# Investigating PEAR1's role in Acute Myeloid Leukemia

By

Thomas J Fernandez

A THESIS

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# Significance of Platelet Endothelial Aggregation Receptor 1 (PEAR1) Mutations in Acute Myeloid Leukemia

## Pathobiology of Acute Myeloid Leukemia

Acute myeloid leukemia (AML) is a malignancy of the stem cell precursors of the myeloid lineage, it starts with genetic variation that has the ability to lead to neoplastic growth as well as clonal proliferation.[1] AML's aggressive and heterogeneous disease state make it arduous to set a reliable clinical course and treatment response.[2]

Further, most AML cases begin with a *de novo* malignancy in otherwise healthy adults.[3] Using objective measurements found in patients during initial diagnosis of AML has been beneficial for prognostication, such as measurements of gene mutation and chromosomal rearrangements, as significant determinants of response and disease outcomes.[5]

Understanding the oncogenic capacity of mutations became easier once reliable animal models became available, with the field eventually developing a two-hit model of leukemogenesis.[3] The model divides somatic mutations in AML into two groups. A class I mutation, activates pro-proliferative pathways, and has to be in co-expressed with a class II mutation, which impacts normal hemopoietic differentiation.[5,6] Common class I mutations in patients occur in genes, such as FLT3, and there are two types of FLT3 mutations - tyrosine kinase domains (TKD), and internal tandem duplications (ITD).[3] Internal tandem duplication mutations of the FLT3 gene (FLT3-ITD mutations) are the most frequent molecular abnormality in AML and are associated with worse overall survival.[5,9] Some common class II mutations like

NPM1 and less seen, CEBPA are more often associated with better patient outcomes.[7] More recently epigenetics regulation can be considered a third class of mutations occurring in DNA-methylation related genes like TET2, IDH1 and IDH2.[4][7]

Metabolic reprogramming, like the mutations found in IDH1 and IDH2 gene, are fundamental traits of cancer. [37] This alteration in metabolism allows rapid proliferation and survival and is characterized by the dysfunction of isocitrate dehydrogenase (IDH1/2) and other metabolic enzymes.[53] Mutations of these genes has been seen in many human cancers including AML.[54] When IDH1/2 genes are mutated, these metabolic enzymes instead of α-ketoglutarate (α-KG), make 2-hydroxyglutarate (2HG) [37-39]. The build-up of this oncogenic metabolite acts as an α-KG antagonist to competitively inhibit α-KG-dependent dioxygenases. This disrupts normal biology of histones, DNA demethylases, resulting in DNA and histone hypermethylation, which is a cause for the altered cell differentiation and eventual tumorigenesis.[48]

Clinical presentation of AML reflects the accumulation of these malignant, not fully differentiated myeloid cells that collect in the bone marrow as well as other organs. If left untreated, death ensues soon after diagnosis. That is why irrespective of the type of AML, the goal of treating AML is to induce complete remission (CR) within first therapy, consolidation or maintenance therapy to follow.[2] Further understanding the type of AML may allow for the development of targeted small molecule inhibitors to further improve patient outcomes.

## AML Classification Systems

The French-American-British classification system for AML was developed in 1976 and defined eight subtypes of AML (M0 through M7) based on morphological characteristics of the leukemic cells within the patient. In an effort to integrate advancements in

diagnosis of AML, World Health Organization (WHO) introduced a new system for 2008.[13] This system included genetic information of mutations and chromosomal rearrangements, as well as clinical presentation such as: therapy related AML, AML with myelodysplasia-related features; myeloid sarcoma.[14] The WHO system has been updated twice since 2008, once 2016 and most recently in 2022.[14]

Eligible patients undergo induction therapy in hopes of achieving complete remission (CR). After this treatment, it is common to have measurable residual disease (MRD) persisting in CR, and if treatment is discontinued relapse will inevitably occur. Therefore, after induction therapy, consolidation therapies are commonly employed in order to eradicate residual disease and achieve lasting remission.[3]

## Small-Molecule Inhibitor Therapies

Further improvements to AML patient outcomes could be achieved through the use of small-molecule inhibitors that target specifically dysregulated genes and pathways found in AML. One early example of this strategy has been targeting of the gain-of-function FLT3-ITD mutation with small molecule FLT3 kinase inhibitors.

Midostaurin is an example of the first generation of small molecule inhibitors for FLT3 AML. Approved by the FDA in 2017, midostaurin is a tyrosine kinase inhibitor (TKI) that can target several receptor and non-receptor tyrosine kinases, including WT FLT3, FLT3-ITD and FLT3-TKD. [8,33] By inhibition of FLT3, midostaurin disrupts critical signaling pathways involved in cell proliferation and survival, leading to increased apoptosis of malignant cells. It is primarily used in the treatment of FLT3 mutation-positive AML and has demonstrated efficacy in combination with standard chemotherapy as well as in the maintenance therapy setting.[33]

Clinical studies have shown that midostaurin improves overall survival in patients with FLT3-mutant AML compared to standard therapy alone.[33]

Quizartinib is a second-generation inhibitor, still for the FLT3-AML, but with highest affinity for the inactive form of the FLT3 receptor [41]. The higher specificity of this TKI helps reduce off target effects which enhances therapeutic efficacy against mutated leukemia cells.[41] This is different from first generation TKI's which were designed to bind both in the on and off conformation of the FLT3 receptor, and thus had less specificity and more toxicity.[43] Quizartinib is considered to be a salvage treatment for AML, patients that have tried other small molecule inhibitor therapies and have not achieved remission. Furthermore, Quizartinib along with other small molecule inhibitors approved for FLT3-TKD/ITD mutated AML, are being explored in combination regimens.[43]

Venetoclax is a targeted small molecule inhibitor, used primarily in the treatment of AML and chronic lymphocytic leukemia (CLL). It's mechanism of action is through inhibition of BCL-2. BCL-2 is an anti-apoptotic protein that helps cells avoid undergoing apoptosis, allowing them to survive and proliferate. By inhibiting BCL-2, venetoclax can cause apoptosis in cancer cells. This mechanism of action makes venetoclax particularly effective in cancers with elevated levels of BCL-2.

Ivosidenib is an oral small-molecule inhibitor that selectively targets mutant isocitrate dehydrogenase 1 (IDH1), an enzyme involved in the Krebs cycle. Mutations in IDH1 lead to the production of 2-hydroxyglutarate (2-HG), an oncometabolite that contributes to leukemogenesis by dysregulating DNA methylation machinery and, thereby, antagonizing normal cellular differentiation.[34] By inhibiting mutant IDH1, ivosidenib reduces the levels of 2-HG, promoting

the differentiation of myeloid cells and inducing anti-tumor effects. It is primarily prescribed for adults with relapsed or refractory (R/RAML) with an IDH1 mutation.[34]

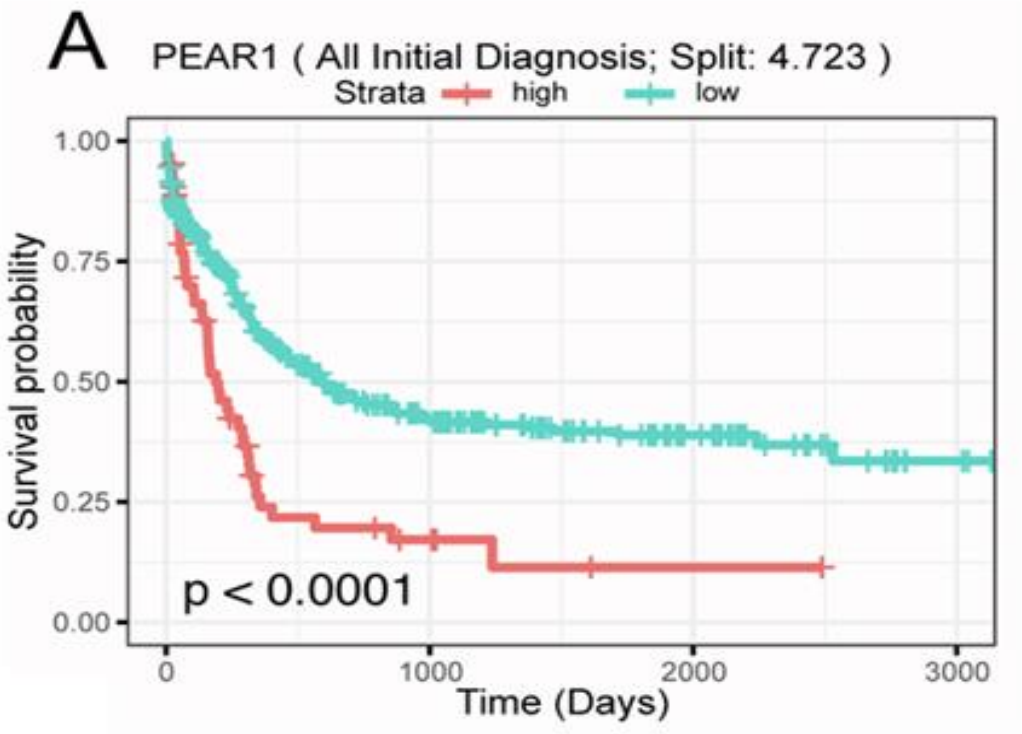
Enasidenib, an inhibitor of mutant IDH2 proteins, induces durable remissions in older patients with newly diagnosed AML.[36] IDH2 mutations in AML cases occur mainly at the conserved arginine residues R140 and R172 inside the enzyme active sites.[37] Enasidenib has been shown to allosterically inhibit IDH2, suppress 2-HG production, and induce cellular differentiation in human IDH2-mutant AML cells.[40] Both mutations are characterized by neomorphic enzymatic activity, R140 and R172 IDH2 mutations are distinct with respect to clinical outcome and molecular classification.[46] In preclinical studies ensidenib showed it could reduce serum (2HG), DNA hypermethylation, and promoted hematopoietic differentiation in R140 and R172 mIDH2 models.[47] In a clinical trial, enasidenib was able to show clinical activity in patients with both R140 and R172 mIDH2 R/RAML.[45]

The literature has shown how mutant IDH 1/2 have a unique epigenetic signature that it could become its own subcategory of AML. [47,48] Part of this signature involves mutations in IDH1/2 and TET2 being mutually exclusive in AML.[48] TET2 is a protein, part of a family of epigenetic regulators. The TET family contains three similar genes encoding DNA dioxygenase: TET1, TET2, and TET3.[49] The TET family mediate the first step in the demethylation process, catalyzing 5-methylcytosine (5mC) into 5-hydroxymethylcytosine (5hmC), 5-formylcytosine (5fC), and 5-carboxycytosine (5caC). The thymine DNA glycosylase (TDG) catalyzes the excision of 5fC and 5caC to create an (AP site), apyrimidinic site. The study also found that mutations in IDH1 and IDH2 were significantly enriched in AML patients with intermediate-risk cytogenetics. These mutations in TET2 and IDH1/2 have been shown to be a loss-of-function

mutation contributing to leukemogenesis through a shared mechanism that disrupts normal DNA demethylation.[48]

## Beat AML

To better understand the mechanisms of action for AML drug response along with clinical outcomes, Oregon Health & Science University (OHSU) started the Beat AML cohort. This cohort consists of 805 AML patients, with research samples from these patients being procured over a span of ten years. These samples were assayed with drug sensitivity testing, clinical annotations, as well as RNA and DNA sequencing. [16] In one aspect of this study, a machine learning approach was used to model factors from the dataset that most significantly impacted on patients' clinical outcomes. This model found that a single gene, PEAR1, was the strongest predictors of patient survival across all age groups. [16]



(Figure 1: Bottomly, D. *et al*. Integrative analysis of drug response and clinical outcome in acute myeloid leukemia. *Cancer Cell* **40**, 850-864.e9 (2022). Originally published in 2022 and currently published by Elsevier (2025), available here unedited through creative commons Attribution 4.0 deed. This graph is depicting how one-gene, PEAR1 heavily stratifies the data for worse survival outcomes in patients)

## Biology of PEAR1

The gene for PEAR1, also named JEDI, was found through gene profiling for a novel receptor in platelets. This was accomplished using RNA from a sample using double-dose plateletpheresis (DDP), a method to retrieve platelets. [18-19] PEAR1 is a single-pass transmembrane receptor, having multiple EGF-like repeats within the extracellular domain. [18-21]


(Figure 2: Nanda, N. *et al.* Platelet Endothelial Aggregation Receptor 1 (PEAR1), a Novel Epidermal Growth Factor Repeat-containing Transmembrane Receptor, Participates in Platelet Contact-induced Activation. *Journal of Biological Chemistry* **280**, 24680–24689 (2005). Originally published in 2005 and currently published by Elsevier (2025), available here unedited through creative commons Attribution 4.0 deed)

Along with hematopoietic stem cells, platelets, megakaryocytes, and endothelial cells can also express PEAR1. [18-21] Dimerized PEAR1 along with other signaling molecules, constitute a signaling complex where platelet integrin αIIbβ3 to be active via a serine/threonine protein kinase (Akt) phosphorylation. [20,23] In addition to platelet aggregation, a role for PEAR1 in regulation of hematopoiesis has also been proposed.[19] Studies have shown the continual upregulation of PEAR1 during human megakaryopoiesis (MK) is a negative regulator of MK progenitor proliferation, but not maturation.[21] Additionally, the knockdown of PEAR1 gene *in vivo* affected the transcriptional regulation of several genes, including PTEN.[21] PTEN is a tumor suppressing gene, and has been observed in disease states like leukemia. [21]

Recently, PEAR1's endogenous ligand was found to be SVEP1.[20] This protein can be found in human plasma, but usually it is thought to be mainly within the (ECM) extra-cellular

matrix of tissues where it is known to be produced, like other ECM proteins.[28] Consistent with PEAR1 activation, both PEAR1 and SVEP1 induced Akt phosphorylation in platelets, as well as platelet aggregation.[20] SVEP1 has also been identified with additional associations of human diseases like pulmonary artery hypertension.[29] Additional studies are needed to fully elucidate the mechanism of PEAR1 and SVEP1 within the pathophysiology of human diseases however.[20]

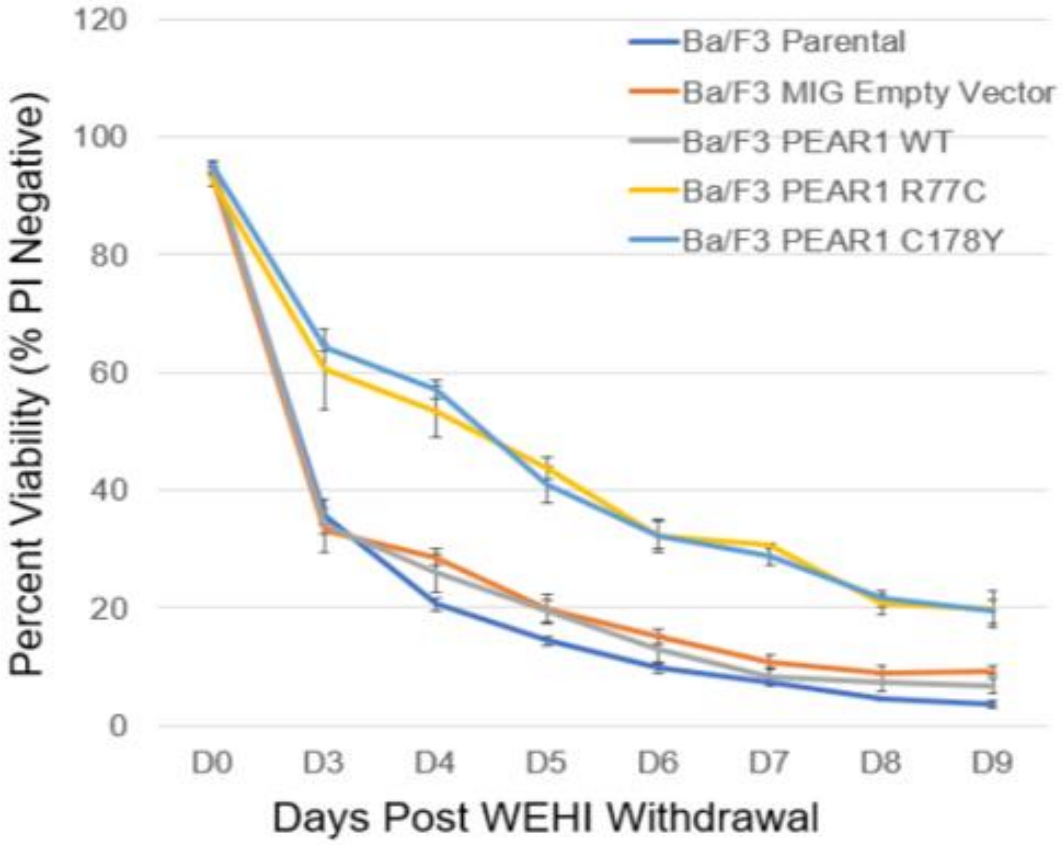
## Mutations in PEAR1

We found two different PEAR1 mutations in AML patients in 805 Beat AML cohort. These mutations were S155L, C178Y, with a third PEAR1 mutation being R77C from The Cancer Genome Atlas (TCGA). All three of these mutations were found in exons that coded for regions in the extracellular portion receptor.

To characterize the phenotype of these mutations, I first virally infected constructs containing WT PEAR1 or each of the mutated versions of PEAR1 into a murine pro B-cell lymphocyte cell line (Ba/F3). Ba/F3 cells rely on Interleukien-3 (IL-3) to survive and proliferate, however, introduction of active signaling oncogenes into Ba/F3 cells can render them independent of the need for IL-3. Hence, I wanted to determine whether PEAR1 mutations have the ability to oncogenically transform Ba/F3 cells to IL-3 independent growth. Initial testing revealed that these PEAR1 mutations conferred a survival advantage compared with parental Ba/F3 cells or those expressing empty vector or PEAR1 WT (Figure 3).

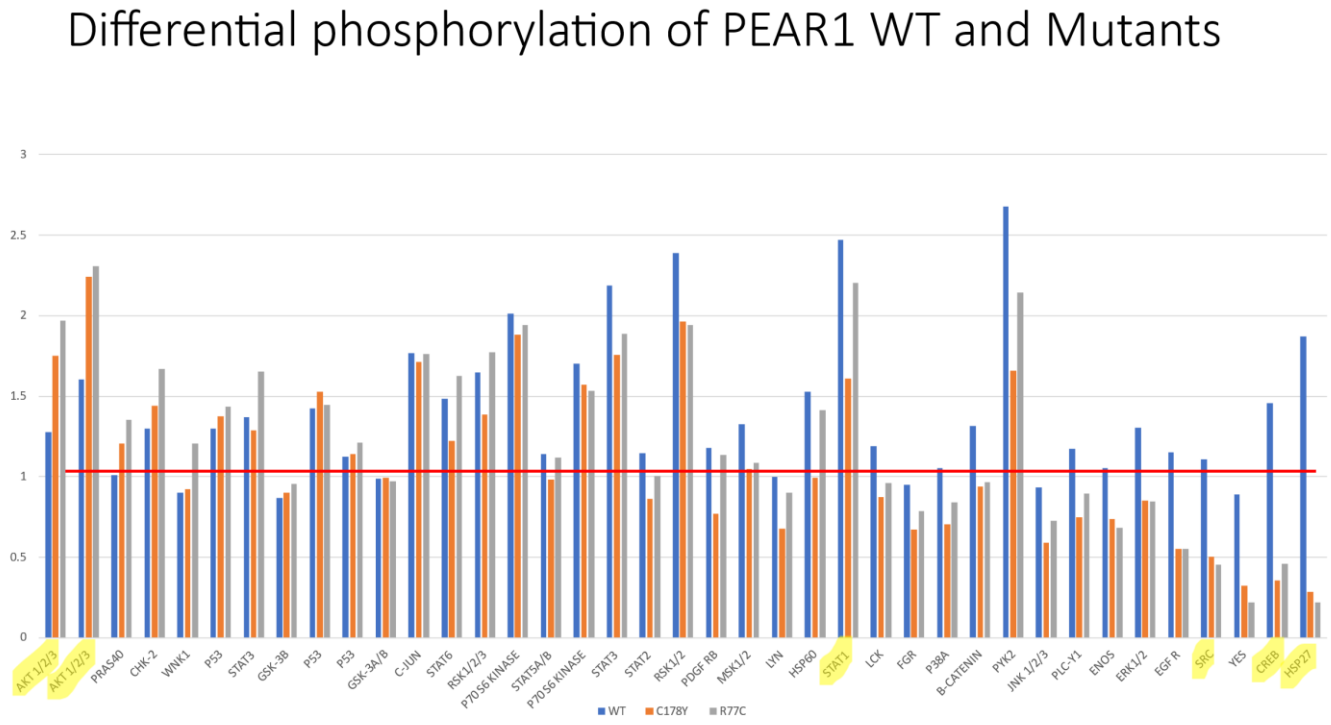
However, these mutations were not able to fully transform the Ba/F3 cells to IL-3 independent growth. Hence, these PEAR1 mutations appeared to an incomplete transforming potential. However, within this IL-3 withdrawal assay, clones expressing weak mutations can sometimes acquire further mutations during the course of the assay, potentially playing a part in

IL-3 independent growth.[25] Consequently, it is important to sequence the transgene before and after the assay to ensure the mutation in question is the sole driver of cell transformation.[25]




(Figure 3. Ba/F3 is a murine pro b cell line reliant on IL-3 to proliferate. These cells were virally infected with PEAR1 constructs that the cells transiently express. WEHI is a media that contains 15% IL-3. Percentage of viability of cells on the y axis, and days of IL-3 withdrawal on the x axis. You can see a phenotype is observable between PEAR1 mutations being able to survive better without IL-3 versus PEAR1 WT)

We next wanted to see whether this survival advantage imparted by PEAR1 mutations compared with WT might result from signaling differences between mutant PEAR1 and WT PEAR1. A proteome profiler, which was a kit with over sixty antibodies lied within several membranes, was used to scan pathway differences between PEAR1 WT, and PEAR1 mutants found in the Beat AML dataset.

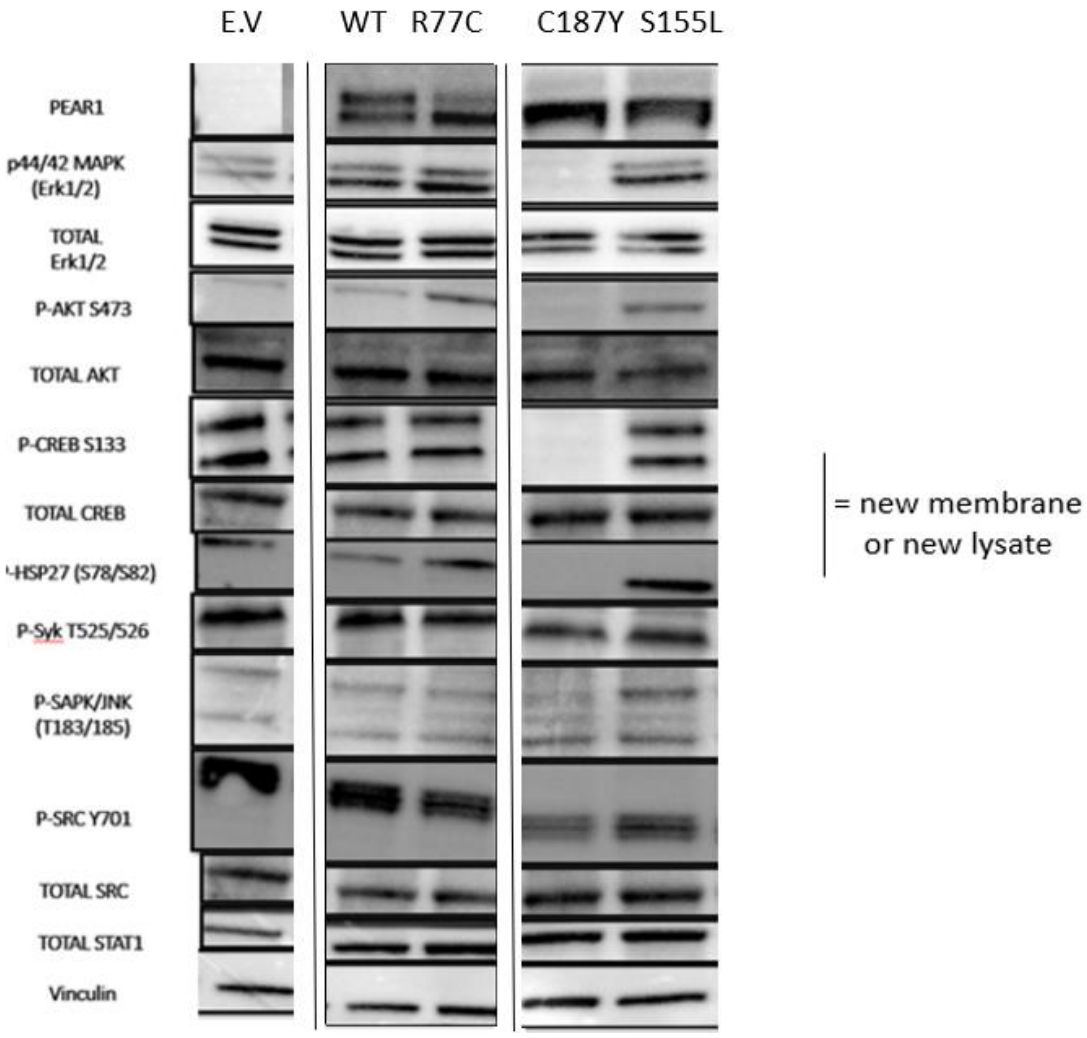



**Mutants show** more activation in AKT, and less activtion for SRC, ERK1/2, CREB, and HSP27

(Figure 4. This experiment was done on human HEK293 cells virally infected with genes of interest that are expressed transiently. The red line is the average signaling based on empty vector. Everything above this line is 1.5 times or 2 times as much as (EV). The overall signaling differences are unremarkable, except for Akt being up both in PEAR1 WT, and even further for PEAR1 mutants)

Because of the unique banding pattern found in PEAR1 R77C mutant, compared to WT, or other mutants found in the study we also wanted to do western blots to assess signaling differences. All the mutants compared to WT have a non-remarkable different signaling pattern. While the total amount of STAT1 increases, phosphorylation did not increase (not pictured). Phosphorylation of STAT1 a well-known growth-signaling, with capacity towards leukemogenesis.[35] We did observe some differences in phosphorylation events between mutant and WT, such as reduced phosphorylation of MAPK and CREB in the C178Y variant, which

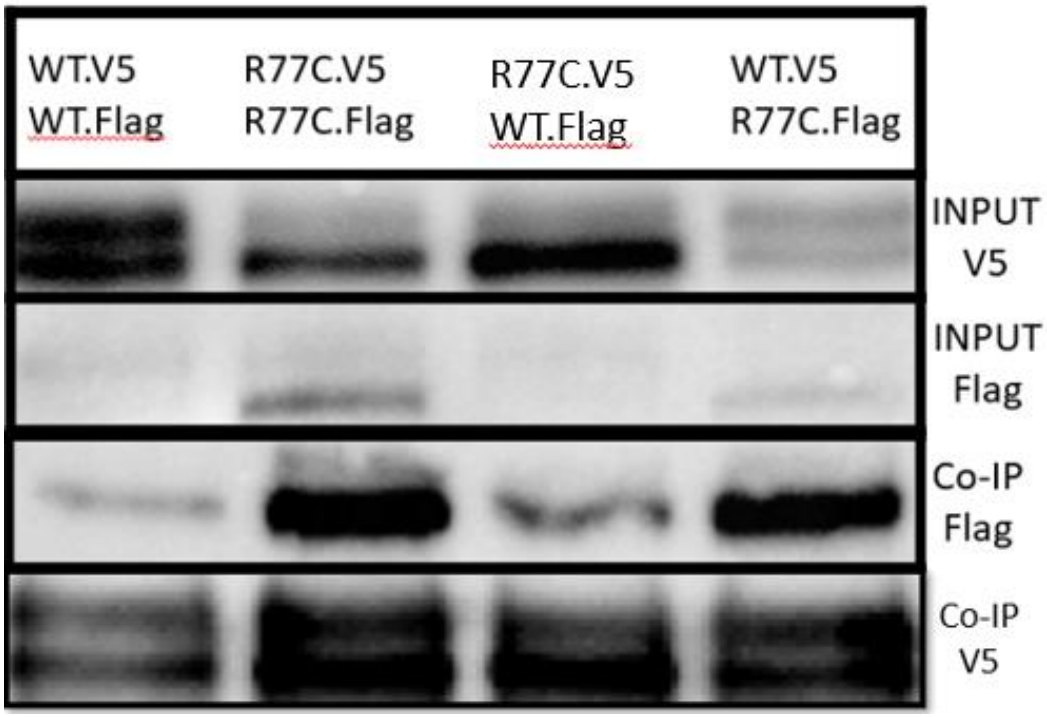
merits further investigation.




(Figure 5: Human HEK293 cells were transiently transfected with constructs expressing wild type (WT) or mutant versions of PEAR1. Equivalent amounts of protein from whole cell lysates were subjected to immunoblot analysis using antibodies specific to the phospho- and total proteins indicated. An empty vector (E.V.) control is also included and vinculin is shown as a loading control. This figure represents cropped lanes from a combination of several different western blots that have been collected into a figure for an initial comparison across these different PEAR1 WT and mutant settings. This experiment needs to be repeated with cell lysates from all conditions loaded onto a single gel and membrane to validate these initially observed patterns.)

To continue investigating the banding pattern found in R77C mutant, we wanted to see if this was potentially a gain of function mutation, potentially leading to increased dimerization of the mutant compared with PEAR1 WT. To address this, a co-immunoprecipitation (co-IP)

experiment was designed where HEK293 cells were virally infected PEAR1 mutants that were tagged using V5 and Flag epitopes (Figure 6).




(Figure 6: Human HEK293 cells were virally infected with PEAR1 constructs with and without epitope tags. INPUT to see proteins in sample before G-beads were introduced)

Results from this experiment indicate that the R77C PEAR1 mutant was more able to interact with itself compared to PEAR WT, suggesting that this mutation may lead to increased PEAR1 dimerization.

## Discussion

Through initial examination of PEAR1 and PEAR1 mutants, we find evidence that PEAR1 may play a role in driving the disease state of acute myeloid leukemia. PEAR1 mutants showed weak IL-3 independent growth. It will be important in the future to perform sequencing

of the cells both before and after IL-3 withdrawal to validate the role of PEAR1 mutations in leading to enhanced Ba/F3 cell survival after IL-3 withdrawal.[25]

The western-blot analysis and other experiments aimed to see signaling differences between PEAR1 WT and mutations illustrated some potentially promising differences, where certain mutations exhibited pathways with more signaling than PEAR1 WT. In addition, the R77C mutation did exhibit increased co-immunoprecipitation compared with PEAR1 WT.

As the stratification of AML subsets increases, it should be noted that classifying patients with AML for diagnostic purposes is different than a system for predicting the outcome of AML in patients.[46] It has been seen in academic health centers treating AML patients that have leukemia-associated variants up to a month after chemotherapy, these variants were associated with increased risk of relapse.[51] These findings illustrate that PEAR1 may be an additional gene that can be involved in driving the disease pathogenesis of AML, which warrants further investigation in future studies.

## MATERIALS & METHODS

### Cell culture

Cells were maintained at 37°C in a humidified incubator at 5% CO2. Cells were grown in media supplemented with 10% fetal bovine serum (Gibco) and 1% penicillin/streptomycin (Gibco).

Ba/F3 cells were grown in RPMI 1640 medium with FBS, L-glutamine, fungizone, penicillin-streptomycin, and 15% IL-3 conditioned media.

HEK-293 cells were grown in D10 medium with FBS, L-glutamine, fungizone, penicillin-streptomycin.

### Ba/F3 Transformation Assay

Gene of interest were virally and transiently infected. Cells were selected for positive infection with puro, then washed and set in medium without WEHI. WEHI is a medium with 15% of IL-3. After three days cells were stained with MTS and read under a plate-reader.

Co-immune Precipitation

HEK-293 cells were virally and transiently infected with a co-transfection titrated at an amount that both flag and v5 to be expressed as equally as possible tested for on western blot expression. The cells were given a couple of days to recover post transfection. Agarose G beads were used, with common antibody, and lysate, to roll around overnight at 4C. Beads were then washed 10x and lysate boiled for ten minutes before western blot.

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