Inhibition of casein kinase 2 sensitizes mantle cell lymphoma to venetoclax through MCL-1 downregulation

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Abstract

BCL-2 family proteins are frequently aberrantly expressed in mantle cell lymphoma (MCL). Recently, the BCL-2-specific inhibitor venetoclax has been approved by the US Food and Drug Administration for chronic lymphocytic leukemia (CLL) and acute myeloid leukemia (AML). In MCL, venetoclax has shown promising efficacy in early clinical trials; however, a significant subset of patients is resistant. By conducting a kinome-centered CRISPR-Cas9 knockout sensitizer screen, we identified casein kinase 2 (CK2) as a major regulator of venetoclax resistance in MCL. Interestingly, CK2 is over-expressed in MCL and high CK2 expression is associated with poor patient survival. Targeting of CK2, either by inducible short hairpin RNA (shRNA)-mediated knockdown of CK2 or by the CK2-inhibitor silmitasertib, did not affect cell viability by itself, but strongly synergized with venetoclax in both MCL cell lines and primary samples, also if combined with ibrutinib. Furthermore, targeting of CK2 reduced MCL-1 levels, which involved impaired MCL-1 translation by inhibition of eIF4F complex assembly, without affecting BCL-2 and BCL-X_L expression. Combined, this results in enhanced BCL-2 dependence and, consequently, venetoclax sensitization. In co-cultures, targeting of CK2 overcame stroma-mediated venetoclax resistance of MCL cells. Taken together, our findings indicate that targeting of CK2 sensitizes MCL cells to venetoclax through downregulation of MCL-1. These novel insights provide a strong rationale for combining venetoclax with CK2 inhibition as therapeutic strategy for MCL patients.

Introduction

Mantle cell lymphoma (MCL) is an aggressive incurable B-cell non-Hodgkin lymphoma (NHL), representing 6-8% of all NHL. Currently, first-line therapy for MCL consists of cytarabine-based chemo-immunotherapy regimens followed by autologous stem cell transplantation in eligible patients. Elderly and frail patients are usually treated with adriamycin and cyclophosphamide-based regimens.[1] However, virtually all patients relapse and these patients receive other/non-cross-resistant chemo-immunotherapy regimens or targeted therapies such as the Bruton's tyrosine kinase (BTK) inhibitor ibrutinib. In addition, anti-CD19 chimeric antigen receptor (CAR) T-cell therapy is now approved for relapsed and refractory (R/R) disease, showing complete responses in approximately 65% of patients.[1] When these therapies fail, treatment options are limited, and therefore there is a high need for novel treatment strategies.

MCL is characterized by the chromosomal translocation t(11;14), resulting in the overexpression of cyclin D1, which facilitates cellular transformation by deregulating the cell cycle.[2] For malignant transformation, additional aberrations are required, including alterations in the mitochondrial apoptotic pathway, such as upregulation of BCL-2, BCL-X_L and Bcl-W and deletion of BIM.[3] Hence, targeting anti-apoptotic BCL-2 family proteins is an attractive option for the treatment of MCL.

Recently, the BCL-2 inhibitor venetoclax was approved by the US Food and Drug Administration for chronic lymphocytic leukemia (CLL) and acute myeloid leukemia (AML).[4] In MCL, venetoclax has also shown promising efficacy in early clinical trials, with complete response (CR) rates of 18-21% and overall response rates (ORR) of 53-75%, depending on the number and the type of prior treatments.[5-7] Combining venetoclax with ibrutinib therapy improved efficacy compared with venetoclax monotherapy, with a CR of 62-71%.[8,9] However, a significant subset of MCL patients is primary refractory or develops resistance during the course of the disease. Therefore, novel combinations need

to be identified to enhance the efficacy of venetoclax in MCL.

Here, we conducted a functional genomic CRISPR-Cas9 knockout sensitizer screen, with a lentiviral guide RNA (gRNA) library that represents all human kinases,[10] and identified casein kinase 2 (CK2) as a major determinant of venetoclax sensitivity in MCL cells. We showed that reduced CK2 expression/activity by RNA interference or by treatment with the CK2 inhibitor silmitasertib synergistically increased the effectiveness of venetoclax due to the reduction in MCL-1 protein levels. These findings indicate that the combination of silmitasertib and venetoclax could represent a promising treatment strategy for MCL.

Methods

For detailed information please see the *Online Supplementary Appendix*.

Cell culture and treatment

The human MCL cell lines Granta-519, Jeko-1, Maver-1, Mino, and Z138 were obtained from DSMZ (Braunschweig, Germany) and cultured in Iscove's modified Dulbecco's medium (IMDM; Invitrogen Life Technologies, Carlsbad, CA, USA). The stromal cell line HS-27a and the kidney cell line HEK-293T/17 were obtained from ATCC (Manassas, VA, USA), and cultured in Dulbecco's Modified Eagle's Medium (DMEM; Invitrogen).

For co-culture assays, bone-marrow stromal cells were seeded in 96-well plates 4 hours (h) prior to the addition of MCL cells, to allow cell attachment. MCL cells were pre-incubated for 2 h with stromal cells, followed by a 3-day co-culture in the presence of the indicated drug concentrations. Cell viability and specific cell death were measured by flow cytometry.

The following small-molecule inhibitors were used: venetoclax/ABT-199, Q-VD-OPh, IPTG (MedChemExpress, Princeton, NJ, USA), silmitasertib/CX-4945, S63845, MK2206 (Selleckchem, Houston, TX, USA), cycloheximide, puromycin (Sigma-Aldrich, St. Louis, MO, USA), and blasticidin (Thermo Fisher Scientific, Waltham, MA, USA).

Patient samples

Patient samples and peripheral blood naïve B cells were obtained as reported previously.[11] This study was approved by the AMC Medical Committee on Human Experimentation. Informed consent was obtained in accordance with the revised Declaration of Helsinki 2008.

Cloning, transfection, and transduction

shRNA targeting *CSNK2A1* (#1: ATTACCTGCAGGTGGAATATT, #2: TGGACAAACTGCTGCGATATG) and a control construct (CCTAAGGTTAAGTCGCCCTCG) were inserted into the Acc65I(Asp718I)/EcoRI site of the pLKO-mCherry-IPTG-3xLacO vector (a gift from Dr. Noam Zelcer and Dr. Jessica Nelson, Department of Medical Biochemistry, Amsterdam UMC, The Netherlands; Sigma #shc334). Lentiviral particles were produced as described previously.[12] Three days after transduction, cells were FACS-sorted using a Sony SH800S cell sorter (Sony Biotechnology, San Jose, CA, USA).

Cells transduced with Lenti-Cas9-Blast (Addgene #52962) were selected for 7 days with blasticidin, 24 h after transduction. Cas9 activity was analyzed by flow cytometry of Lenti-Cas9-reporter-transduced cells (Addgene #67980) using the FACSCanto II flow cytometry system (BD Biosciences, San Jose, CA, USA).

Synthetic lethality screen

The screening procedure was performed as outlined in Figure 1C. (See *Online Supplementary Methods* for full details.)

Real-time-quantitative polymerase chain reaction

Real-time-quantitative polymerase chain reaction (RT-qPCR) was essentially performed as described previously.[11]

Flow cytometry

Cell viability and (specific) apoptosis were analyzed by flow cytometry using 7-aminoactinomycin-D (7-AAD; Invitrogen) staining. Early apoptosis was determined using Annexin V staining and cell cycle distribution was analyzed using bromodeoxyuridine (BrdU) incorporation performed as previously described.[11]

Immunoblotting

Immunoblotting was essentially performed as described previously.[11,12]

Gene expression profile analysis and gene set enrichment analysis

The gene expression profiling data of B cells from 165 MCL patients (GSE93291, GSE132929)[13,14] and 10 healthy donors (GSE28491, E-MTAB-1771), publicly available and deposited in the NIH Gene Expression Omnibus and the EMBL European Bioinformatics Institute databases, respectively, were analyzed using the R2 Genomics Analysis and Visualization Platform (http://r2.amc.nl). The GSE93291 dataset was analyzed for gene set enrichment using the gene set enrichment analysis (GSEA) application (version 4.0.3).[15]

Statistical analysis

Statistical analyses were performed using log-rank tests for Kaplan-Meier analysis, 2-tailed Student *t* tests for comparisons between 2 groups, and two-way analysis of variance (ANOVA) with Tukey, Šidák or Dunnett's multiple comparison post-hoc tests as indicated in the figure legends for comparisons between more than 2 groups.

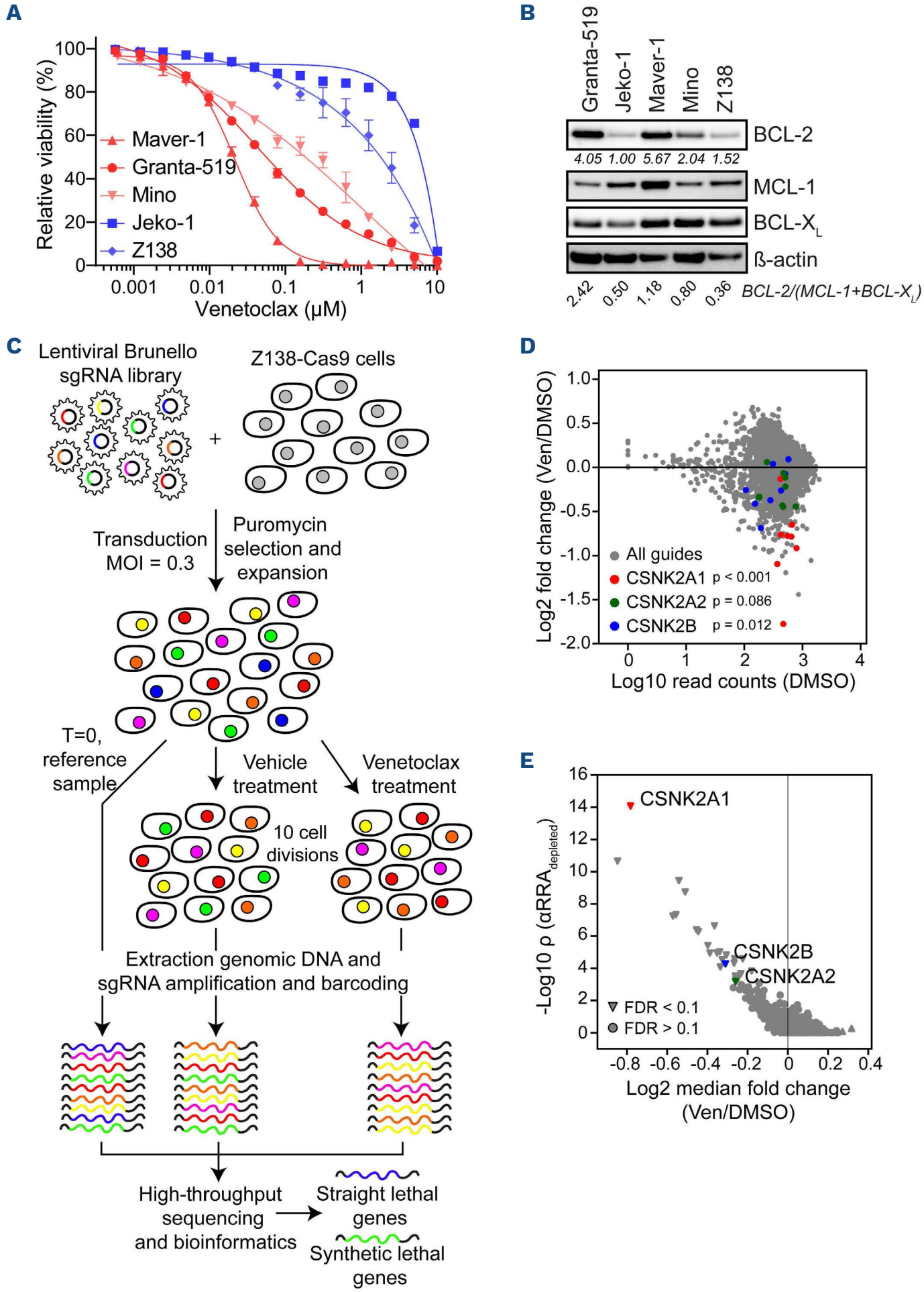

Figure 1. A kinome-centered synthetic-lethal-screen with venetoclax identifies casein kinase 2 knockout as potential sensitizer to venetoclax in Z138. (A) Mantle cell lymphoma (MCL) cell lines were treated with increasing concentrations of venetoclax for 3 days. Viability was assessed by flow cytometry. The mean±standard deviation (SD) of triplicate cultures of an experiment, representative for at least three independent experiments, is shown. (B) Immunoblot analysis of BCL-2, MCL-1, and BCL-X_L protein expression in MCL cell lines. β-actin was used as a loading control. Representative immunoblots of three independent experiments are shown. Upper numbers depict the BCL-2 expression levels corrected for β-actin; lower numbers the relative expression of BCL-2 over MCL-1 and BCL-X_L-combined, corrected for β-actin, as quantified by ImageJ. Equal protein was loaded. (C) Schematic outline of the kinome-centered CRISPR-Cas9 synthetic lethal screen performed in Z138 cells stably expressing Cas9. The drop-out of the blue sgRNA in the vehicle (DMSO)-treated cells identifies its target gene as a straight lethal gene, the exclusive drop-out of the green sgRNA in the venetoclax-treated cells identifies its target gene as a synthetic lethal or sensitizer gene for venetoclax. (See main text for further details.) (D) Distribution of individual gRNA from the synthetic lethality screen, with gRNA targeting the casein kinase 2 (CK2) subunits color-coded subunits. (E) Volcano plot showing the genes specifically depleted from the venetoclax-treated cells in the synthetic lethal screen (drop-outs), with significant genes shown as a triangle.

Results

A kinome-centered CRISPR screen identifies CK2 as potential target for venetoclax sensitization of MCL cells

To assess their sensitivity to venetoclax, five MCL cell lines (*Online Supplementary Table S1*) were treated with increasing concentrations of venetoclax, and their viability was analyzed after 3 days. In line with previous reports,[16-18] two cell lines, Jeko-1 and Z138, were relatively resistant to venetoclax, with IC_{50} values approximately 100 times higher compared to the IC_{50} of the other three cell lines, Granta-519, Maver-1 and Mino (Figure 1A). These IC_{50} largely correspond to the protein expression of BCL-2 (Figure 1B): the venetoclax-resistant cell lines Jeko-1 and Z138 express relatively low BCL-2 protein levels.

Next, we performed an unbiased functional genomic CRISPR knockout screen with the Brunello kinome-centered library to identify kinases that, given their druggability, may serve as targets to sensitize MCL cells to venetoclax.[10] For this CRISPR screen, the Z138 cell line was selected, being a venetoclax-insensitive cell line with high Cas9 activity after transduction with a Cas9 construct (*Online Supplementary Figure S1A*). The abundance of the different gRNA was assessed before initiating drug treatment (T_0) and after passaging the cells for 10 population doublings in the presence of either DMSO (T_1 DMSO) or the IC^{30} of venetoclax (500 nM, T_1 Ven). Then, enrichment or depletion was determined using the DESeq2 and MAGeCK pipelines (Figure 1C).[12] The performed screen was of high quality, given the strong correlation between the three independent replicates (*Online Supplementary Figure S1B*) and the selective dropout of the gRNA against reference essential genes compared to non-essential genes in the control cells (T_1 DMSO) *versus* T_0 samples (*Online Supplementary Figure S1C*).[12]

Comparison of the two treated experimental arms (T_1 Ven *vs*. T_1 DMSO) identified genes that preferentially affected viability and cell growth in venetoclax-treated cells over DMSO-treated cells (Figure 1D, E). The highest ranked gene was *CSNK2A1*, with 7 out of 8 guides significantly depleted and with a median fold change of 0.58. In addition, the gRNA targeting *CSNK2A2* and *CSNK2B* were also significantly depleted (Figure 1D, E). These genes encode the catalytic α- and α'-subunits, and the regulatory β-subunits of CK2, respectively. CK2 is a pleiotropic serine/threonine protein kinase comprising two β-subunits and two of the α-subunits, resulting in either $\alpha_2\beta_2$, $\alpha\alpha'\beta_2$, or $\alpha'_2\beta_2$ configurations.[19,20] It is involved in a broad array of cellular processes, including cell cycle progression, survival and differentiation, and is a regulator and/or mediator of several oncogenic pathways, such as Wnt-, PI3K/Akt- and NF-κB signaling.[19,20]

CK2 is aberrantly expressed in MCL and high expression of CK2 is associated with inferior prognosis for MCL patients

CK2 overexpression has been reported in several hematologic malignancies, including MCL, CLL, diffuse large B-cell lymphoma (DLBCL), and multiple myeloma (MM).[20-23] In line with this, comparison of primary MCL *versus* naïve B cells, either from publically available microarray datasets or RT-qPCR, revealed a significant upregulation of *CSNK2A1* and *CSNK2B* RNA expression in MCL cells (Figure 2A, B; *Online Supplementary Figure S2A*). Moreover, CK2 expression measured in excisional lymph node biopsy material (tumor content ≥60%) of 122 MCL patients that were all treated with rituximab plus cyclophosphamide, doxorubicin, vincristine, and prednisone (R-CHOP) (GSE93291) revealed a 5-year survival of 0% *versus* 72% in the patients with the highest *CSNK2B* expression and lowest *CSNK2B* expression, respectively (Figure 2C).[13] *CSNK2A1* expression in the same cohort showed a similar trend, although this was not significant (*Online Supplementary Figure S2B*). Taken together, our results indicate that CK2 is aberrantly expressed and associated with an aggressive disease course.

Targeting of CK2 sensitizes MCL cells to venetoclax

To evaluate CSNK2A1 as a hit from the CRISPR screen, the venetoclax-insensitive cell lines Z138 and Jeko-1, which both express high levels of CK2α (*Online Supplementary Figure S3A, B*), were transduced with different IPTG-inducible shRNA targeting CSNK2A1 (Figure 3A). These shRNA induced efficient knockdown of CK2α protein levels in both cell lines, without affecting the highly homologous CK2α' protein encoded by the *CSNK2A2* gene. Viability was only modestly affected (Figure 3B), supporting our screening results, in which *CSNK2A1* KO did not affect cell growth in the absence of venetoclax (*Online Supplementary Figure S2C*). However, both Z138 and Jeko-1 cells became significantly sensitized to venetoclax upon *CSNK2A1* silencing, most prominently in Jeko-1, resulting in a 60% increased cell death compared to control cells (Figure 3C).

We next investigated the effects of silmitasertib (CX-4945), a specific and selective CK2 inhibitor with proven safety and tolerability profiles in several phase I and II clinical trials.[24-27] Upon silmitasertib treatment, phosphorylation of Akt S129, which is exclusively phosphorylated by CK2, was effectively reduced in the MCL cell lines (Figure 4A).[28,29] However, cell viability was only decreased at concentrations above 3 μM, with IC_{50} values as high as 8-10 μM (Figure 4B). Silmitasertib also decreased cell viability of primary samples only at concentrations in the micromolar range. The silmitasertib-sensitivity of the primary MCL samples roughly corresponded to *CSNK2* RNA expression (*Online Supplementary Figure S4A*).

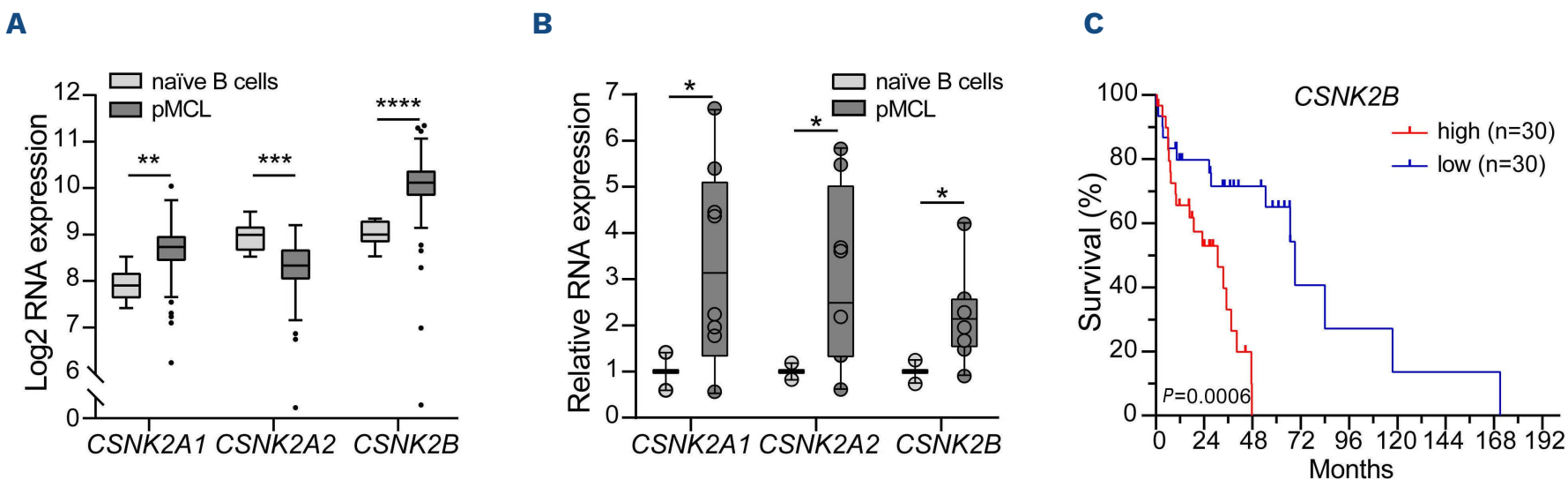

Figure 2. Casein kinase 2 is aberrantly expressed and high expression is associated with poor prognosis in mantle cell lymphoma. (A) RNA expression of the casein kinase 2 (CK2) subunits in 165 primary mantle cell lymphoma (MCL) samples from the microarray datasets GSE93291 and GSE132929, compared to naïve B-cell samples from 10 healthy donors from the microarray datasets GSE28491 and E-MTAB-1771 (***P*<0.01, ****P*<0.001, *****P*<0.0001; ANOVA with Tukey multiple comparison test). (B) mRNA expression of the CK2 subunits in primary MCL samples (n=8) and in naïve B cells from healthy donors (n=2) as determined by quantitative polymerase chain reaction (qPCR) (**P*<0.05; unpaired Student *t* test). (C) Kaplan-Meier analysis depicting overall survival of patients with MCL from the GSE93291 micro-array dataset divided into the highest and lowest quartile of *CSNK2B* expression (*P*=0.0006; log-rank test).

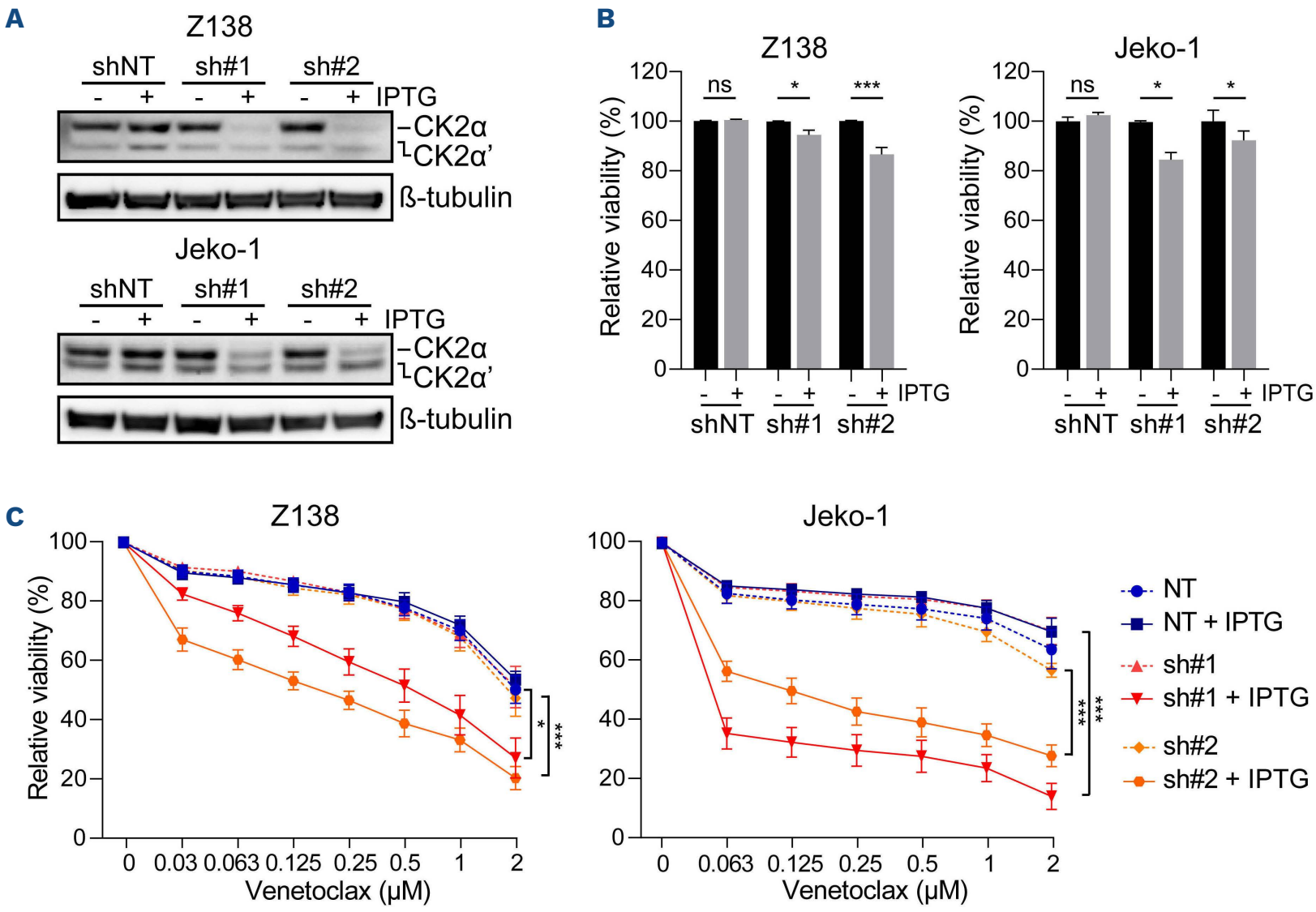

Figure 3. shRNA-mediated casein kinase 2 knockdown sensitizes mantle cell lymphoma cells to venetoclax. (A) Immunoblot analysis of casein kinase 2 (CK2)α and CK2α' (*CSNK2A2*) protein expression in Z138 and Jeko-1 cells transduced with pLKO-3xLacO-mCherry plasmids encoding two independent shRNAs targeting *CSNK2A1* or a scrambled non-targeting shRNA (NT). Cells were treated with IPTG for 3 days. ß-tubulin was used as a loading control. Representative immunoblots of three independent experiments are shown. (B) Flow cytometry analysis of the effect of IPTG-induced *CSNK2A1* knockdown on cell viability at 7 days of culture, normalized to the untreated condition. The mean±standard error of mean (SEM) of at least four independent experiments performed in triplicate is shown (**P*<0.05; paired Student *t* test). (C) Flow cytometry analysis of the effect of venetoclax treatment on cell viability in *CSNK2A1* knockdown cells, normalized to the untreated condition. Cells were treated for 4 days with IPTG, followed by 3 days treatment with both IPTG and venetoclax. Percentage viable cells was normalized to the untreated condition. The mean±SD of triplicate cultures of an experiment, representative of at least three independent experiments, is shown.

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Figure 4. Casein kinase 2 inhibition by silmitasertib sensitizes mantle cell lymphoma cells to venetoclax. (A) Immunoblot analysis of p-Akt S129 and total Akt protein expression in the mantle cell lymphoma (MCL) cell lines Z138, Jeko-1, Mino and Granta-519 treated with 4 μM silmitasertib for 4 hours. β-actin was used as a loading control. Representative immunoblots of three independent experiments are shown. (B) Flow cytometry analysis of the effect of 3-day treatment with indicated concentrations of silmitasertib on cell viability of MCL cell lines. The mean±SD of triplicate cultures, of an experiment representative of at least two independent experiments, is shown. (C) Flow cytometry analysis of the effect of 3-day treatment with venetoclax, silmitasertib or the combination of both on cell viability of MCL cell lines. It should be noted that the concentration of venetoclax is in the low μM range for the insensitive cell lines Z138 and Jeko-1, and in the low nM range for the sensitive cell lines Mino and Granta-519. The upper panel depicts the percentage viable cells compared to the untreated condition; blue is no effect, red is strong effect. The lower panel depicts the delta-Bliss values, which is the observed additional effect of the combination over the expected (calculated) effect; grey is no additional effect, green is strong synergy. The graphs depict the effect of 3-day venetoclax treatment on cell viability on MCL cell lines treated in combination with different concentrations of silmitasertib, normalized to the condition without venetoclax. The mean±SD of triplicate cultures of an experiment, representative of at least three independent experiments, is shown (***P*<0.01, ****P*<0.001, *****P*<0.0001; ANOVA with Šidák multiple comparison test of the highest concentration silmitasertib compared to venetoclax only). (D) Flow cytometry analysis of the effect of 2-day treatment with venetoclax (1.6-25 nM), silmitasertib (2.5-10 μM) or the combination of both on the apoptosis of 6 primary MCL samples. Black bars depict the apoptosis as observed in the experiment±SD of triplicate cultures, the grey bars the expected apoptosis based upon the effects of single treatment (**P*<0.05, ***P*<0.01, ****P*<0.001, *****P*<0.0001, ns=not significant; ANOVA with Dunnett's multiple comparison test). (E) The expected and observed values of (D). ***P*<0.01, paired Student *t* test.

To assess potential synergy between venetoclax and silmitasertib in MCL, cells were treated with increasing doses of either venetoclax, silmitasertib, or a combination of both, and cell survival was determined (Figure 4C). Synergy was assessed using the Bliss independence model: silmitasertib and venetoclax were highly synergistic in all MCL cell lines tested, with ΔBliss scores up to 55 in Jeko-1 (Figure 4C), indicating 55% more cell death in the cells treated with the combination of drugs than expected based upon the effects of single treatment of the drug.[30] In agreement, treatment of 6 primary MCL samples with the combination of venetoclax and silmitasertib also resulted in more cell death than predicted, with ΔBliss scores ranging from 6 to 18 (Figure 4D, E). As the IC_{50} of silmitasertib was only reached in the high micromolar range, indicating off-target effects, assessment of synergy using the Chou Talalay method was not considered appropriate.[31] The zero interaction potency (ZIP) model[32] revealed synergy scores similar to those of the Bliss independence model (*Online Supplementary Figure S4B*).

Since clinical trials frequently evaluate venetoclax in combination with ibrutinib,[8,9,33] we also determined whether targeting CK2 can sensitize MCL cells to venetoclax in the presence of ibrutinib (*Online Supplementary Figure S4C, D*). Indeed, both CK2 knockdown (KD) and inhibition also potentiated the activity of venetoclax combined with ibrutinib. Interestingly, in Jeko-1, this potentiation by silmitasertib was even stronger for the combination compared to venetoclax alone. Please note that ibrutinib treatment neither affected cell viability by itself nor sensitized the cell lines to venetoclax. This is in line with the observation that, also in patients, ibrutinib does not directly target cell viability, but rather mobilizes the malignant cells from the protective lymphoid organs to the peripheral blood.[34,35]

To assess whether the venetoclax-sensitizing effect of CK2 silencing or inhibition is a cytostatic or cytotoxic effect, apoptosis and cell cycle were analyzed using Annexin V staining and BrdU labeling, respectively. Treatment with venetoclax significantly increased the percentage of Annexin V-positive cells in both *CSNK2A1* KD cells and silmitasertib-treated cells, whereas the cell cycle was only modestly affected (*Online Supplementary Figure S5A, B*).

CK2 inhibition down-regulates MCL-1 levels

To investigate the mechanism(s) through which loss of CK2 activity sensitizes MCL cells to venetoclax, we evaluated changes in protein levels of BCL-2, MCL-1, and BCL-X_L. The ratio of expression of BCL-2 over MCL-1 and BCL-X_L combined has been described as an important determinant of venetoclax sensitivity in MCL,[3,16,17] and also corresponds with venetoclax sensitivity in the cell lines used in this study (Figure 1B). Both CK2 silencing and inhibition resulted in markedly reduced MCL-1 levels in MCL cells, wheras BCL-2 and BCL-X_L protein levels were unaffected (Figure 5A, B; *Online Supplementary Figure S6A*). Also in primary MCL samples, silmitasertib treatment decreased MCL-1 protein levels without altering BCL-X_L levels (Figure 5C).

To assess whether the reduction in MCL-1 levels was sufficient to sensitize MCL cells to venetoclax, we applied a BH3-mimetic specific to MCL-1, S63845, in combination with venetoclax to the MCL cells, and cell survival was assessed. Indeed, upon MCL-1 inhibition, MCL cells became significantly sensitized to venetoclax, as observed by the shift in the response curve (Figure 5D; *Online Supplementary Figure S6B*). These results confirm that MCL-1 is a major regulator of venetoclax resistance and that CK2-mediated MCL-1 regulation affects venetoclax sensitivity.

CK2 regulates MCL-1 translation

Next, we investigated the mechanism of CK2-mediated MCL-1 protein expression. MCL-1 expression is tightly regulated at multiple levels, involving transcriptional, trans-

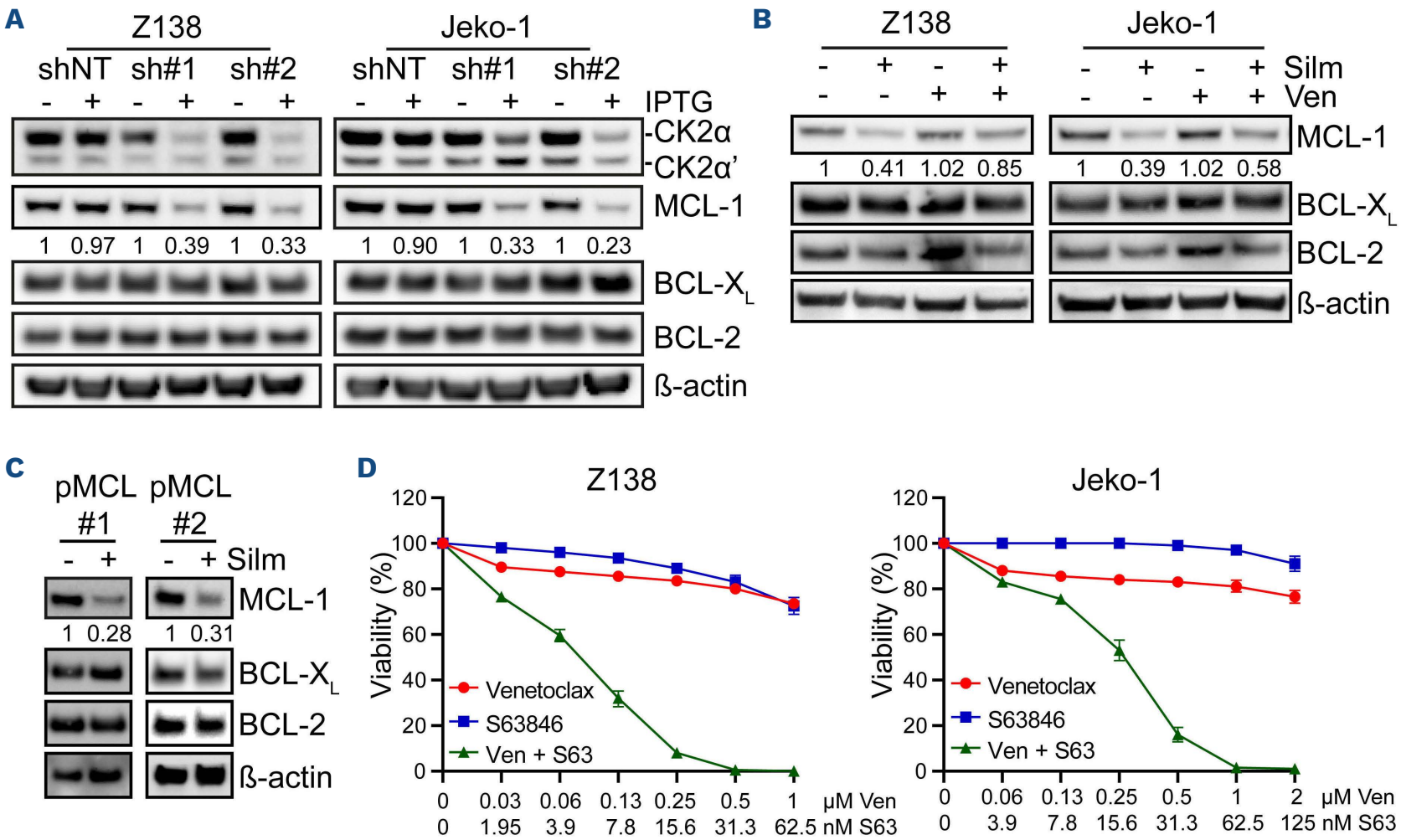

Figure 5. Casein kinase 2 regulates MCL-1 protein levels. (A) Immunoblot analysis of casein kinase 2 (CK2)α, MCL-1, BCL-X_L and BCL-2 protein expression after CSNK2A1 knockdown in Z138 and Jeko-1 cells. Cells were treated with IPTG for 3 days. β-actin was used as a loading control. Representative immunoblots of three independent experiments are shown. (B) Immunoblot analysis of MCL-1, BCL-X_L and BCL-2 protein expression in Z138 and Jeko-1 cells pre-treated with 10 μM Q-VD-OPh for 1 hour (h), followed by 24 h treatment with 4 μM silmitasertib and/or 1 μM venetoclax. β-actin was used as a loading control. Representative immunoblots of three independent experiments are shown. (C) Immunoblot analysis of MCL-1, BCL-X_L and BCL-2 protein expression in two primary MCL samples pre-treated with 10 μM Q-VD-OPh for 1 h, followed by 24-h treatment with 4 μM silmitasertib and/or 1 μM venetoclax. β-actin was used as a loading control. (D) Flow cytometry analysis of the effect of 3-day treatment with indicated concentrations of venetoclax, S63845 or the combination of both on the cell viability of MCL cell lines Z138 and Jeko-1. The mean±SD of triplicate cultures of an experiment, representative of at least three independent experiments, is shown.

lational, and post-translational processes.[36] First, we monitored *MCL1* mRNA levels by RT-qPCR, and in none of the MCL cell lines were alterations in the expression levels of *MCL1* observed upon CK2 targeting, arguing against transcriptional regulation (Figure 6A, B). Next, we assessed whether CK2 inhibition decreased protein stability of MCL-1, by blocking translation with cycloheximide (CHX) treatment. The MCL-1 protein half-life was unaffected by CK2 inhibition in both Z138 and Jeko-1 (Figure 6C), suggesting that CK2 regulates MCL-1 protein levels by controlling translation. In agreement, GSEA of an MCL patient cohort of 122 patients (GSE93291) revealed that the expression of genes involved in protein translation was strongly enriched in patients with high *versus* low *CSNK2A1* expression (*Online Supplementary Figure S7A*). Taken together, these data indicate that CK2 is not involved in MCL1 expression or MCL-1 protein stability and proteasomal degradation, but rather affects MCL-1 translation.

The rate of MCL-1 translation is, amongst others, dependent upon the eukaryotic translation initiation factor 4F (eIF4F) complex.[37-41] Association of this complex with mRNA depends on the cap-binding factor eIF4E, which is inhibited by eIF4E-binding protein (4EBP). The mTOR-dependent phosphorylation of 4EBP releases eIF4E to increase translation initiation, and p38- and extracellular signal-regulated kinase (ERK)-dependent phosphorylation of eIF4E further enhances formation of the eIF4F complex.[39] Furthermore, the phosphorylation of ribosomal protein S6 has been implicated in translation initiation. In line with this, both CK2 silencing and inhibition clearly reduced activation of the Akt/mTOR-, p38- and/or the ERK-pathways and the subsequent phosphorylation/activation of S6 and the eIF4 complex (Figure 6D; *Online Supplementary Figure S7B*), indicating their possible role in CK2-controlled MCL-1 translation. The importance of the Akt pathway is further supported by the synergy observed between the Akt inhibitor MK2206 and venetoclax in Jeko-1 and Mino (*Online Supplementary Figure S7C*). However, Akt inhibition did not result in clear synergy with venetoclax in any of the tested cell lines, indicating that, for example, the p38 and ERK pathways are also involved in the synergy observed between CK2 inhibition and venetoclax. In sum-

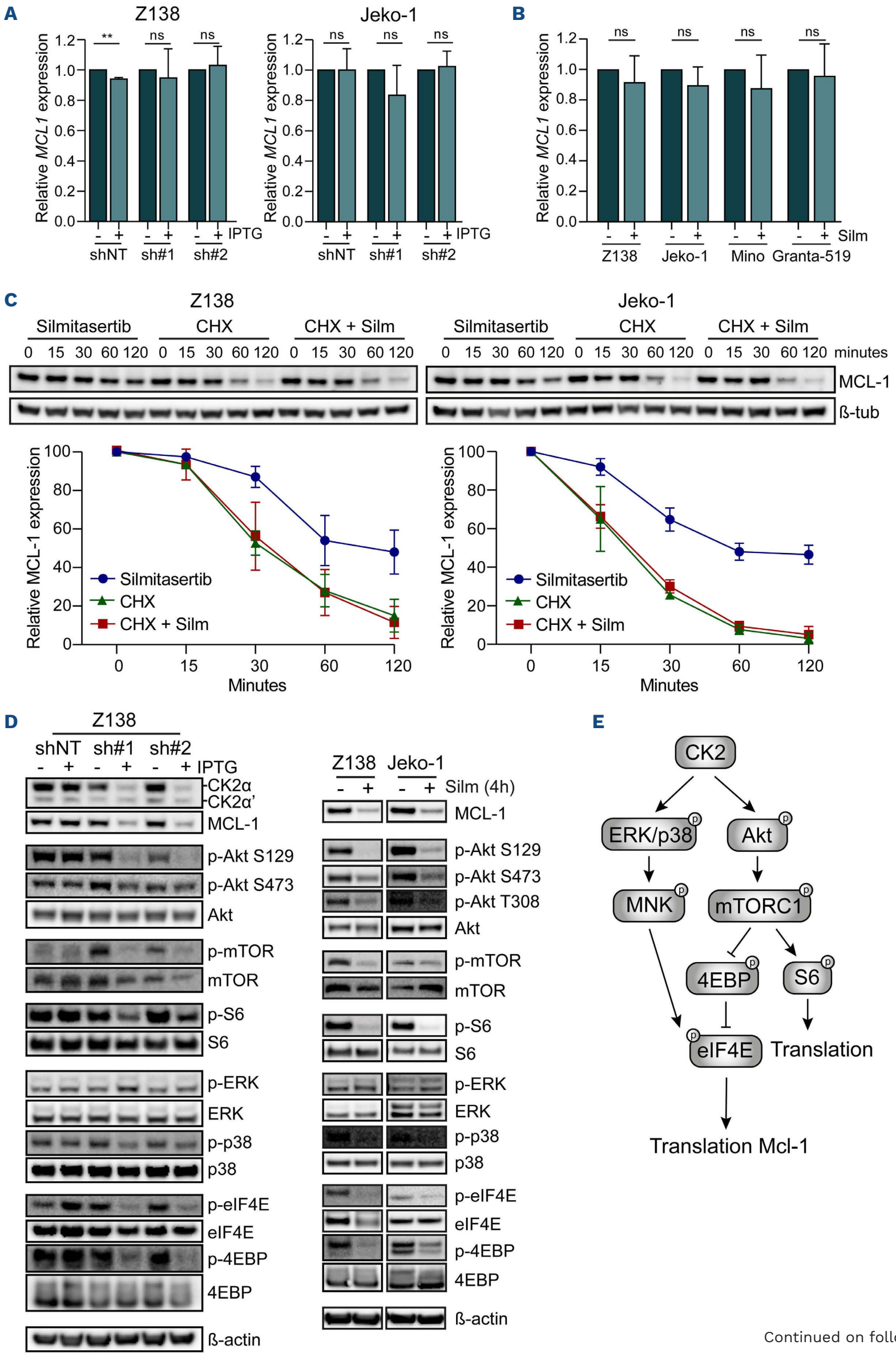

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Figure 6. Casein kinase 2 (CK2) mediates MCL-1 protein levels via assembly of the translation machinery. (A) *MCL1* mRNA expression after *CSNK2A1* knockdown as determined by real-time quantitative polymerase chain reaction (RT-qPCR), normalized to the untreated condition. *B2M* and *HPRT* were used as input controls. The mean±Standard Error of Mean (SEM) of at least three independent experiments performed in triplicate is shown (**P*<0.05; paired Student *t* test). (B) *MCL1* mRNA expression after 24-hour (h) treatment with 4 μM silmitasertib as determined by RT-qPCR, normalized to the untreated condition. *B2M* and *HPRT* were used as input controls. The mean±SEM of at least three independent experiments performed in triplicate is shown (**P*<0.05; paired Student *t* test). (C) Immunoblot analysis and its quantification of MCL-1 protein stability in Z138 and Jeko-1 cells after 4 μM silmitasertib treatment, 200 μg/mL cycloheximide (CHX) treatment, or the combination of both for the indicated time points. Cells were pre-treated with 10 μM Q-VD-OPh for 1 h. β-tubulin (β-tub) was used as a loading control. Representative immunoblots of at least three independent experiments are shown. Bar graphs depict the mean protein levels of at least three independent experiments, as quantified by ImageJ. (D) Immunoblot analysis of protein expression of the indicated proteins in Z138 *CSNK2A1* knockdown cells treated with IPTG for 3 days (left panel) or in Z138 and Jeko-1 cells pre-treated for 1 h with 10 μM Q-VD-OPh, followed by 4 h silmitasertib (4 μM) (right panel). β-actin was used as a loading control. Representative immunoblots of three independent experiments are shown. If an antibody combination did not allow re-probing of the same blot, e.g., for loading or non-phospho controls, the same samples were analyzed by immunoblotting of a parallel gel. (E) A schematic representation of the (putative) signaling pathway underlying CK2-mediated MCL-1 translation.

mary, these data indicate that CK2 sensitizes MCL cells to venetoclax by downregulation of MCL-1 levels via interference with the assembly of the eIF4F complex and thus the translation of MCL-1 (Figure 6E).

CK2 inhibition overcomes microenvironmental venetoclax resistance

Microenvironment-mediated drug resistance is commonly observed in MCL, also for venetoclax.[3,16] Direct contact with other cells in the lymph node and bone marrow (cell adhesion-mediated drug resistance), as well as paracrine soluble factors such as cytokines and chemokines result in reduced drug efficacy. To study microenvironment-mediated venetoclax resistance, we co-cultured MCL cell lines with the bone marrow stromal cell line HS-27a in the presence of venetoclax. Indeed, the stromal cells protected the MCL cells from venetoclax-induced cytotoxicity (Figure 7A). Importantly, when venetoclax was combined with CK2 silencing or inhibition, this protective effect of the stromal cells was largely abolished, without compromising the viability of the stromal cells (Figure 7B, C; *Online Supplementary Figure S8*). This further enhances the therapeutic potential of this combination.

To investigate the mechanism underlying the stromal cell-mediated venetoclax resistance and the ability of CK2 inhibition to overcome this, we determined the BCL-2 family protein levels in MCL cells co-cultured with stromal cells (Figure 7D). Interestingly, co-culture with stromal cells upregulated MCL-1 levels in MCL cells without affecting BCL-2 and BCL-X_L levels, and this MCL-1 upregulation was counteracted by CK2 inhibition. Taken together, our data indicate that repression of MCL-1 translation by CK2 inhibition prevents stroma-mediated upregulation of MCL-1, thereby sensitizing MCL cells to venetoclax.

Discussion

Whereas venetoclax shows promising anti-tumor activity in MCL patients, a substantial proportion of patients resists the treatment. Our kinome-centered CRISPR-Cas9 venetoclax-sensitizer screen identified the genes encoding the subunits of CK2 as prominent determinants of venetoclax sensitivity in MCL. We observed a strong synergistic effect between venetoclax and CK2 knockdown or inhibition in both venetoclax-sensitive and venetoclax-insensitive MCL cell lines, also in the presence of ibrutinib, and this synergistic effect can be attributed to CK2-mediated regulation of MCL-1 translation. Importantly, combining CK2 inhibition with venetoclax could also overcome microenvironmental protection to venetoclax, providing a clear rationale for combining venetoclax with a CK2 inhibitor in MCL patients.

CK2 is a ubiquitously expressed and constitutively active kinase that is involved in a broad array of cellular processes, including cell cycle progression, survival and differentiation.[19,20] CK2 is an attractive kinase to target in cancer, as cancer cells have been shown to be more sensitive to CK2 inhibition than their non-malignant counterparts.[20] CK2 is an attractive target also in MCL, as we and others have shown that the CK2 subunits are frequently over-expressed in MCL cells compared to naïve B cells from healthy donors (Figure 2A, B; *Online Supplementary Figure S2A*).[21,22] Furthermore, we show that *CSNK2B* expression is strongly associated with poor patient survival (Figure 2C), suggesting a possible role for CK2 in the aggressive disease course of MCL.

Using CK2 knockdown, and supported by the use of a specific CK2 inhibitor, our study demonstrates that CK2 is an important regulator of BCL-2 family proteins and thereby venetoclax sensitivity in MCL. This finding may have broader implications, for example, for treatment of other B-cell malignancies. In line with this, Lazaro-Navarro *et al.*[42] recently reported synergy between venetoclax and silmitasertib in B-cell precursor acute lymphoblastic leukemia (BCP-ALL) cells. Furthermore, based on the use of rather non-specific CK2 inhibitors such as TBB, apigenin and quercetin, several prior studies have suggested a possible role for CK2 in the expression of BCL-2 family proteins in various other cell types.[23,43-48] For example,

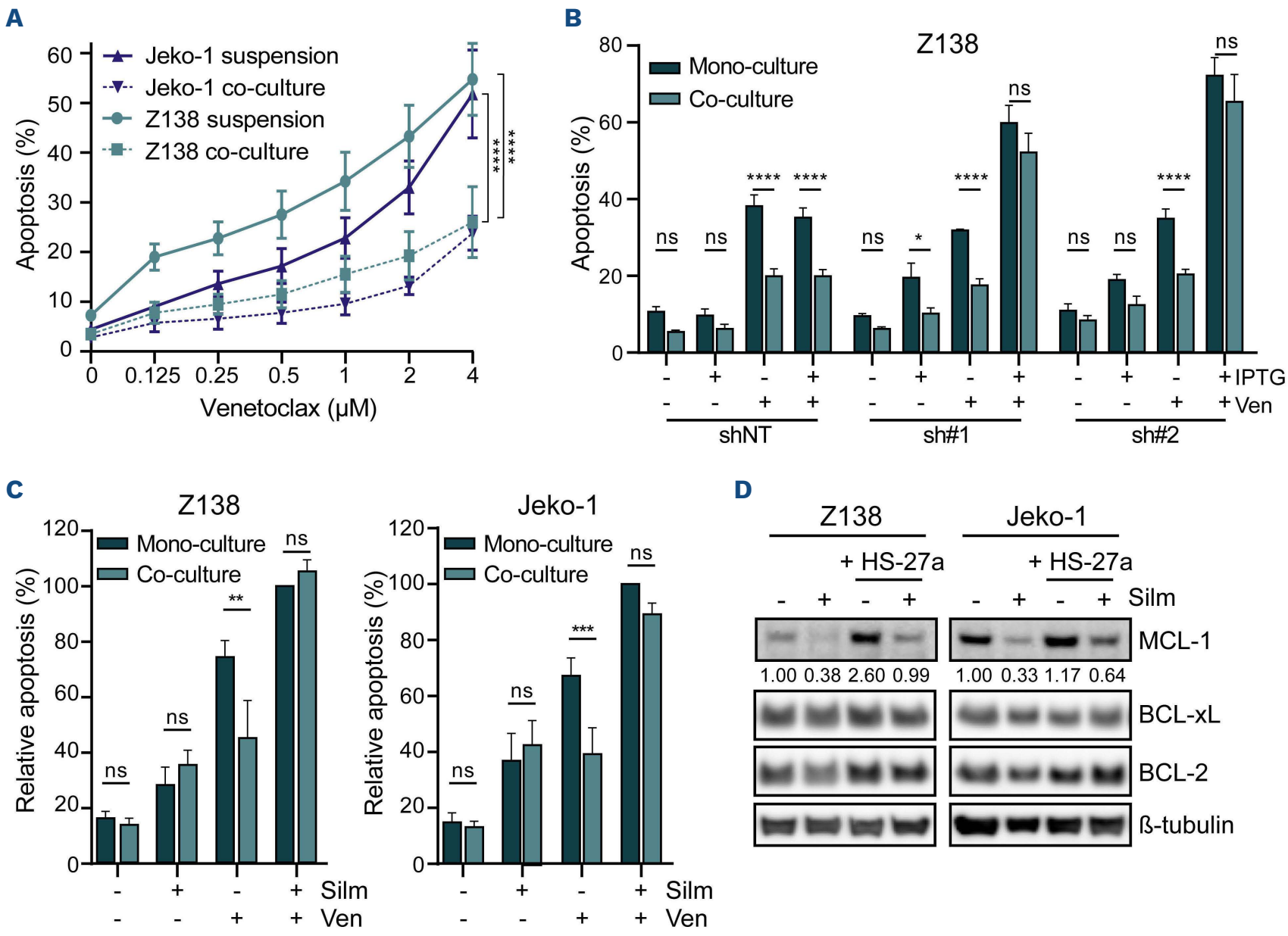

Figure 7. Casein kinase 2 inhibition overcomes microenvironmental venetoclax resistance. (A) Flow cytometry analysis of the effect of a 3-day co-culture with the human stromal cell line HS-27a on venetoclax-mediated toxicity of Z138 and Jeko-1. Data are presented as mean±SEM of at least four independent experiments performed in triplicate. (B) Flow cytometry analysis of the effect of *CSNK2A1* knockdown on HS-27a-mediated venetoclax resistance in Z138 cells. Cells were treated for 4 days with IPTG, followed by 3-days treatment with IPTG and/or 1 μM venetoclax. Data are presented as mean±Standard Error of Mean (SEM) of at least three independent experiments performed in triplicate. (C) Flow cytometry analysis of the effect of casein kinase 2 (CK2) inhibition on HS-27a-mediated venetoclax resistance in Z138 and Jeko-1 cells. Cells, cultured alone or co-cultured with HS-27a cells, were treated for 3 days with either 1 μM venetoclax, 4 μM silmitasertib or both. Data are presented as mean±SEM of three independent experiments performed in triplicate. (D) Immunoblot analysis of anti-apoptotic BCL-2 family protein expression in MCL cells treated with silmitasertib, cultured alone or co-cultured with HS-27a cells. MCL cells were pre-treated with 10 μM Q-VD-OPh for 1 h, followed by 1-h pre-incubation on HS-27a cells before 4 μM silmitasertib was added for 24 h. β-tubulin was used as a loading control. Representative immunoblots of two independent experiments are shown. MCL-1 protein expression is quantified by ImageJ and corrected for ß-tubulin levels. **P*<0.05; ***P*<0.01; ****P*<0.001, *****P*<0.0001; two-way ANOVA with Šidák multiple comparison test.

Russo *et al.*[47] have shown that quercetin synergizes with the dual BCL-2 and BCL-X_L inhibitor ABT-737 in primary CLL cells, involving quercetin-mediated downregulation of MCL-1. However, quercetin, apart from being an antioxidant, is a broad spectrum kinase inhibitor that targets, amongst others, PI3K, AMPK and several MAPK, besides CK2,[48] whereas for silmitasertib several studies have shown that off-target effects are negligible.[26,27]

The observed synergistic effect between venetoclax and CK2 knockdown or inhibition is best explained by the specific reduction in MCL-1 protein levels after CK2 inhibition or knockdown; the levels of BCL-X_L and BCL-2 were not altered (Figure 5A-C, Figure 8). Elevated MCL-1, and BCL-X_L, levels have frequently been described as a prominent determinant of primary venetoclax sensitivity in MCL, and, moreover, upregulation of these proteins has been reported as a mechanism of resistance to prolonged venetoclax treatment.[3] The up-regulated MCL-1 and BCL-X_L proteins serve as a buffer for the released BH3-only proteins upon venetoclax treatment, resulting in reduced BAK/BAX oligomerization and thus reduced apoptosis (Figure 8). Accordingly, venetoclax combined with MCL-1 inhibitors demonstrated strong synergy in several pre-clinical MCL models (Figure 5D; *Online Supplementary Figure S6B*).[3,16,17] However, *in vivo* toxicities such as cardiotoxicity have thus far hampered the development and clinical approval of MCL-1 therapy.[3] Indirect inhibition of MCL-1 by targeting a tumor-specific molecule such as CK2

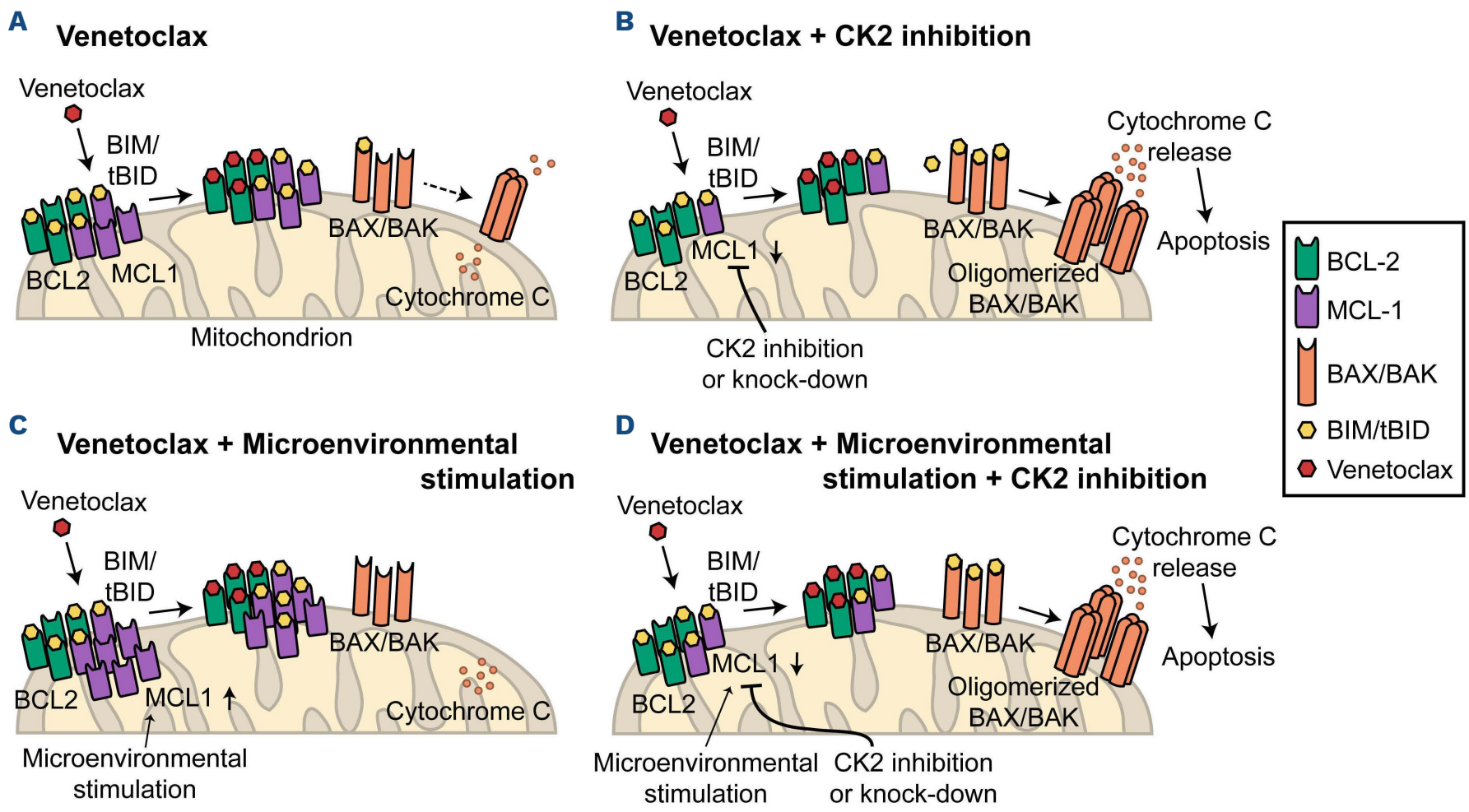

Figure 8. Casein kinase 2 inhibition sensitizes cells to venetoclax and overcomes microenvironmental venetoclax resistance. A schematic representation of the mechanism underlying the prevention of microenvironmental venetoclax resistance by casein kinase 2 (CK2) inhibition. (A) Venetoclax binds to the BH3 domain of BCL-2 with subsequent release of pro-apoptotic proteins such as BIM or truncated Bid (tBid). The pro-apoptotic proteins are then sequestered by MCL-1 or free to bind BAX/BAK, which then oligomerize and initiate the apoptosis cascade. (B) CK2 inhibition or knockdown reduces MCL-1 levels, resulting in increased levels of free pro-apoptotic proteins, and thus increased induction of apoptosis. (C) Microenvironmental stimulation enhances MCL-1 levels, thereby increasing the buffer for the pro-apoptotic proteins and thus inducing resistance to venetoclax-mediated apoptosis. (D) CK2 inhibition or knockdown counteracts the microenvironmental-mediated MCL-1 upregulation, thereby resensitizing the cells for venetoclax.

might prevent these toxicities, especially since silmitasertib showed a safe tolerability profile in clinical trials (NCT03904862, NCT03897036, NCT03571438, NCT04668209, NCT04663737).[24,25] In other hematologic malignancies, MCL-1 is also an important determinant in venetoclax resistance and, moreover, CK2 was frequently found to be over-expressed.[3,19-21] Combined with the recently reported synergy between silmitasertib and venetoclax in BCP-ALL cells,[42] this highlights the possibilities for a combination therapy of CK2 inhibition and venetoclax in other hematologic malignancies as well, and might expand the application of venetoclax to MCL-1-dependent malignancies such as multiple myeloma.

Our data indicate that CK2 regulates MCL-1 levels in MCL via its translation machinery in an mTOR-mediated fashion. Targeting CK2 did not result in diminished MCL-1 RNA levels or protein stability (Figure 6B, C), whereas phosphorylation of translation-regulatory proteins, such as S6, eIF4E and 4EBP, was affected (Figure 6D; *Online Supplementary Figure S7C*). This is considered to specifically affect mRNA translation of, amongst others, MCL-1; elevated eIF4E phosphorylation correlates with high MCL-1 protein levels and overexpression of a phosphomimetic S209D eIF4E variant increases translation of only a limited number of proteins, among which MCL-1.[37,38] Furthermore, we have previously shown that macrophages affect MCL-1 translation in CLL cells via the eIF4E-axis[40] and others have reported that inhibition of eIF4F assembly in DLBCL cells diminishes MCL-1 levels and synergizes with venetoclax.[41] In the pre-B ALL cell line NALM6, silmitasertib treatment was reported to enhance proteasomal degradation of MCL-1.[42] However, similar to our findings in MCL, in HEK cells Gandin *et al.*[49] also observed that CK2 controls 4EBP phosphorylation and eIF4F complex assembly. In addition, the expression of genes involved in protein translation correlates with CSNK2A1 expression in MCL patients (*Online Supplementary Figure S7A*). Notably, in several other cell types CK2 has been shown to phosphorylate components of other translation initiation complexes, thereby also affecting global translation.[49-53] Taken together, our data indicate that CK2-mediated regulation of MCL-1 protein levels in MCL is due to the altered activity of translation machinery.

In conclusion, we have shown that aberrant expression of some CK2 subunits in MCL correlates with inferior patient prognosis and that CK2 inhibition strongly sensitizes MCL cells to venetoclax, also in a lymphoid organ-mimetic co-culture setting (Figure 8). These pre-clinical findings

strongly support further clinical investigation of combination therapy of silmitasertib and venetoclax in MCL patients. Our data indicate that MCL-1 downregulation is the main mechanism underlying the observed synergy, which is in accordance with previous studies showing that MCL-1 downregulation results in sensitization to venetoclax. CK2 inhibition might provide a less toxic therapeutic strategy to sensitize MCL cells to venetoclax as compared to MCL-1 inhibitors, making this a promising combination therapy for patients with MCL, and possibly other hematologic malignancies.

Disclosures

No conflicts of interest to disclose.

Contributions

YJT designed the research, performed experiments, analyzed the data, and wrote the manuscript; MFMdR designed the research and performed bioinformatics; NS performed experiments; RLB and MJK provided materials; JG, EE and STP analyzed the data and revised the manuscript; APK supervised the study, analyzed data and revised the manuscript; MS designed and supervised the study, analyzed data and wrote the manuscript. All authors edited the manuscript.

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Data-sharing statement

The Perl, R, and Python scripts used for analyzing the CRISPR screen are available in the public GitHub repository (https://github.com/MFMdeRooij/CRISPRscreen). Raw data and the list of genes for which their targeting gRNAs were depleted in the venetoclax-treated arm are provided in Online Supplementary Table S3. Expression profile data analyzed in this study were obtained from Gene Expression Omnibus (GEO) at GSE93291, GSE132929, and GSE28491 and from the EMBL European Bioinformatics Institute database at E-MTAB-1771.

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