (BD Biosciences).

**Wnt-5a treatment**

$10^7$ primary CLL cells per sample (thawn 2 h before experiment) were incubated 2 hours at 37°C and 5% $CO_2$ with recombinant Wnt-5a (200 ng/ml, 645-WN-010, R&D Systems) or corresponding volume of PBS/0.1% BSA in RPMI-1640 supplemented with 10% FBS and 1% antibiotics and further processed by Western blotting.

**Statistical analysis and data visualization**

The differences in expression (non-paired samples), *IGHV* identity, migration and phosphorylation increase were evaluated by Mann-Whitney test. Expression in paired samples was evaluated by Wilcoxon signed rank test. Overall survival (defined as disease-specific survival), time to first treatment and time to second treatment (defined as interval between start of the first and the second therapy line) were analyzed and evaluated by Gehan-Breslow-Wilcoxon test. Differences in aberrations' frequency and in disease progression were tested by Fisher's exact test. Association between two variables was tested by Spearman correlation. *COBLL1* evaluation as an independent prognostic factor was performed by univariate and multivariate Cox regression analyses.

To analyze *COBLL1*-linked signaling pathways, CLLE-ES dataset was used (www.icgc.org).[13] The RPKM values (reads per kilobase per million mapped reads) from RNASeq01 data were extracted for all expressed genes. Spearman correlation of *COBLL1* (ENSG00000082438) with remaining genes was applied. By hypergeometric testing, significantly associated KEGG pathways were identified.

The standard level of statistical significance was $P \leq 0.05$. All statistical tests were performed as two-sided.

**Supplementary References**


1. Plevova K, Francova HS, Burckova K, et al. Multiple productive immunoglobulin heavy chain gene rearrangements in chronic lymphocytic leukemia are mostly derived from independent clones. Haematologica 2014;99(2):329–338.
2. Baliakas P, Iskas M, Gardiner A, et al. Chromosomal translocations and karyotype complexity in chronic lymphocytic leukemia: A systematic reappraisal of classic cytogenetic data. Am J Hematol 2014;89(3):249–255.
3. Baliakas P, Hadzidimitriou A, Sutton LA, et al. Clinical effect of stereotyped B-cell receptor immunoglobulins in chronic lymphocytic leukaemia: A retrospective multicentre study. Lancet Haematol 2014;1(2):e74–e84.
4. Malcikova J, Smardova J, Rocnova L, et al. Monoallelic and biallelic inactivation of TP53 gene in chronic lymphocytic leukemia: Selection, impact on survival, and response to DNA damage. Blood 2009;114(26):5307–5314.
5. Pospisilova S, Gonzalez D, Malcikova J, et al. ERIC recommendations on TP53 mutation analysis in chronic lymphocytic leukemia. Leukemia 2012;26(7):1458–1461.
6. Baliakas P, Hadzidimitriou A, Sutton L, et al. Recurrent mutations refine prognosis in chronic lymphocytic leukemia. Leukemia 2015;29(2):329–336.
7. Janovska P, Poppova L, Plevova K, et al. Autocrine signaling by Wnt-5a deregulates chemotaxis of leukemic cells and predicts clinical outcome in chronic lymphocytic leukemia. Clin Cancer Res 2016;22(2):459–469.
8. Kaucká M, Krejčí P, Plevová K, et al. Post-translational modifications regulate signalling by Ror1. Acta Physiol 2011;203(3):351–362.
9. Arquint C, Sonnen KF, Stierhof YD, Nigg EA. Cell-cycle-regulated expression of STIL controls centriole number in human cells. J Cell Sci 2012;125(Pt 5):1342–1352.
10. Cervenka I, Valnohova J, Bernatik O, et al. Dishevelled is a NEK2 kinase substrate

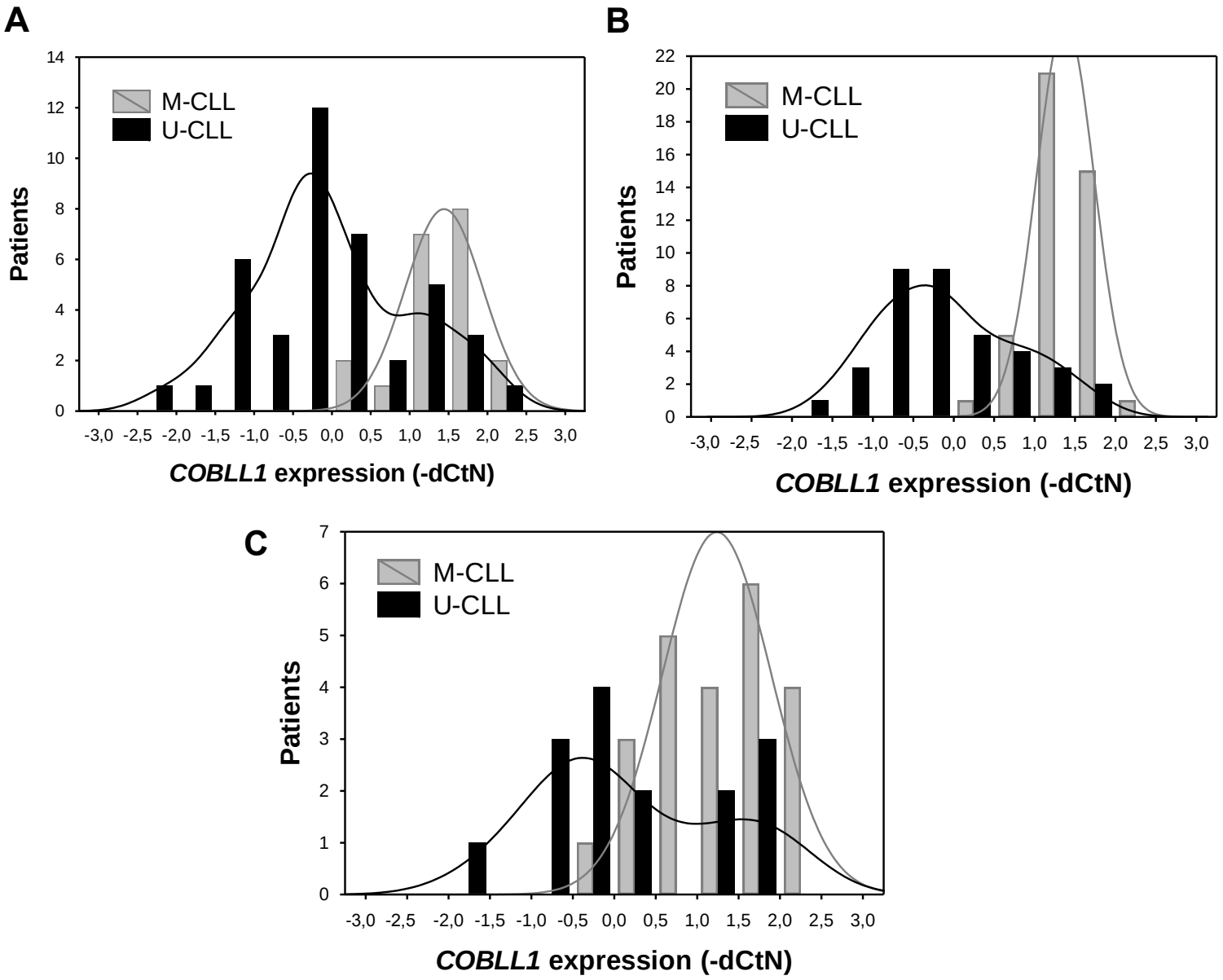
controlling dynamics of centrosomal linker proteins. Proc Natl Acad Sci U S A 2016;113(33):9304–9309.

11. Bryja V, Schulte G, Arenas E. Wnt-3a utilizes a novel low dose and rapid pathway that does not require casein kinase 1-mediated phosphorylation of Dvl to activate β-catenin. Cell Signal 2007;19(3):610–616.

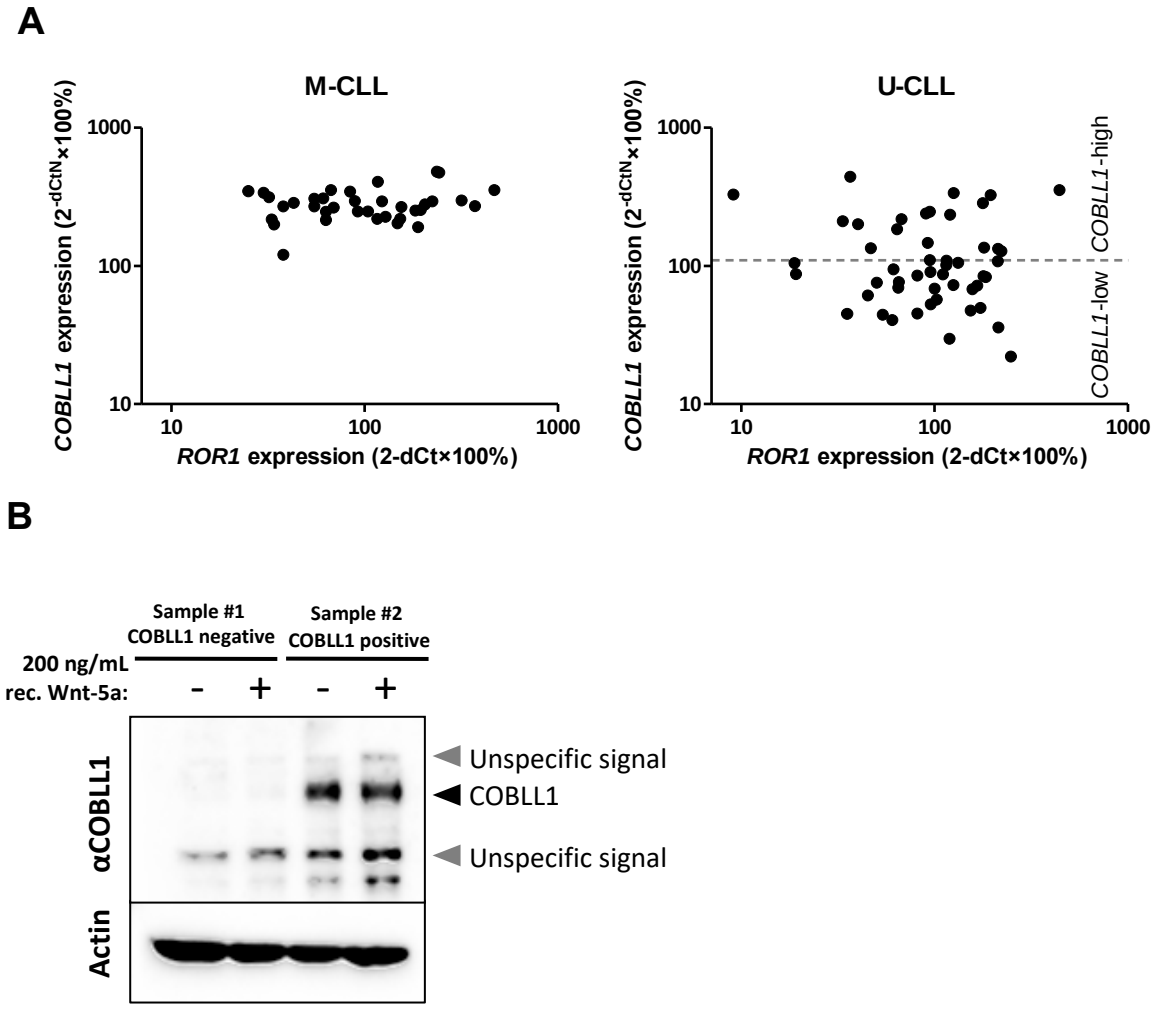
12. Plesingerova H, Librova Z, Plevova K, et al. COBLL1, LPL and ZAP70 expression defines prognostic subgroups of chronic lymphocytic leukemia patients with high accuracy and correlates with IGHV mutational status. Leuk Lymphoma 2017;58(1):70–79.

13. Ramsay AJ, Martínez-Trillos A, Jares P, Rodríguez D, Kwarciak A, Quesada V. Next-generation sequencing reveals the secrets of the chronic lymphocytic leukemia genome. Clin Transl Oncol 2013;15(1):3–8.

## Supplementary Figures



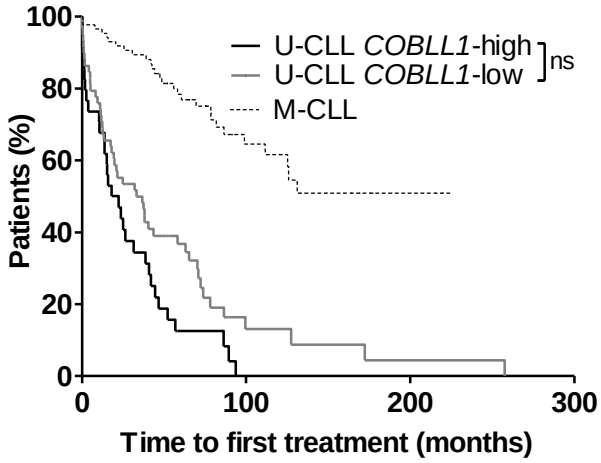

**Supplementary Figure 1: Distribution of *COBLL1* expression.** Histogram of *COBLL1* expression is plotted using -dCtN values. Data were further transformed using mean expression and standard deviation of U-CLL samples and merged into single cohort. ■ M-CLL, ■ U-CLL. **A.** Cohort A. 20 M-CLL, 41 U-CLL. **B.** Cohort B. 43 M-CLL, 36 U-CLL. **C.** Cohort C. 23 M-CLL, 15 U-CLL.



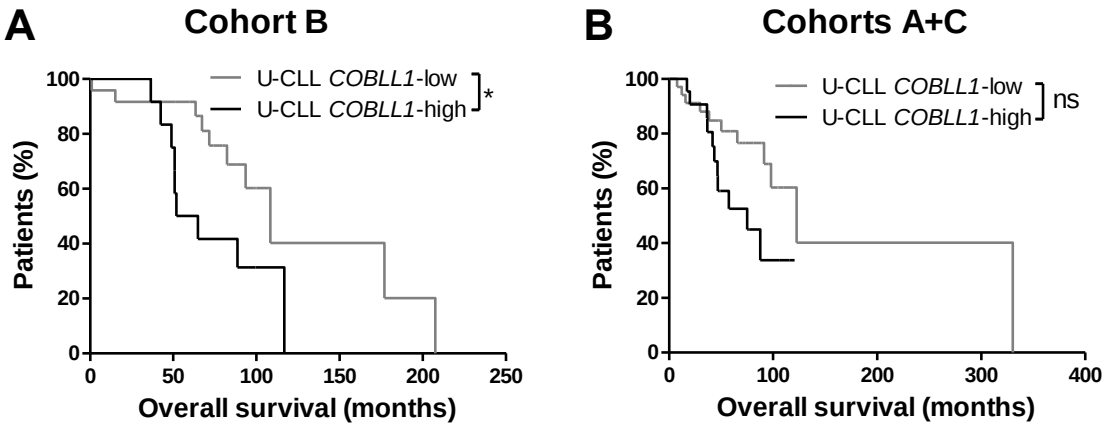

**Supplementary Figure 2: Connections between COBLL1 and ROR1.**

**A. *COBLL1* and *ROR1* expression does not correlate.** Expression of *COBLL1* and *ROR1* was analyzed and correlated in 34 M-CLL (left, R=0.1090, P=0.5267) and 42 U-CLL patients (right; R=-0.1411, P=0.3727). Spearman correlation.

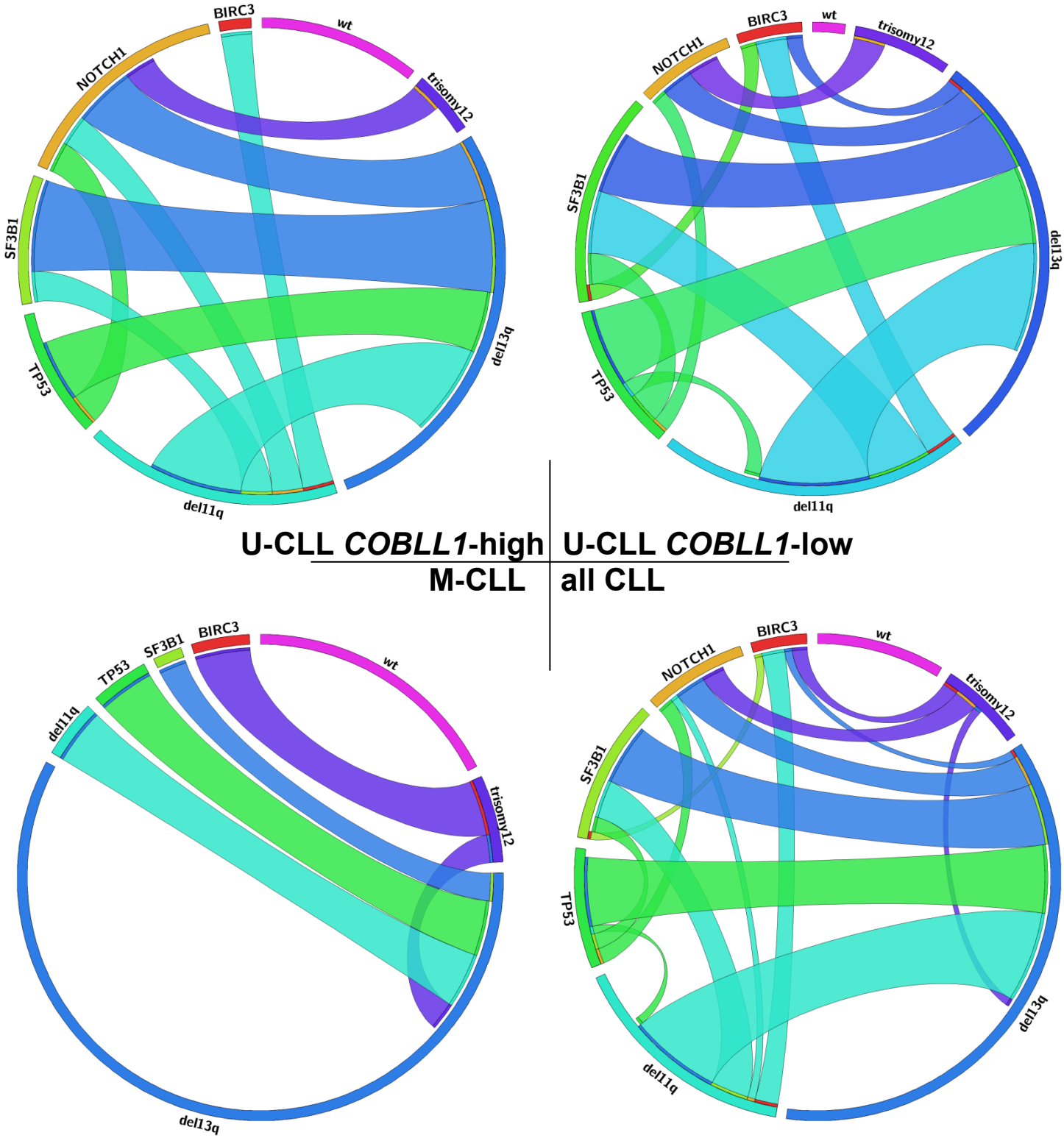
**B. COBLL1 is not influenced by Wnt-5a treatment.** Recombinant Wnt-5a treatment (2h) did not induce any changes in COBLL1 levels or mobility, analyzed by western blotting analysis. A representative result is presented – analysis was performed in primary CLL samples with negative, low or high expression of COBLL1, in both M-CLL and U-CLL samples. No consistent effect was detected.



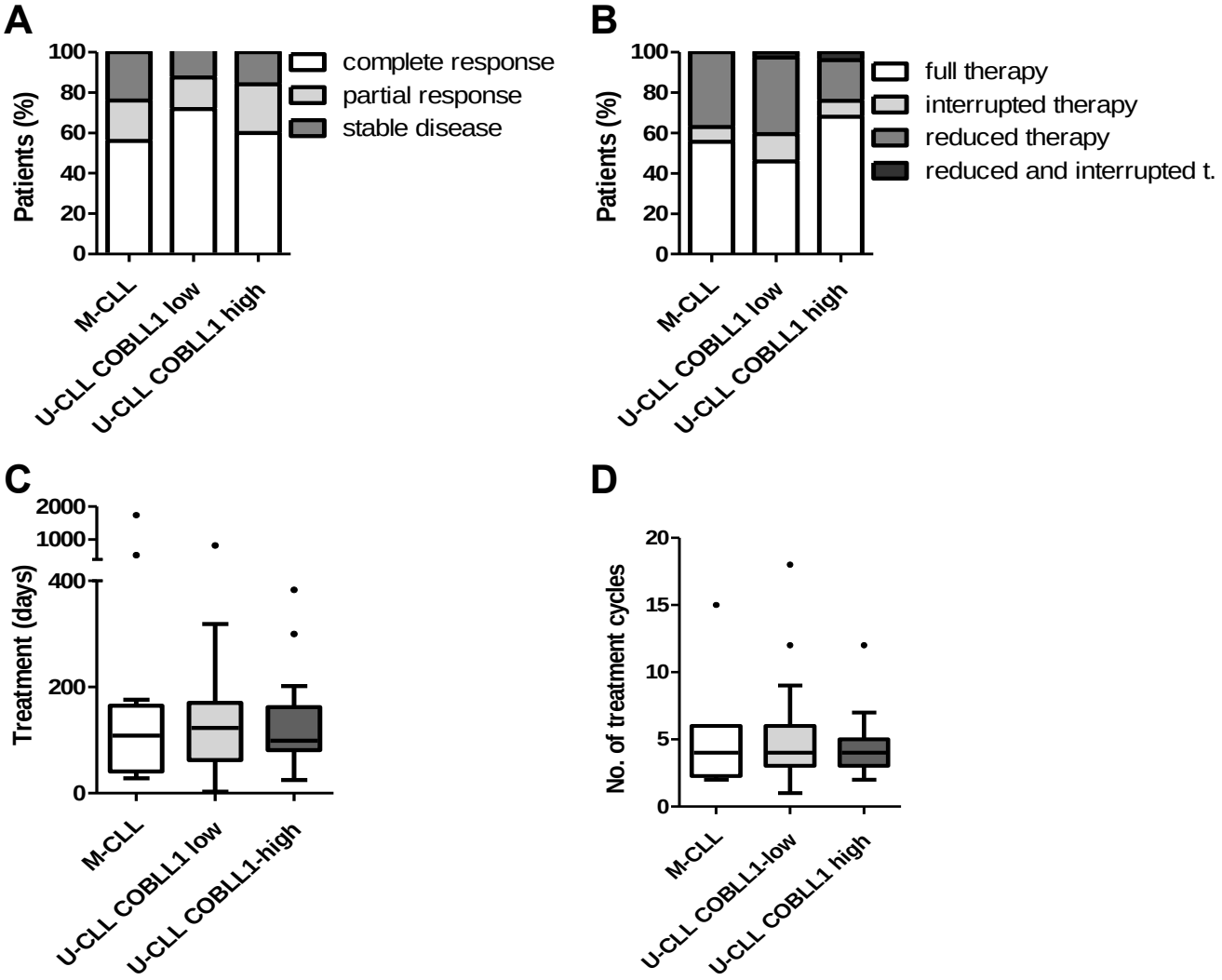

**Supplementary Figure 3: U-CLL *COBLL1*-high show non-significant trend to shorter time to first treatments than U-CLL *COBLL1*-low.** Median U-CLL *COBLL1*-high 20 months, U-CLL *COBLL1*-low 33 months, M-CLL not reached. U-CLL *COBLL1* vs U-CLL *COBLL*1-low P=0.1348, U-CLL *COBLL1*-high and U-CLL *COBLL1*-low vs M-CLL both P<0.0001. ns – not significant. Gehan-Breslow-Wilcoxon test.



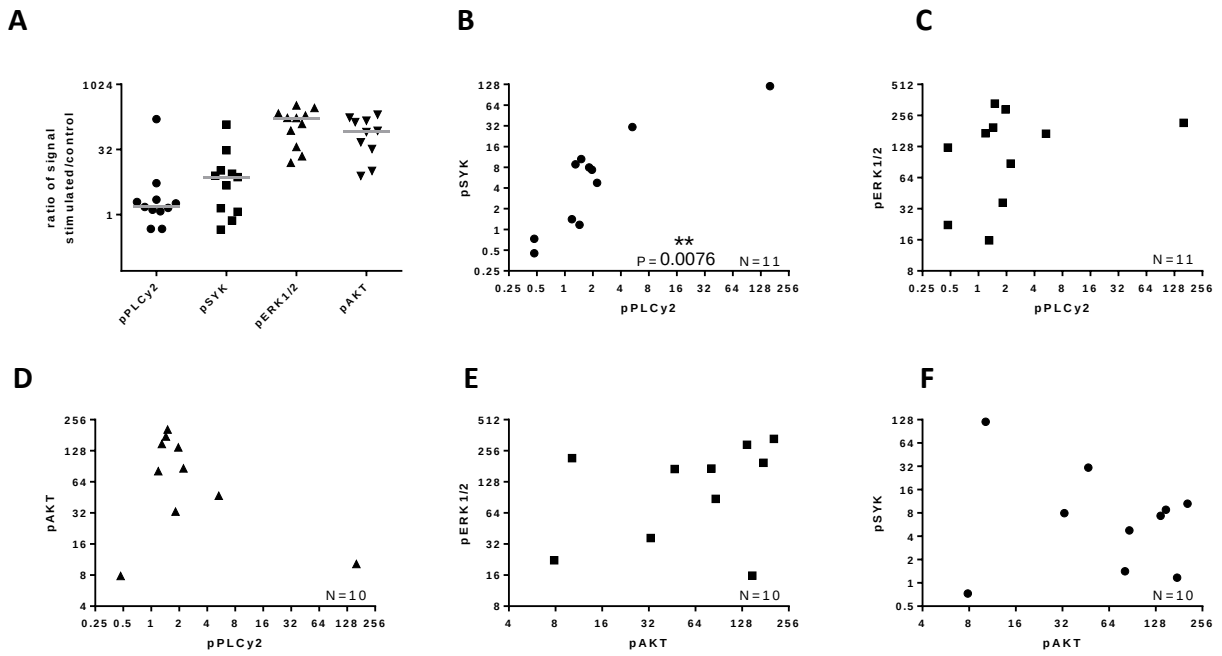

**Supplementary Figure 4: U-CLL *COBLL1*-high patients show shorter overall survival in independent cohorts. A.** Cohort B. 24 U-CLL *COBLL1*-low (median 108 months), 12 U-CLL *COBLL1*-high (median 58 months), P=0.0314. **B.** Cohorts A+C. 34 U-CLL *COBLL1*-low (median 123 months), 22 U-CLL *COBLL1*-high (median 75 months), P=0.0946. ns – not significant. Gehan-Breslow-Wilcoxon test.




**Supplementary Figure 5: Distribution of aberration in CLL cells.** Genomic aberrations in 107 samples (41 M-CLL, 37 U-CLL *COBLL1*-low, 29 U-CLL *COBLL1*-high) were analyzed by I-FISH [del(17p), del(11q), trisomy 12, del(13q)] and sequencing (*TP53*, *BIRC3*, *NOTCH1*, *SF3B1* mutation). *TP53* defect was characterized as *TP53* mutation, deletion or both.




**Supplementary Figure 6: Treatment in CLL patients.** A total amount of 84 treated CLL patients (25 M-CLL, 33 U-CLL *COBLL1*-low, 26 U-CLL *COBLL1*-high) was analyzed. Patients did not differ in their response to therapy (**A**; complete response, partial response, stable disease), if categorized as full, reduced, interrupted or reduced therapy (**B**), in number of administrated treatment cycles (**C**) or in length of their treatment (**D**). Box-and-Whisker plots show quartiles and median, • represent outliers.




**Supplementary Figure 7: pAKT and pERK1/2 exhibit different response to BCR stimulation in CLL cells than pPLCγ2 and pSYK.** Fresh primary cells (N = 10/11) were stimulated according to the same protocol as samples analyzed by flow cytometry. Cells were lyzed in 1x Laemmli buffer supplemented by protease and phosphatase inhibitors. Quantification of response to anti-IgM stimulation was performed based on western blotting analysis. The signal was quantified by ImageJ software. Ratio of signal of the stimulated and unstimulated sample was calculated. **A.** Increase in the phosphorylation of PLCγ2 (pY1217), pSYK (pY525/526), pAKT (pS473) and pERK1/2 (pT202/Y204) in response to anti-IgM stimulation in the individual patients. Note much higher increase of pERK1/2 and pAKT signal upon IgM stimulation in comparison to pPLCγ2 and pSYK. **B.-F.** Values shown in A were plotted against each other and correlation of individual phospho-signalswas performed by GraphPad software (Spearman correlation). Only pPLCγ2 and pSYK values correlate. For the correlation summary see Fig. 5C.

## Supplementary Tables

**Supplementary Table 1: Genes correlating with *COBLL1* in U-CLL.** Table is in separate xls file.

**Supplementary Table 2: Pathways of components correlating with *COBLL1* expression in U-CLL.**

| KEGG pathway | P-value | Genes correlating with *COBLL1* expression in U-CLL | | |
|---|---|---|---|---|
| | | N | Positive correlation | Negative correlation |
| Inositol phosphate metabolism | 0.0003 | 9 | *IMPA2, ITPKB, PIK3C3, PIP4K2A, PTEN, SYNJ2* | *PIP5KL1, PI4K2A, PLCD3* |
| Small cell lung cancer | 0.0005 | 11 | *AKT3, BIRC3, CDK6, E2F1, IKBKB, ITGA2B, ITGB1, MAX, PTEN, RARB, SKP2* | |
| Valine, leucine and isoleucine degradation | 0.0009 | 7 | *ACAD8, ACADSB, BCKDHB, MCCC2, OXCT1, PCCB* | *OXCT2* |
| Ubiquitin mediated proteolysis | 0.0018 | 14 | *ANAPC13, ANAPC5, BIRC3, CUL4A, CUL4B, NEDD4L, SKP2, UBE2E2, UBE2K, UBE2I, UBE2W, UBE3C, VHL* | *SIAH1* |
| Biotin metabolism | 0.0022 | 1 | | *HLCS* |
| Toll-like receptor signaling pathway | 0.0025 | 11 | *AKT3, CD86, CTSK, IKBKB, IRAK4, LY96, MAP2K1, MAP2K4, MAP3K7, MYD88* | *FOS* |
| Prostate cancer | 0.0026 | 10 | *AKT3, E2F1, GSK3B, IKBKB, MAP2K1, PTEN, TCF7* | *CREB3L2, HSP90AA1, PDGFRB* |
| Glioma | 0.0030 | 8 | *AKT3, CDK6, E2F1, MAP2K1, PTEN* | *PDGFRB, CALML6, CAMK2D* |
| Ubiquinone and other terpenoid-quinone biosynthesis | 0.0031 | 2 | *COQ2, COQ3* | |
| Phosphatidylinositol signaling system | 0.0031 | 9 | *IMPA2, ITPKB, PIK3C3, PIP4K2A, PTEN, SYNJ2* | *CALML6, PI4K2A, PLCD3* |
| Pancreatic cancer | 0.0049 | 8 | *AKT3, ARHGEF6, CDK6, E2F1, IKBKB, JAK1, MAP2K1* | *CALML6* |
| Pathogenic Escherichia coli infection | 0.0052 | 7 | *ARPC5L, ITGB1, LY96, NCK2* | *NCL, TUBA1A, TUBA1C* |
| Pantothenate and CoA biosynthesis | 0.0056 | 3 | *ENPP1* | *PPCDC, UPB1* |
| Pathways in cancer | 0.0059 | 24 | *AKT3, BIRC3, CDK6, E2F1, EGLN1, ETS1, GSK3B, IKBKB, ITGA2B, ITGB1, JAK1, JUP, MAX, MAP2K1, PTEN, RARB, SKP2, TCF7, TPM3, VHL* | *FOS, HSP90AA1, PDGFRB, PLD1* |
| Progesterone-mediated oocyte maturation | 0.0068 | 9 | *AKT3, ANAPC13, ANAPC5, CCNB3, CCNA2, MAP2K1, PRKACB, RPS6KA3* | *HSP90AA1* |
| Selenocompound metabolism | 0.0070 | 3 | *MTR, PAPSS2, SEPSECS* | |
| Synthesis and degradation of ketone bodies | 0.0070 | 2 | *OXCT1* | *OXCT2* |
| **B cell receptor signaling pathway** | **0.0077** | **8** | ***AKT3, DAPP1, GSK3B, IKBKB, NFATC2, MAP2K1, VAV2*** | ***FOS*** |
| Shigellosis | 0.0077 | 7 | *ARPC5L, ATG5, IKBKB, ITGB1, WASF1* | *ELMO1, NOD2* |