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Inhibition of MYC translation through targeting of the newly identified PHB-eIF4F complex as therapeutic strategy in CLL

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Abstract:
Dysregulation of mRNA translation, including preferential translation of mRNA with complex 5'-UTRs such as the MYC oncogene, is recognized as an important mechanism in cancer. In this study, we show that both human and murine chronic lymphocytic leukemia (CLL) cells display a high translation rate, which can be inhibited by the synthetic flavagline FL3, a prohibitin (PHB)-binding drug. A multiomics analysis consisting of pulsed SILAC, RNA sequencing and polysome profiling performed in CLL patient samples and cell lines treated with FL3 revealed the decreased translation of the MYC oncogene and of proteins involved in cell cycle and metabolism. Furthermore, inhibition of translation was associated with a block of proliferation and a profound rewiring of MYC-driven metabolism. Interestingly, contrary to other models, the RAS-RAF-(PHBs)-MAPK pathway is neither impaired by FL3 nor implicated in translation regulation in CLL cells. Here, we rather show that PHBs are directly associated with the translation initiation complex and can be targeted by FL3. Knock-down of PHBs resembled FL3 treatment. Importantly, inhibition of translation was efficient in controlling CLL development *in vivo* either alone or combined with immunotherapy. Finally, high expression of translation initiation-related genes and PHBs genes correlated with poor survival and unfavorable clinical parameters in CLL patients. In conclusion, we demonstrated that translation inhibition is a valuable strategy to control CLL development by blocking the translation of several oncogenic pathways including MYC. We also unraveled a new and direct role of PHBs in translation initiation, thus creating new therapeutic opportunities for CLL patients.

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Inhibition of MYC translation through targeting of the newly identified PHB-eIF4F complex as therapeutic strategy in CLL

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ABSTRACT

Dysregulation of mRNA translation, including preferential translation of mRNA with complex 5'-UTRs such as the MYC oncogene, is recognized as an important mechanism in cancer. In this study, we show that both human and murine chronic lymphocytic leukemia (CLL) cells display a high translation rate, which can be inhibited by the synthetic flavagline FL3, a prohibitin (PHB)-binding drug. A multiomics analysis consisting of pulsed SILAC, RNA sequencing and polysome profiling performed in CLL patient samples and cell lines treated with FL3 revealed the decreased translation of the MYC oncogene and of proteins involved in cell cycle and metabolism. Furthermore, inhibition of translation was associated with a block of proliferation and a profound rewiring of MYC-driven metabolism. Interestingly, contrary to other models, the RAS-RAF-(PHBs)-MAPK pathway is neither impaired by FL3 nor implicated in translation regulation in CLL cells. Here, we rather show that PHBs are directly associated with the translation initiation complex and can be targeted by FL3. Knock-down of PHBs resembled FL3 treatment. Importantly, inhibition of translation was efficient in controlling CLL development *in vivo* either alone or combined with immunotherapy. Finally, high expression of translation initiation-related genes and PHBs genes correlated with poor survival and unfavorable clinical parameters in CLL patients. In conclusion, we demonstrated that translation inhibition is a valuable strategy to control CLL development by blocking the translation of several oncogenic pathways including MYC. We also unraveled a new and direct role of PHBs in translation initiation, thus creating new therapeutic opportunities for CLL patients.

Key points

- Inhibition of translation initiation prevents CLL growth *in vitro* and *in vivo*, through targeting the MYC oncogene
- PHBs directly interact with the translation initiation machinery, filling a gap in the understanding of the crucial roles of these proteins

INTRODUCTION

In human chronic lymphocytic leukemia (CLL), the lymph node (LN) microenvironment sustains the proliferation of leukemic cells through different stimuli, such as the activation of B-cell receptor (BCR),[1] or Toll-Like receptors (TLRs).[2] Targeting their downstream signaling led to major breakthrough in the standard of care in CLL.[3] The oncogene MYC represents another interesting target in CLL, as MYC aberrations are detected in CLL cells and associated with Richter Transformation, a rare but aggressive complication of CLL.[4-6] In addition, its expression is upregulated in the LN microenvironment,[7-9] especially at the translational level.[10]

Recently, translation initiation was recognized as one "*Achilles' heel*" of cancer cells. Indeed, increase in global translation rate and aberrant translation of specific oncogenic transcripts seems to be a common feature for a large variety of tumors.[11,12] Interestingly, translation is also a nexus of resistance to several kinase inhibitors.[13] Particularly in CLL, several reports demonstrated a dysregulation of translation, either through mutations in ribosomal proteins[14-17] or through activation of translation by microenvironment-mediated stimuli.[10,18] In addition, ZAP-70 expression also contributes to aberrant translation, through direct interaction with ribosomal proteins.[19] The pathways and mechanisms leading to increased translation are not fully understood yet. Some studies demonstrated the efficacy of inhibiting translation in CLL, using different types of translation inhibitors.[20-22] However, there is still a debate on the mechanisms of action of the different molecules used, and only little evidence of their efficacy *in vivo.*

Here we investigated translation in CLL and its inhibition. We used the translation inhibitor FL3, which previously showed an anti-tumor activity both *in vitro* and *in vivo*.[13,23,24] This molecule, from the flavagline family, was shown to bind prohibitins (PHBs).[25] These scaffold proteins are found in several cellular sub-localizations that dictate their activity.[26] At the membrane, they are required for the RAF activation by RAS in a large variety of cancers,[27] leading to the phosphorylation of eukaryotic initiation factor 4E (eIF4E) through the MAPK pathway, and ultimately resulting in increased translation. By binding to PHBs, FL3 was shown to prevent the activation of RAF and therefore decreases the translation.[28]

In the present paper, we showed that aberrant translation is indeed a feature of human and murine CLL. We demonstrated that inhibition of translation in CLL targets the MYC oncogene leading to decreased proliferative capacities and reversion of metabolic rewiring. Finally, we identified PHBs as direct interactors of the eIF4F machinery and as crucial factors for translation in CLL cells.

METHODS

Animal experiments

All experiments involving laboratory animals were conducted in a pathogen-free animal facility with the approval of the Luxembourg Ministry for Agriculture. Mice were treated in accordance with the European guidelines. C57BL/6 mice were purchased from Janvier Labs. Eμ-TCL1 mice (called TCL1) were kindly given by Pr. Carlo Croce and Pr. John Byrd.[29] CLL progression was monitored by determining the percentage of $CD5^+CD19^+$ CLL cells in the peripheral blood using flow cytometry (FC).

Patient samples

All experiments involving human samples were conducted in accordance with the declaration of Helsinki, approved by the institutional review board (Jules Bordet Institute Ethics Committee) or the Luxembourg Comité National d'Ethique et de Recherche. Samples were collected from CLL patients after written informed consent. For the cohort of 144 patients used in Figure 7, all patients had a $CD19^+CD5^+CD23^+$ phenotype and a Catovsky score of 4/5 or 5/5. All tested prognostic factors were proven to be significant predictors of treatment-free survival (TFS) and overall survival (OS), indicating that our cohort is representative of a CLL population (**TableS1**).

OPP Protein Synthesis Assay

O-propargyl-puromycin (OPP, Bioconnect) was added into cell culture at a final concentration of 20µM for 30min. The viability staining was performed at 4°C for 30min with a Zombie Fixable Viability Kit (BioLegend). The cells were fixed for 15min at room temperature with 3.7% PFA, and permeabilized with 0.5% Triton X-100 in PBS for 15min at room temperature. The detection of the OPP was performed according to the manufacturer's protocol, using the Click-iT technology (Life Technologies). The cells were further subjected to antibody staining and analyzed by FC.

Pulsed SILAC assay

The SILAC labeling medium used was the following: RPMI 1640 for SILAC (Thermofisher) containing 10% of dialyzed serum, 1% of Pen/Strep, unlabeled leucine, either "heavy" amino acids [0.398mM L-($^{13}C_6$,$^{15}N_4$)-arginine and 0.798mM L-($^{13}C_6$,$^{15}N_2$)-lysine] or "medium-heavy" amino acids [0.398mM L-($^{13}C_6$)-arginine and 0.798mM L-(D_4)-lysine]. 10x10^6 of MEC-1 or OSU-CLL cells were resuspended at a concentration of 0.6x10^6 cells/ml, and treated as indicated for 8h. 20x10^6 patient CLL cells were resuspended at a concentration of 10x10^6 cells/ml, and treated as indicated for 16h. At the end of the labeling, the cells were washed 3 times in cold PBS and snap frozen, before proceeding to protein extraction and to mass spectrometry analysis.

The detailed material and methods can be find as supplementary information.

Data sharing statement

RNA-seq data may be found at the NCBI GEO database under the accession number GSE221880. For more details, contact corresponding authors at etienne.moussay@lih.lu or jerome.paggetti@lih.lu

RESULTS

Translation is increased in CLL cells and can be inhibited by FL3

First, using publicly available gene expression data sets, we showed that translation-related genes are upregulated in leukemic B cells compared to healthy B cells, in both human and mice, resulting in a functional enrichment in translation (**Fig.1A**). We also observed increased levels of proteins responsible for translation initiation in CLL cells from patients (**Fig.1B**). Therefore, we quantified translation by OPP incorporation (**Fig.1C**) and confirmed that protein translation is increased in CLL cells compared to B cells from healthy donors and from CLL patients (**Fig1.D-E**). To investigate whether an increased translation is also a feature observed in a CLL murine model, we analyzed the translation rate in different cell populations from the spleen of leukemic mice. We observed a higher translation rate in CLL cells ($CD19^+CD5^+$) compared to normal B cells ($CD19^+CD5^-$) and to both $CD8^+$ and $CD4^+$ T lymphocytes. Interestingly, $FOXP3^+$ regulatory T cells also showed a higher translation rate compared to other T cells (**Fig.1F**). Next, in order to verify that TLR and BCR activation induce translation, we treated CLL cells with the TLR9-agonist Class B CpG ODN-2006 or with an anti-IgM antibody. Indeed, the cellular activation increased translation, as attested by increased OPP incorporation (**Fig.1G, Fig.S1A**). Additionally, we showed that treatment of patient samples with the synthetic flavagline FL3 decreased translation (**Fig.1G, Fig.S1A**). Accordingly, in human (MEC-1, OSU-CLL, HG-3, WA-OSEL and PGA-1) and murine (TCL1-355)[30] CLL cell lines, incubation with FL3 led to a significant decrease in translation starting from 6nM, after only 3h of treatment (**Fig.1H-I, Fig.S1B-F**). The reduction in translation after FL3 treatment was also confirmed by measuring the incorporation of the methionine analogue HPG (**Fig. S1G**). Active translation is characterized by the interaction between the translation initiation factors eIF4E and eIF4G, which can be detected by proximity ligation assay (PLA, **Fig.1J**). Indeed, we observed an increased interaction between eIF4E and eIF4G in activated CLL patient cells and cell lines, which was decreased upon FL3 treatment (**Fig.1J, Fig.S1H-I**). Finally, the effect of FL3 on translation was analyzed by polysome profiling. We observed a decreased polysome peak amplitude and an accumulation of RNA in subpolysome fractions, demonstrating a significant reduction in translation efficiency (**Fig.1K, Fig.S1J**). Altogether, these results confirm the

relevance of studying translation in CLL, and demonstrates that FL3 can efficiently inhibit translation in human and murine CLL cells.

Multiomics analysis *revealed that inhibition of translation affects proteins involved in translation, cell cycle regulation, MYC and other oncogenic pathways*

As flavaglines were shown to reprogram the translational landscape rather than completely inhibit protein translation,[31] we wanted to understand more precisely the effect of FL3 in CLL cells. For this purpose, we performed pulsed SILAC assay on 5 patient samples and CLL cell lines (**Fig.2A**). First, we confirmed the increase in protein synthesis following activation in patient CLL cells, representing 16% of the detected proteins (**Fig.S2A-B, TableS2)**. This includes proteins involved in translation and activating signaling pathways (**Fig.S2C**). In resting samples, FL3 treatment led to decreased protein synthesis for 17% of the detected proteins (**Fig.S2B-S2D, TableS2**). However, the effect was more striking when CLL cells were activated and treated with FL3, with 31% of the detected proteins showing a decrease in their translation rate (**Fig.2B, Fig.S2B-S2E, TableS2**). Treatment of MEC-1 and OSU-CLL cell lines led to a similar effect (51% and 30% of the detected proteins) (**Fig.2C, Fig.S2B-S2F, TableS3**). The ontology analysis revealed that FL3 treatment affected common pathways in patient samples and cell lines, including proteins involved in translation and cell cycle regulation (**Fig.2D, Fig.S2G**). In addition, proteins regulated by several known oncogenes in CLL, such as the targets of MYC and NFKB, displayed a decreased synthesis (**Fig.2E-F**). Thus, our pulsed SILAC experiments suggested a decreased MYC activity in FL3-treated cells. Translation of MYC is reportedly increased upon CLL stimulation, contributing to leukemia development and proggression.[18] RNA-sequencing followed by GSEA confirmed the repression of translation-related genes and MYC target genes in FL3-treated cells (**Fig.2G-H, Fig.S2H, TableS4**). Furthermore, transcription factor enrichment analysis confirmed the involvement of MYC/MAX in the regulation of these repressed genes by FL3 (**Fig.2I)**. Ontology analysis indicated that deregulated MYC-target genes were mainly involved in metabolism-related biological processes (**Fig.S2I-J**). We thus hypothesized that FL3 could directly inhibit MYC translation (undetectable in pulsed SILAC assay). Indeed, treatment with FL3 resulted in a rapid loss of the MYC protein (**Fig.2J, Fig.S2K**) whereas its mRNA level was increased (**Fig.2K**). In addition, FL3 led to decreased levels of other oncogenes, such as ETS-1 (**Fig.S2L-M**). Finally, polysome profiling confirmed the lower abundance of Myc transcripts in the polysome fraction of both human and murine cells treated with FL3 (**Fig.2L, TableS4**) confirming the reduction in MYC translation. Preranked-GSEA indicated that FL3 predominantly repressed translation of genes involved in metabolism (glycolysis, fatty acid metabolism and oxidative phosphorylation, **Fig.2M**).

Altogether, these data show that FL3 specifically inhibits the translation of proteins involved in translation, cell cycle regulation and MYC oncogenic pathways.

Targeting of MYC translation is associated with decreased proliferative capacities, and reversion of metabolic rewiring

Based on the previous results, we decided to investigate the biological consequences of inhibiting translation. First, we measured cell viability of CLL cells, healthy PBMCs, MEC-1 and Eμ-TCL1 primary murine cells (**Fig.3A, Fig.S3A**). Interestingly, healthy PBMCs were less sensitive to FL3 compared to patient leukemic cells (IC_{50} at 72h: 118.2nM and 11.7nM respectively). In addition, FL3 induced more cell death in CLL cells from patients than in B cells from healthy donors at the same dose (**Fig.3B**), confirming the therapeutic window for treating CLL with inhibitors of translation. Cell growth of human and murine CLL cell lines was strongly impaired by very low doses of FL3 (from 6nM, **Fig.3C, Fig.S3B**). Drug withdrawal experiments indicated that 4 days of treatment were necessary for total impairment of cell recovery (**Fig.3D, Fig.S3C**). We also showed a limited effect of FL3 on apoptosis at an early time point, appearing only for higher doses and after 48h of treatment (**Fig.3E, Fig.S3D**). On the contrary, CFSE assay revealed a block of proliferation even with low FL3 doses (**Fig.3F, Fig.S3E**). However, no difference in cell cycle phase distribution was observed, indicating a complete block of the cell cycle rather than the inhibition of a

specific phase (**Fig.S3F**). The decreased expression of several targets involved in cell proliferation (CDK4, BIRC5, MCL1) in cells treated with FL3 was validated at protein level (**Fig.S3G).**

Data in figure 2 strongly suggests impairment of cell metabolism as majority of the MYC target genes, which show decreased transcription/translation, are associated with metabolic pathways. We performed stable isotope tracing using [U-^{13}C]-glucose and [U-^{13}C]-glutamine (**Fig.3G**). Following [U-^{13}C]-glucose tracing (depicted in orange), we observed a reduction in glycolytic flux and PDH flux (**Fig.3G, Fig.S3H**). The pentose phosphate pathway and purine/pyrimidine synthesis were also affected by FL3 (**Fig.3G**), contributing to the block in proliferation observed in previous experiments. In addition, the production of lactate from pyruvate is decreased in FL3-treated cells (**Fig.3G**), as LDHA, the enzyme catalyzing this reaction and target of MYC, was also decreased (**Fig.S3G**). [U-^{13}C] glutamine tracing (in green), revealed that FL3 inhibits glutaminolysis, in line with the role of MYC in this context.[32,33] In addition, the TCA cycle activity was markedly reduced (**Fig.3G, Fig.S3I**). As expected, following translation inhibition, we observed an accumulation of proteinogenic amino acids following FL3 treatment (**Fig.S3J**). To confirm the involvement of MYC in the metabolic reprogramming observed upon FL3 treatment, we used specific inhibitors (10058-F4 and 10074-G5) blocking MYC transcriptional activity. We validated their efficacy in our cells (**Fig.S3K**), and repeated the metabolic tracing. MYC inhibition led to similar metabolic changes to FL3 treatment except for glycolysis (**Fig.S3L-M**). This suggests that FL3-mediated metabolism rewiring is mainly due to MYC inhibition but other proteins may also contribute partially to this effect.

In conclusion, through the targeting of MYC and other factors, FL3 treatment leads to the arrest of the proliferative capacities of CLL cells and results in major changes in cellular metabolism.

Prohibitins interact directly with the eIF4F translation initiation machinery, and FL3 binding disrupts this complex

We then investigated FL3's mechanism of action in CLL cells. Through its binding to PHBs, FL3 was shown to block RAS-RAF-MAPK pathway activation.[27,34] In patient CLL cells, activation led to increased phosphorylation of RAF1, ERK1/2 and eIF4E (**Fig.4A, Fig.S4A**). Surprisingly, the phosphorylation of RAF1 and ERK1/2 was further increased upon FL3 treatment whereas eIF4E phosphorylation was decreased (**Fig.4A, Fig.S4A-B**), demonstrating that translation inhibition was not caused by the impairment of RAF1-ERK1/2 signaling. To understand whether the decrease in eIF4E phosphorylation is responsible for the phenotype observed in FL3-treated cells, we used the MAP kinase-interacting kinases (MNK) inhibitors eFT-508 (Tomivosertib) and CGP57380, as MNK1/2 are the main kinases phosphorylating eIF4E.[35] Treatment with inhibitors completely abolished eIF4E phosphorylation without affecting cell growth and translation rate (**Fig.4B-D, Fig.S4C-H**), showing that eIF4E phosphorylation status is not responsible for the decreased translation observed upon FL3 treatment. In addition, phosphorylation of MNK is not affected by FL3 treatment (**FigS4I**). 4E-BP1 is a major repressor of translation by complexing with eiF4E.[36] Phosphorylation of 4E-BP1 upon activation of the mTORC1 pathway leads to decreased interaction with eIF4E, promoting translation initiation.[37] We observed that p-4E-BP1 and 4E-BP1 protein levels were unaffected by FL3 and the interaction between eIF4E and 4E-BP1 was even slightly decreased upon FL3 treatment (**Fig.4E-F, Fig.S4J-K**). Cap-binding assay indicated that FL3 does not impair eIF4E binding to the cap but strongly reduces the ability of eIF4G to interact with cap-bound eIF4E, with no effect on 4E-BP1 (**Fig.S4L**). These data suggest a direct targeting of the eIF4F translation initiation complex by FL3, rather than deregulation of upstream signaling pathways.

Using DARTS assay, we confirmed that FL3 binds to PHBs in CLL cells (**Fig.4G**), but not to eIF4A **(Fig.S4M**), which was shown to be the target of other flavaglines such as rocaglamide A (RocA) or silvestrol.[23,26,38] In addition, we tested whether other molecules targeting PHBs, not related to flavaglines, as IN44 and Fluorizoline, were associated with a defect in translation. Indeed, treatment of CLL cells with IN44 and Fluorizoline at sub-lethal doses was associated with a decreased translation rate (**Fig.S4N-O**). Altogether, these data point to a direct role of PHBs in the translation initiation machinery. In addition, we

quantified PHBs levels in patient cells and observed that PHBs are more abundant in CLL cells than in healthy donors B cells, both at mRNA and protein levels (**Fig.4H-I**).

Considering all these results, we hypothesized that PHBs interact with the translation initiation machinery. By immunoprecipitation (IP) of the endogenous eIF4E protein in MEC-1 cells, and of the tagged version of eIF4E and eIF4G in transfected HEK-293T cells, we showed their interaction with PHB (**Fig.4J-K**). Using PLA, we demonstrated the cytoplasmic interaction of PHB and PHB2 with eIF4E, eIF4G and eIF4A in patient CLL cells and MEC-1 cells (**Fig.4L-M, Fig.S4P-T**). Interestingly, these interactions increased upon activation, and decreased upon treatment with FL3. In order to understand the stoichiometry of these interactions, we performed NanoBRET experiments (**Fig.4N**). BRET ratios were measured in HEK-293T cells transfected with plasmids encoding PHB fused with the nanoluciferase (NLF) and either eIF4E or HaloTag (HT, control) fused with the NeonGreen (NG), in N- or C-terminal. The higher ratio observed when using PHB(NLF) and eIF4E(NG), compared to HT(NG), indicates that PHB and eIF4E indeed interact (**Fig.S4U**). The plateau reached when using increasing amounts of NG-eIF4E, and the linear curve when using the NG-HT control, confirmed the specificity of the observed interaction (**Fig.4N**). These experiments proved the interaction of PHB with the eIF4F complex.

Finally, we used the other flavaglines RocA and silvestrol, known to destabilize the eIF4F complex by binding to eIF4A. Both molecules decreased translation rate and cell proliferation, similar to FL3 treatment, however they induced much higher rates of apoptosis (**Fig.S4V-X**). In addition, they led to decreased phosphorylation of eIF4E, without affecting RAF/ERK pathway (**Fig.S4Y**), which is comparable to FL3 treatment. The similarity between FL3 and RocA/silvestrol confirms that FL3 is also destabilizing the eIF4F complex, but by binding to PHBs instead of eIF4A.

Silencing of prohibitins inhibits translation and replicates the effects of FL3 treatment

The interaction of PHBs with the members of the translation initiation machinery strongly suggests a direct role of PHBs in translation. To validate this hypothesis, we knocked-down (KD) PHBs using shRNA against either PHB or PHB2, as validated at the mRNA and protein levels (**Fig.5A, Fig.S5A-C**). First, we investigated translation and protein interactions and we could confirm that PHBs KD decreased translation rate in CLL cells, reduced the formation of the eIF4E/eIF4G complex, and inhibited eIF4E phosphorylation (**Fig.5B-C, Fig.S5D-E**). In addition, PHBs KD was associated with a decrease in cell growth and proliferation (**Fig.5D-E**), while not inducing any cell death **(Fig.S5F)**. KD of PHBs was also associated with a decrease in MYC expression and activity (**Fig.5F**, **Fig.S5G**). Finally, we repeated the targeted metabolomics analysis with glucose or glutamine tracing in PHBs KD cells. We observed an impact of the KD similar to treatment with FL3, as exemplified by a reduction of glycolysis and TCA cycle, a decreased synthesis of purines and pyrimidines (**Fig.5G**), and an accumulation of proteinogenic amino acid (**Fig.S5H**). In conclusion, KD of PHBs replicated all the effects observed with FL3. Altogether, using different experimental strategies, we demonstrated for the first time a direct role for PHBs in translation.

FL3 alone or in combination with immunotherapy controls CLL development in vivo

Next, we tested the efficiency of translation inhibition to control CLL development *in vivo*. For this, we performed adoptive transfer of Eμ-TCL1 splenocytes into C57BL/6 mice and treated recipient mice with FL3 or vehicle. The analysis of $CD19^{+}CD5^{+}$ CLL cells in the peripheral blood (PB) indicated that FL3 treatment significantly controlled the development of the disease (**Fig.6A-B**). This correlated with an increased survival of the mice (median survival of 62d in FL3-treated vs 48d in vehicle, **Fig.6C**). In a second independent cohort, we analyzed the splenocytes after 15 days of treatment. The percentage of CLL cells in the spleen was again significantly decreased (**Fig.5D**), confirming the efficiency of FL3 to control CLL progression *in vivo*. FL3 treatment inhibited the translation rate in the CLL cells *in vivo* but had no effect on non-leukemic B cells ($CD19^{+}CD5^{-}$, **Fig.6E**). Interestingly, the high translation rate of Tregs was inhibited by FL3 treatment *in vivo*, while their percentage was significantly reduced (**Fig.6D-E**). In addition, CLL cells expressing high levels of the inhibitory immune checkpoint PD-L1 displayed a higher translation rate

(**Fig.6F**). Finally, we observed that combining FL3 with anti-PD1 therapy resulted in a better outcome *in vivo* (**Fig.6G-H**). Altogether, these data indicate that FL3 is efficient *in vivo* by targeting the malignant cells, but also probably by removing the brakes on cell-mediated anti-tumor immunity.

Expression of translation-related genes correlates with disease progression and poor survival in CLL patients

To validate the importance of translation in CLL, we quantified the expression of 6 genes from the translation initiation machinery (*eIF4E1, eIF4E2, eIF4G1, eIF4G2, eIF4A1, eIF4A2*) along with *PHB and PHB2* by RT-qPCR in a cohort of CLL patients (n=144). First, using Cox regression analysis, we identified *eIF4E2 and eIF4G2* expression as predictors of overall survival (OS, hazard ratio>1 and p-value<0.05), a high expression was related with poor OS (**Fig.7A-B**, median survival of 174mo vs 397mo, **Fig.S6A**). When combining these genes, the 8-gene translation signature was also linked to OS (**Fig.7C**) and the higher hazard ratios were obtained when combining two genes (**Fig.7D**, HR > 1.5). We made similar observations when analyzing the treatment-free survival (TFS). We identified that *eIF4G2, eIF4E2, eIF4A1 and PHB2* transcripts expression, as well as the translation gene signature and multiple gene combinations, was related with TFS in these patients (**Fig. 7E-F, Fig.S6B-D**). We then analyzed the expression of single or multiple genes in groups of patients segregated by the classical prognostic parameters. Single-gene analysis confirmed the increased expression of *eIF4E1*, *eIF4E2, eIF4G1, eIF4G2, PHB and PHB2* in unfavorable groups (e.g. $ZAP70^+$ vs $ZAP70^-$) and sub-groups (e.g. $IGHV_M$ LPL^+ vs $IGHV_{UM}$ LPL^-) (**Fig.7G, Fig.S6E**), while logistic regression identified differentially expressed gene signatures (**Fig.S6F**). Within groups of patients, the level of expression of *eIF4E2* was linked to OS and TFS (**Fig.7H-I, FigS6G**). Altogether, our data confirmed the relevance of translation in CLL as the expression of selected genes is related to survival and clinical parameters.

DISCUSSION

Although often overlooked in favor of transcriptional alterations, the relevance of translation defects in cancer has been long-established. Such importance can be illustrated by the tight association between ribosomopathies and cancer susceptibility.[39] In addition, increased expression of several translation initiation factors has been described in a large variety of neoplasms.[11] This overexpression can be associated with either overall increase in translation or with alteration of specific mRNA translation. Here, we showed through gene expression analysis, that translation-related genes are upregulated in leukemic B cells compared to healthy B cells, in both human and mice, which correlate with higher the translation rate observed in CLL cells. In addition to patient samples, EBV-infected human cell lines[40] were used in this study for functional and gene expression studies. Aware that they might not fully represent CLL cells, we validated all key experiments with patient samples and with the new murine cell line TCL1-355. This corroborates the interest of targeting translation in this neoplasm. Activation signals converge to upregulation of translation and inhibiting this common endpoint appears to be more promising compared to targeting different pathways independently. In addition, oncogenes are more susceptible to dysregulation of translation, due to the complex 5' structure found in the mRNAs of these genes. Our multiomics analysis demonstrated that translation inhibition affects the translation of the oncogene MYC, which is associated with decreased proliferation capacity and a switch in metabolism. Thus, inhibiting translation allows targeting the leukemic process at different levels. MYC translation is highly regulated at the translational level[10,41] and previous studies demonstrated that eIF4A inhibition targets MYC translation in CLL.[22,42] Here, we show that targeting translation through PHB leads to a similar effect. In addition, we demonstrated that MYC inhibition is responsible for major metabolism reprogramming.

The exact mechanism of action of flavaglines is still under debate. Surprisingly, despite having biological effects in the nanomolar range of concentration, the mechanistic studies on flavagline molecules are often conducted in the micromolar range, thus one has to be careful when interpreting the data, as increased dose of the drug often lead to off-target effects. The known targets of flavaglines are PHBs,[26,43]

eIF4A[26,44] and DDX3.[19] By binding to eIF4A, flavaglines stimulate the RNA-binding function of eIF4A, which prevents RNA/eIF4A dissociation, and therefore incorporation of free eIF4A in a new eIF4F complex.[23,45] Through targeting of PHBs, FL3 was believed to act on translation indirectly, through the inhibition of the RAS-RAF-MAPK pathway, ultimately leading to decrease eIF4E phosphorylation.[27,34,43,46,47] Thanks to DARTS analysis, we proved that FL3 targets PHBs in CLL cells. In addition, the present study demonstrated that FL3 treatment is not associated with decreased phosphorylation of RAF1 and ERK1/2 in CLL cells. We also showed that in any case the level of eIF4E phosphorylation (downstream of RAF1/ERK signaling) does not account for translation rate or cell proliferation. This may be seen as a contradiction with some reports on the role of eIF4E phosphorylation on cancer,[48-50] nevertheless elegant work demonstrated that defects in eIF4E phosphorylation are not associated with impaired translation nor cell growth.[51] The decreased eIF4E phosphorylation rather reflects the dissociation of the translation initiation machinery. Indeed, eIF4E-eIF4G interaction is crucial for MNKs-driven eIF4E phosphorylation.[52] Then, we showed that 4E-BP1 is also not affected in FL3-treated cells, demonstrating that the effect of FL3 on translation does not involve upstream pathways.

Therefore, we hypothesized that PHBs are directly involved in translation. We used different approaches to demonstrate the interaction of PHBs with the eIF4F complex. Then, by using shRNA, we showed that KD of PHBs mirror FL3 treatment. This discovery represents an important breakthrough that provides key biological insights necessary to understand both PHBs biology and PHB-targeting drugs. However, many questions remain open. Future work must determine whether the interaction between PHBs and the eIF4F machinery is limited to CLL cells, what factors govern this interaction and how FL3 impairs it. Finally, whether PHBs are general players in translation or limited to certain circumstances (oncogenic process, hypoxia) remains to be elucidated.

We showed that CLL cells and Treg have a higher translation rate *in vivo* compared to normal B cells and conventional $CD4^+$ T cells respectively. Moreover, inhibition of translation led to an efficient control of the disease following adoptive transfer of Eμ-TCL1 diseased splenocytes. In FL3-treated mice, we observed a decrease in translation rate in the leukemic cells and in $CD8^+$, $CD4^+$ T lymphocytes and Tregs. Interestingly, the translation rate in non-leukemic B cells was not decreased by FL3 treatment. In addition, only CLL cells and Tregs were negatively impacted by translation inhibition, indicating a specific effect of the drug. We recently demonstrated the crucial role of Tregs in the development of CLL,[53,54] thus the possibility to target both CLL cells and Tregs appears promising for the therapeutic potential of translation inhibition in CLL. Interestingly, Tregs rely on a non-canonical translation initiation machinery[55] and on the ribosome biogenesis factor Nocl4,[56] suggesting that FL3 might also be able to target specific translation mechanisms that do not use the canonical factors. In addition, we demonstrated that FL3 treatment in combination with immunotherapies targeting PD1 is more efficient than single therapies. Future studies should compare the advantage of inhibiting translation relative to current treatments. As translation is the nexus of resistance to several therapies,[13] the possibility to combine inhibition of translation with standard of care in CLL represents a promising approach, particularly in a malignancy characterized by relapse and refractory disease.

In conclusion, we have highlighted the importance of the deregulation of translation in CLL, unveiled a direct role of PHBs in translation, and demonstrated that translation inhibition is efficient in controlling CLL development in a preclinical model. We also found a correlation between translation-related/PHBs gene expression, prognostic markers, and survival in a cohort of CLL patients. Current therapies targeting downstream of the BCR constituted a major advance in the standard of care in CLL. However, resistance to these therapies remains an important pitfall. The identification of translation as novel therapeutic target could be key to establish effective therapeutic strategies for high-risk CLL patients.

Supplementary Materials: Supplemental methods, figures and tables are available online.

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Authorship Contributions: AL designed and performed experiments, analyzed results and wrote the manuscript. VK, EV, SG, IFB, SP, CD, MW, EG, and GP performed experiments and analyzed data. AB, NA, NEH, and PC performed HPG assay, Polysome Profiling and analysis, and provided expertise on translation. PM and TH performed DARTS assay and analysis. DPH and GD performed Pulsed SILAC experiments and analysis. SC and DGE provided materials and expertise. PN performed statistical analysis of gene expression data in CLL patients. MB and JM performed metabolomics experiments and analyzed data. MS and AC performed BRET experiments and analyzed data. BS provided CLL patient samples, cDNA from cohort of CLL patients, patients' data and expertise for analyses. LD synthetized FL3 and provided his expertise on the molecule. LY, JHF, SC, GB, provided CLL patient samples and expertise on hemato-oncology. EM and JP designed and supervised the study, performed bioinformatics analyses, analyzed results, and wrote the final version of the manuscript. All authors revised the manuscript.

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Figures legends

Figure 1. Translation is increased in CLL cells and can be inhibited by FL3

(A) Preranked gene set enrichment analysis from public datasets indicating an enrichment in translation in CLL cells compared to healthy B cells in Human (NCBI GEO, GSE67640, GSEA, left panel, and EIF4A2 and EIF4G2 gene expression in healthy donor B cells (HD) vs CLL cells, middle panels), and in mouse (GSE175564, TCL1 cells compared to C57BL/6 B cells, right panel). **(B)** Western-blot analysis of phospho-eIF4E, eIF4E, eIF4A, eIF4G and HSC70 proteins in B cells from healthy donors and CLL patients. **(C)** Schematic representation of the OPP incorporation assay to evaluate translation rate. **(D-F)** Determination of translation rate by OPP assay in B cells from healthy donors and CLL patients (D, n=3), in normal B cells and CLL cells from CLL patients (E, n=5) and in T- and B-cell subsets from the spleen of sick recipient mice, after transfer of Eμ-TCL1 splenocytes (F, n=5). Left panel: representative plots, right panel: quantification **(G-I)** Determination of translation rate by OPP assay in patients CLL cells activated (Activ.) or not (Rest.) with CpG ODN-2006, and treated with DMSO or 100nM FL3 for 16h (left panel: representative plots, right panel: quantification, n=21) (G), and in MEC-1 (H) or TCL1-355 (I) cells treated with DMSO or FL3 for 3h (0-50nM) (left panel: representative plots, right panel: quantification, n=3). **(J)** Schematic representation of proximity ligation assay (PLA) and determination of translation rate, based on PLA detection of eIF4E/eIF4G interaction, in patient CLL cells activated or not with CpG ODN-2006 and treated with DMSO or 100nM FL3 for 3h (Rest.:858 cells, Activ.: 695 cells, Activ.+FL3: 357 cells). **(K)** Schematic representation of polysome profiling, representative plot and translation efficiency in MEC-1 cells treated with DMSO or 50nM FL3 for 24h.

Figure 2. Multiomics analysis revealed that inhibition of translation affects proteins involved in translation, cell cycle regulation, MYC and other oncogenic pathways

(A) Schematic representation of Pulsed SILAC assay. **(B)** Volcano plot showing differentially translated proteins (DTP) in CpG ODN-2006-activated patients CLL cells treated with DMSO or 100nM of FL3 for 16h, with FDR<0.2 and $\log_2$FC>1 (n=5). **(C)** Volcano plot showing DTP between MEC-1 treated with DMSO or 50nM of FL3 for 8h, with FDR<0.05 and $\log_2$FC>1. (n=3). **(D)** Heatmap depicting the ontology terms enriched in proteins with decreased translation in CpG ODN-2006-activated patient CLL cells, MEC-1 and OSU-CLL treated with FL3. **(E)** Heatmap depicting the upstream factors that regulate the expression of proteins with decreased translation in CpG ODN-2006-activated patient CLL cells, MEC-1 and OSU-CLL treated with FL3. **(F)** Heatmap showing the expression of DTP from selected pathways (identified in D-E) in CLL patient samples. **(G-H)** GSEA plots obtained from gene expression data generated from MEC-1 (G) and TCL1-355 cells (H) treated with DMSO or 50nM FL3 for 24h. **(I)** Transcription factor enrichment analysis (top10) for DEGs downregulated by FL3. **(J)** Western-blot analysis of MYC proteins in patients CLL cells activated (Activ.) or not (Rest.) with CpG ODN-2006 and treated with DMSO or FL3 for 24h, and in MEC-1 and OSU-CLL cell lines treated for 3h. **(K)** *Myc* mRNA levels in cell lines treated with DMSO or 50nM FL3 for 3h by RT-qPCR (n=3). **(L)** Myc mRNA ratio of expression in polysome versus subpolysome fractions of cells treated with DMSO or 50nM FL3 for 24h. **(M)** Heatmap showing GSEA normalized enrichment scores (NES) from polysome profiling RNA sequencing. **P*<.05,** *P*<.01,*** *P*<.001.

Figure 3. Targeting of MYC translation is associated with decreased proliferative capacities, and reversion of metabolic rewiring

(A) Viability of PBMCs from healthy donors (HD), CLL patient cells, MEC-1, and primary Eμ-TCL1 splenocytes treated 72h with FL3 (0-500nM) assessed by CCK8 assay. **(B)** Viability of sorted $CD19^+$ B cells from HD and CLL patients treated with FL3 (0-100nM) for 48h (n=3, Apotracker™ green and 7AAD staining). **(C)** Growth of MEC-1 cells treated with FL3 (0-50nM) for 4 days (n=3). **(D)** Growth of MEC-1 cells after drug withdrawal.

The cells were treated (red, then green after withdrawal) with 50nM FL3 or DMSO (blue) for 96h, before being washed and resuspended at $0.1x10^6$ cells/ml without the drug at day 0. The growth was assessed for 5d after drug withdrawal. **(E)** Percentage of apoptotic cells after 3h, 24h or 48h of treatment with FL3 (0-50nM) in MEC-1 cells determined by Apotracker™ green and PI staining. **(F)** Determination of the proliferation of MEC-1 cells based on CFSE assay (left panel: representative plot, right panel: quantification, n=3). **(G)** Metabolomic isotopologue analysis of MEC-1 and TCL1-355 cells treated with DMSO or 50nM FL3 for 24h in the presence of [U-^{13}C]-glucose (orange) and [U-^{13}C]-glutamine (green). Relative metabolic fluxes are indicated with grey for unlabeled fraction, in orange or green for labeled fractions (n=3). **P*<.05,** *P*<.01,*** *P*<.001,**** *P*<.0001.

Figure 4. Prohibitins interact directly with the eIF4F translation initiation machinery, and FL3 binding disrupts this complex

(A) Western-blot analysis of p-RAF1, RAF1, p-ERK1/2, ERK1/2, p-eIF4E and eIF4E proteins in patient CLL cells activated or not (resting) with anti-IgM, and treated with DMSO or 100nM FL3 for 3h, and MEC-1 and TCL1-355 cells treated with 50nM FL3 for 3h. **(B-D)** MEC-1 cells treated with MNK1/2 inhibitor eFT-508 (0-10µM) for 24h were analyzed by western-blot for p-eIF4E and eIF4E levels (B), for proliferation (C) and protein synthesis (D, OPP assay), n=3. **(E-F)** Western-blot analysis of p-4E-BP1 and 4E-BP1 (E) and PLA detection of eIF4E/4E-BP1 interaction (DMSO: 583 cells, FL3: 612 cells, n=3) (F) in MEC-1 cells treated with DMSO or 50nM FL3 for 3h. **(G)** Schematic representation of DARTS assay (upper panel). PHB and PHB2 stability in presence of pronase in MEC-1 cells treated with DMSO or 100nM FL3 for 3h. Middle panels: % of PHB or PHB2 remaining at different concentration of pronase (ND: not digested), n=3; lower panel: representative western-blot. **(H)** Western-blot analysis of PHB and PHB2 proteins in healthy donors (HD) and patients CLL cells. **(I)** PHB gene expression in HD B cells vs patients CLL cells (GSE67640 data set). **(J)** Western-blot analysis of eIF4E and PHB proteins after immunoprecipitation of eIF4E in MEC-1 cells. **(K)** Western-blot analysis of His-tag, HA-tag and Myc-tag after immunoprecipitation of His-tag and HA-tag in HEK-293T cells overexpressing His-eIF4G, HA-eIF4E and Myc-PHB. **(L-M)** PLA detection of eIF4E/PHB or eIF4G/PHB interactions in CLL patient cells activated or not with CpG ODN-2006 and treated with DMSO or 100nM FL3 for 16h (K) and MEC-1 cells with 50nM FL3 for 3h (left panel: representative image, right panel: quantification, L-upper: Rest.: 1147 cells, Activ.: 1223 cells, Activ+FL3: 1097 cells; L-lower: Rest.: 1297 cells, Activ.: 1195 cells, Activ+FL3: 1155 cells; M-upper: DMSO: 419 cells, FL3: 411; M-lower: DMSO: 53, FL3: 52; n=3). **(N)** Schematic representation of NanoBRET assay and BRET ratio measured in HEK-293T cells transfected with increasing amount of plasmids encoding PHB fused with the nanoluciferase (NLF) and either eIF4E or HaloTag (HT) fused with the NeonGreen (NG). **P*<.05,*** *P*<.001,**** *P*<.0001.

Figure 5. Silencing of prohibitins inhibits translation and replicates the effects of FL3 treatment

MEC-1 cells were transfected with plasmids encoding shRNA against Scramble (shCtrl), PHB (shPHB) and PHB2 (shPHB2) and analyzed 72-96h after transfection **(A)** Gene expression measured by RT-qPCR (72h, n=3). **(B)** Determination of translation rate (OPP assay, 72h, n=3). **(C)** PLA detection of eIF4E/eIF4G interaction (72h, shCtrl: 117 cells, shPHB: 127 cells, shPHB2: 92 cells, n=3). **(D)** Growth of cells up to 96h after transfection (n=4). **(E)** Determination of proliferation (CFSE assay, 96h, n=3). **(F)** Western-blot analysis of MYC protein (72h). **(G)** Metabolomic isotopologue analysis of cells (72h, tracers incubated after 48h) in the presence of [U-^{13}C]-glucose (orange) and [U-^{13}C]-glutamine (green). Relative metabolic fluxes are indicated with grey for unlabeled fraction, in orange or green for labeled fractions. **P*<.05,** *P*<.01,*** *P*<.001,**** *P*<.0001.

Figure 6. FL3 alone or in combination with immunotherapy controls CLL development *in vivo*

(A-B) Percentage (A) and number (B) of $CD19^+CD5^+$ CLL cells in the peripheral blood (PB) of C57BL/6 mice after adoptive transfer of splenocytes from a sick Eμ-TCL1 mouse, and treated with vehicle (n=9) or FL3

(n=8). **(C)** Survival of mice from panel A-B. **(D)** Percentage of CLL cells ($CD19^+CD5^+$), B cells ($CD19^+CD5^-$), $CD8^+$ T cells ($CD3^+CD8^+$), $CD4^+$ T cells ($CD3^+CD4^+$) and Treg ($CD4^+FOXP3^+$) in the spleen of mice treated with vehicle or FL3, 17d after adoptive transfer of splenocytes from a sick Eμ-TCL1 mouse (n=5) **(E)** Determination of the translation rate in cells from panel D. **(F)** Determination of the translation rate in PD-$L1^{high}$ or PD-$L1^{low}$ $CD19^+CD5^+$ CLL cells from the spleen of C57BL/6 mice after TCL1 adoptive transfer (n=4). **(G-H)** Percentage of CLL cells in the blood at the indicated time points (G) and spleen at euthanasia (H) of C57BL/6 mice after adoptive transfer of splenocytes from a sick Eμ-TCL1 mouse, and treated with vehicle, FL3, anti-PD1 antibody or the combination FL3/anti-PD1. **P*<.05,** *P*<.01,*** *P*<.001,**** *P*<.0001.

Figure 7: Expression of translation-related genes correlates with disease progression and poor survival in CLL patients

Gene expression analysis was performed by RT-qPCR for 8 genes involved in translation in a cohort of 144 CLL patients. The relation between gene expression and survival was evaluated by Cox univariate regression analysis. Gene expression in clinical groups was evaluated by differential expression analysis for single genes or by logistic regression (LR) analysis for multiple genes. **(A and E)** Calculated hazard ratios >1 (red dots, *P*-value<0.05) indicate an increased risk for patients with high single-gene expression in term of overall survival (OS) (A) and treatment-free survival (TFS) (E). **(B and F)** Relation between high or low *eIF4E2* gene expression and OS (B) or TFS (F). Low and High groups are of identical size (n=72). **(C)** Relation between high or low combined 8-gene expression and OS. **(D)** Calculated hazard ratios >1 (red dots, *P*-value<0.05) indicate an increased risk for patients with high multiple gene expression in term of OS**. (G)** Standardized expression of single genes in groups of patients according to prognostic markers (CytoG unfav.: cytogenetics unfavorable, group size indicated in each panel). **(H-I)** Relation between high or low *eIF4E2* gene expression and OS in $IGHV_{UM}$ or LPL^+ CLL patients (H), and TFS in Binet A CLL patients (I). **P*<.05,** *P*<.01,*** *P*<.001.

Figure 1

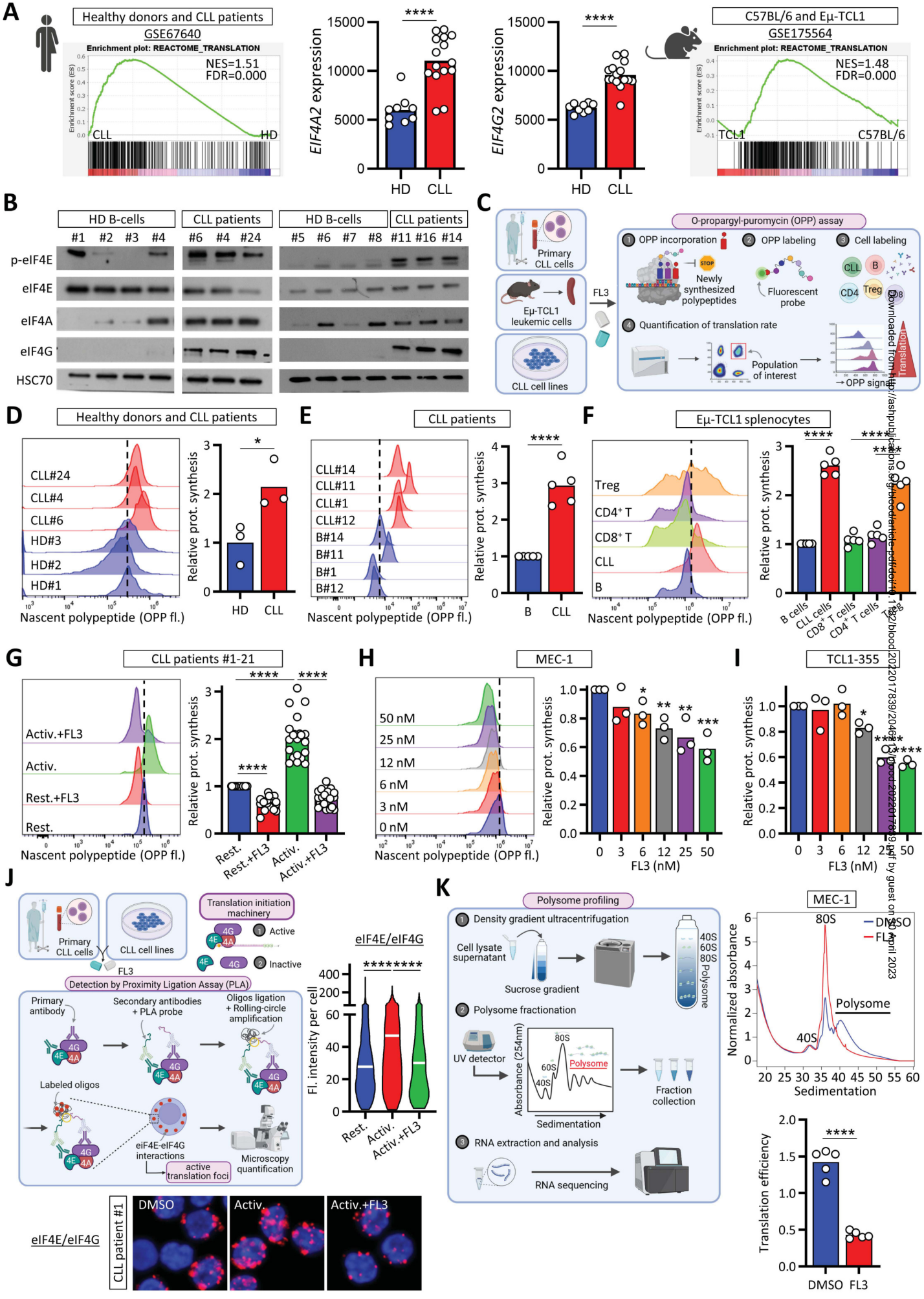
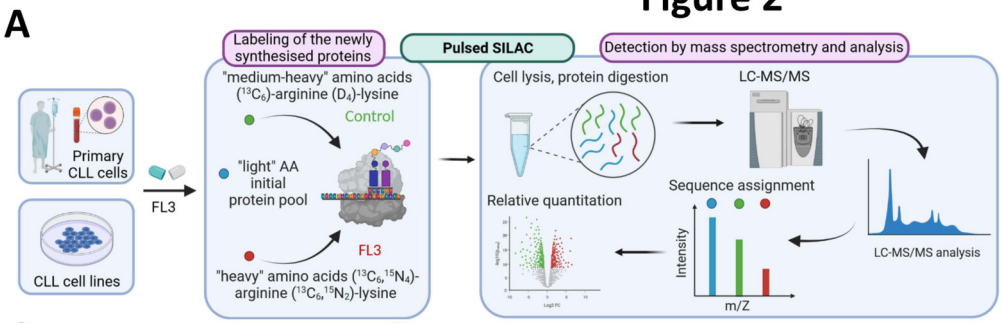
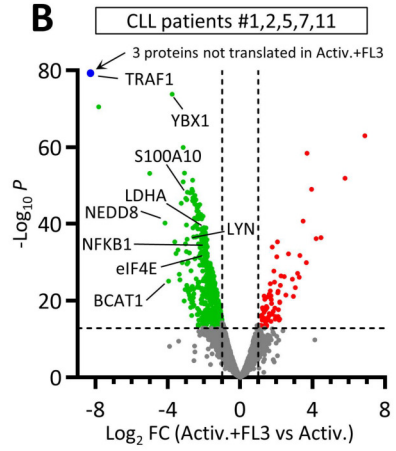
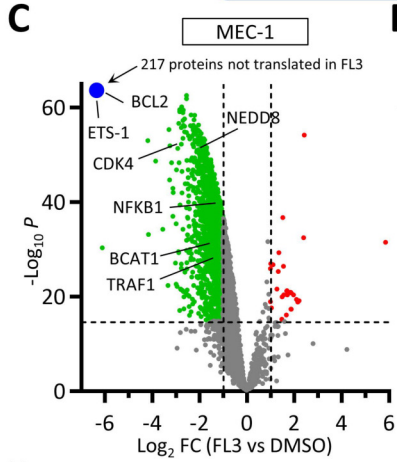
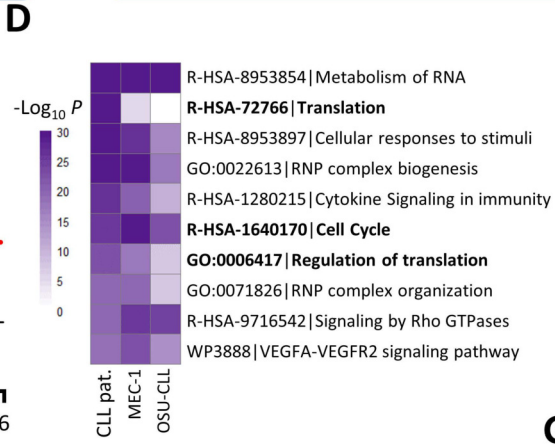


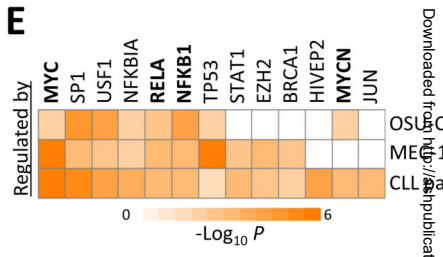
Figure 2

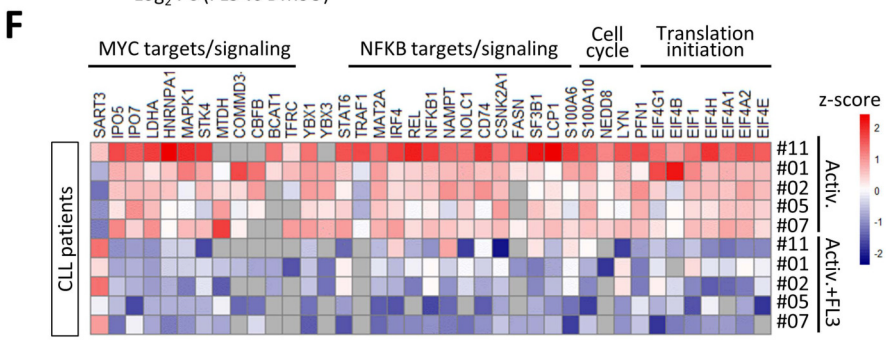


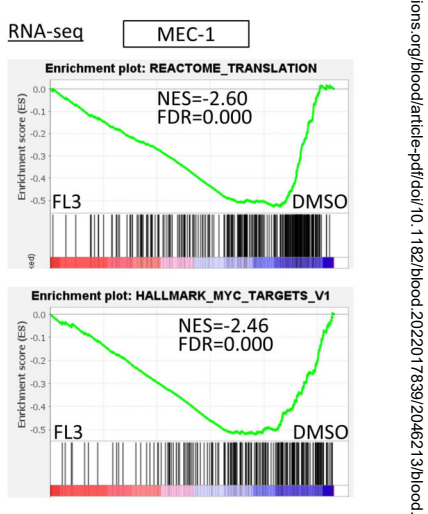


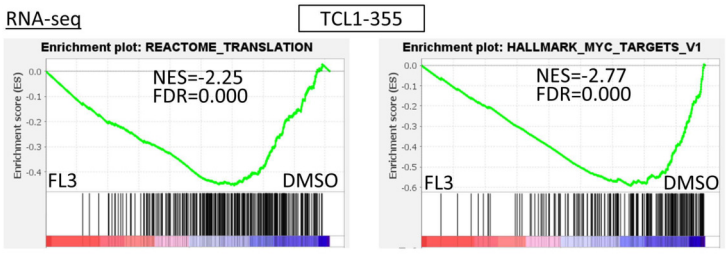


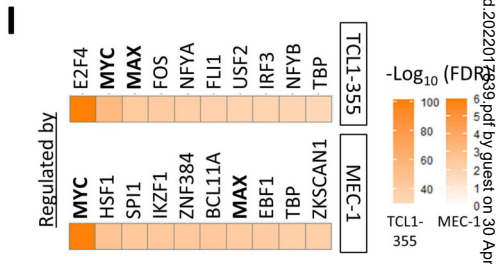


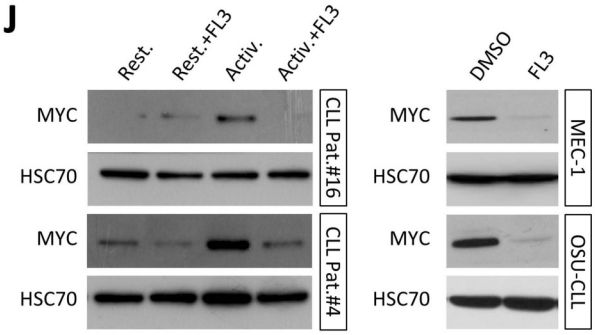


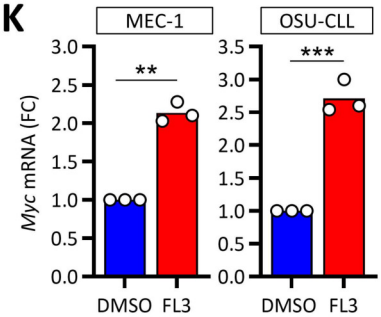


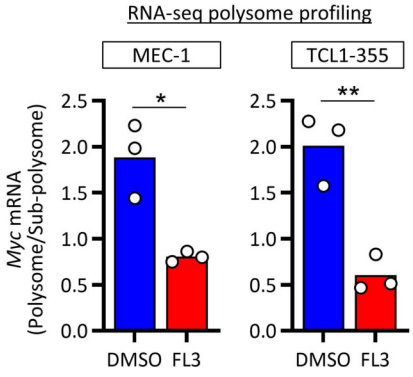












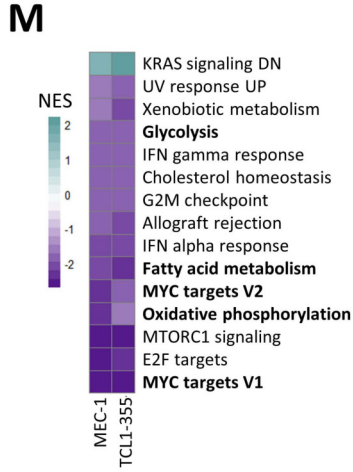
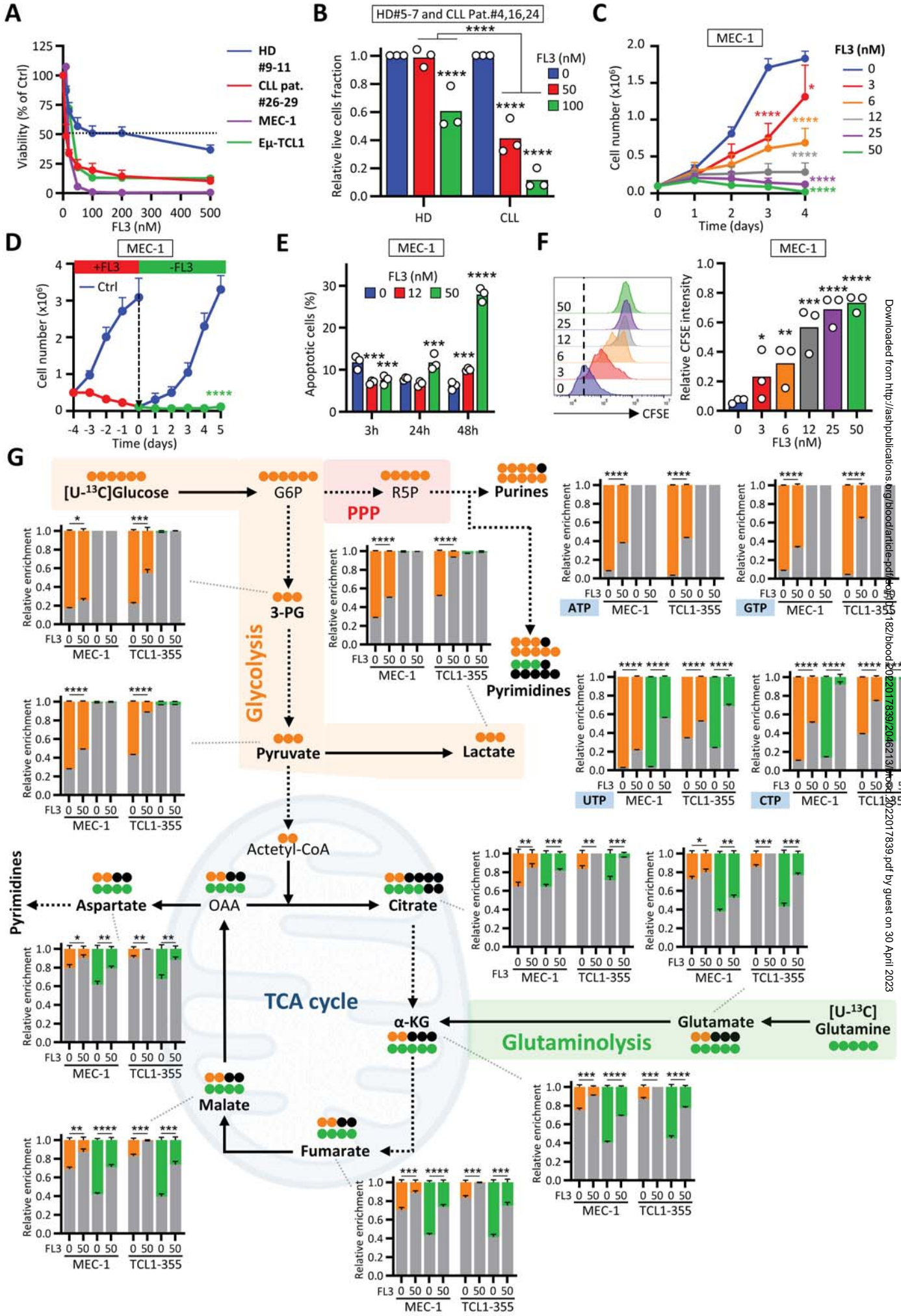


Figure 3



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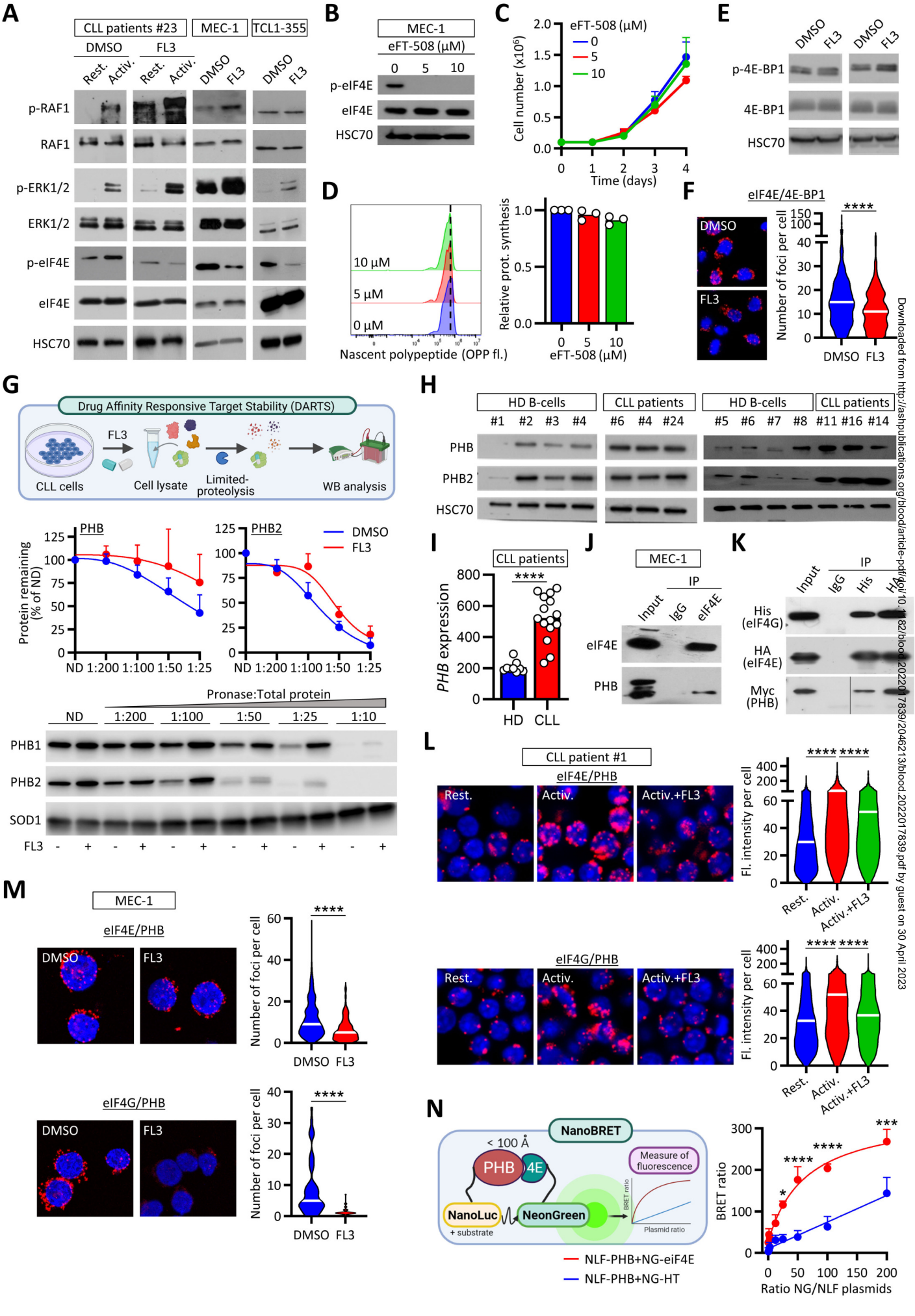

Figure 5

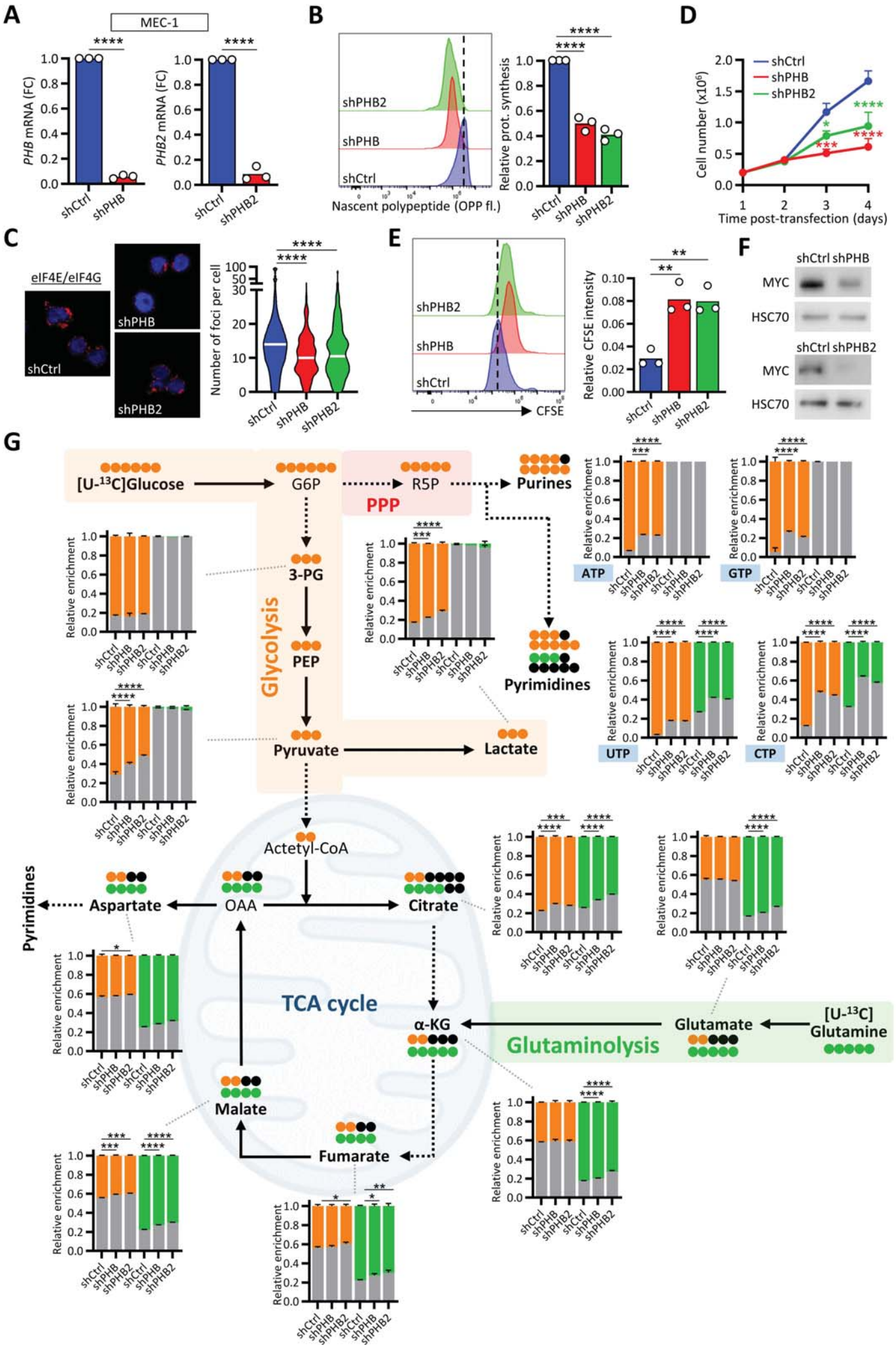

Figure 6

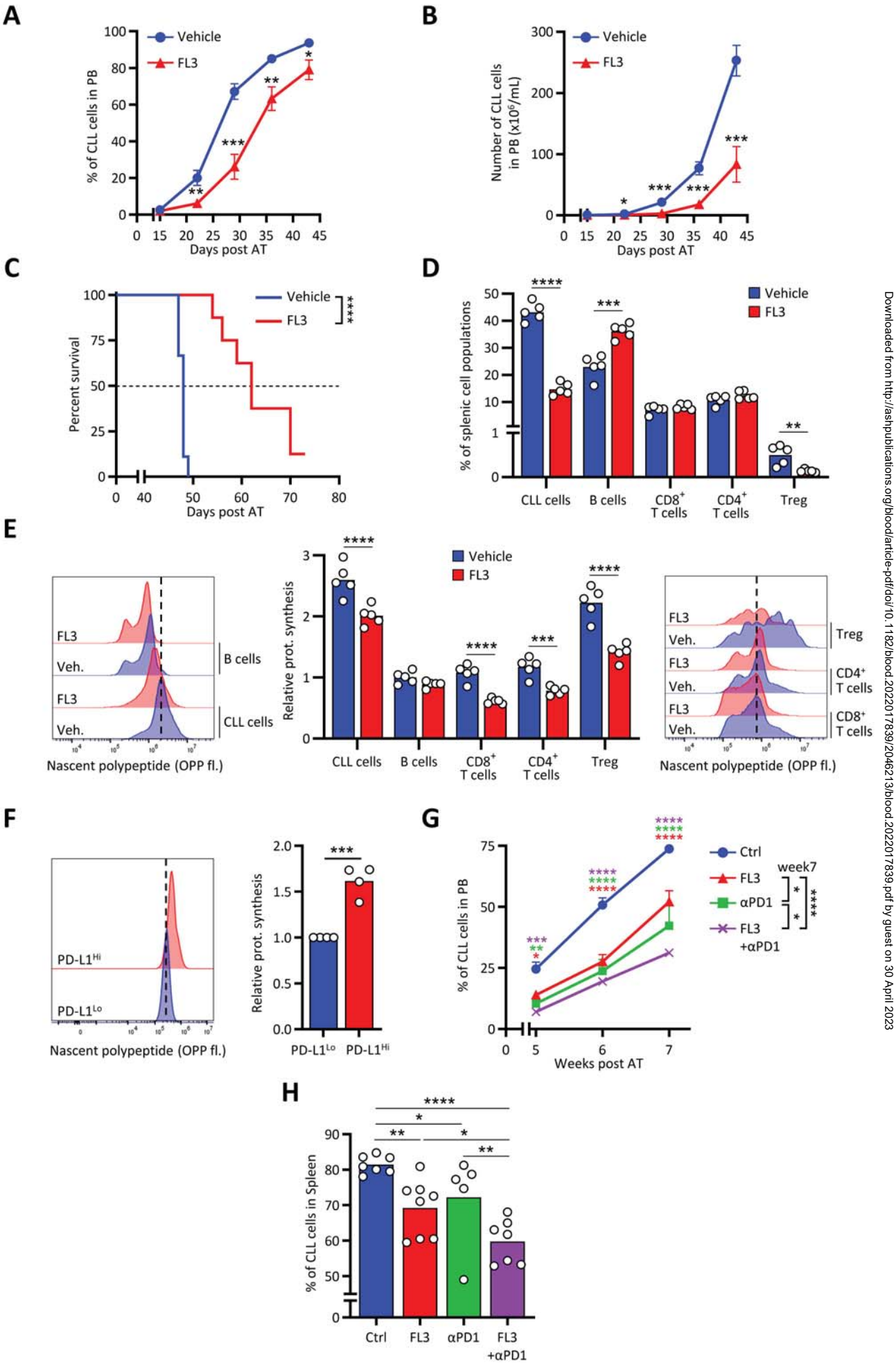

Figure 7