



# The methyltransferase PRMT1 regulates $\gamma$ -globin translation

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Induction of fetal hemoglobin to overcome adult  $\beta$ -globin gene deficiency is an effective therapeutic strategy to ameliorate human  $\beta$ -hemoglobinopathies. Previous work has revealed that fetal  $\gamma$ -globin can be translationally induced *via* integrated stress signaling, but other studies have indicated that activating stress may eventually suppress  $\gamma$ -globin expression transcriptionally. The mechanism by which  $\gamma$ -globin expression is regulated at the translational level remains largely unknown, limiting our ability to determine whether activating stress is a realistic therapeutic option for these disorders. In this study, we performed a functional CRISPR screen targeting protein arginine methyltransferases (PRMTs) to look for changes in  $\gamma$ -globin expression in K562 cells. We not only discovered that several specific PRMTs may block  $\gamma$ -globin transcription, but also revealed PRMT1 as a unique family member that is able to suppress  $\gamma$ -globin synthesis specifically at the translational level. We further identified that a non-AUG uORF within the 5' untranslated region of  $\gamma$ -globin serves as a barrier for translation, which is bypassed upon PRMT1 deficiency. Finally, we found that this novel mechanism of  $\gamma$ -globin suppression could be pharmacologically targeted by the PRMT1 inhibitor, fumamidine dihydrochloride. These data raise new questions regarding methyltransferase function and may offer a new therapeutic direction for  $\beta$ -hemoglobinopathies.

A switch from fetal globin (HbF, consists of two  $\alpha$  and  $\gamma$  subunits) to adult globin (HbA, consists of two  $\alpha$  and  $\beta$  subunits) is a key step of human erythropoiesis during development. However, inherited mutations in the  $\beta$  subunit globin gene render either a dysfunction or a decrease of  $\beta$ -globin protein, thereby causing hemoglobinopathies including  $\beta$ -thalassemia and sickle cell disease (SCD) (1, 2). Studies have shown a potential therapeutic efficacy of elevating fetal  $\gamma$ -globin in adults pharmacologically as a replacement for the dysfunctional  $\beta$  subunit to ameliorate hemoglobinopathies

(3–5). In this respect, a promising strategy is to induce  $\gamma$ -globin gene transcription in adult erythroid cells through blocking the activity of repressors of  $\gamma$ -globin transcription (6–9), such as methyltransferases that either mediate DNA or histone methylations (10–14). On the other hand, however, studies have pointed out that the final production of  $\gamma$ -globin protein does not always correlates with the transcription of  $\gamma$ -globin gene (15) and that  $\gamma$ -globin protein alters even before any changes in the  $\gamma$ -globin mRNA level happen (16). These works strongly suggest that despite transcriptional regulation, the posttranscriptional regulation, for example, translation of  $\gamma$ -globin mRNA is also a key determinant of the fetal globin production. A recent work has suggested that  $\gamma$ -globin is notably controlled at the translational level by integrated stress response (ISR) pathway (17). Nevertheless, it remains unclear how this translational regulation is achieved. Furthermore, others also addressed that inducing the stress signaling may eventually render a transcriptional suppression of the  $\gamma$ -globin gene (18). Therefore, whether activating integrated stress response can serve as a therapy for hemoglobinopathies requires a careful investigation, and a new strategy that can induce  $\gamma$ -globin synthesis both transcriptionally and translationally is urgently needed.

Protein arginine methyltransferases (PRMTs), consisting of nine family members including type I PRMTs (PRMT1, 2, 3, 4, 6, and 8) and type II PRMTs (PRMT5 and 9), are a group of catalytic enzymes that modify the arginine methylation on key proteins involved in the gene expression at both transcriptional and posttranscriptional levels (19). Due to its role in mediating the histone H4R3me2s, a transcriptional repressive mark on several loci including the  $\gamma$ -globin gene, PRMT5 has been discovered as a key suppressor of  $\gamma$ -globin gene expression, which can be utilized as a target for treating  $\beta$ -thalassemia (10, 20). Although most PRMTs methylate glycine- and arginine-rich motifs, and often share substrates, various PRMT members are not redundant in eukaryotic cells. This is likely due to a substrate preference *in vivo*, for example, *Prmt4* (also named *Carm1*)-knockout cells exhibit hypomethylation in polyadenylate-binding protein 1 (PABP1) (21), while PRMT6

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silencing mainly resulted in a repression of histone H3 arginine 2 (H3R2) methylation (22). In this respect, whether the vast majority of other PRMTs can also participate in regulating  $\gamma$ -globin expression remains largely unknown.

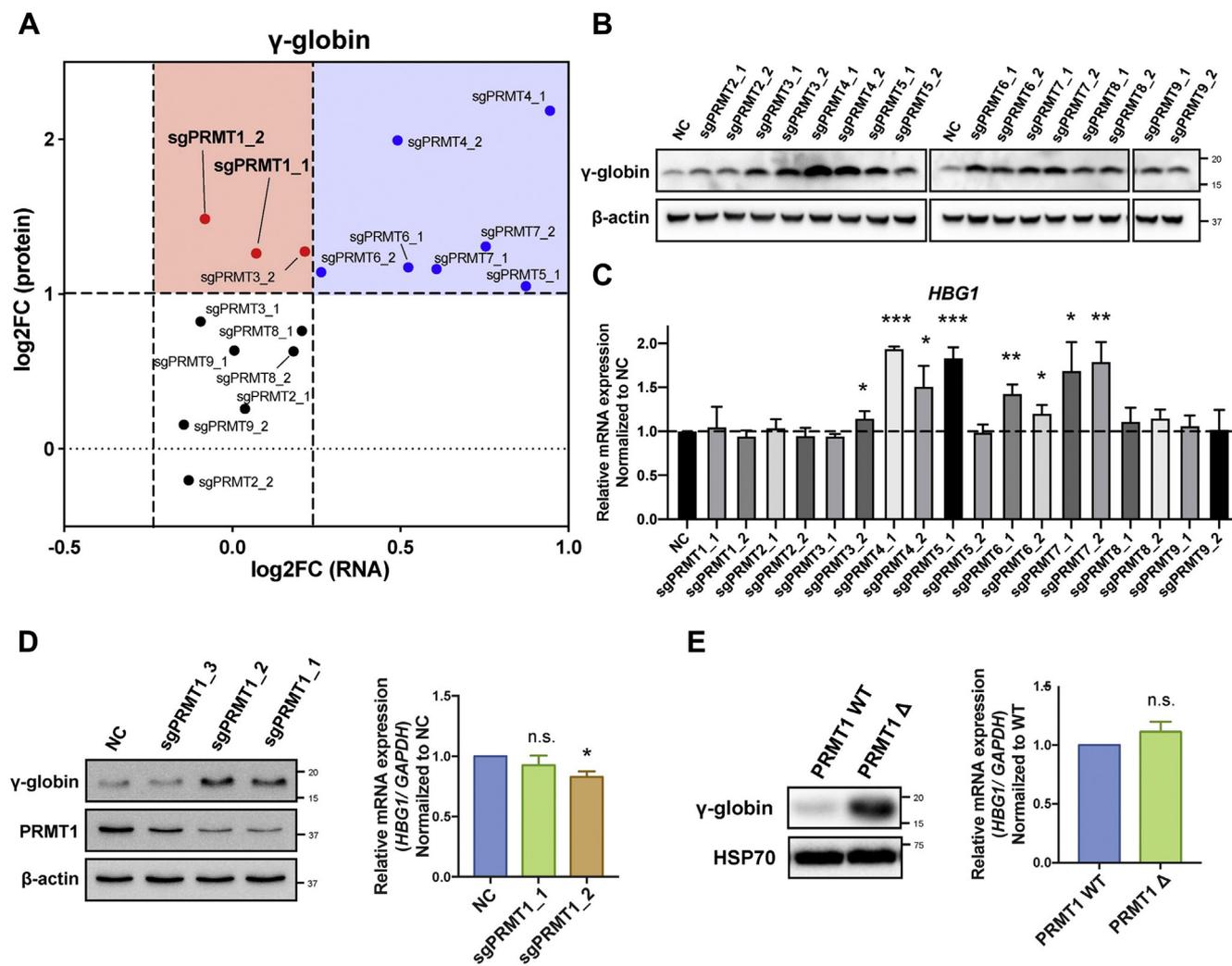
In this study, to investigate the functions of PRMTs in  $\gamma$ -globin expression, we performed a systematic silencing of each individual PRMTs. Several PRMTs have shown potential in suppressing  $\gamma$ -globin transcription. Surprisingly, PRMT1, a major methyltransferase, catalyzing the mono- or asymmetric dimethylations of a large number of substrates, is a key PRMT that regulates  $\gamma$ -globin expression at the translational level. We show that blocking PRMT1 mediates the bypassing of a noncanonical upstream open reading

frame (uORF) that represses  $\gamma$ -globin mRNA translation, which may associate with the activity of an ATP-dependent RNA helicase DDX3. Therefore, pharmacologically targeting PRMT1 may serve as a novel therapeutic strategy in treating  $\beta$ -thalassemia.

## Results

### PRMT1 is a unique protein arginine methyltransferase that regulates $\gamma$ -globin expression at the protein level via its catalytic activity

To investigate the role of each PRMTs in  $\gamma$ -globin expression, we first performed PRMTs silencing in human K562 cells

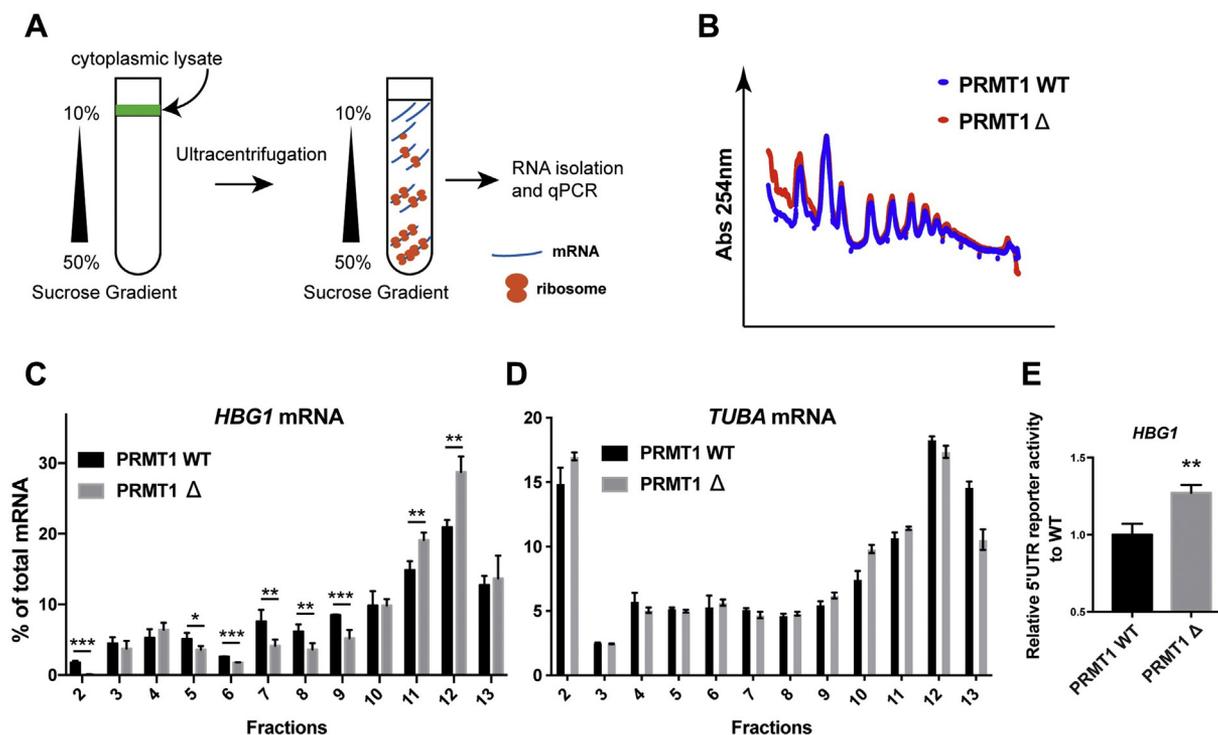


**Figure 1. PRMT1 is the only protein arginine methyltransferase that regulates  $\gamma$ -globin expression at the protein level via its catalytic activity.** *A*, the log<sub>2</sub> fold changes (FC) of  $\gamma$ -globin protein and mRNA expressions upon each individual PRMT knockdown are plotted. Each individual PRMT is silenced by two CRISPRi sgRNAs, and their  $\gamma$ -globin protein and mRNA expressions are normalized to those of a nontarget control (NC). *Red dots* represent sgRNAs that only alter  $\gamma$ -globin expression at the protein level; *blue dots* represent sgRNAs that regulate  $\gamma$ -globin expression at both mRNA and protein levels. Protein quantifications were performed using Image J. The correlation between log<sub>2</sub>FC (protein) and log<sub>2</sub>FC (RNA) is calculated using Pearson Correlation test,  $r = 0.627$ ,  $p = 0.0041$ . *B*, representative western blots of  $\gamma$ -globin and actin in K562 cells stably silencing each individual PRMTs. *C*, RT-qPCR analysis of relative  $\gamma$ -globin mRNA expression in K562 cells stably silencing each individual PRMTs compared to NC. *D*, *Left*: Representative western blots of  $\gamma$ -globin, PRMT1, and actin upon PRMT1 silencing. *Right*: Quantitative PCR with reverse transcription (RT-qPCR) analysis of  $\gamma$ -globin mRNA (*HBG1*) levels upon PRMT1 silencing. *E*, *Left*: Representative western blots of  $\gamma$ -globin and HSP70 in K562 cells overexpressing wild-type PRMT1 (PRMT1 WT) and catalytic center-deleted PRMT1 (PRMT1  $\Delta$ ). *Right*: RT-qPCR analysis of  $\gamma$ -globin mRNA expression in K562 cells overexpressing PRMT1 WT and PRMT1  $\Delta$ . All values represent the mean  $\pm$  s.d. of three biological repeats. n.s., not significant,  $p > 0.05$ ; \*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$  compared with the indicated control. Statistical testing was performed using the two-sided Student's *t*-test.

using two CRISPR interference (CRISPRi) single guide RNAs (sgRNA) targeting each individual PRMTs in a dCas9-dependent manner. Despite the second sgRNA of PRMT5 failed to inhibit PRMT5 expression, all other sgRNAs successfully blocked the expression of each individual PRMTs (Fig. S1A). Overall, we observed a significant correlation between  $\gamma$ -globin protein alterations and its mRNA expression changes (Fig. 1A, Pearson Correlation  $r = 0.627$ ,  $p = 0.0041$ ), suggesting that the regulation of PRMTs on  $\gamma$ -globin expression, if there is any, may happen at its mRNA expression level. Particularly, in accordance to previous publications (10, 11), silencing PRMT4 (also named CARM1) and PRMT5 results in a significant upregulation of  $\gamma$ -globin protein (Fig. 1, A and B) through inducing  $\gamma$ -globin gene transcription (Fig. 1C). PRMT6 and 7 silencing also rendered the upregulation of  $\gamma$ -globin expression at both mRNA and protein level to different extents, suggesting their putative roles in regulating  $\gamma$ -globin expression at its mRNA expression level; while PRMT2 and 9 silencing has no effect (Fig. 1, A–C). Intriguingly, PRMT1 turned out to be the best candidate from the PRMT family that regulates  $\gamma$ -globin at the posttranscriptional level: more than 2.5-fold increase in  $\gamma$ -globin protein, but no effect on  $\gamma$ -globin mRNA expression was observed when PRMT1 was silenced by both sgRNAs (Fig. 1, A and D). Furthermore, the effect on  $\gamma$ -globin protein upregulation correlates with the knockdown efficiency (Fig. 1D). Other

PRMTs including PRMT3 and 8 may render a slight augment in  $\gamma$ -globin protein without significant alterations on their mRNA expressions (Figs. 1, A–C and S1B).

PRMT1 is a major methyltransferase that catalyzes the mono- or asymmetric dimethylations of a large number of substrates including histones, transcription factors, and RNA-binding proteins. Therefore, PRMT1 may be involved in various gene expression processes, such as transcription and translation, through its catalytic activity. To understand whether the effect of PRMT1 in suppressing  $\gamma$ -globin protein expression is through its catalytic activity, we generate a mutant PRMT1 form of PRMT1 in which five amino acids (GSGTG, aa86–90) located in the S-adenosyl-L-methionine binding motif were deleted (PRMT1  $\Delta$ ) in K562 cells. This PRMT1 mutant showed a dominant-negative effect on asymmetric dimethylation of arginine residues in protein (Fig. S1C). Noteworthy, K562 cells express sufficient amount of  $\gamma$ -globin mRNA, which may serve as a suitable model to study the posttranscriptional regulation (especially the mRNA translation) of  $\gamma$ -globin. Interestingly, compared with the wild-type PRMT1, overexpressing the S-adenosyl-L-methionine-binding motif deleted PRMT1 results in a profound upregulation of  $\gamma$ -globin protein expression, without a significant alteration in  $\gamma$ -globin mRNA level (Fig. 1E). These results demonstrated that PRMT1 is a unique methyltransferase that regulates  $\gamma$ -globin post-transcriptionally, potentially at the translational level.



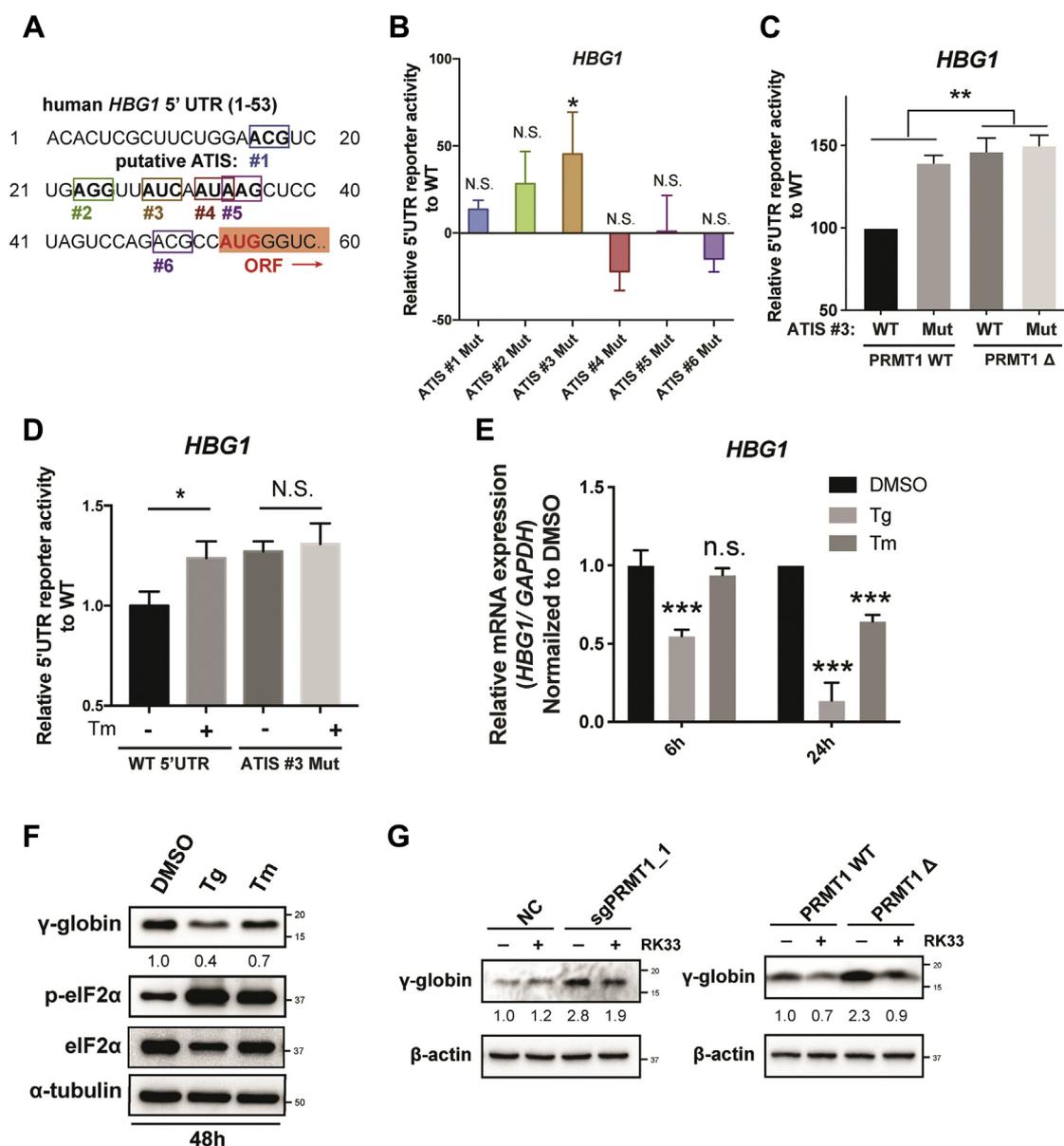
**Figure 2. PRMT1 suppresses  $\gamma$ -globin mRNA translation.** A, experimental workflow for investigating mRNA translational status by sucrose gradient fractionation assay. B, representative polysome trace of K562 cells overexpressing PRMT1 WT and PRMT1  $\Delta$ . C, RT-qPCR analysis of  $\gamma$ -globin mRNA levels in fractions, percentage of  $\gamma$ -globin mRNA distributed in each fraction against total  $\gamma$ -globin mRNA are shown. D, RT-qPCR analysis of  $\alpha$ -tubulin mRNA (*TUBA*) levels in fractions, percentage of  $\gamma$ -globin mRNA distributed in each fraction against total  $\gamma$ -globin mRNA are shown. E,  $\gamma$ -globin mRNA 5' UTR reporter activity in K562 cells overexpressing PRMT1 WT and PRMT1  $\Delta$ . All values represent the mean  $\pm$  s.d. of three biological repeats. \*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$  compared with the indicated control. Statistical testing was performed using the two-sided Student's *t*-test.

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### PRMT1 reprograms $\gamma$ -globin expression at the mRNA translation level

To further investigate whether  $\gamma$ -globin is regulated by PRMT1 at the translation level, we examined the distribution of  $\gamma$ -globin mRNAs in polysomes (translationally active ribosome fractions) on sucrose gradient fractionation (Fig. 2, A and B). Compared with its wild-type control, catalytic dead PRMT1 does not affect the overall distribution of mRNAs in monosome and polysome fractions (Fig. 2B), suggesting that

abrogating the catalytic activity of PRMT1 does not alter global protein synthesis in K562 cells. By contrast, we observed a higher percentage of  $\gamma$ -globin (*HBG1*) mRNA accumulated in the translationally active heavy polysome fractions (fractions 11 and 12), while reduced in the translationally inactive monosome or light polysome fractions (fractions 5–9) in catalytic-dead PRMT1-expressing cells (Fig. 2C). This shift in mRNA distribution toward the heavy polysomes was not detected for the control  $\alpha$ -tubulin mRNA (Fig. 2D), suggesting



**Figure 3. PRMT1 deficiency promotes  $\gamma$ -globin mRNA translation through bypassing a noncanonical uORF.** A, the sequences of the 5' UTR of human  $\gamma$ -globin mRNA are shown. Six putative upstream alternative translation initiation sites (ATIS) in the 5' UTR of  $\gamma$ -globin mRNA are highlighted in boxes. B, percentage increase in the 5' UTR reporter activity of human  $\gamma$ -globin uORF-luciferase with loss-of-function (LOF) point mutations in the six putative upstream ATIS over that with wild-type in K562 cells. ATIS #1: "ACG" to "ACC", #2: "AGG" to "AGC", #3: "AUC" to "ACC", #4: "AUA" to "CUA", #5 "AAG" to "AAC", #6: "ACG" to "ACC". C, relative 5' UTR reporter activity of  $\gamma$ -globin uORF-luciferase with a LOF point mutation in ATIS #3 in K562 cells overexpressing PRMT1 WT and PRMT1  $\Delta$ . D, Relative  $\gamma$ -globin 5' UTR reporter activities in K562 cells overexpressing PRMT1 WT and PRMT1  $\Delta$ , with and without 5  $\mu$ M tunicamycin (Tm) treatment. E, RT-qPCR analysis of  $\gamma$ -globin mRNA expressions in K562 cells treated with DMSO, 2  $\mu$ M of thapsigargin (Tg), or 5  $\mu$ M of Tm for 6 h and 24 h. F, representative western blots of  $\gamma$ -globin, p-eIF2 $\alpha$ , eIF2 $\alpha$ , and  $\alpha$ -tubulin in K562 cells treated with DMSO, 5  $\mu$ M of Tm, or 2  $\mu$ M Tg for 48h. G, representative western blots of  $\gamma$ -globin and  $\beta$ -actin in K562 cells with PRMT1 silencing (left) and PRMT1 catalytic-dead mutant overexpression (right) with or without DDX3 inhibition using RK33. All values represent the mean  $\pm$  s.d. of three biological repeats. n.s., not significant,  $p > 0.05$ ; \*  $p < 0.05$ , \*\*  $p < 0.01$  compared with the indicated control. Statistical testing was performed using the two-sided Student's *t*-test.

that PRMT1 selectively represses the translation of  $\gamma$ -globin mRNA. We next sought to address the mechanism by which  $\gamma$ -globin mRNA translation is suppressed by PRMT1. The translation of mRNAs is often regulated by the 5' untranslated region (5'UTR), which can be composed of several regulatory elements (23). To explore whether the 5' UTR of  $\gamma$ -globin mRNA is responsible for the translation regulation mediated by PRMT1, we cloned the 5'UTR of  $\gamma$ -globin mRNA before the open reading frame (ORF) of the firefly luciferase reporter gene. The reporter activities (Firefly/Renilla) of these constructs were measured and normalized to their luciferase mRNA levels. We observed that the  $\gamma$ -globin 5'UTR reporter activity was increased significantly when the catalytic activity of PRMT1 is abrogated (Fig. 2E), suggesting that PRMT1 may control  $\gamma$ -globin mRNA translation *via* the regulatory element within its 5'UTR.

#### **PRMT1 deficiency promotes $\gamma$ -globin mRNA translation through bypassing a noncanonical uORF**

We then analyzed the 5'UTR sequence of the  $\gamma$ -globin mRNA. Notably, the 5'UTR of human  $\gamma$ -globin, but not  $\beta$ -globin mRNA, contains six putative alternative translation initiation sites (ATIS), which are all out of frame of the AUG initiation codon of the main ORF (Figs. 3A and S2A). If ribosome initiates from these upstream ATISs resulting in the translation of upstream open reading frames (uORFs), the translation of the downstream ORF will be often repressed. Recently, the application of ribosome profiling has revealed thousands of novel initiation events at non-AUG codons (24, 25). Indeed, these putative ATISs within  $\gamma$ -globin 5'UTR are all composed of near-cognate non-AUG codons, one nucleotide different from AUG, which may initiate translation at a reduced efficiency compared with AUG codons. On the other hand, it has been proposed that the initiation from a non-AUG codon can become more efficient when a strong thermostable hairpin structure is present near the codon (26, 27). Interestingly, we observed that the 5'UTR of  $\gamma$ -globin mRNA may form a strong hairpin structure (Fig. S2B), which can increase the recognition of these ATISs by the ribosome and thus suppress the translation of downstream main ORF of  $\gamma$ -globin.

In order to determine whether these ATISs are indeed functional in repressing  $\gamma$ -globin mRNA translation, we mutated each of the six ATISs without notably affecting the hairpin structure of  $\gamma$ -globin 5'UTR (Fig. S2C) and cloned these 5'UTR sequences into the firefly reporter construct. The reporter activities (Firefly/Renilla) of these constructs in K562 cells were measured and normalized to their luciferase mRNA levels. Although we cannot exclude the possibility that these mutations may affect the binding of some transcription factors or chromatin regulators, their mRNA expression levels remained unchanged (Firefly/Renilla mRNA ratios are shown in Fig. S2D). Interestingly, only disrupting the third ATIS (AUC codon) resulted in a significant upregulation of the  $\gamma$ -globin 5'UTR reporter activity, while the others did not (Fig. 3B). For decades, studies have provided evidences that specific nucleotide flanking the initiation codon namely the "Kozak

sequence" (28) may largely influence the recognition of non-AUG initiation codons, where the A or G nucleotide at position -3 and G nucleotide at position +4 stabilize the interaction of preinitiation complex (PIC) with the initiation codon (29, 30). Consistent with this model, the Kozak sequence flanking the AUC codon in the  $\gamma$ -globin 5'UTR exhibits the highest strength (Fig. S2E). When mutating the "AUC" initiation codon to the canonical "AUG" codon, we observed a further decrease in the 5'UTR reporter activity (Fig. S2E). These results suggest that the AUC codon initiates a uORF that represses the translation of downstream main ORF of  $\gamma$ -globin.

To explore whether PRMT1 suppresses  $\gamma$ -globin mRNA translation through the AUC-mediated uORF, we transfected the 5'UTR reporter constructs into cells stably expressing wild-type and catalytic-dead PRMT1. Intriguingly, mutating the AUC ATIS notably increased the reporter activity in the wild-type cells, while was not able to further enhance the reporter activity in the catalytic-dead PRMT1-expressing cells (Fig. 3C), indicating that PRMT1 deficiency drives the translation of  $\gamma$ -globin mRNA through bypassing the AUC-mediated uORF.

#### **Activating integrated stress response (ISR) can induce $\gamma$ -globin translation through uORF bypass, but may also render $\gamma$ -globin mRNA reduction**

Previous studies have addressed that activation of the ISR pathway increases the fetal hemoglobin at the translational level (17, 31). An outstanding question remaining is whether this translation upregulation upon ISR is through bypassing AUC-mediated uORF within  $\gamma$ -globin 5'UTR. In this respect, we discovered that tunicamycin-induced ISR enhanced the activity of the reporter containing the wild-type  $\gamma$ -globin 5'UTR, but not the AUC codon-mutated one, demonstrating that ISR activation-mediated  $\gamma$ -globin mRNA translation is achieved through bypassing the AUC-mediated uORF (Fig. 3D). However, it is also important to note that activating an important node of the stress response, HRI- eIF2 $\alpha$  signaling may also inhibit  $\gamma$ -globin mRNA expression (18). This is because long-term stress may shut down the synthesis of essential transcription factors important for  $\gamma$ -globin transcription. Therefore, it is likely that short-term stress can indeed increase  $\gamma$ -globin translation through bypassing the uORF, but long-term stress may eventually result in  $\gamma$ -globin reduction due to transcriptional suppression. To understand whether this is the case, we performed tunicamycin treatment for 24 h and also extended the treatment to 48 h. In accordance to previous publications, short-term (24-h) induction of ISR significantly upregulated  $\gamma$ -globin protein (Fig. S2F). Interestingly, at early timepoint (6 h), ISR activation by tunicamycin did not affect  $\gamma$ -globin mRNA expression, while a significant reduction in  $\gamma$ -globin mRNA expression was observed at 24-h by both tunicamycin and thapsigargin treatment (Fig. 3E). Importantly, as a result of the transcription suppression, the  $\gamma$ -globin protein abundance was eliminated, even reduced upon prolonged stress (48-h) (Fig. 3F). These

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results suggest that even though ISR activation stimulated the translation of  $\gamma$ -globin, the transcription of which was repressed. Thus, activating stress might not be beneficial for  $\gamma$ -globin synthesis eventually, and a strategy to induce uORF-bypassing without reducing  $\gamma$ -globin mRNA expression is urgently needed.

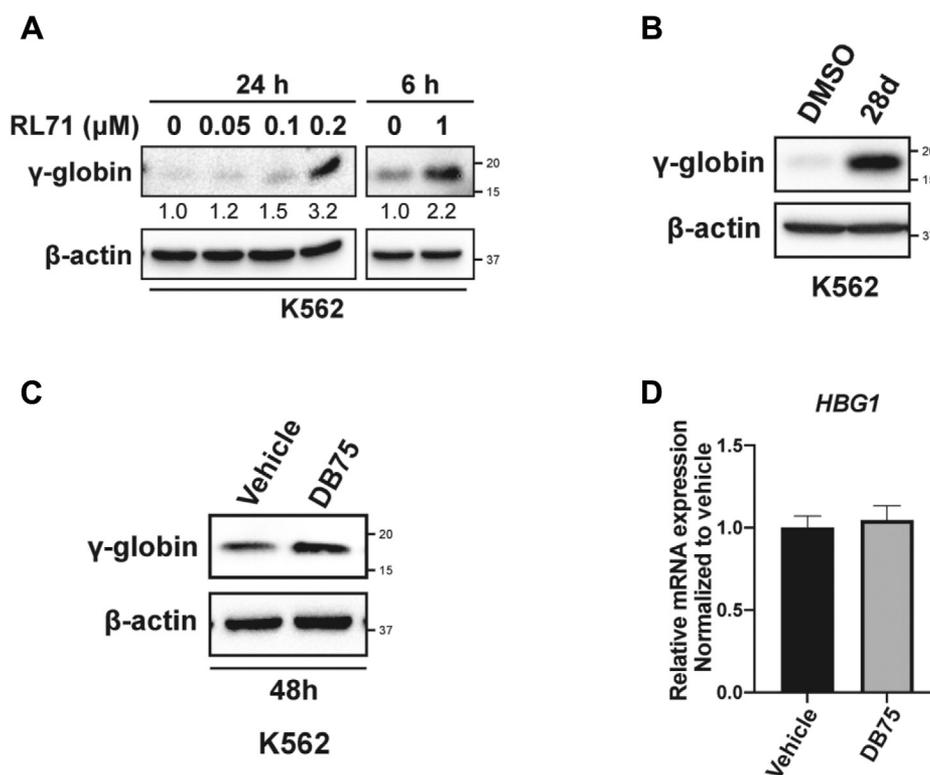
### Silencing PRMT1 stimulates uORF bypassing associated with DDX3

The next key question is: what is the underlying mechanism of PRMT1-mediated uORF bypassing? To answer this question, we explored some translation regulators involved in uORF bypassing that may act as a substrate of PRMT1. Interestingly, we observed that PRMT1 could directly interact with an ATP-dependent RNA helicase, DDX3 (Fig. S3A), and mediated its methylation (Fig. S3B). DDX3 is well conserved from yeast to mammal and of great importance for mRNA translation (32). A recent study demonstrated that the activity of DDX3 is required for unwinding the RNA structures in 5' untranslated regions (5' UTR) of mRNAs, which is key for bypassing the translation from AITS and subsequently mediating protein synthesis from the main ORF (33). We therefore questioned whether as a substrate of PRMT1, DDX3 is a key downstream player responsible for PRMT1-mediated  $\gamma$ -globin translational inhibition. Intriguingly, inhibiting DDX3 activity through a specific inhibitor RK33 (34, 35) significantly reverted

the increase in  $\gamma$ -globin protein abundance mediated by either silencing PRMT1 or expressing its enzyme-dead mutant (Fig. 3G), demonstrating that PRMT1-blockage-mediated  $\gamma$ -globin mRNA translation increase requires DDX3 activity. Furthermore, we have mutated two putative PRMT1-mediated arginine methylation sites on DDX3 [reported previously (36)] and observed that these mutants on DDX3 (R101 K, R632 K) resulted in a notable increase in the  $\gamma$ -globin protein abundance (Fig. S3C). Together, these results suggest that PRMT1 may inhibit  $\gamma$ -globin translation through mediating DDX3 arginine methylation even though the precise role of PRMT1 in  $\gamma$ -globin translation merits further study.

### Pharmacologically targeting type I PRMTs induces $\gamma$ -globin expression

As a barrier of  $\gamma$ -globin translation, AUC-mediated uORF can be bypassed by ISR activation (Figs. 3D and S2F). Therefore, we sought to test whether a compound targeting the ATPase Sarcoplasmic/Endoplasmic Reticulum  $\text{Ca}^{2+}$  Transporting 2 (ATP2A2) thus inducing ISR can stimulate  $\gamma$ -globin production in a short-term manner. Exposure to RL71, an ATP2A2 specific inhibitor (37), led to an induction of  $\gamma$ -globin protein expression, at as early as 6-h treatment in K562 cells (Fig. 4A). Nevertheless, due to the fact that long-term stress may not be beneficial in  $\gamma$ -globin synthesis, we sought to design a rational pharmacological approach to induce  $\gamma$ -globin expression at



**Figure 4. Pharmacologically targeting PRMT1 and activating ISR induce  $\gamma$ -globin protein expression.** A, representative western blots of  $\gamma$ -globin and actin in K562 cells treated with an ER-stress inducer RL71 (a compound targeting the endoplasmic reticulum  $\text{Ca}^{2+}$  transportation) at indicated concentration for 24 h and 6 h respectively. B, representative western blots of  $\gamma$ -globin and actin in K562 cells treated with DMSO or 1  $\mu\text{M}$  28d for 48 h. C, representative western blots of  $\gamma$ -globin and actin in K562 cells treated with vehicle or 8  $\mu\text{M}$  PRMT1 inhibitor DB75 for 48 h. D, RT-qPCR analysis of relative  $\gamma$ -globin mRNA expression in K562 cells treated with 8  $\mu\text{M}$  PRMT1 inhibitor DB75 for 48 h. All experiments were performed with three biological repeats.

both mRNA and protein levels to ameliorate hemoglobinopathies. Given the finding that several PRMTs (e.g., PRMT4, PRMT6, etc.) suppress  $\gamma$ -globin transcription (Fig. 1A) and that PRMT1 guides a new layer of uORF-mediated translation control of  $\gamma$ -globin, we utilized a newly developed compound that is able to target PRMT1 (28days). Compound 28d is a highly potent type I PRMT inhibitor, which suppresses PRMT1 and PRMT6 activity at an IC<sub>50</sub> of approximately 1 nM and PRMT4 at 60 nM (38). We found that 28d successfully inhibited the catalytic activity of PRMT1 assessed by the significant reduction of histone H4R3me2a modification, without affecting PRMT1 protein itself (Fig. S4). Interestingly, 28d was very potent at upregulating the  $\gamma$ -globin protein abundance in K562 cells (Fig. 4B), suggesting that targeting type I PRMTs pharmacologically may be a potential therapeutic strategy for hemoglobinopathies, with an advantage of stimulating  $\gamma$ -globin synthesis at both mRNA and protein levels. More specifically, we also employed a selective PRMT1 inhibitor, furamidine dihydrochloride (DB75), which does not target PRMT4, 5, and 6, to test if it also possesses a role in regulating  $\gamma$ -globin synthesis. As a result, we discovered that specifically targeting PRMT1 significantly increases  $\gamma$ -globin protein abundance without affecting its mRNA expression (Fig. 4, C and D), confirming our finding that PRMT1 controls  $\gamma$ -globin synthesis specifically at the translation level.

## Discussion

As is known for decades, the erythroid program of gene expression is determined by several essential transcription factors during erythropoiesis, and therefore, there is a historical focus on transcription as the primary regulatory mechanism. Nevertheless, emerging evidence has demonstrated that protein levels correlate poorly with transcript expression (39), and translational control may play a critical role in regulating many erythroid mRNAs during terminal differentiation. Compared with transcriptional regulation, mRNA translation allows for a relatively more rapid production of functional protein from the existing transcriptome and thus balances the protein synthesis in erythroid cells in response to the environmental changes, e.g., growth factor stimulation and iron shortage. Interestingly, our work and others' (11) have addressed that one subunit of the predominant type of protein that erythroid cells synthesize,  $\gamma$ -globin, is profoundly controlled at the translational level through the ISR. Our work further addressed that the 5'UTR of  $\gamma$ -globin mRNA harbors a non-AUG alternative translation initiation codon, which mediates the translation of a uORF that act as a suppressor of the translation of  $\gamma$ -globin ORF. It is also important to note that the translation of other globin subunits, such as  $\alpha$ -globin, is not induced upon ISR activation (HRI-eIF2 $\alpha$  pathway activation), instead, like many other proteins, its protein synthesis is significantly repressed upon stress. This regulation is functionally important, as it helps erythroid cells inhibit the accumulation of free globin subunits in respond to heme or iron shortage and mitigate oxidative stress (40). Therefore, it remains poorly understood why unlike that of  $\alpha$ - and  $\beta$ -globin,

the translational regulation of fetal  $\gamma$ -globin acts oppositely, which needs further investigation.

On the other hand, although ISR can stimulate  $\gamma$ -globin translation, long-term stress may also render a reduction in  $\gamma$ -globin mRNA expression (Fig. 3E). Therefore, another outstanding question is: what are the upstream regulators of the uORF-mediated  $\gamma$ -globin translation besides ISR? Indeed, ISR and the eIF2 $\alpha$  signaling activation seems to be important for  $\gamma$ -globin production (Fig. 3), yet the uORF composition of  $\gamma$ -globin (only one non-AUG mediated uORF) differs from that of canonical eIF2 $\alpha$  targets such as ATF4, which harbors two AUG-mediated uORFs in its 5'UTR. Till now the detailed mechanism of which eIF2 $\alpha$  regulates the translation of mRNA harboring only one uORF remains unclear. Therefore, it is likely that other components of the translation machinery are also involved in bypassing the uORF of  $\gamma$ -globin. Our work provided an example of the uORF bypassing through a specific ATP-dependent RNA helicase DDX3, which may facilitate  $\gamma$ -globin uORF bypass, as it is capable of controlling translation through regulating the usage of near-cognate upstream initiation codons that are proximal to secondary structure (33). The activity of DDX3 could be suppressed by the post-translational modification mediated by PRMT1, thus contributing to PRMT1-mediated  $\gamma$ -globin translation. These data would provide a new therapeutic window for targeting  $\gamma$ -globin translation without inducing unwanted stress.

Proteomics studies have revealed that several components of the translation machinery and RNA binding proteins are often methylated (41–44), and therefore members of the PRMT family have been shown to participate in the protein synthesis process *via* their catalytic activities. For example, PRMT5 can regulate IRES-dependent translation of specific transcripts (e.g., *MYC* and *CDKN1B*) through the methylation of hnRNP A1 (45); PRMT3 can methylate ribosomal protein S2, which is required for its efficiency in 40S ribosomal subunit assembly (46, 47). Our work here provided a mechanism that the methylation of DDX3 by PRMT1 may suppress the cap-dependent translation of  $\gamma$ -globin mRNA through the engagement of a non-AUG uORF. Indeed, a recent work has also identified that PRMT1 mediates the methylation of the translation initiation factors, leading to an alteration in the transcriptome of murine osteosarcomas (36). These studies suggest that there is a broad impact of PRMTs on protein synthesis important for various physiological processes, and misregulation of which may render human diseases (48). In this regard, our work together with previous discoveries that several PRMTs can regulate  $\gamma$ -globin transcription suggests that compounds targeting PRMTs may have the potential of inducing  $\gamma$ -globin expression at both the transcriptional and translational level, offering a therapeutic approach that can ameliorate human hemoglobinopathies.

## Experimental procedures

### Cell cultures, plasmids, and treatment

Human K562 and HEK-293T cells were cultured according to ATCC recommendations. K562 CRISPRi stable cell lines

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were created by infecting with Lentiviral constructs pLV-hU6-EF1a-GFP-Puro (with sgRNA cloned) together with the packaging plasmids psPAX2 and the envelope plasmids pMD2.G. sgRNA sequences are provided in Table S1. The PRMT1 catalytic-dead mutant (PRMT1 $\Delta$ ) was created by deleting amino acids 86–90 located in its S-adenosyl-L-methionine-binding motif. K562 stable cell lines were generated by overexpressing WT PRMT1 and PRMT1 $\Delta$  followed by FACS sorting. The oligonucleotides used to generate the deletion are provided in Table S2.

In total, 5  $\mu$ g/ml of Tunicamycin (Tm) and 2  $\mu$ M of thapsigargin (Tg) were used to induce ISR pathway in K562 cells for indicated time. DMSO was used as vehicle control, and the final concentration of DMSO used in cell culture medium was 1/2000(v/v).

For compounds treatment, RL71 and 28d were obtained from Dr Qiang Xu (37) and Dr Cheng Luo (38), respectively. Furamide (DB75) was purchased from Sigma. A 10 mM stock solution of RL71 and a 100 mM stock of DB75 were dissolved in H<sub>2</sub>O; a 50 mM stock solution of 28d was prepared in DMSO. Working solutions of all reagents were prepared by dilution in RPMI 1640 immediately prior to use. K562 cells were spun down, replaced with media containing indicated inhibitors, and cultured for indicated time. The final concentration and treating time were provided in figure legends.

### qRT-PCR

Total RNA was isolated from cells by RNeasy mini kit (Qiagen). cDNA was generated and analyzed by quantitative real-time PCR using PowerUP SYBR Green master mix reaction on Applied Biosystems QuantStudio 6 Flex Real-Time PCR System (Thermo Fisher). Q-RT-PCR primers are provided in Table S3.

### Western blot analysis

Cellular proteins were extracted by RIPA lysis buffer with the addition of Complete Mini Proteasome inhibitors (Roche) using standard procedures with commercial antibodies for PRMT1 and asymmetric dimethyl arginine motif (Cell Signaling Technology),  $\gamma$ -globin (Santa Cruz Biotechnology),  $\beta$ -actin (Thermo Fisher), p-eIF2 $\alpha$  Ser51 (Cell Signaling Technology), eIF2 $\alpha$  (Cell Signaling Technology),  $\alpha$ -tubulin (Cell Signaling Technology), H3 (Cell Signaling Technology), H4R3me2a (Active Motif). Protein quantifications were performed using Image J software.

### Luciferase reporter assay

K562 cells ( $1 \times 10^6$ ) were transfected with 4  $\mu$ g of pGL3 (Firefly luciferase) constructs containing full-length or mutant  $\gamma$ -globin 5' UTR and 200 ng of pRL (Renilla luciferase) plasmid using electroporation kit-V(Lonza) for 24 h. Half of the cells were processed with the Dual-luciferase Reporter Assay System (Promega); the other half were collected for RNA purification followed by twice Turbo DNase treatment to exclude the effect of transfected DNA, followed by reverse transcription and qPCR analyses. The Firefly to Renilla reporter ratio

was normalized to the ratio of their mRNAs. The oligonucleotides used to generate the mutant form of  $\gamma$ -globin 5'UTR are provided in Table S2.

### Polysome fractionation

PRMT1 WT and PRMT1  $\Delta$  cells were treated with 100  $\mu$ g/ml cycloheximide (Sigma) for 5 min at 37 °C, washed once with ice-cold PBS containing 100  $\mu$ g/ml cycloheximide, and lysed in 300  $\mu$ l of polysome lysis buffer for 30 min on ice. The supernatant was isolated and adjusted by OD260 and loaded onto 10%–50% sucrose gradients before centrifuged with a SW41Ti rotor at 38,000 rpm for 2.5 h at 4 °C. Samples were fractionated on an ISCO gradient fractionation system and fractions 2–14 were used for Q-RT-PCR analysis.

### Statistical analysis

All experiments were performed at least three independent biological repeats. Statistical significance between experimental groups was determined as described in the figure legends.

### Data availability

All data are contained within the article.

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*Supporting information*—This article contains [supporting information](#).

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*Authors contribution*—Y. W. and J. L. performed the experiments, analyzed the data, and drafted the article; X. L., M. L., and X. P. provided technique support; C. L. provided the PRMT inhibitors; Y. X. and Q. Z. supervised the study.

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*Abbreviations*—The abbreviations used are: 5'UTR, 5' Untranslated Region; ATF4, Activating Transcription Factor 4; ATIS, Alternative Translation Initiation Sites; ATP2A2, ATPase Sarcoplasmic/Endoplasmic Reticulum Ca<sup>2+</sup> Transporting 2; DDX3, Dead-Box Polypeptide 3; eIF2 $\alpha$ , Translation Initiation Factor 2; HbA, Adult Hemoglobin; HbF, Fetal Hemoglobin; HRI, Heme-Regulated Inhibitor; ISR, Integrated Stress Response; PABP1, Polyadenylate-Binding Protein 1; PIC, Preinitiation Complex; PRMT1, Protein Arginine Methyltransferase 1; SCD, Sickle Cell Disease; uORF, upstream Open Reading Frame.

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