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PRINCIPAL INVESTIGATOR: David Shechter, PhD

CONTRACTING ORGANIZATION: Albert Einstein College of Medicine, Inc

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13. SUPPLEMENTARY NOTES

14. ABSTRACT: Mutations in Nucleophosmin (NPM1) are found in up to 35% of adult patients with acute myeloid leukemia (AML). NPM1 is a ubiquitously expressed pentameric histone chaperone primarily localized to the nucleolus. The most common mutation in NPM1 seen in AML patients is in the C-terminus (NPM1c), which leads to cyto-plasmic mislocalization of the protein. However, the mechanisms by which NPM1c transforms hematopoietic cells and confers leukemic self-renewal are poorly understood. An often-overlooked role of NPM1 is that of a histone chaperone. Histone chaperones function by preventing histone aggregation, facilitating their cytoplas-mic-nuclear transport, and deposition or removal from DNA. NPM1 binds core histones as well as linker his-tone (H1). Histones are a fundamental component of chromatin, which is the physiologic form of the eukaryotic genome. H1 dynamically associates with chromatin, promoting compaction into higher order structures and generally repressing gene expression. We showed that the enzyme TTLL4 post-translationally glutamylates NPM1 to alter its histone chaperone function. We hypothesize that aberrant glutamylation of the NPM1c mu-tant alters histone deposition at leukemogenic gene loci, thereby increasing expression and promotes leuke-mogenesis. In this project, we are testing how, when, and what TTLL4 and NPM1c do to promote leukemogen-esis and how TTLL4 may be an effective drug target.

15. SUBJECT TERMS

NONE LISTED

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1. INTRODUCTION

Mutations in Nucleophosmin (NPM1) are found in up to 35% of adult patients with acute myeloid leukemia (AML). NPM1 is a ubiquitously expressed pentameric histone chaperone primarily localized to the nucleolus. The most common mutation in NPM1 seen in AML patients is in the C-terminus (NPM1c), which leads to cytoplasmic mislocalization of the protein. However, the mechanisms by which NPM1c transforms hematopoietic cells and confers leukemic self-renewal are poorly understood. An often-overlooked role of NPM1 is that of a histone chaperone. Histone chaperones function by preventing histone aggregation, facilitating their cytoplasmic-nuclear transport, and deposition or removal from DNA. NPM1 binds core histones as well as linker histone (H1). Histones are a fundamental component of chromatin, which is the physiologic form of the eukaryotic genome. H1 dynamically associates with chromatin, promoting compaction into higher order structures and generally repressing gene expression. We showed that the enzyme TTLL4 post-translationally glutamylates NPM1 to alter its histone chaperone function. We hypothesize that aberrant glutamylation of the NPM1c mutant alters histone deposition at leukemogenic gene loci, thereby increasing expression and promotes leukemogenesis. In this project, we are testing how, when, and what TTLL4 and NPM1c do to promote leukemogenesis and how TTLL4 may be an effective drug target.

2. KEYWORDS

AML; glutamylation; post-translational modification; leukemia; gene expression; histone chaperone; Nucleophosmin

3. ACCOMPLISHMENTS

What were the major goals of the project?

The Statement of Work is Shown below with Accomplishment percentage

Specific Aim	Timeline	Site	Accomplished
Obtain HRPO Approval	Months 1-4	Einstein	100%
Obtain ACURO Approval	Months 2-3	Einstein	100%
Aim 1: Test the effect of the NPM1c mutation on both its glutamylation and its histone chaperone function			
Milestone(s) Achieved: 1.1 Test NPM1/NPM1c glutamylation in OCI-AML3 cells cells and identify sites of glutamylation (*Homo sapiens* Cell line Commercial Source: https://www.dsmz.de/collection/catalogue/details/culture/ACC-582)	Months 1-9	Dr. Shechter	100%

1.2 NPM1/NPM1c/and glutamylated isoform histone binding qualitative measurements, including competition binding experiments	6-9	Dr. Shechter	50%
1.2 NPM1 Quantitative Binding Analysis using Surface Plasmon Resonance or Isothermal Titration Calorimetry; partially dependent upon identification of glutamylation sites in Aim 1.1	9-15	Dr. Shechter	25%
1.3 Histone chaperone assays to test glutamylation function	12-18	Dr. Shechter	50%
Total Time to Completion of Aim 1 *AML cells, derived from OCI-AML3, were obtained from the Goodell Lab. OCI-AML3 were originally obtained from DSMZ (German repository) and will be validated.*	18		70%
Aim 2: Define how NPM1c glutamylation modulates its regulation of HOX genes			
2.1 Examine the role of NPM1c glutamylation on its cellular histone binding and localization (*Homo sapiens* Cell line Commercial Source: https://www.dsmz.de/collection/catalogue/details/culture/ACC-582)	12-18	Dr. Shechter	25%
2.2 Test the role of NPM1c glutamylation on leukemogenic self-renewal gene chromatin and expression Test the effects of NPM1c glutamylation on chromatin assembly in OCI-AML3 cells with CRISPR-Cas9 knockout of TTLL4 glutamyl-transferase (Flag-ChIP Seq and ChIP-qPCR for H1 isoforms and core histones) (*Homo sapiens* Cell line Commercial Source: https://www.dsmz.de/collection/catalogue/details/culture/ACC-582)	12-24 12-36	Dr. Shechter	25%
Time to Completion of Aim 2	36		25%
Aim 3: **Test the transformation potential conferred by TTLL4 and its glutamylation of NPM1c**			
Milestone(s) Achieved: 3.1 Test the role of NPM1c glutamylation on cellular transformation, self-renewal, and			50%

differentiation in OCI-AML3 cells with degron-NPM1c -qRT-PCR for HOXA/B genes and MEIS1 in TTLL4 CRISPR knockout OCI-AML3 cells -CCP5 overexpression studies in OCI-AML3 degron-NPM1c cells -generation of NPM1cEtoD glutamylation-incompetent variants (*Homo sapiens* Cell line Commercial Source: https://www.dsmz.de/collection/catalogue/details/culture/ACC-582)	1-6 3-4 5-8	Dr. Gritsman/Dr. Shechter Dr. Gritsman Dr. Gritsman Dr. Shechter	
3.2 Determine whether TTLL4 is required for leukemia-initiating activity of NPM1c mouse hematopoietic progenitors -breeding of NPM1^{frt-CA}/+;Flpo-ER mice (both alleles need to be heterozygous, so one generation of breeding is sufficient) -TTLL4 inducible CRISPR knockout in NPM1^{frt-CA}/+;Flpo-ER mouse hematopoietic progenitors and serial replating assays -leukemia-initiation and propagation experiments with NPM1^{frt-CA}/+;Flpo-ER progenitors transduced with TTLL4 shRNA Total number of mice to be used in Aim 2: 842 (424 mice from NPM1^{frt-CA}/+;Flpo-ER line, including breeders, culls, and experimental mice + 408 B6.SJL mice for transplantation experiments)	4-10 12-24 12-36	Dr. Gritsman Dr. Gritsman Dr. Gritsman	30%
3.3 Test the roles of NPM1c glutamylation in AML initiation and progression -proliferation and differentiation assays of primary AML patient samples and healthy donor bone marrow mononuclear cells with TTLL4 deletion	12-24	Dr. Gritsman	0%
-in vivo inducible TTLL4 CRISPR knockout in NPM1c AML patient-derived xenograft mice	18-36	Dr. Gritsman	0%

Total number of mice to be used in Aim 3: 66 NSG mice (2 different patient AML samples x 3 gRNAs each x 11 mice/condition = 66 mice total)			
Time to Completion of Aim 3	36		

What was accomplished under these goals?

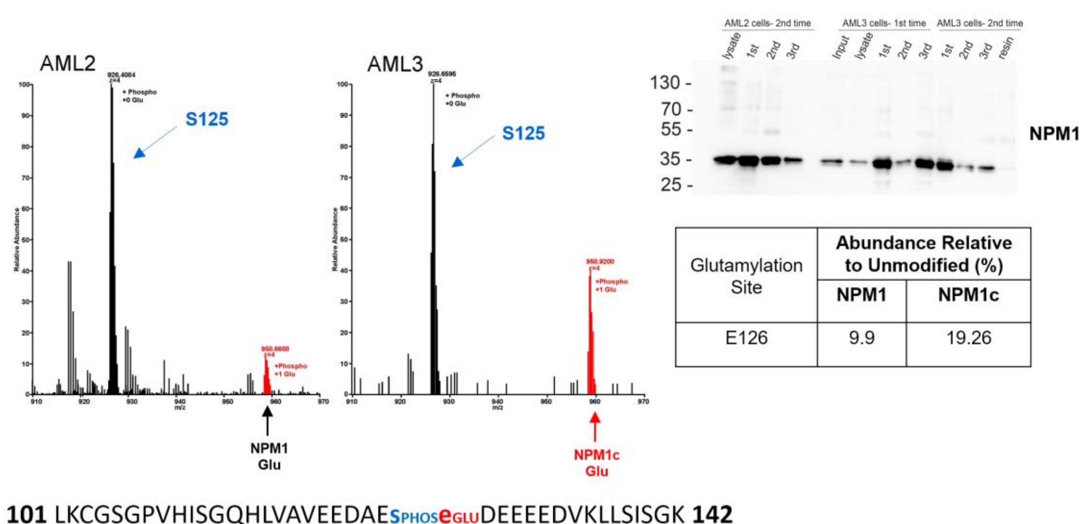
Aim 1: Test the effect of the NPM1c mutation on both its glutamylation and its histone chaperone function

Milestone(s) Achieved:

1.1 Test NPM1/NPM1c glutamylation in OCI-AML2 and AML3 cells and identify sites of glutamylation

To accomplish this goal, we pursued two sub goals. Last year we reported *in vitro* identification of glutamylation sites after we treated recombinant, highly purified wildtype NPM and mutant NPM1c with TTLL4 enzyme. We confirmed glutamylation by western blot and then subjected the recombinant protein to mass spectrometry analysis. We showed that mutant NPM1c was both a better substrate for glutamylation and also had new glutamylation sites (*not shown, in 2022 report*).

In this project year, we successfully purified endogenous NPM1 and NPM1c from OCI-AML2 and OCI-AML3 cells, respectively. Subjecting them to mass spectrometry analysis revealed constitutive phosphorylation on Ser125 in both cell lines. There was a single site of glutamylation, on the adjacent E126. This had double the abundance on the NPM1c mutant than on the wildtype protein, consistent with our *in vitro* results and with our hypothesis. Importantly, these sites (S125 and E126) lie directly in the A2 acidic stretch recently implicated in NPM1c leukemogenic and chromatin binding function (1). Altogether, our results in this subaim significantly advance the project.



Glutamylation Site	Abundance Relative to Unmodified (%)	
	NPM1	NPM1c
E126	9.9	19.26

Figure 1. NPM1 and NPM1c glutamylation sites. Purified NPM1 and NPM1c (upper right western blot) was subject to mass spectrometry analysis. The main sites of modification were on the peptide 101-142 (sequence at the bottom). S125 was phosphorylated in 95% of both AML2 and AML3 samples. E126 was glutamylated in ~10% of NPM1 protein and doubled to 20% in NPM1c.

1.2 NPM1/NPM1c/and glutamylated isoform histone binding qualitative measurements, including competition binding experiments

To accomplish this goal, we completed some experiments and others are in progress. First, we performed histone:DNA disaggregation assays; these experiments test relative affinity for histones. We demonstrated that NPM1c and glutamylated NPM1c both had increased affinity for histones and had greater disaggregation activity; this is consistent with our hypothesis (Figure 2). Competition binding studies are pending.

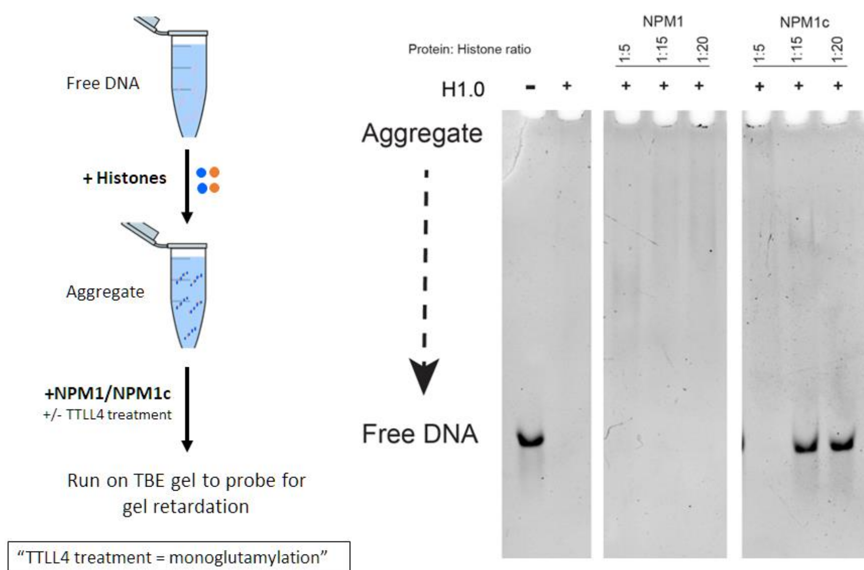


Figure 2. NPM1c has increased histone affinity. Histone:DNA disaggregation assay revealed that NPM1c (and glutamylated NPM1c, not shown) have increased histone affinity, consistent with our hypothesis.

We established new histone chaperone assays that we are now prepared to use with NPM1, +/- post-translational gutamylation. Our recent biochemistry preprint (2) on a related project on other histone chaperones showed that we can use these assays to probe consequences of glutamylation.

Importantly, prior to conducting these experiments, we set out to test the specific sites of modification through mutagenesis. Based on the PTM sites identified in Aim 1.1, we have prepared recombinant wildtype NPM1, NPM1E126A and E126D (phosphomimetic) mutants and the same corresponding mutations in the NPM1c protein. Purification of these proteins is currently ongoing. We will: 1) test them for TTLL4 activity, the mutants should not be modified; 2) test them, alongside wildtype protein +/- glutamylation, in the histone disaggregation and linker histone deposition and removal assays. These experiments will directly test the mechanistic role for glutamylation directly in histone chaperone assays. As the kinase for site of phosphorylation (S125) is unknown, analysis of its role is currently planned for future studies beyond the scope of this project.

1.3 Quantitative Binding Studies using Surface Plasmon Resonance or Isothermal Titration Calorimetry

We have prepared recombinant NPM1 and NPM1c tail domain, in vitro glutamylated them, and produced and purified recombinant ligand (linker and core histones). We have also performed quantitative binding assays with synthetic ligands with increasing glutamylation states (0, 1, 2, and 4) on a related NPM2 peptide (Figure 3**)** and preprint (2)**.** Technical challenges with the surface plasmon resonance setup and instrument have limited our ability to perform this experiment with NPM1 and NPM1c, so we will be approaching this with two qualitative approaches: 1) differential scanning fluorimetry/protein thermal shift analysis to determine increased stability of linker histones upon NPM1/NPM1c peptide or glutamylated peptide binding; 2) competition linker-histone pulldown experiments between NPM1, NPM1c, mutants, and glutamylated forms. Both of these experiments are exhibited with NPM2 and Nap1 chaperones in our recent preprint (2), showing confidence that we can perform them for this project.

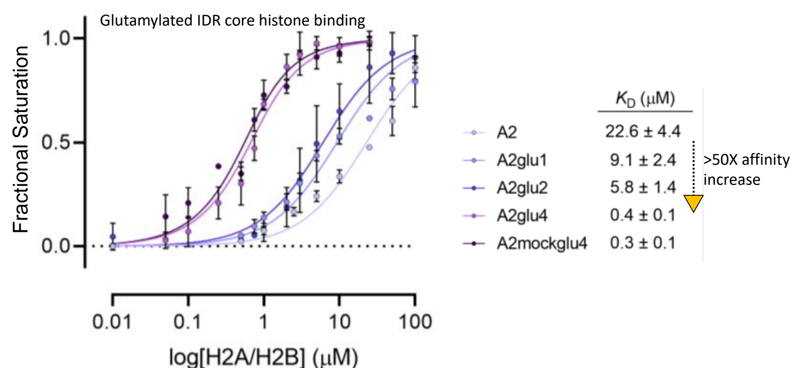


Figure 3. Glutamylation promotes stepwise increased histone affinity. Quantitative binding assay with NPM2 synthetic peptides with 0, 1, 2, or 4 post-translational glutamylations added.

Aim 2: Define how NPM1c glutamylation modulates its regulation of HOX genes

First, we initiated these Aim 2 and 3 goals by establishing a constitutive CRISPRi-knockdown for TTLL4 in both the AML3 NPM1c-degron cells and in OCI-AML2 (NPM1 wildtype) cells. This successfully led to loss of NPM1c glutamylation, but not protein (Figure 4a-c). To improve temporal resolution of the loss of glutamylation, we spent significant effort working towards an inducible CRISPRi-mediated knockdown of the TTLL4 glutamyl-transferase in the NPM1c-degron OCI-AML3 cells. Unfortunately, multiple independent approaches were unsuccessful, so we are returning to the constitutive CRISPRi knockdown for our cell-line studies.

We also recently prepared NPM1c E126D (charged and non-glutamylatable) and E126A (uncharged), and S125D (phosphomimic) and S125A (non-phosphorylatable) mutant overexpression constructions. We will use these constructs to rescue OCI-AML3-NPM1cdegron cell dTAG (degrader) treatment and protein destruction of NPM1c.

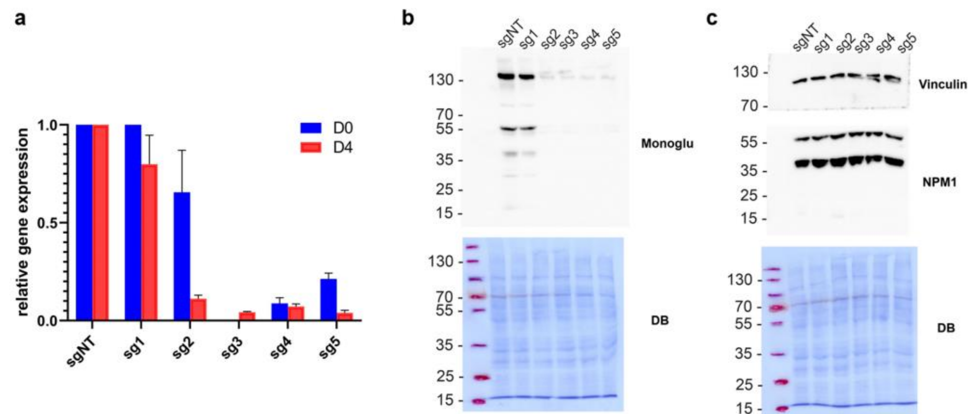


Figure 4. CRISPRi knockdown of TTLL4 in OCI-AML3 cells. A. 5 guide RNAs and a non-targeting (NT) guide were used, 4 out of 5 guides had robust loss of TTLL4 gene expression. **B.** guides 2-5 all caused loss of monoglutamylation; DB = direct blue total membrane stain. **C.** Loading Control blots for b.

2.1 Examine the role of NPM1c glutamylation on its cellular histone binding and localization

Using the approaches outlined above, we have started establishing immunofluorescence experiments for NPM1 and NPM1c subcellular localization. A few technical challenges with mounting the suspension cells on coverslips for IF, but these have recently been overcome. We will perform the proposed experiments in the early part of year 3.

2.2 Test the role of NPM1c glutamylation on leukemogenic self-renewal gene chromatin and expression

We successfully piloted ChIP-qPCR for H3K4me3 at HOXA loci (not shown). Otherwise, *nothing to report*

Aim 3: Test the role of NPM1c glutamylation on cellular transformation, self-renewal, and differentiation in OCI-AML3 cells

3.1 Test the role of NPM1c glutamylation on cellular transformation, self-renewal, and differentiation in OCI-AML3 cells

To establish a system usable both for cultured AML2 and AML3 cells and ultimately for primary cells, we setup CRISPR knockout studies. Due to difficulties with lentiviral expression of Cas9 in OCI-AML3 degron cells, we instead used OCI-AML3 cells with constitutive Cas9 expression, which we transduced with a lentivirus expressing sgRNAs targeting TTLL4 or a non-targeting sgRNA with an RFP tag. The transduced cells were sorted for RFP and then tested in proliferation, apoptosis, and differentiation assays. Both Cell Titer Glo proliferation assays and single cell colony assays revealed a significant decrease in proliferation by OCI-AML3 cells after TTLL4 editing, with an increase in both apoptosis and myeloid differentiation (see progress report from

Year 1). We confirmed editing of TTLL4 by multiple sgRNAs using amplicon sequencing and confirmed that editing of TTLL4 leads to decreased mono-glutamylation of NPM1c by Western analysis.

Before we had completed the mass spectrometry identifying only a single site of glutamylation, we generated an NPM1c EtoD glutamylation-incompetent variant by mutating many Glu sites on NPM1c to Asp. Using lentiviral transduction, we overexpressed either NPM1c or the NPM1c EtoD mutant construct in OCI-AML3 degron cells. After antibiotic selection for transduced cells, we treated the cells with dTag to degrade the endogenous NPM1c, which has an $FKBP12^{F36V}$ degron tag. We did not observe any differences in proliferation between overexpression of the EtoD mutant and overexpression of unaltered NPM1c. However, this experiment had some technical limitations, as we did not observe complete degradation of the endogenous NPM1c with addition of dTag (Figure 5), which makes the data difficult to interpret. In addition, we did not observe the expected decrease in NPM1c glutamylation in cells transduced with the EtoD mutant. This suggests that the wrong glutamylation sites on NPM1c may have been mutated in this case. We have now repeated this experiment two more times with similar results. Therefore, we have moved forward with studies targeting just the E126 site, as elaborated on in Aim 2. We will also probe potential crosstalk between S125ph and E126glu.

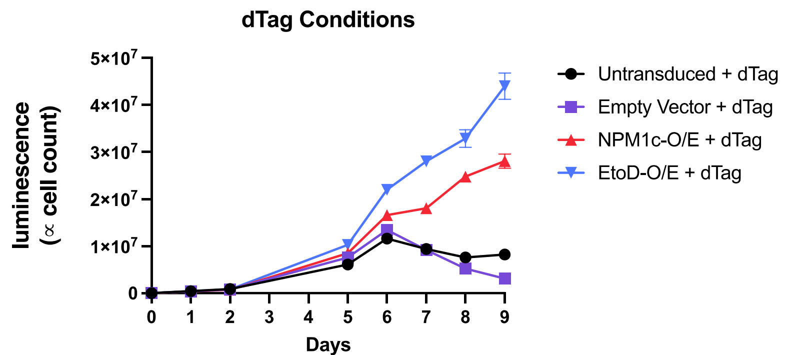


Figure 5. OCI-AML3-degron knockdown/rescue with NPM1c-E-to-D mutants. OCI-AML3-NPM1c-degron cells were treated with dTAG-13 to remove NPM1c protein. Then the cells were transfected with plasmids expressing NPM1c-wildtype or NPM1c-E-to-D glutamylation site mutants. Note that our control experiments (not shown) did not show loss of glutamylation, so we are repeating this work with new constructs specifically mutated at NPM1cE126D.

3.2 Determine whether TTLL4 is required for leukemia-initiating activity of NPM1c mouse hematopoietic progenitors

We have completed breeding of $NPM1^{frt-CA}$/+;Flpo-ER mice, and are currently optimizing approaches for CRISPR editing of TTLL4 in $NPM1^{frt-CA}$/+;Flpo-ER hematopoietic progenitors for colony assays and transplantation (see future plans for details).

We are also performing CRISPR editing of TTLL4 in WT mouse hematopoietic progenitors *ex vivo* by electroporation with recombinant Cas9 and TTLL4 sgRNAs and non-targeting sgRNAs, followed by colony assays to examine effects on clonogenic activity, differentiation, and self-renewal. In our preliminary experiment, we observed no difference in colony formation between TTLL4 KO and control hematopoietic progenitors, as expected (Figure 6).

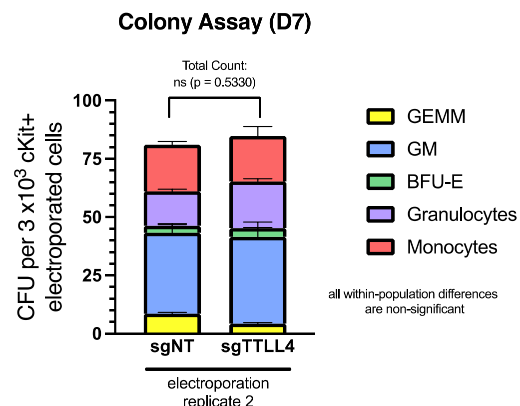


Figure 6. HSPC TTLL4 CRISPR knockout showed no difference in hematopoietic progenitors. Mouse HSPC cells treated with TTLL4 gRNA and Cas9 by electroporation were subject to colony assays. Legend indicates colony abundance of each population, no differences observed.

What opportunities for training and professional development has the project provided?

Nothing to report

How were the results disseminated to communities of interest?

Nothing to report

What do you plan to do during the next reporting period to accomplish the goals?

Aim 1

We will finish the proposed biochemistry experiments, including NPM1 glutamylation binding and histone chaperone assays.

Aim 2

We are also currently piloting ChIP approaches in the OCI-AML3 cells. We plan to perform genome wide chromatin studies upon TTLL4 knockdown in year 3.

Similarly, we prepared an inducible CCP5 (deglutamylase) overexpression construct; we expect this inducible expression to phenocopy the TTLL4 loss.

Finally, based on our mass spectrometry in OCI-AML3 (NPM1-mutated) and OCI-AML2 (NPM1-WT) cells, we designed and produced a more accurate EtoD mutant version of NPM1c, with a point mutation in residue E126A and E126D (see Aim 2 progress). We will then perform degradation of endogenous NPM1c and replace with NPM1c or NPM1c-EtoD mutant expression (see Figure 5). These non-glutamylatable mutant NPM1c constructs will directly test the role of glutamylation. Alternatively, we will use CRISPR HDR editing to mutate the NPM1c genomic sites (S125 and E126).

Aim 3

We have generated the $NPM1^{frt\text{-}CA}$/+;Flpo-ER;Cas9-lox-STOP-lox/+;Mx1-Cre mouse strain, and it has been approved by IACUC at Albert Einstein College of Medicine and ACCURO. The mice will be treated with tamoxifen to activate NPM1c and with polyI-polyC to induce Mx1-Cre. We will then perform electroporation hematopoietic progenitors from $NPM1^{frt\text{-}CA}$/+;Flpo-ER;Cas9-lox-STOP-lox;Mx1-Cre with TTLL4 sgRNAs or a non-targeting sgRNA to delete TTLL4. These edited hematopoietic progenitors will then be transplanted into sub-lethally irradiated B6.SJL mice. We will use this experimental system to assess the requirement for TTLL4 in NPM1c leukemia development.

We are also performing CRISPR editing of TTLL4 in $NPM1^{frt\text{-}CA}$/+;Flpo-ER and WT mouse hematopoietic progenitors *ex vivo* by electroporation with recombinant Cas9 and TTLL4 sgRNAs and non-targeting sgRNAs, followed by colony assays to examine effects on clonogenic activity, differentiation, and self-renewal.

We are also in the process of performing CRISPR editing of TTLL4 using the same Cas9 and TTLL4 sgRNA electroporation approach in human NPM1c AML patient samples and in healthy donor CD34 cells for colony assays and for xenotransplantation of edited AML patient samples into NSG mice.

How were the results disseminated to communities of interest?

Nothing to report

4. IMPACT

What was the impact on the development of the principal discipline of the project

Nothing to report

What was the impact on other disciplines?

Nothing to report

What was the impact on technology transfer?

Nothing to report

What was the impact on society beyond science and technology

Nothing to report

5. CHANGES/PROBLEMS

What was the impact on the development of the principal discipline of the project

Nothing to report

6. PRODUCTS

Publications, websites, technologies

Nothing to report

Patent applications

We filed a *provisional* patent on preparing small molecule inhibitors of the TTLL4 enzyme. The internal claim number is AET-03260.

Other products

Nothing to report

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Name:	*David Shechter, PhD*
Project Role:	*PD/PI*

ORCID:	0000-0001-9388-6004
Person Months worked:	1.2 months
Contribution to Project:	Dr. Shechter is a project supervisor. He provided project guidance and data analysis.
Funding Support:	Current other support from NIH/NIGMS (R01GM135614 and R01GM108646), Irma T. Hirschl Fund

Name:	*Kira Gritsman, MD, PhD*
Project Role:	*Co-Investigator*
ORCID:	0000-0002-1367-1167
Person Months worked:	0.6 months
Contribution to Project:	Supervisor, provides project guidance and data analysis
Funding Support:	Current other support from NIH/NIDDK (R01DK056638-22, R01DK130895), American Cancer Society (134154-RSG-19-130-01-DDC), iOnctura

Name:	*Humaira Ilyas, PhD*
Project Role:	*Postdoctoral Associate*
ORCID:	
Person Months worked:	9.6 months
Contribution to Project:	Dr. Ilyas performed experiments and data analysis for Aims 1 and 2
Funding Support:	Current other funding from NIH/NIGMS (R01GM135614)

Name:	*Alexandra Schurer*
Project Role:	*MSTP Student*
ORCID:	

Person Months worked:	0 - Ms. Schurer is currently supported by other funding sources (American Cancer Society, #134154-RSG-19-130-01-DDC)
Contribution to Project:	Ms. Schurer performed all experiments for Aim 3 and some for Aim 2
Funding Support:	American Cancer Society, #134154-RSG-19-130-01-DDC

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

Yes. Dr. Kira Gritsman has two new grants from NIH/NIDDK (R01DK130895 and R01DK056638-22) and new research funding from iOnctura. This does not affect her effort on the current award.

What other organizations were involved as partners?

Organization name: University of Virginia, Chemistry Department (Donald Hunt's lab)

Location of organization: Charlottesville, VA, USA

In-kind support: to identify sites of post-translational glutamylation, partner performed mass spectrometry analysis of NPM1 and NPM1c

8. SPECIAL REPORTING REQUIREMENTS

Nothing to report

9. APPENDICIES

Nothing to report

References

1. Uckelmann, H. J., Haarer, E. L., Takeda, R., Wong, E. M., Hatton, C., Marinaccio, C. *et al.* (2023) Mutant NPM1 Directly Regulates Oncogenic Transcription in Acute Myeloid Leukemia Cancer discovery **13**, 746-765 10.1158/2159-8290.Cd-22-0366
2. Lorton, B. M., Warren, C., Ilyas, H., Nandigrami, P., Hegde, S., Cahill, S. *et al.* (2023) Glutamylation of Npm2 and Nap1 acidic disordered regions increases DNA charge mimicry to enhance chaperone efficiency bioRxiv 2023.2009.2018.558337 10.1101/2023.09.18.558337