PUM1 mediates the posttranscriptional regulation of human fetal hemoglobin

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Key Points

• PUM1, an RNA-binding protein, is a novel target of EKLF that binds to fetal γ-globin mRNA and impairs its stability and translation.
• Elevated HbF levels are observed upon PUM1 knockdown ex vivo and in an individual harboring a novel PUM1 mutation in the RNA-binding domain.

The fetal-to-adult hemoglobin switching at about the time of birth involves a shift in expression from γ-globin to β-globin in erythroid cells. Effective re-expression of fetal γ-globin can ameliorate sickle cell anemia and β-thalassemia. Despite the physiological and clinical relevance of this switch, its posttranscriptional regulation is poorly understood. Here, we identify Pumilo 1 (PUM1), an RNA-binding protein with no previously reported functions in erythropoiesis, as a direct posttranscriptional regulator of β-globin switching. PUM1, whose expression is regulated by the erythroid master transcription factor erythroid Krüppel-like factor (EKLF/KLF1), peaks during erythroid differentiation, binds γ-globin messenger RNA (mRNA), and reduces γ-globin (HBG1) mRNA stability and translational efficiency, which culminates in reduced γ-globin protein levels. Knockdown of PUM1 leads to a robust increase in fetal hemoglobin (~22% HbF) without affecting β-globin levels in human erythroid cells. Importantly, targeting PUM1 does not limit the progression of erythropoiesis, which provides a potentially safe and effective treatment strategy for sickle cell anemia and β-thalassemia. In support of this idea, we report elevated levels of HbF in the absence of anemia in an individual with a novel heterozygous PUM1 mutation in the RNA-binding domain (p.(His1090Profs*16); c.3267_3270delTCAC), which suggests that PUM1-mediated posttranscriptional regulation is a critical player during human hemoglobin switching.

Introduction

β-globin switching from fetal γ-globin (HBG1 and HBG2) to adult β-globin (HBB) is a developmental process that occurs at around the time of birth in erythrocytes, and the mechanisms that allow γ-globin re-expression in adult erythrocytes are exploited as an effective therapeutic strategy to ameliorate sickle cell anemia and β-thalassemia.1 Unlike transcriptional and epigenetic regulation, posttranscriptional regulation of β-globin switching is poorly understood, with few reports on its physiological and clinical relevance.2-9 Here, we report that Pumilo 1 (PUM1), an RNA-binding protein with no previously reported functions in

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Figure 1.
erythropoiesis, is a novel target of the erythroid master transcription factor erythroid Kräppel-like factor (EKLF/KLF1) and has a role in the posttranscriptional regulation of fetal hemoglobin (HbF).

**Methods**

Human blood samples were collected after approval by the Institutional Review Board at Cleveland State University. Eklfl and Eklf murine erythroid cells, human umbilical cord blood–derived erythroid progenitor 2 (HUDEP2) cells, and the K562 cell line were grown and differentiated as described previously. Primary adult human CD34+ cells (hematopoietic stem and progenitor cells [HSPCs]) from mobilized peripheral blood collected via apheresis from healthy adult donors were purchased from Yale Cooperative Center of Excellence in Hematology and were expanded and differentiated as described before.

Click-iT Nascent RNA Capture Kit (Thermo Fisher Scientific) was used according to the manufacturer’s protocol to assess transcript stability. Polyribosome fractionation in HUDEP2 cells was performed as described previously. RNA immunoprecipitation (RIP) was performed using RIPA-b+ PUM1 polyclonal antibody and primer set (Millipore Sigma) and Magna-RIP RNA-Binding Protein Immunoprecipitation Kit (Millipore Sigma) according to the manufacturer’s instructions. Detailed protocols for the above techniques and for lentiviral transductions to knock down and overexpress PUM1, quantitative reverse transcriptase polymerase chain reaction, western blotting, flow sorting, F-cell staining, Hb high-pressure liquid chromatography (HPLC), and statistics, along with the list of antibodies and primers are available in the supplemental Materials and Methods.

**Results and discussion**

We previously created an ex vivo primary cell culture system to expand our understanding of how EKLF mediates the precise changes leading to terminal erythropoiesis and enucleation. RNA sequencing analysis on the Eklfl and Eklf murine erythroid cells and chromatin immunoprecipitation sequencing analysis in human erythroid cells identified a novel EKLF target, PUM1, which is upregulated specifically during erythroid terminal differentiation (Figure 1A-B). PUM1, a member of the Pumilio RNA-binding protein (PUF) family of sequence-specific RNA-binding proteins, acts as a posttranscriptional repressor by binding to the 3’ untranslated region (3’-UTR) of messenger RNA (mRNA) targets and impairing their stability and/or translational efficiency. Consistent with these functions, we observed PUM1 in the cytoplasm before and after erythroid terminal differentiation (supplemental Figure 1).

EKLF is a known regulator of Hb switching, so to identify the functions of PUM1 in erythroid terminal differentiation, we first tested its ability to regulate globin levels. Fetal γ-globin is expressed from 2 genes, HBG1 (γγ) and HBG2 (γδ); HBG1 has 2 core PUM1 consensus binding sites in its 3’-UTR, but HBG2 and other fetal and adult globins do not (supplemental Figure 2). Because HbF comprises < 1% of total Hb in adult erythroid cells, we asked whether PUM1 suppressed γ-globin expression specifically in these cells. We tested this by knocking down PUM1 in HUDEP2 immortalized human erythroid progenitor cells that exhibit an adult Hb profile. We observed a modest increase (~2.5-fold) in levels of the γ-globin transcript (supplemental Figure 3), but the increase in the γ-globin protein levels after PUM1 knockdown was more than 12-fold by western blot analysis, and HPLC showed 22.6% HbF levels in PUM1 knockdown cells compared with no detectable HbF in control cells (Figure 1C-D). Similar results were also observed in primary erythroid cells derived from human adult HSPC CD34+ cells (Figure 1E-F; supplemental Figure 4A-B). Conversely, overexpression of PUM1 in K562 erythroleukemia cells that express high endogenous HbF led to a reduction of γ-globin (Figure 1G).

Our data underline the importance of the fine-tuned homeostasis required to maintain PUM1 protein levels, because we observed that even slight perturbations in PUM1 levels result in gross γ-globin changes, as was previously reported in patient mutations that reduced PUM1 levels by 25% in other tissues. Importantly, PUM1 knockdown did not affect the progression of erythropoiesis either in primary adult human HSPCs or in HUDEP2 cells (Figure 1H; supplemental Figure 5A-B). Furthermore, as shown in supplemental Figure 6, knockdown of PUM1 did not lead to changes in the levels of known γ-globin regulators such as EKLF, BCL11A, and ZBTB7A.

PUM1 regulates gene expression in mammals by degrading target mRNA and/or inhibiting translation of target mRNAs.

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*Figure 1. EKLF upregulates PUM1 during erythroid terminal differentiation, PUM1 knockdown robustly increases the levels of γ-globin protein and HbF without altering β-globin, and PUM1 overexpression decreases the levels of γ-globin protein. (A) RNA sequencing analysis of Eklfl and Eklf murine Extensively Self Renewing Erythroblasts (ESRE) shows Pum1 transcript levels during expansion and differentiation (n=3). (B) The human PUM1 gene sequence with transcriptional start at position +1 (hg19; chr1 [p35.2]). Transcription factor–binding motifs for EKLF, p300, histone marks H3K4me3 and H3K4me2, and RNA Pol II binding are shown. (C) Western blot analysis of HUDEP2 cell extracts harvested after infection with either PUM1 or non-target control (NTC) lentiviral short hairpin RNAs (shRNAs). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the loading control. Quantitation of the indicated proteins by western blot analysis of HUDEP2 cell extracts upon PUM1 knockdown is shown on the right (n = 3). (D) HPLC analysis of Hb in HUDEP2 cells after infection with either PUM1 or NTC lentiviral shRNAs was performed to quantitate the percentages of HbF and adult hemoglobin (HbA) upon erythroid terminal differentiation (day 10). (E) Results of quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) assay show the extent of PUM1 knockdowns after lentiviral transfection of the 3 PUM1 shRNAs in adult human primary HSPCs (n = 2). (F) Western blot analysis of adult human primary HSPC extracts harvested on day 11 of erythroid differentiation after infection with either PUM1 or NTC lentiviral shRNAs, GAPDH was used as the loading control. Quantitation of the indicated proteins by western blot analysis of PUM1 knockdown cell extracts upon PUM1 knockdown is shown on the right (n = 2). (G) Western blot analysis of erythroleukemia cell line K562 cells with and without PUM1 overexpression (OE), GAPDH was used as the loading control. Quantitation of the indicated proteins by western blot analysis of K562 cell extracts upon PUM1 overexpression is shown on the right (n = 3). (H) Flow cytometry analysis of adult primary human HSPCs at day 7 of erythroid differentiation using human CD71 and glycophorin A (hGlyA) antibodies shows that PUM1 knockdown does not impair the progression of erythroid differentiation. Gates are drawn based on unstained and single-color controls. Population percentages within each gate are indicated. Data were analyzed by using a two-sided Student t test. Bar graphs show mean ± standard deviation (SD). *P < .05; **P < .005; ***P < .0005. FITC, fluorescein isothiocyanate; NS, not significant; PE, phycoerythrin.
Figure 2. PUM1 binds to γ-globin mRNA and decreases γ-globin (HBG1) mRNA stability and translational efficiency, and the blood from an individual harboring a novel heterozygous mutation in PUM1 RNA-binding domain displays elevated percentages of HbF and F cells. (A) qRT-PCR analysis of RNA levels of the indicated transcripts in the immunoprecipitated eluates after RIP of PUM1 in HUDEP2 cells (n = 4). Bar graphs show mean ± standard error of the mean. (B) qRT-PCR analysis of 5-EU incorporated mRNAs of indicated genes at 0-hour pulse and 24-hour chase time points after incorporation in HUDEP2 cells infected with either PUM1 or NTC lentiviral shRNAs showed an increase in the stability of HBG1 but not HBG2 or HBB globins at the 24-hour time point upon PUM1 knockdown (n = 2). Bar graphs show mean ± SD. (C) Polysome fraction analysis in HUDEP2 cells infected with either PUM1 or NTC lentiviral shRNAs showing relative distribution of HBG1 mRNA in each of the 14 fractions. Fraction 8 corresponds to the monosome fraction (80S), whereas fractions 10 to 14 correspond to the polysome fractions. A decrease in the distribution
Native RIP of PUM1 pulled down γ-globin mRNA compared with β-globin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA (Figure 2A; supplemental Figure 7), thus confirming a unique and direct role for PUM1 in regulating γ-globin. We then performed a nascent mRNA degradation assay in which we pulsed HUDEP2 cells with ethylene uridine (EU) ribonucleotide homologs and then washed and analyzed the newly synthesized EU-incorporated mRNA at different time points. The EU-incorporated HBG1, HBG2, and HBB β-type globin mRNA levels were reduced over time in the control cells, but only HBG1 mRNA levels were relatively stabilized after PUM1 was knocked down. We observed this after either a short 24-hour chase or a longer 3-day chase, suggesting that PUM1 specifically mediates HBG1 mRNA degradation (Figure 2B; supplemental Figure 8A). The delay in the degradation of HBG1 upon PUM1 knockdown is further captured in a time course of EU incorporation and chase (supplemental Figure 8B). Next, polysome profiling of the control and PUM1 knocked-down HUDEP2 cells showed a specific increase in the HBG1 mRNA levels in polyribosomal fractions compared with the monosomal fractions, suggesting that HBG1 mRNA, unlike HBG2 and HBB mRNA, is more actively translated under reduced PUM1 levels (Figure 2C-D; supplemental Figure 9A-D). These results demonstrate that PUM1 regulates HBG1 at the level of mRNA stability and also at translation and thus serves as a posttranscriptional regulator of γ-globin in adult human erythroid cells.

The diverse roles of PUM1 in human pathology imply that it has distinct cell type-dependent roles during development.20 Therefore, we investigated whether patient mutations in PUM1 could result in high HbF levels. We identified a 5-year-old child with PUM1-associated developmental disability, ataxia, and seizure (PADDAS) who harbored a novel heterozygous PUM1 mutation (p.(His1090Profs*16)); c.3267_3270delTCAC). The mutation, a frameshift in the RNA-binding domain, introduces 16 new amino (p.(His1090Profs*16); c.3267_3270delTCAC). The mutation, a frameshift in the RNA-binding domain, introduces 16 new amino acids and a premature stop codon (Figure 2E). We observed elevated HbF levels in the patient (above the accepted reference range), with a more than 10-fold increase over the levels in healthy parents, as analyzed by HPLC and by modified Kleihauer-Betke staining for F cells (Figure 2F-G). The complete blood count in the patient suggested that elevated HbF was not due to anemia (supplemental Figure 10).

Here we identify PUM1 (an RNA-binding protein with no previously reported roles in erythropoiesis) as a direct posttranscriptional regulator of Hb switching. The change in the ratio of $\gamma$γ(γ-globin) to $\beta$β(β-globin) from 3:1 at birth to 2:3 in the small amount of HbF present in the blood of adults may be explained by the existence of exclusive regulatory mechanisms for these 2 genes (such as the PUM1-mediated targeting of HBG1 alone), although how PUM1 itself is regulated during this developmental window is unknown.24 An investigation of available databases identifies an increase in PUM1 transcript levels as the site of erythropoiesis shifts from the fetal liver to bone marrow, similar to previously identified regulators of Hb switching such as ZBTB7A/LRF (supplemental Figure 11).25 Investigation will be required to decipher whether, in addition to direct posttranscriptional regulation, indirect mechanisms could also contribute to the robust induction of γ-globin observed upon PUM1 knockdown in erythroid cells. Future studies will probe the potential for erythroid-specific enhancers of PUM1 expression as an EKLF target.

The HbF induction (~22% HbF) observed after PUM1 knockdown exceeds the levels considered therapeutic in patients with sickle cell anemia.26-29 although it is lower than the level of induction observed after modulation of targets such as BCL11A and ZBTB7A/LRF (ranging from ~23% to 73% HbF in cultured erythroid cells22,29). Because our studies indicate that PUM1 knockdown does not affect progression through erythroid terminal differentiation, that PUM1 knockout mice are viable with no reported erythroid defects,31 and that PUM1 functions as a cytoplasmic regulator, we propose that it could potentially serve as a safe and effective non–gene-altering target toward ameliorating β-thalassemia and sickle cell disease.

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**Authorship**

Contribution: M.N.G. and M.R. conceptualized the study and wrote the manuscript; R.E., A.R.D., R.M.G., and M.R. performed quantitative reverse transcriptase polymerase chain reaction assays and western blots; R.E., A.R.D., and R.M.G. performed the differentiation assays and cell culturing; M.N.G. performed the Click-iT pulse-chase experiments and RNA immunoprecipitation; S.E.A. performed the immunofluorescence experiment; R.A.W. genotyped the mutations...
of the patients; S.G. and O.Y.M. ran the fluorescence-activated cell sorted (FACS) samples and helped analyze the FACS data; A.O.-A. helped interpret the deidentified patient data; A.G. performed the polysome fractionation; A.G., A.A.K., M.R., and M.N.G. analyzed the data; Y.M. and U.A.G. helped with the HPLC analysis; and M.R. and M.N.G. processed raw data and created the figures.

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