UBIQUITIN-LIKE POST-TRANSLATIONAL MODIFICATIONS MAY MODULATE THE PATHOGENESIS OF CHRONIC LYMPHOCYTIC LEUKEMIA MEDIATED BY RRAS2

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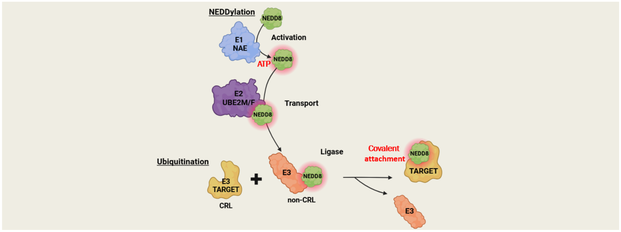
INTRODUCTION

Chronic lymphocytic leukemia (CLL) is the most common adult leukemia, marked by the accumulation of mature B cells. **Overexpression** of the ***RRAS2*** gene, present in **82%** of cases, is a key molecular driver and can induce CLL even without mutations.

Additionally, **increased** ubiquitin-like post-translational modifications (**UBL-PTMs**) and elevated translation of specific oncogenes play essential roles in maintaining the leukemic phenotype.

Furthermore, inhibition of **eIF4A1**, a key translation initiation factor, modulates the expression of oncogenes such as **RRAS2**, **MYC**, and **MCL1**. Treatment with the eIF4A1 inhibitor **Rohinitib (RHT)** led to reduced RRAS2 protein levels without significant changes in its mRNA, indicating translational repression.



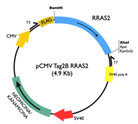
METHODS

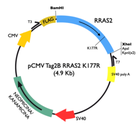
We have cloned the ORF of RRAS2 and generated **mutants** of the lysines aberrantly modified in CLL. Preliminary **transfection** experiments have been performed in HEK293T cells.

Transcription of the RRAS2 gene was also analyzed by RT-qPCR and luciferase assays. Then, MEC-1 cell line and ex-vivo experiments on B lymphocytes from patients are utilized to try to extrapolate the data.

Sensitivity of RRAS2 modifications to MLN4924 or MLN7243 (Fold variation with respect to untreated)

Protein Name	Site	MLN4924	TAK243	Peptide
RRAS2	177	-4.7	1.1	K*FQEQECPPSPEPTR
RRAS2	106	-1.5	2.0	GSFEEIYK*FQR





OBJECTIVE

Our objective is to shed light on the role of the aberrant UBL-PTM of RRAS2 found in B-CLL cells on the pathogenesis mediated by RRAS2, in search for **new therapeutic targets**.

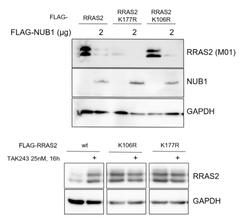
RESULTS

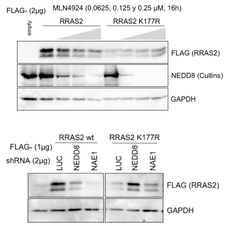
RRAS2 exhibit several **lysines** overmodified in **CLL**, being **K177** the most **sensitive** to **NEDDylation** inhibition with **Pevonedistat** (MLN4924), but not to the ubiquitin inhibitor TAK243. As Pevonedistat has shown therapeutic value in CLL, we wondered whether the NEDDylation at K177 could be enhancing the pathogenic role of RRAS2.

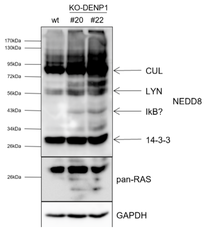
NEDDYLATION AT K177 MODULATES RRAS2 STABILITY AND REGULATES ITS UBIQUITIN-MEDIATED DEGRADATION IN HEK293T CELLS

We first analyzed the **stability** of **RRAS2** wild type or its K177R mutant when transfected into **HEK293T** cells. While **wt** RRAS2 protein accumulation **decreased** in response to the NEDDylation inhibitor Pevonedistat, **K177R** mutant was **unaffected** or even exhibited a slight **stabilization**. A similar response was observed when NEDDylation was impaired by genetic means, including **RNA interference** constructs against NEDD8 or the NEDD8 activation enzyme NAE1.

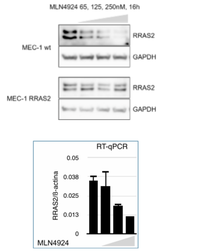
On the contrary, an **increase** in overall **NEDDylation** in HEK293T cell induced by deletion of the de-NEDDylation enzyme SENP8 induced the **accumulation** of **RRAS.** These data suggest that RRAS2 is both NEDDylated and ubiquitinated and that both modifications may compete for the same lysine targets. Moreover the NEDD8-degrader enzyme **NUB1** induced a **reduction** in **RRAS2** protein accumulation, while the ubiquitin activator enzyme (UBA) inhibitor **TAK243 stabilized** the **protein**.

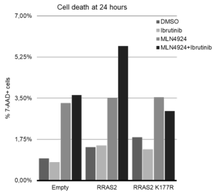


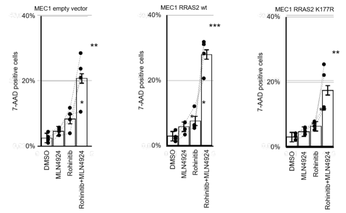


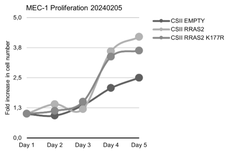


RRAS2 OVEREXPRESSION IN MEC-1 CELLS INCREASES SENSITIVITY TO COMBINATION THERAPIES; PEVONEDISTAT REDUCES RRAS2 LEVELS THROUGH TRANSCRIPTIONALREGULATION AND CHANGES ITS SUBCELLULAR LOCALIZATION









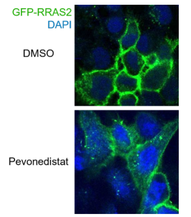
In the CLL cell line **MEC-1**, **RRAS2** protein **decrease** after **Pevonedistat** treatment came along with a down regulation of its mRNA, suggesting a possible additional **regulation** at the **transcriptional level**.

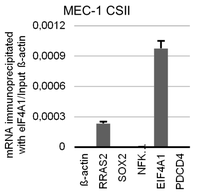
Functionally, our data demonstrated a **higher sensitivity** of **RRAS2-overexpressing MEC-1** cells to specific **combinational treatments** like **Ibrutinib + Pevonedistat** or **Rohinitib + Pevonedistat**, just as CLL cells do.

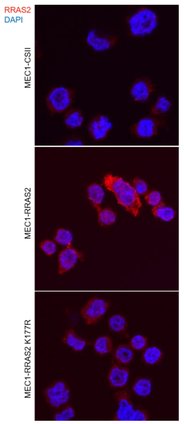
Interestingly, **K177R** mutation of RRAS2 **hindered** such **sensitivity**. **MEC-1-RRAS2** cells exhibit higher mRNA expression of BCL2 and EIF4A1 as well as an **increase** in general **RNA transcription**.

In addition, our preliminary data in HeLa cells show how Pevonedistat induces a **redistribution** of RRAS2 from the membrane to a more **cytoplasmic location**.

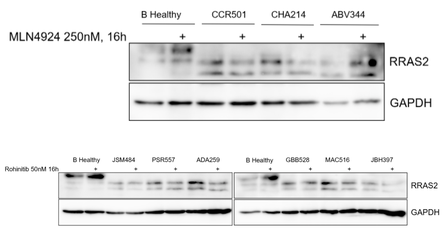
RRAS2 mRNA could be detected in **eIF4A1 pulldowns** from MEC-1 cells, suggestion of its translation.

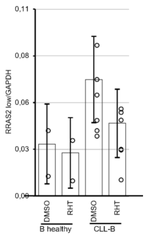


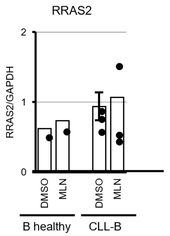


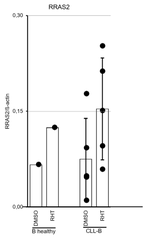


DIFFERENTIAL REGULATION OF RRAS2 PROTEIN AND mRNA LEVELS IN CLL PATIENT SAMPLES









To validate these findings in primary cells, we treated B lymphocytes from CLL **patients** and healthy donors with **Pevonedistat** or **Rohinitib**. While Pevonedistat led to variable effects on RRAS2 protein levels across CLL samples, **Rohinitib** consistently **reduced RRAS2 protein** expression without significantly altering mRNA levels.

This **discrepancy** between **mRNA** and **protein** supports a regulatory mechanism beyond **transcription**. Together, these data point to a complex, multi-layered regulation of RRAS2 in CLL involving NEDDylation, transcriptional control, and EIF4A1-mediated post-transcriptional mechanisms.

CONCLUSION

Our work suggests a regulatory role of **NEDDylation** over RRAS2 CLL pathogenesis at different upstream and downstream levels. However, there is a discrepancy between the different cellular models used.

In parallel, our findings support the role of **eIF4A1**-mediated translation in controlling RRAS2 expression, with **RHT** treatment reinforcing its potential as a modulator of oncogenic protein synthesis in CLL.

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