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GAPDH is involved in the heme-maturation of myoglobin and hemoglobin

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Abstract

GAPDH, a heme chaperone, has been previously implicated in the incorporation of heme into iNOS and soluble guanylyl cyclase (sGC). Since sGC is critical for myoglobin (Mb) heme-maturation, we investigated the role of GAPDH in the maturation of this globin, as well as hemoglobins α, β, and γ. Utilizing cell culture systems, we found that overexpression of wild-type GAPDH increased, whereas GAPDH mutants H53A and K227A decreased, the heme content of Mb and Hbα and Hbβ. Overexpression of wild-type GAPDH fully recovered the heme-maturation inhibition observed with the GAPDH mutants. Partial rescue was observed by overexpression of sGCβ1 but not by overexpression of a sGCΔβ1 deletion mutant, which is unable to bind the sGCα1 subunit required to form the active sGCα1β1 complex. Wild type and mutant GAPDH was found to be associated in a complex with each of the globins and Hsp90. GAPDH at endogenous levels was found to be associated with Mb in differentiating C2C12 myoblasts, and with Hbγ or Hbα in differentiating HiDEP-1 erythroid progenitor cells. Knockdown of GAPDH in C2C12 cells suppressed Mb heme-maturation. GAPDH knockdown in K562 erythroleukemia cells suppressed Hbα and Hbγ heme-maturation as well as Hb dimerization. Globin heme incorporation was not only dependent on elevated sGCα1β1 heterodimer formation, but also influenced by iron provision and magnitude of expression of GAPDH, d-aminolevulinic acid,

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AUTHOR CONTRIBUTIONS

Arnab Ghosh and Dennis J. Stuehr designed the experiments. Arnab Ghosh and Dennis J. Stuehr analyzed the data and wrote the manuscript. Arnab Ghosh, Blair Tupta, Eric Stuehr and Mamta P. Sumi performed all cell culture and biochemical studies. Elizabeth A. Sweeny and Brandon Smith generated stable cell lines expressing GAPDH and its mutants.

DISCLOSURES

The authors declare no conflict of interest.

SUPPORTING INFORMATION

Additional supporting information may be found in the online version of the article at the publisher's website.

and FLVCR1b. Together, our data support an important role for GAPDH in the maturation of myoglobin and γ, β, and α hemoglobins.

Keywords

dimerization; GAPDH; globin; heme-maturation

1 | INTRODUCTION

Hemeproteins play multiple roles in cellular processes including respiration, sensing of diatomic gases, signal transduction, regulation of transcription, translation, and various mitochondrial functions.[1–5] The strong affinity of heme toward molecular oxygen makes it possible for two hemeproteins, hemoglobin and myoglobin, to function as major oxygen transporters.[6–11] While the expression and maturation of these globins occurring in erythropoiesis or during myogenesis are subject to multiple levels of regulation,[12–19] our understanding of maturation of these globins is still incomplete. Despite being two of the oldest globins known to protein science,[7,20–25] the heme-maturation of these two proteins is still not fully understood, and we do not know the critical details of their heme insertion steps or what partner proteins may assist these globins during such processes.

Previous studies[26,27] on hemoglobin (Hb) and myoglobin (Mb) maturation have implicated chaperon hsp90 and its co-chaperons or independently functioning small chaperons, e.g., AHSP to be involved in their heme-maturation.[28,29] However since recent studies on GAPDH have given unequivocal evidence for its role in the heme-insertion of some key hemeproteins[30–32] and the fact that hsp90 and GAPDH often co-immunoprecipitate in molecular complexes with other proteins[33,34] and also with such hemeproteins,[32] led us to investigate the role of GAPDH in hemoglobin and myoglobin maturations. Based on the current findings of glycolytic enzyme GAPDH in heme delivery, it has been termed as a heme-chaperone and is attributed to bind and transfer labile heme to downstream target proteins.[31,32,35] Previously GAPDH has been implicated in iNOS heme-insertion[30] and our more recent study indicates that it also transfers the heme to sGCβ1.[32] Some previous reports also indicate that GAPDH can interact with Hb.[36] Together with these mounting evidences and the fact that sGC was crucial for fetal hemoglobin maturation (Hbγ)[37] and that an active sGC is needed for heme-maturation of Mb,[27] we were inquisitive to determine the role of GAPDH in the maturation of these globins.

In this study we expressed GAPDH and its mutants, H53A or K227A transiently or as stable lines, along with Hbαβ or Mb and determined its effect on globin heme and also compared the ability of GAPDH to form a complex with hsp90 and the globins. The GAPDH mutant H53A displays a decreased heme-binding affinity[31] while the K227A is known to act as a dominant negative inhibitor of iNOS heme-insertion and also displays reduced heme-binding properties.[30] Since the heme-binding function of GAPDH is an essential aspect that enables its heme-delivery, we used these mutants alongside WT GAPDH to determine their impact on heme-maturation of these cytosolic globins. To ascertain the physiological relevance of GAPDH function in promoting globin heme-insertions during

red cell maturation or muscle formation, we first tested GAPDH-globin associations by doing IPs in differentiating erythroid progenitors, HiDEP-1[26,38] or in differentiating C2C12 myoblasts[27,39] and then determined the impact of silencing the endogenous GAPDH in differentiating K562 cells {expressing fetal (Hbγ) and alpha (Hbα)},[40] or C2C12 myoblasts expressing Mb.[27] Lastly we determined the impact of overexpressing GAPDH or sGC to reverse the downregulation of globin heme caused by the GAPDH mutants H53A or K227A and studied the involvement of ferrous ion (Fe^{2+}) plus delta-aminolevulinate (D-Ala)[13,41] and the mitochondrial heme exporter, FLVCR1b[42,43] on globin maturation taking Mb as a target globin.

2 | MATERIAL AND METHODS

2.1 | Reagents

All chemicals were purchased from Sigma (St. Louis, MO) and Fischer chemicals (New Jersey). NO donor, 2,2′-(Hydroxynitrosohydrazino) *bis*-ethanamine, (NOC-18 or DETA NONOate), phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX), protein synthesis inhibitor (cycloheximide), hemin and sGC activator BAY 41–2272 were all purchased from Sigma. Fetal bovine serum and AB human serum were purchased from Atlanta Biologicals. HA-tagged GAPDH construct and its mutants HA-H53A and HA-K227A were obtained from Dr. Stuehr's lab (Cleveland Clinic). Stable siRNA resistant GAPDH (SRG) cell line in HEK293 cells expressing Myc tagged GAPDH or its mutants Myc-H53A and Myc-K227A were generated in Dr. Stuehr's lab and used in our studies. Myc-DDK tagged human hemoglobin alpha (Hbα), beta (Hbβ), human skeletal muscle myoglobin expression construct and Myc-tagged mitochondrial heme transporter cDNA construct, FLVCR1b were purchased from OriGene (Rockville, MD, USA). sGCβ1 deletion constructs (sGC-β1 Δ204–244 + Δ379–408) was a gift from Dr. Andreas Papapetropolous (Athens University, Athens, Greece). Small interfering RNA (siRNA) specific to human GAPDH (D-001830–01) or mouse GAPDH (L-040917–00-00005) was purchased from GE Healthcare Dharmacon (Lafayette, CO, USA). Human immortalized erythroid progenitor cells, HiDEP-1 were obtained from Dr. Y. Nakamura (RIKEN BioResource Center, Japan). Chemicals and growth factors required for culture and differentiation of human erythroid progenitor cells, e.g., dexamethasone (DEX), doxycycline (DOX), stem cell factor (SCF) and erythropoietin (EPO) were purchased from StemCell technologies (Cambridge, MA) while insulin, heparin and holo-transferrin was purchased from Sigma. HEK 293T, HEK 293, K562 and C2C12 cells were purchased from American Type Culture Collection (ATCC; Manassas, VA, USA). cGMP ELISA assay kits were obtained from Cell Signaling Technology (Danvers, MA, USA), Protein G-sepharose beads were purchased from Sigma and molecular mass markers were purchased from Bio-Rad (Hercules, CA, USA).

2.2 | Antibodies

Antibodies were purchased from different sources. Table S1 describes various types of antibodies used and its source.

2.3 | siRNA resistant GAPDH (SRG) cell line production

The siRNA resistant GAPDH (SRG) cell lines were made by transfecting HEK293 cells with Myc-tagged human GAPDH variants containing five silent point mutations which together render them resistant to human GAPDH targeting siRNA (Dharmacon D-001830–01). Hence the GAPDH siRNA can no longer bind the altered mRNA sequence of the GAPDH variants and thus the exogenous protein cannot be silenced. Stably transfected cells were selected for by growth in DMEM supplemented with 10% FBS and 400 μg/ml G-418 until all non-transfected control cells were dead. Expression of Myc-tagged GAPDH was confirmed by western blot and cells were maintained in media containing 400 μg/ml G-418 for all experiments. In all experiments involving silencing of endogenous GAPDH by siRNA, the culture media was supplemented with 1 mM sodium pyruvate to reduce effects of toxicity.[44]

2.4 | Cell culture, transient transfection, growth/differentiation of cells, and gene silencing by siRNA

All cell lines were grown and harvested as previously described.[26,45] Cultures (50%–60% confluent) of HEK 293T cells were transiently transfected with either Myc-Mb or Myc-Hb-αβ along with wild type GAPDH construct or its mutants (H53A or K227A) and expressed for 42 h before harvest. In parallel experiments HEK 293 cells stably expressing GAPDH or its mutants H53A or K227A were similarly transfected with Mb or Hb-αβ before harvest. In order to determine the recovery of the globin heme from the impact of GAPDH mutants H53A or K227A, separate experiments were done where the globins were transfected with GAPDH or sGCβ1 or its deletion construct, sGC-β1 Δ204–244 + Δ379–408 in HEK stables expressing H53A or K227A, allowed to express for 42 h before harvest. In experiments to ascertain the effect of Fe^{2+} + D-Ala, FLVCR1b and GAPDH on globin heme-maturation, or to determine whether Fe^{2+} + D-Ala can rescue the globins from the negative impact of H53A or K227A, Mb + FLVCR1b were co-transfected ∓ GAPDH in HEK cells or Mb + FLVCR1b were expressed in HEK stables and after 24 h were given 100 μM ferric citrate and 1 mM D-Ala for varying time periods between 1 and 16 h before harvest. For silencing of endogenous GAPDH, 80 nM of human GAPDH specific siRNA (Dharmacon) was transiently transfected in HEK cells for 24 h prior to transfection of Myc-Mb/Myc-Hbαβ which along with the siRNA, was allowed to express for additional 42 h before cell harvest. Similarly control scrambled siRNA was also transfected in parallel followed by globin transfections. *Culture and differentiation of erythroid progenitor cells*: Immortalized human erythroid progenitor cells HiDEP-1 cells were cultured as described earlier[26] and following protocols obtained from Dr. Y. Nakamura (RIKEN BioResource Center, Japan). In brief the HiDEP-1 cells were allowed to proliferate vigorously in Stem Span SFEM media (StemCell Technologies) containing EPO (3 IU/ml), SCF (50 ng/ml), DEX (10^{-6}M) and DOX (1 μg/ml). The cells were then split into desired number of T75 flasks and induced to differentiate by switching to differentiation media (IMDM media from Sigma, I:3390) containing 2% FBS + 3% AB human serum (Atlanta Biologicals), EPO (3 IU/ml), insulin (10 μg/ml), holo-transferrin (500 μg/ml), and heparin (3 U/ml). The cells were allowed to differentiate under these conditions and at intervals between 0 and 16 days, the cells were then harvested for immunoprecipitation (IP) assays. *Culture and differentiation of K562 and C2C12 cells*: K562 cells were cultured as previously described.[26] In order to determine

the role of GAPDH in Hbγ heme-maturation, K562 cells expressing basal levels of Hb-γ were treated with SA for 72 h, while parallel cultures were SA treated and simultaneously transfected with human GAPDH siRNA for 72 h to silence the endogenous GAPDH, followed by treatment with cycloheximide (10 μg/ml) for 30 min and then given hemin (5 μM) for additional 3 h before being harvested. In other experiments the K562 cells were transfected with GAPDH siRNA or transfected with scrambled siRNA and cultured for 24–72 h and then given 50 μM of hemin to allow for differentiation for 24–72 h before harvest. In separate experiments K562 cells were treated with variable doses of NO donor NOC-18 (0–100 μM) for 24 h and the effect of NO on Hbγ maturation determined.

For C2C12 culture, the myoblasts were induced to differentiate into myotubes between 0 and 96 h by growing in media containing 2% horse serum, with media changed every 48 h.[27] To investigate the effect of GAPDH silencing on Mb heme-maturation, mouse specific GAPDH siRNA (Dharmacon) was used to downregulate the endogenous GAPDH during C2C12 differentiation. The C2C12 myoblasts were transfected with 70 nM of GAPDH siRNA or control scramble siRNA for 48 h and were then induced to differentiate to myotubes for additional 96 h. The cells were imaged every 24 h before being harvested.

At the point of cell harvest, the monolayers were washed twice with 4 ml cold PBS containing 1 mg/ml of glucose, and cells on each plate were collected by scraping in presence of 250 μl of classical lysis buffer (40 mM EPPS buffer pH 7.6, 10% Glycerol, 3 mM DTT, 150 mM NaCl and 1% NP40) or at times cell lysis buffer from cGMP estimation kit (Cell Signaling Technology) was used. The collected cells were lysed by 3 cycles of freeze-thawing (in liquid nitrogen and at 37°C, respectively). The lysates were centrifuged for 30 min at 4°C and the supernatants were collected and stored at −80°C. Total protein contents of the supernatants was determined using the Bio-Rad protein assay kit. In all cases wherever applicable cell supernatants were assayed for protein expression by western blot, binding assays by IPs, depiction of heme-maturation by heme staining and soret absorption by UV-visible spectroscopy, as indicated.

2.5 | Western blots, heme staining and immunoprecipitations (IPs)

Western blots were performed using standard protocols as previously mentioned. For westerns, 50–80 μg of the supernatants were run on SDS-PAGE (8% or 15%), transferred to the same PVDF membrane, probed with a specific antibody and developed using ECL reagent. In all cases β-actin was used as a loading control. Multiple protein detection was achieved by stripping the membranes and re-probing with specific antibodies. Heme staining of cell supernatants from differentiating K562 (75 μg) or C2C12 cells (350 μg) or from transfected HEK cell supernatants (150 μg) cells was done as previously described.[26,27] For immunoprecipitations (IP), 600 μg of the total cell supernatant was precleared with 20 μl of protein G-sepharose beads (Amersham) for 1 h at 4°C, beads were pelleted, and the supernatants incubated overnight at 4°C with 3 μg of the indicated antibody (Myc/DDK/sGCβ1/Mb antibodies). Protein G-sepharose beads (20 μl) were then added and incubated for 1 h at 4°C. The beads were micro-centrifuged (6000 rpm), washed three times with wash buffer (50 mM HEPES pH 7.6, 100 mM NaCl, 1 mM EDTA and 0.5% NP-40) and then boiled with SDS-buffer and centrifuged. The supernatants were then loaded on

SDS-PAGE gels and western blotted with specific antibodies. Band intensities on westerns were quantified using Image J quantification software (NIH).

2.6 | cGMP enzyme-linked immunosorbent assay

The cGMP concentration in various cell supernatants made from intact cells that had been given NO from NO donor NOC-18 (variable concentrations between 0 and 100 μM as indicated) or sGC activator BAY 41–2272 in the presence of 250 μM IBMX, was estimated using the cGMP ELISA assay kit (Cell Signaling Technology).[46] The estimated cGMP concentrations as determined by ELISA was a measure of sGC activity in the cells and were determined from the mean values of three independent experiments ($n = 3$).

2.7 | UV-visible absorption spectroscopy

UV-visible absorption spectra of cell supernatants were recorded at room temperature between 350 and 700 nm on a Shimadzu spectrophotometer (Shimadzu, Kyoto, Japan). Equal amounts of total protein supernatants were used for respective wavelength scans. The heme content for Mb or Hb was determined from the Soret absorption peak at 409 (for Mb)/414 (for Hb) by using the extinction coefficient of 179 000 M^{-1} cm^{-1} (for Mb at 409 nm)/342 500 M^{-1} cm^{-1} (for Hb at 414 nm) and a manipulation to account for the variable absorbance contributions that were attributable to sample turbidity. This involved creating a baseline for each scan by drawing a line that connected the absorbance values at 380 and 470 nm. The additional Soret absorbance at 409/414 nm above this baseline was then used to calculate the Mb/Hb heme content.[27]

3 | RESULTS

3.1 | Impact of wild type GAPDH and its mutants H53A or K227A on globin heme-maturation

In order to determine the impact of GAPDH on globin heme-maturation we transfected myc-tagged Myoglobin (Mb) or hemoglobin (Hb α and β) constructs with HA-tagged GAPDH or its mutants H53A and K227A in HEK 293T cells. As depicted by western blots and in-gel heme-stains in Figures 1 and 2A,B, overexpression of GAPDH increased the heme levels of Mb and Hb, while GAPDH mutants H53A and K227A significantly downregulated the globin heme without changes in protein expression of the transfected globins. Immunoprecipitation (IPs) assays and its corresponding mean densitometries as shown in Figures 1 and 2C,D suggests that GAPDH or its mutants-globin or hsp90-globin interactions are at its peak with H53A mutant when the heme of the globins is the lowest (Figures 1B and 2B), in agreement to our previous findings that client hemeprotein-hsp90 interactions increase with heme-depletion of the client hemeproteins.[45,47] The GAPDH mutant-Hb (H53A and K227A) interactions seem to be in an irreversibly locked state (Figure S1) as it cannot be dissociated with even hemin supplementation, suggesting a blockage in Hb heme-insertion.[26] Repeating these experiments by determining how stable expression of GAPDH or its mutants would impact globin heme, we generated stable lines of GAPDH and its mutants (H53A and K227A) in HEK293 cells. As depicted in Figure 3, Mb or Hb (α and β) were transiently transfected in these lines and the effect on globin heme was determined. These results of globin transfections in stable lines corroborated

with what we found with transient transfections of GAPDH and its mutants. As shown in Figures 3A–D and S2, GAPDH upregulated, while H53A and K227A downregulated myoglobin and hemoglobin heme-maturations. The globin-hsp90 interactions were in line with client protein-hsp90 interactions where interactions increased with globins becoming heme-free (apo) and had the highest impact for H53A mutant (Figure 3C,F). Together our data indicates that cellular GAPDH is involved in heme-maturation of Mb and Hb (α and β), while mutants H53A and K227A can inhibit these globin heme-maturations.

3.2 | Impact of silencing endogenous GAPDH on stable lines expressing GAPDH or its mutants H53A or K227A on Mb and adult Hb (α and β) heme-maturations

To more clearly determine the influence of the expressed GAPDH or its mutants in the stable lines on the globin heme, we silenced the endogenous GAPDH by SiRNA in the stable lines. As depicted in Figure 4, the harvested cells were assayed for protein expression by westerns and heme estimations by in gel heme stains of corresponding cell supernatants (Figure 4A–E). Our data indicates that silencing of GAPDH by siRNA in H53A and K227A stable lines elevates the dominant negative effect of these mutants on Mb or Hb heme, relative to stable lines expressing Mb or Hb without GAPDH siRNA. This suggests that endogenous GAPDH is intricately involved in the maturation of Mb and Hb (α and β).

3.3 | GAPDH binding to fetal (γ) and alpha Hb (α) during differentiation of erythroid progenitors (HiDEP-1) and its impact on fetal (γ)/alpha Hb (α) heme-maturation by silencing of GAPDH

Performing IPs on differentiating erythroid progenitor cells (HiDEP-1, Figure 5A,B)[26] expressing Hbα and Hbγ, revealed that both fetal (γ) and alpha Hb (α) binds to GAPDH (Figure 5A–C). Interestingly Hbα differentiation involved the alpha hemoglobin stabilizing protein (AHSP, the small chaperon specific for Hbα heme-maturation) which was in complex with GAPDH and this AHSP was absent from the fetal (γ) Hb immunoprecipitates (Figure 5A,B). Similarly hsp90 was associated with Hbγ pull downs but was absent from the Hbα complexes (Figure 5A,B), suggesting specific complexes exist during maturation of these two globins in red cells.[23,40,48,49] The densitometries of bound proteins revealed that while GAPDH bound to Hbγ/α peaked at the initial phase of differentiation (day 2, Figure 5A–C), chaperones hsp90 or AHSP association with respective globins (γ or α) peaked between 4 and 6 days (Figure 5C), suggesting that differences may exist in the functions of GAPDH and these chaperons during heme-maturation of erythroid cells.

Having determined the existence of these complexes during HIDEP-1 differentiation we wanted to further elucidate the role of GAPDH in fetal (γ) Hb heme-maturation. For this we took K562 cells treated them with heme-biosynthesis inhibitor, succinyl acetone (SA) for 72 h and simultaneously silenced the endogenous GAPDH by siRNA (80 nM). The cells were then given micromolar amounts of hemin (5 μM) for additional 3 h before harvest and cell supernatant generation. Non-SA treated and SA treated cells without GAPDH siRNA were used as controls. As depicted by heme-staining (Figure 5D,E), cells without GAPDH siRNA readily showed heme-insertion into the fetal (γ) Hb and those cells with their endogenous GAPDH silenced displayed marked inhibition of fetal (γ) Hb heme-insertion, without change in their Hb protein levels (Figure 5D,E). To further test the role of GAPDH

in fetal (γ) Hb heme-maturation we differentiated K562 cells by hemin (50 μM) treatment from 0 to 3 days and simultaneously used either GAPDH siRNA or scrambled siRNA as controls. This way doing GAPDH silencing by siRNA on K562 cells was much feasible on a shorter time period of differentiation (3 days) relative to a much longer period of 16 days for erythroid progenitors like HiDEP-1.[26] As depicted in Figure 5F,G, GAPDH silencing during differentiation significantly downregulated fetal (γ) Hb and alpha Hb (α) heme-maturations and also hindered their ability to form dimers (Figure 5F). The heme soret peak of Hb (α and γ) (Figure 5H) from the corresponding spectral traces also suggested significant inhibition of Hb heme-insertion during silencing of GAPDH. Together our data indicates that GAPDH can associate with both fetal (γ) and alpha Hb (α) during differentiation of erythroid progenitors, suggesting that GAPDH-Hb interactions are physiologically relevant as it occurs during red cell maturation, lowering of GAPDH protein levels by siRNA in K562 cells inhibits heme-insertion into the fetal (γ) and alpha Hb (α), and the subsequent dimerization (Hbγ-γ/Hbα-α/Hbγ-α) of these globins during erythroid differentiation.

3.4 | GAPDH silencing in differentiating C2C12 myoblasts inhibits Mb heme-maturation

In order to determine the physiological relevance of GAPDH promoted heme-maturation of Mb, we lowered the endogenous GAPDH protein levels by siRNA in differentiating C2C12 muscle myoblasts and determined the effect on muscle Mb heme-maturation (Figure 6). As depicted in Figure 6A–C, lowering of GAPDH levels in C2C12 myoblasts inhibited heme-maturation of muscle Mb without affecting the induction in Mb protein levels in differentiating myoblasts (Figure 6B,C) or affecting myotube formation in differentiated cells (Figure 6A). Performing IPs in differentiating C2C12 cells revealed that GAPDH was associated strongly to Mb during the initial 0–24 h, while hsp90 association with Mb peaked between 48 and 72 h and the protein complexes dissociated from Mb by 96 h when myoblasts had differentiated into myotubes (Figure 6A,D,E). Together these data suggest that GAPDH is involved in the heme-maturation of muscle Mb during myoblast differentiation and GAPDH or hsp90 association with Mb resembles the association of these proteins with Hbα/γ happening during red cell maturation (Figure 5A–C) where GAPDH-Hb interactions peaked in the initial phase (0–2 days) while hsp90-Hb associations summited in the later phases (4–6 days) of maturation.

3.5 | Impact of overexpressing GAPDH or sGCβ1 to overcome the dominant negative effect of GAPDH mutants and the importance of mitochondrial heme, FLVCR1b and GAPDH on improving heme-insertion in a target globin

In an earlier study[27] we found that an active sGC is required for Mb heme-maturation. Moreover cGMP was found to be critical for fetal hemoglobin maturation (Hbγ).[37] This led us to determine the actual role of sGC in fetal (γ) Hb maturation. As depicted in Figure S3, we found that in K562 cells an activated sGCα1β1 heterodimer is a prerequisite for fetal (γ) Hb maturation during differentiation induced by either nitric oxide (NO) or hemin. Probing into the sGCα1β1 heterodimer status of stable lines GAPDH, H53A and/K227A expressing Mb, we found that a strong heterodimer exists for the GAPDH line and this is relatively weak the other two mutants H53A and K227A (Figure S4A). The sGC activation pattern of these lines by BAY 41–2272 also mirrored this finding (Figure S4B). Given the role of GAPDH and sGC in globin maturation, we tested a role of these two proteins to overcome

the dominant negative effect of GAPDH mutants H53A or K227A on Mb and Hb (α and β) heme-maturations. Mb or Hb were expressed in stable lines expressing GAPDH mutants H53A or K227A along with GAPDH, sGCβ1 or its deletion mutant sGCΔβ1 (sGC-β1 Δ204–244 + Δ379–408) which is unable to bind the sGCα1 subunit.[27] As depicted in Figure 7A–D we found that overexpressing GAPDH or sGCβ1 in these stable lines can fully (GAPDH) or partly (sGCβ1) overcame the dominant negative effect of H53A or K227A on Mb and Hb heme. The mechanistic implications of these results may imply that both GAPDH and sGCβ1 may elevate the sGCα1β1 heterodimer status of the stable lines H53A and K227A thereby overcoming the inhibitory impact on globin heme-maturation by these mutant lines. These data highlight the role of GAPDH and the need to elevate the sGC heterodimer as a prime requirement for globin maturation.

In order to further determine the efficacy of heme-insertion on a target globin, we first performed experiments where we tested the ability of mitochondria generated heme to rescue Mb from the inhibitory effect of GAPDH mutants H53A or K227A (Figure S5). Transient expression of Mb in the stable lines of GAPDH, H53A and K227A followed by supplementation with ferric citrate and D-Ala between varying time points (1–16 h) showed that the Mb heme plateaued between 3 and 16 h for GAPDH stable line. However this heme-insertion was inhibited in Mb expressed in H53A or K227A (Figure S5), which suggested that only addition of iron plus D-Ala alone cannot rescue the inhibition of globin heme and upregulation of enzymes like GAPDH is needed to reverse this effect as we earlier determined (Figure 7A–D). In order to further elucidate the role of other contributing factors on globin heme-maturation we then transfected Mb in HEK cells as a target globin, in the presence or absence of mitochondrial heme-exporter FLVCR1b and/GAPDH, with 100 μM ferric citrate and 1 mM D-Ala supplementation after 24 h of transfection for additional 16 h before cell harvest (Figure 7E,F). Overexpressing FLVCR1b with Mb and adding the supplements ferric citrate plus D-Ala increased the Mb heme-insertion nearly two-fold relative to controls without these supplements, but there was a greater increase in Mb heme-insertion with ferric citrate plus D-Ala, even in the absence of FLVCR1b. However the combination of FLVCR1b, GAPDH and Ferric citrate plus D-Ala gave the highest increase in Mb heme-insertion relative to controls which suggests that generated mitochondrial heme (via ferric citrate and D-Ala addition), FLVCR1b and GAPDH are potent factors which act in a concerted manner to enhance Mb heme-maturation (Figure 7E,F). Our findings therefore suggest that there is additional synergy between mitochondrial heme, its transporter FLVCR1b and heme-delivery by GAPDH during a globin heme-maturation event.

4 | DISCUSSION

Our current study shows for the first time that a rather unlikely protein GAPDH is intricately involved in the heme-maturation of Mb and Hb, and the fact that it is involved in the maturation of all four globins (alpha, adult, fetal Hb and Mb) suggests that it can be a key regulator of globin heme-maturation.[31,35] Based on the results and our current concepts we construct a model (Figure 8) which incorporates heme-maturation of four globins, involves GAPDH along with chaperons and co-chaperons which are either common to the heme-insertion process or are very specific to a particular globin. GAPDH can bind heme from

intracellular sources (acquired heme)[31] or bind the heme synthesized in the mitochondria. Heme bound to GAPDH constitutes the labile heme pool,[50,51] and this can be used to deliver heme to downstream target proteins to enable their heme-maturations.[35,52] The Mb and Hb heme-maturation processes also require an active sGCα1β1 heterodimer making cGMP and this induction maybe translational or transcriptional.[27,37,40] GAPDH itself can deliver heme to sGCβ1[32] and thus can regulate these processes. Overexpressing GAPDH or sGCβ1 can completely or partly rescue Hb/Mb heme-maturations from the inhibitory effect of GAPDH mutants H53A and K227A (Figures 7 and 8), thereby suggests that these proteins are indispensable for globin maturations. The apo-globins (heme-free) bind to specific chaperons/co-chaperons,[26,27] with GAPDH and hsp90 being common chaperons which bind to both apo-Hb or apo-Mb. GAPDH may transfer its bound heme to the globins directly via conformational changes happening during protein-protein interactions[53] or indirectly by transferring its heme to hsp90, which uses its ATPase function to hydrolyze ATP and push heme into its client proteins,[45] resulting in heme-containing Hb dimers/tetramers or Mb monomers. These possibilities arise from the different time points of association of GAPDH and hsp90/AHSP to Hbγ/α or to Mb during erythroid (Figure 5A–C) or myoblast differentiation respectively (Figure 6D,E). The fact that GAPDH associations with the Hb or Mb peak at the initial stages while hsp90 association with these globins peak at the later stages of two diverse differentiation processes, implies that both GAPDH and hsp90 follow a common mechanism during heme-maturation of these globins. We also determined factors like D-Ala and ferric citrate (causing mitochondrial heme generation) can enhance heme-insertion into a target globin like Mb. Moreover a combination of overexpressed GAPDH, FLVCR1b (Figure 7E,F) and supplementation of D-A la plus ferric citrate gives the maximum heme-insertion into the target Mb, suggesting that these processes act in a concerted way to enhance heme-insertion into the globins and are important for cellular heme homeostasis.[51,54,55]

GAPDH is an ancient protein and is best known for its glycolytic function and plays a key role in the production of ATP and pyruvate. However, various non-glycolytic functions have been attributed to GAPDH, and studies suggest a dynamic subcellular localization for GAPDH,[56] which is in line with GAPDH's new found role in heme binding and trafficking.[50,51] GAPDH has been earlier implicated in heme-insertion for iNOS,[30] where silencing of GAPDH led to downregulation of iNOS heme-insertion and its heme-dependent activity. Later studies involving knockdown of the yeast homologue of GAPDH, TDH3, led to increased labile heme and at the same time reduced the transcriptional activity of the nuclear, heme-dependent transcription factor Hap1.[50] The implications of these findings were made clearer when it was found that GAPDH can bind and transfer heme.[57,58] Recent findings by Sweeny et al.[31] showed that Histidine-53 residue of GAPDH can axially ligate to the bound heme, and this heme binding is drastically reduced in the H53A mutant. Our finding that H53A mutant drastically downregulates Hb and Mb heme-insertion parallels the earlier find by Sweeny et al.[31] where iNOS heme-insertion and its heme-dependent activity was inhibited when expressed in cells containing the H53A variant. So the effect of downregulation of Hb and Mb heme in cells by overexpression of H53A (Figures 1 and 2) or by stable expression in HEK cells (Figure 3) can be directly attributed to the signature heme-binding role of GAPDH which is reduced in this mutant. The K227A

mutant which has been shown to display reduced heme-binding properties and inhibits iNOS heme-insertion,[30] also showed downregulation of Hb and Mb heme but to a lesser extent relative to H53A. Likewise the extent of hsp90-Hb/Mb interactions which we found to be markers of heme-free client proteins were more potent for H53A relative to K227A, as it showed stronger binding between the corresponding globin and hsp90 (Figures 1C,D, 2C,D, and 3C,F). Understanding how the H53A and the K227A mutant actually work inside the cells to downregulate the Hb and Mb heme-maturations is rather speculative at this stage and would require further investigations. However it is conceivable that if GAPDH and hsp90 have similar roles in client protein heme-insertions, then these mutants can work by strong binding to the endogenous GAPDH, thus sequestering it or can bind tightly to the client globin thus blocking its heme-maturation by GAPDH or hsp90, similar to the non-hydrolysable ATP analog, D88N-hsp90 mutant, which does so by binding strongly to the client protein, thereby sequestering the endogenous hsp90's ATPase function.[45] Likewise these processes can occur both ways and the H53A on being low on its heme may bind and sequester the heme-bound GAPDH as well as bind tightly to the client globin to inhibit its heme-maturation. On the other hand while the K227A which cannot acetylate or bind to Siah1, thus cannot undergo nuclear translocation,[44,59] can be largely cytosolic, thereby sequestering the endogenous GAPDH. Our current study now opens up these prospects for further study.

It is well established that interplay between molecular chaperons is both cyclical and sequential to enable client protein maturation or initiate a misfolded protein response in healthy or diseased states respectively.[60–62] While cycling of molecular chaperons is a predictable event in the chaperoning of proteins, our current study suggests a unique synergistic role of an active sGC, heme bound-GAPDH and the molecular chaperon hsp90 or globin specific chaperons (e.g., AHSP) to enable heme-maturations of target globins.[26,27] This places globin maturations in a different class and indicates a tight regulation, which is in accord with previous concepts in globin maturation.[12,13] We found that silencing of GAPDH has a drastic inhibitory impact on the globin heme-maturations (Figures 4–6), and endogenous GAPDH silencing on stable lines, H53A or K227A further elevated the negative impact of these mutants on globin heme (Figure 4), suggesting that this heme-chaperon has a significant role in globin heme-insertions. The fact that GAPDH itself enables heme-insertion of sGCβ1 (Figure 8)[32] and since an active sGCα1β1 heterodimer is absolutely essential for Mb and Hb heme-maturation (Figures 6 and S3)[27] further elevates the role of GAPDH in globin maturation. Investigating the role of chaperon hsp90, we earlier determined it to be vital in enabling Hb heme-maturation both in erythroid and non-erythroid cells and Mb maturation in myoblasts,[26,27] where its ATPase function was critical and inhibiting this by drugs (radicicol, novobiocin or AUY-922) stalled the heme-insertion into these globins. Comparing the relative contribution of GAPDH and hsp90 towards heme-maturation of fetal (γ) and alpha (α) Hb in differentiating erythroid progenitors or in the maturation of Mb in C2C12 myoblasts we found that GAPDH associates strongly with these globins at the initial phase of maturation (day 2 for HiDEP-1 and 0–1 days for C2C12), while chaperon AHSP or hsp90 peaked in the later phases (4–6 days for HiDEP-1 and 0–1 day for C2C12) (Figures 5C and 6D,E). These data suggest different roles for GAPDH and AHSP/hsp90 in the corresponding globin's heme-maturation. GAPDH may

have a more basic role of allocating its bound heme to the chaperon machinery in the initial phases, or GAPDH can directly transfer its bound heme to the apo-globins during the initial association. However further studies are needed to authenticate these processes during erythropoiesis or muscle formation. Nonetheless the fact that all three factors, namely an active sGC heterodimer buildup, a heme-bound GAPDH and an active hsp90/AHSP with its chaperon machinery working in conjunction is what enables a globin heme-maturation event. Any inhibition of these factors/enzymes would partially or completely stall the heme-maturation event. Here GAPDH seems to have a major impact in its role since only a partial silencing of GAPDH by siRNA in differentiating K562 cells or C2C12 myoblasts drastically inhibits the heme-insertion into the fetal and alpha Hb or in Mb (Figures 5D–H and 6). This may be attributed to the fact that GAPDH being the initial heme transfer protein during these differentiation processes, even a modest downregulation in its protein levels can severely impact the downstream events. Thus GAPDH's role seems central to the regulation of heme-maturation in these globins.

In mammalian cells heme-biosynthesis and export of newly made heme from the mitochondria to the cytosol for insertion into proteins is a well-coordinated process. Although little is known about the processes of heme export, recent evidences suggest that mitochondrial heme exporter proteins FLVCR1b[42,43,55] and cytosolic heme exporter FLVCR1a[55,63] play significant roles in transporting heme outside the mitochondria or from inside the cell to the outside. These heme transporters are reported from erythroid cells and homologs of these transporters are believed to be present in non-erythroid cells. In order to understand how the dynamics of heme-biosynthesis and transport relates to the role of GAPDH in globin maturation we tried several permutations to determine what combination of a heme precursor (D-Ala), an iron source (ferric citrate), FLVCR1b and GAPDH can cause maximum heme-insertion on a target globin like Mb (Figure 7). Overexpression of FLVCR1b with D-Ala plus ferric citrate did not increase the Mb heme, relative to D-Ala and ferric citrate supplementation alone. However when GAPDH was overexpressed along with the exporter and supplemented with D-Ala plus ferric citrate, it produced the maximum heme-insertion into Mb, suggesting that a fine balance exits between heme-biosynthesis, its export protein and ultimate heme binder/transfer protein, GAPDH. These factors are thus coordinated to regulate the labile heme pool in mammalian cells which is vital for maintaining cellular heme homeostasis.[55] Thus a combination of D-Ala and Fe^{2+}, FLVCR1b and GAPDH are potent factors which can cause the maximum heme-insertion or remove the inhibition of globin heme-maturation in a target globin. These findings can help identify potential targets in modulating hemoglobin production.

sGC activated by nitric oxide (NO) generated from NOS enzymes makes cGMP thereby constituting the NO-sGC-cGMP signal axis.[52] The generated cGMP acts as a second messenger to initiate a cascade of downstream events to cause vasodilation in the vasculature[52,64,65] or bronchodilation in the lungs.[66,67] Our current study has important biomedical relevance as it is focused on globin maturation. Recent studies with aggressive cancer cells suggest that Mb and Hb are expressed by such cells[68,69] which are needed for survival of these cells. Moreover circulating tumor cells which cause metastasis by breaking off from tumors and spread it to other parts of the body via blood stream have been implicated to express Hbβ which is important in the survival of such CTCs.[70] Our current

and past studies link the activation of sGC to maturation of the globins (Mb/Hb), thereby contributing to the formation of a novel NO-sGC-Globin axes and brings this pathway in a “new light” whose significance has thus far not been explored in cancer cells. Since GAPDH is central to heme-maturation of iNOS, sGC and the globins (current study), its earlier defined role in cancer progression,[71,72] needs to be critically looked at with a fresh perspective for better therapeutic intervention.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations:

AARSD1	alanyl-tRNA synthetase domain containing 1
Aha1	activator of hsp90 ATPase protein1
AHSP	alpha hemoglobin stabilizing protein
D-Ala	delta aminolevulinic acid
DEX	dexamethasone
DOX	doxycycline
EPO	erythropoietin
HA	hemagglutinin
Hb	hemoglobin
HiDEP-1	human iPS cell-derived erythroid progenitor cell line-1
iNOS	inducible nitric oxide synthase
Mb	myoglobin
NO	nitric oxide
SA	succinyl acetone
SCF	stem cell factor
sGC	soluble guanylyl cyclase

STIP1	stress induced phosphoprotein 1

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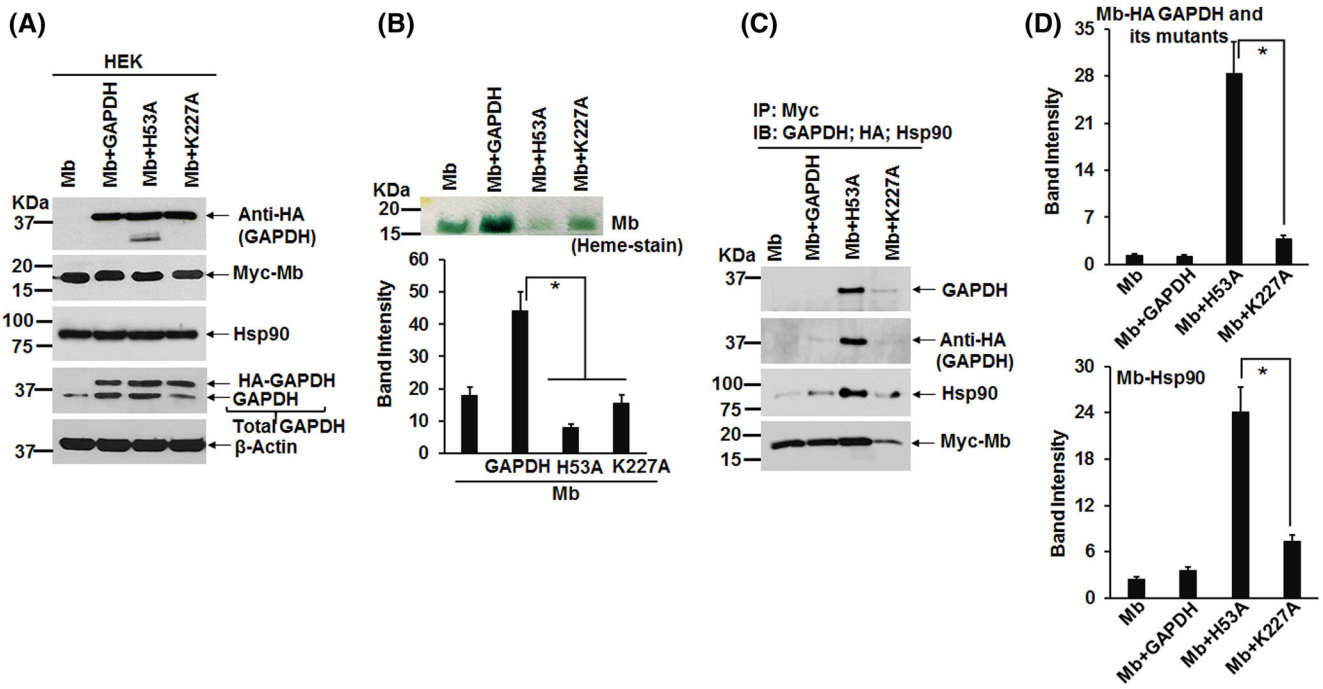

FIGURE 1.

Impact of GAPDH on myoglobin heme-maturation. Myoglobin (Mb) and GAPDH or its mutants H53A and K227A were transiently transfected in HEK 293T cells to determine the effect on Mb heme. (A) Protein expression by western blotting. (B) Representative heme-stain of Mb heme from generated cell supernatants and corresponding densitometries of heme-stains determined from the mean of three independent experiments ($n = 3$). (C) IPs were performed to determine the extent of heme-free (apo) Mb in the generated cell supernatants. (D) Mean densitometries from corresponding IPs ($n = 3$) comparing Mb bound to GAPDH or hsp90. All values depicted are mean $n = 3$, ± SD. *$p < .05$, by one-way ANOVA. Wherever applicable molecular weight markers (KDa) are depicted at the left of gel bands throughout the figure legends

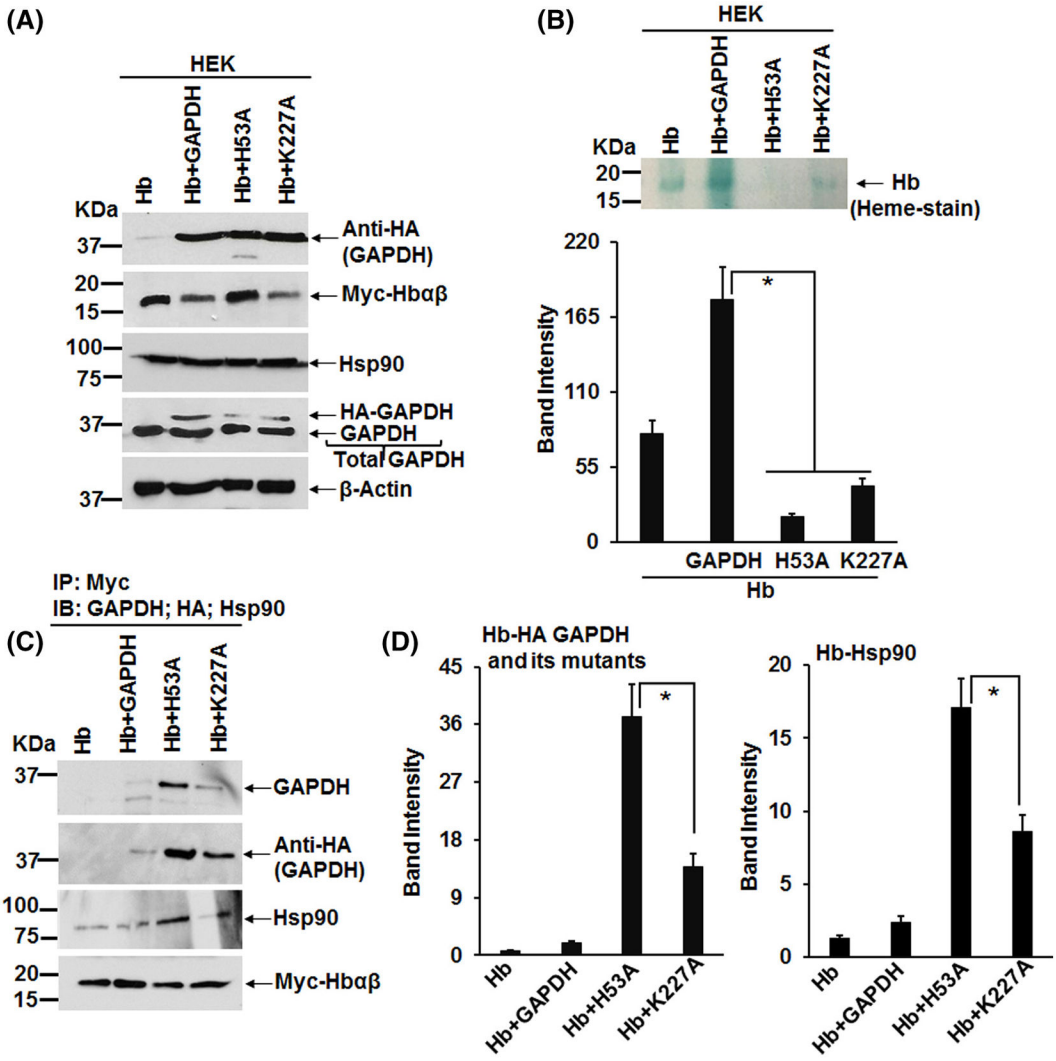

FIGURE 2.

Effect of GAPDH on hemoglobin heme-maturation. Hemoglobin (Hb) and GAPDH or its mutants H53A and K227A were transiently transfected in HEK 293T cells to determine the effect on Hb (α and β) heme. (A) Protein expression by western blotting. (B) Representative heme-stain of Hb heme from generated cell supernatants and corresponding densitometries of heme-stains determined from mean of three independent experiments ($n = 3$). (C) IPs were performed to determine the extent of apo-Hb in the generated cell supernatants. (D) Mean densitometries from corresponding IPs ($n = 3$) comparing Hb bound to GAPDH or hsp90. All values depicted are mean $n = 3$, ± SD. *$p < .05$, by one-way ANOVA

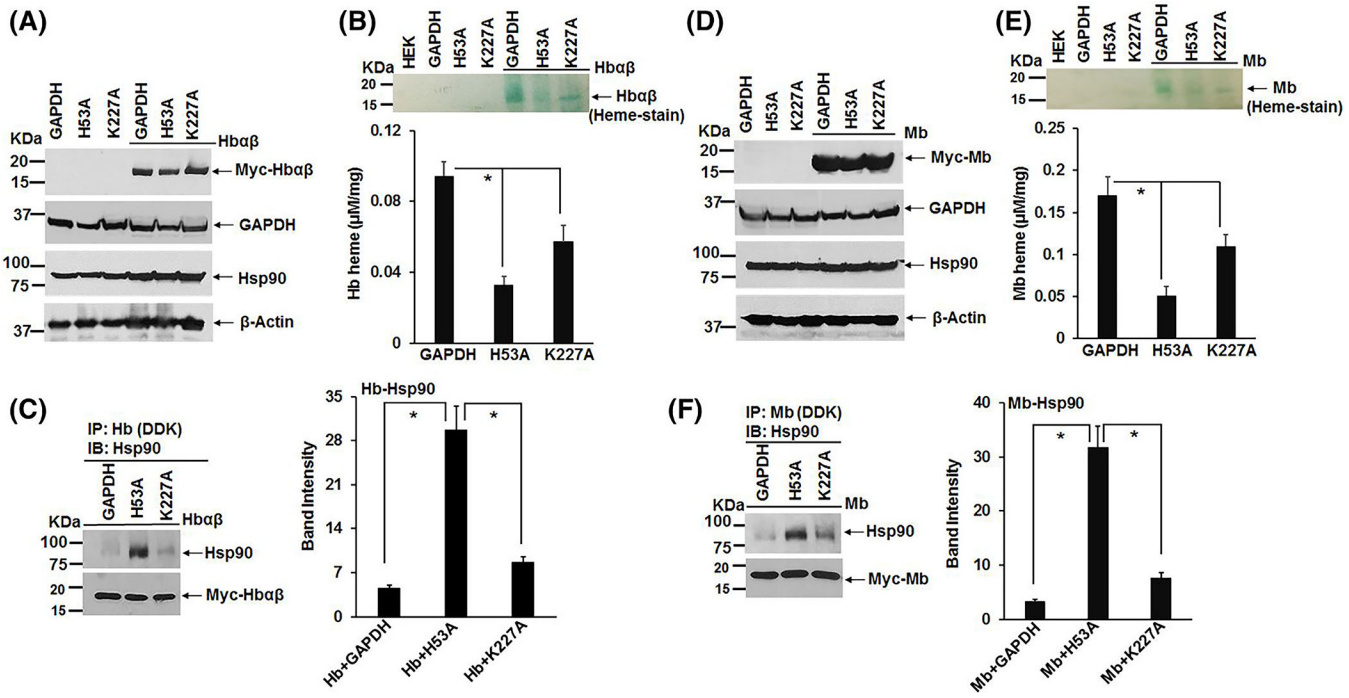

FIGURE 3.

Stable lines expressing GAPDH mutants H53A and K227A downregulates Mb and Hb heme-maturations. We transiently expressed Mb or Hbα & Hbβ in HEK stable lines expressing GAPDH and its mutants H53A and K227A. After 42 h of transient expression, the cells were harvested and generated supernatants assayed for protein expression by westerns, heme estimations of the globins were determined by in gel heme-stains and heme contents calculated from corresponding UV-visible spectra. IPs were performed to determine apo-globin hsp90 interactions. (A, D) Western blots of indicated proteins. (B, E) Heme-stain of Hb/Mb and calculated mean heme content of Hb/Mb from corresponding UV-visible spectra as indicated. (C, F) IPs depicting hsp90 bound to apo-Hb/apo-Mb expressed in various stable lines as indicated and mean densitometries from corresponding IPs ($n = 3$) comparing apo-Hb/apo-Mb bound to hsp90. All values depicted are mean $n = 3$, ± SD. *$p <$ .05, by one-way ANOVA

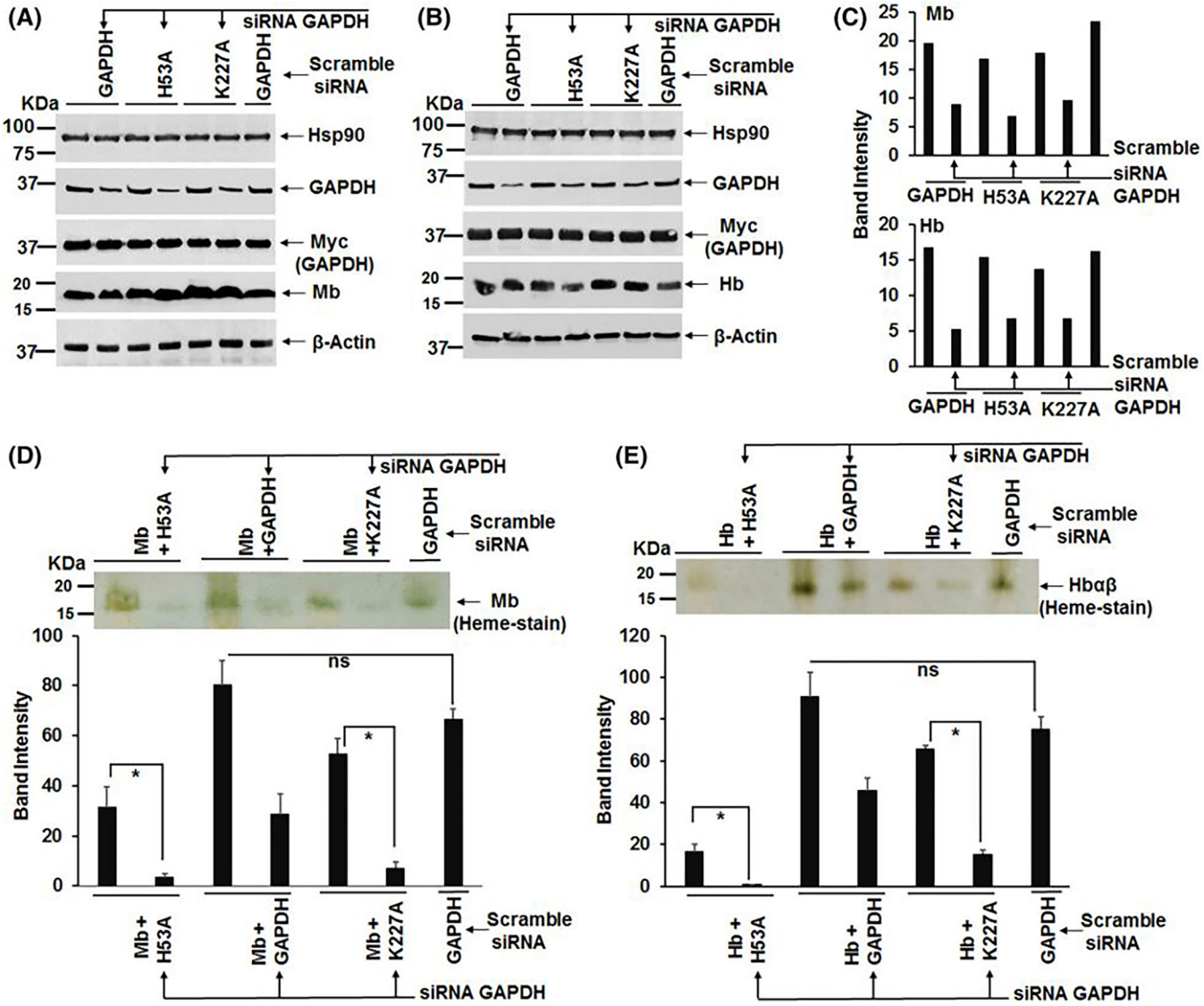

FIGURE 4.

Silencing of GAPDH by siRNA in H53A and K227A stable lines elevates the dominant negative effect of these mutants on globin heme. We silenced the endogenous GAPDH in stable lines by GAPDH SiRNA and the harvested cells were assayed for protein expression by westerns and globin heme levels were analyzed by heme stain. Scramble siRNA was used as a control. (A, B) Protein expression by westerns as indicated. (C) Densitometries of GAPDH siRNA as indicated in panels (A) and (B). (D, E) Representative heme stains of the globins as indicated and depicted densitometries of heme-stains derived from the mean of three independent experiments ($n = 3$). All values depicted are mean $n = 3$, ± SD. *$p < .05$, by one-w ay ANOVA

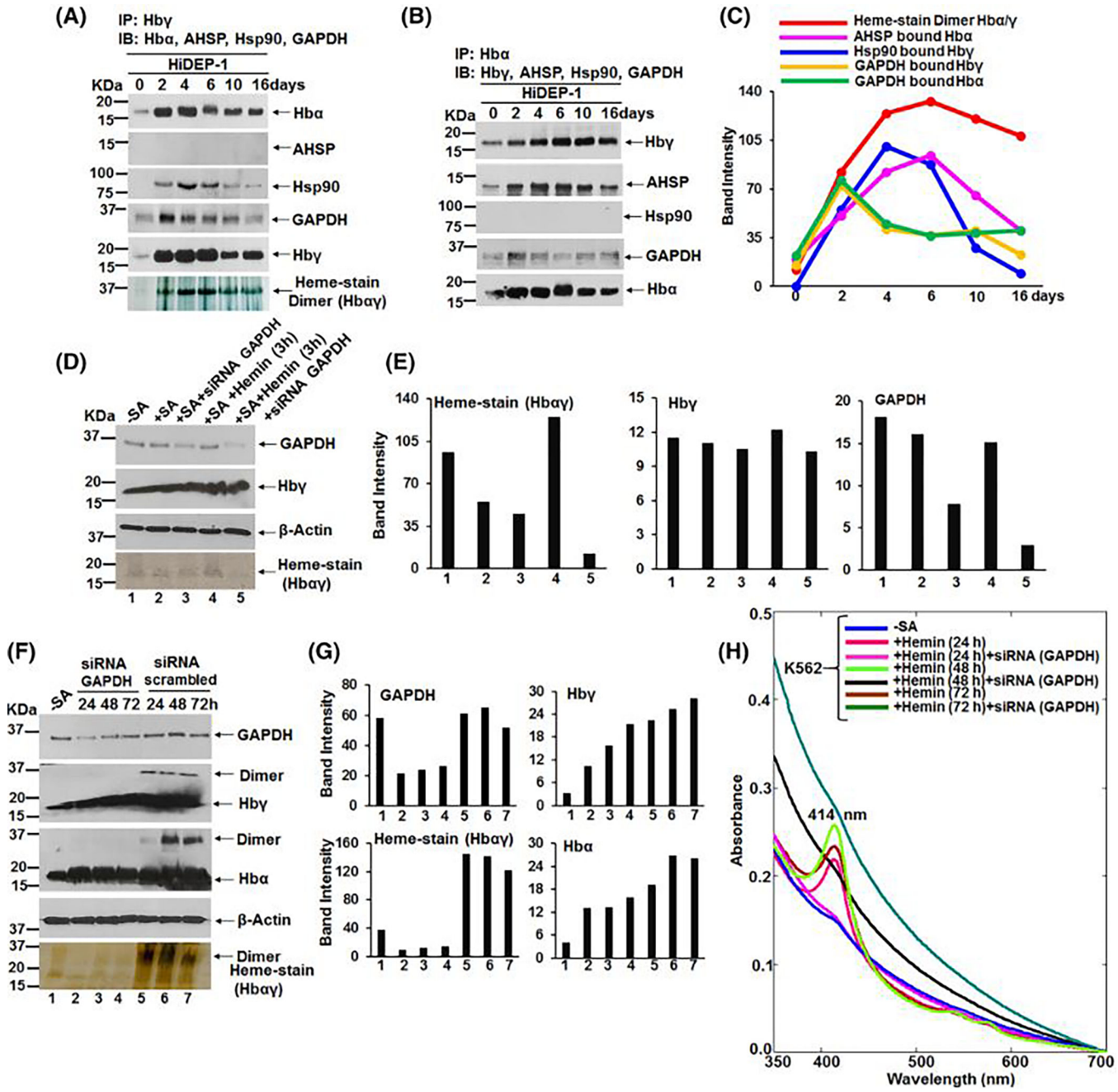

FIGURE 5.

GAPDH associates with Hb (γ) and Hb (α) in differentiating erythroid progenitors (HiDEP-1) and silencing of GAPDH in K562 cells downregulates fetal (γ) and alpha (α) Hb heme-maturations. We performed IPs during differentiation of erythroid progenitor cells (HiDEP-1) which express Hbα and Hbγ, and found that both fetal (γ) and alpha (α) Hb binds to GAPDH. In order to further elucidate the role of GAPDH in fetal (γ) Hb heme-maturation we treated K562 cells with SA for 72 h and did simultaneous silencing of GAPDH by siRNA (80 nM). The cells were then given micromolar amounts of hemin (5 μM) for additional 3 h before harvest and cell supernatant generation. SA treated cells

with GAPDH siRNA were used as controls. In other experiments the K562 cells were transfected with GAPDH siRNA or transfected with scrambled siRNA and cultured for 24–72 h and then given 50 μM of hemin to allow for differentiation for 24–72 h before harvest. (A, B) IPs depicting GAPDH bound to Hbγ or Hbα during HiDEP-1 differentiation and heme-stain for Hbγ/α during differentiation. (C) Densitometries comparing Hbα/γ heme-stain dimer to AHSP/Hsp90 and/or GAPDH bound to Hbα/γ as depicted in panels (A, B). (D) Representative westerns and heme-stains under indicated conditions depicting the effect of silencing GAPDH on Hbαγ heme-maturation. (E) Densitometries of Hbαγ heme-stain, and expression levels of Hbγ and GAPDH depicted in panel D. (F) Representative westerns depicting inhibition of Hbα/γ dimer formation by silencing GAPDH during K562 differentiation. Corresponding heme-stains show inhibition of heme-insertion into Hbα/γ under similar conditions. (G) Densitometries of Hbαγ heme-stain, and expression levels of Hbα/γ and GAPDH as depicted in panel F. (H) Corresponding UV-visible spectra of Hbγ/α during K562 cell differentiation ∓ GAPDH siRNA

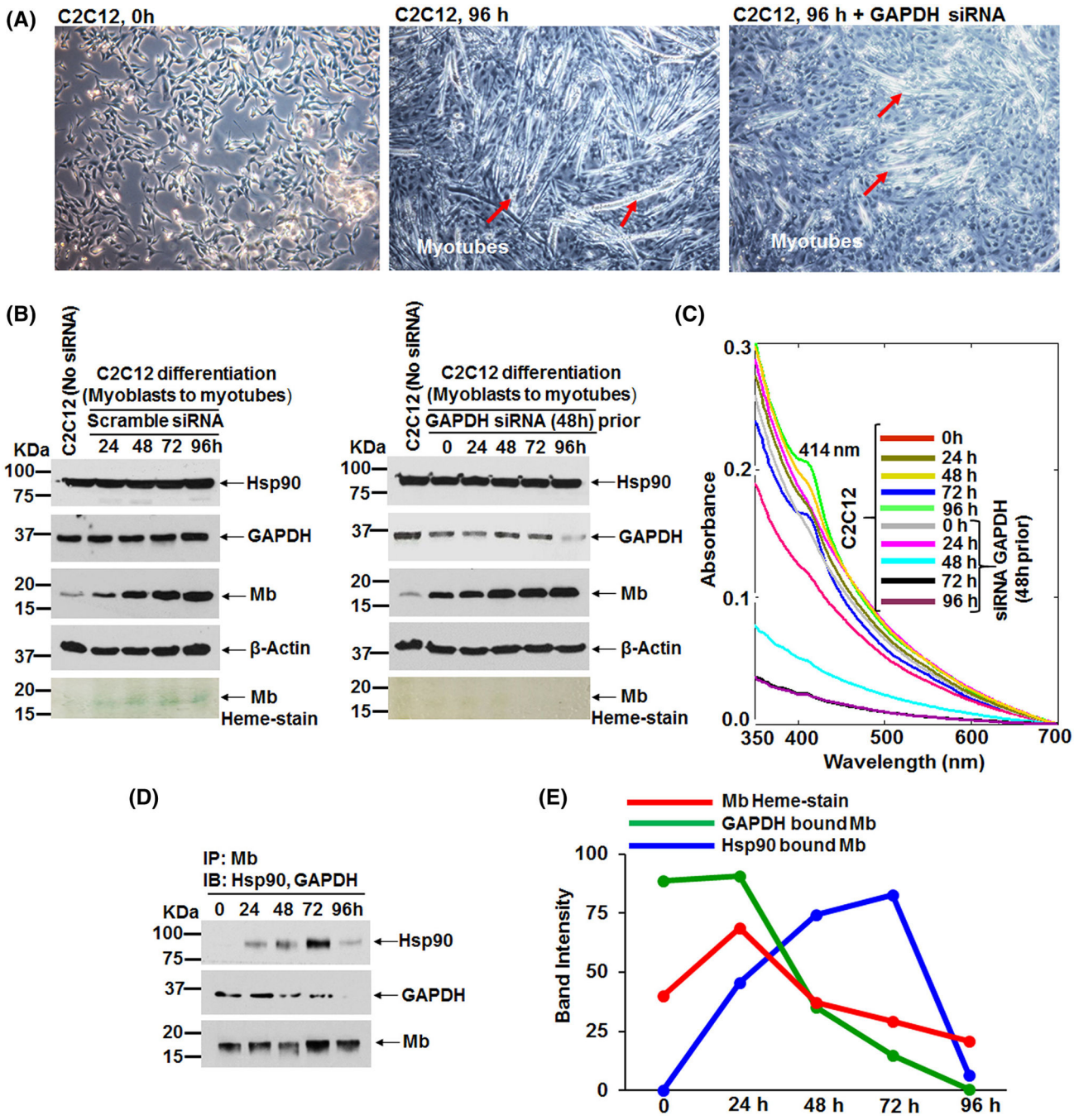

FIGURE 6.

GAPDH silencing in differentiating C2C12 myoblasts inhibits Mb heme-maturation. C2C12 myoblasts were cultured and transfected with mouse GAPDH siRNA or scramble control siRNA (70 nM) for 48 h followed by induction of differentiation by culturing in differentiation media for additional 96 h, the cells were imaged each day before being harvested. Generated supernatants were analyzed for protein expression by western blotting, heme-stain or UV-visible spectra to detect Mb heme, and IPs were performed on differentiating myoblasts between 0 and 96 h. (A) Images at 5× (Bright field) at 0 and

96 h under indicated conditions showing differentiation of myoblasts (0 h) into myotubes (96 h). Red arrows indicate myotube formation. (B) Corresponding protein expression and Mb heme-stains under indicated conditions ∓ GAPDH siRNA. (C) Corresponding UV-visible spectra of Mb during C2C12 cell differentiation ∓ GAPDH siRNA. (D) IPs depicting GAPDH or Hsp90 bound to Mb during C2C12 differentiation. (E) Densitometries comparing Mb heme-stain to Hsp90 and/or GAPDH bound to Mb as depicted in panel D

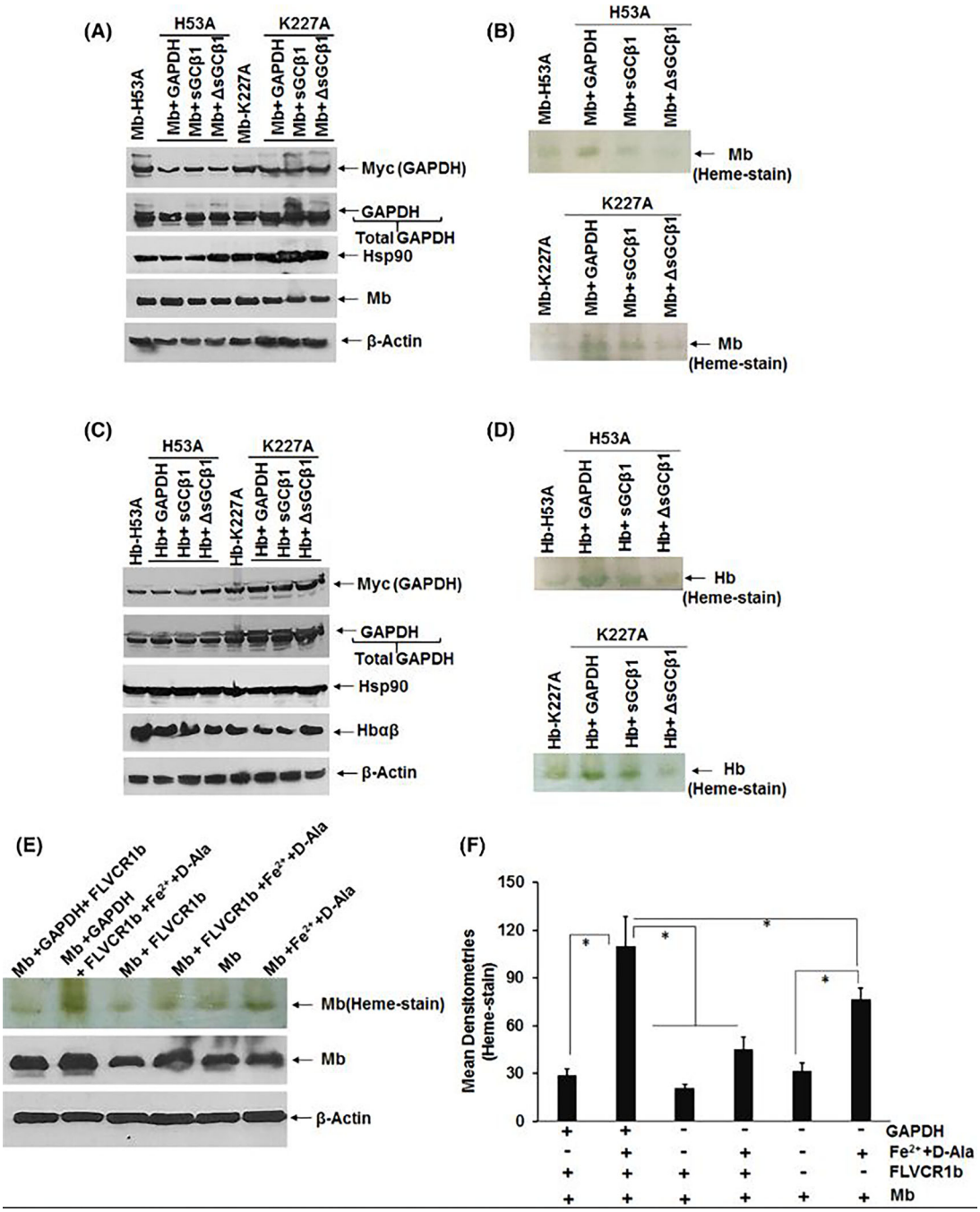

FIGURE 7.

GAPDH and sGCβ1 rescues the globin heme from the dominant negative effects of GAPDH mutants while a combination of GAPDH, FLVCR1b along with mitochondria generated heme can increase the heme-insertion on a target globin. To determine the effect on globin heme, Mb or Hb were expressed in stable lines expressing GAPDH mutants H53A or K227A along with GAPDH or sGCβ1/sGCΔβ1. In order to further determine the efficacy of heme-insertion on a target globin, separate experiments were performed where Mb, ∓ FLVCR1b and ∓ GAPDH were transfected in HEK cells with supplementation of 100 μM

ferric citrate plus 1 mM D-Ala for 16 h before cell harvest. The generated supernatants were assayed for protein expression by westerns and the globin heme was analyzed by heme-stain. (A–D) Representative western blots of indicated proteins and heme-stains of Hb/Mb as indicated. (E) Representative westerns and heme-stains as indicated. (F) Mean densitometries of depicted heme-stains from $n = 3$ repeats ± SD. *$p < .05$, by one-way ANOVA

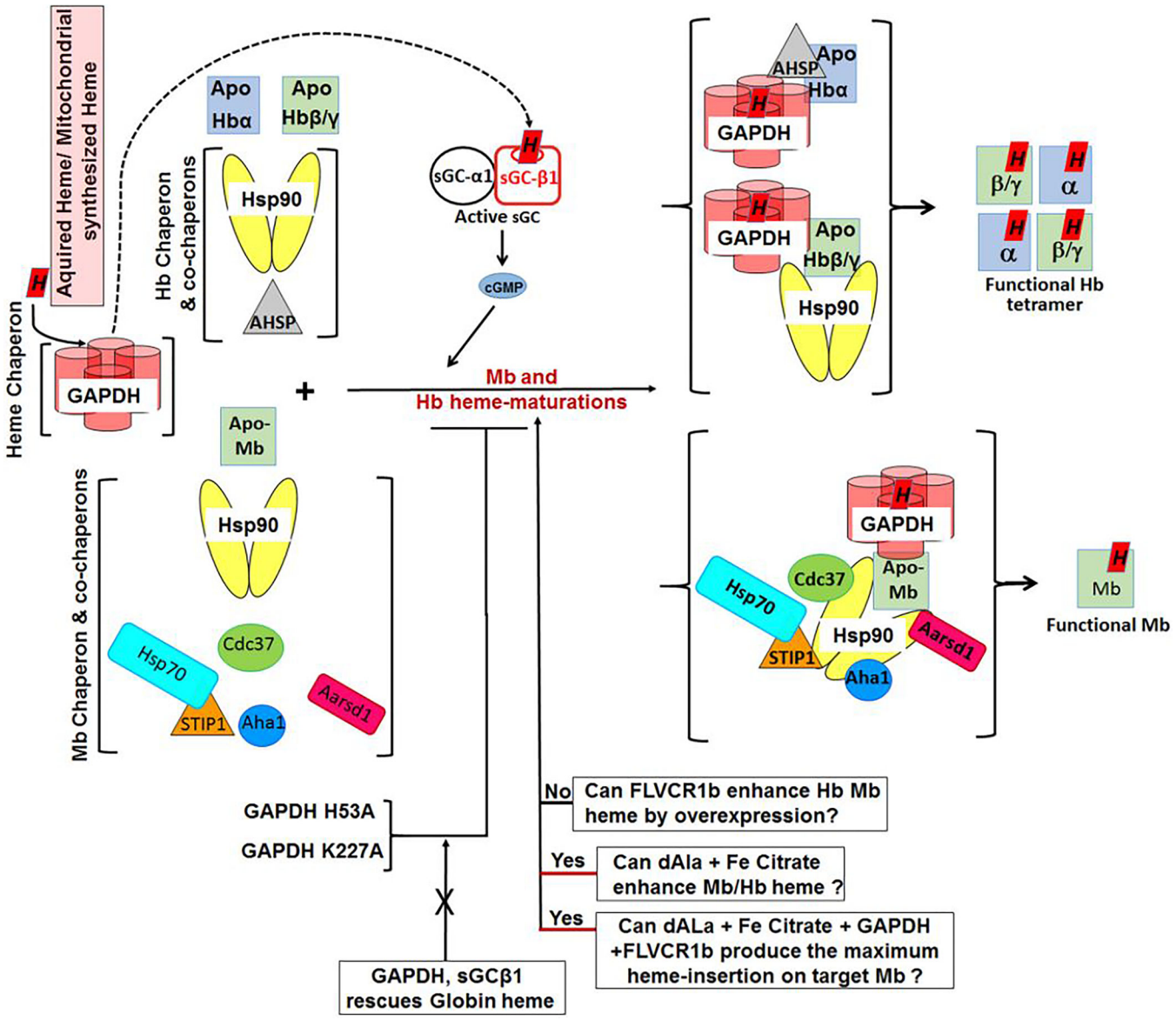

FIGURE 8.

Model depicting the role of GAPDH on globin heme-maturation. Cellular GAPDH acting as heme chaperon is involved in heme-maturation of Mb and alpha, beta (adult) and gamma (fetal) Hb (α, β, γ), along with specific chaperons/co-chaperons for the globins as indicated. The apo-Hbα/apo-Hbβ/γ or the apo-Mb proteins bind to specific chaperons/co-chaperons[26,27] with GAPDH and hsp90 being common chaperons to both apo-Hb or apo-Mb. The Mb and Hb heme-maturation processes require an active sGCα1β1 heterodimer making cGMP and this induction maybe transcriptional. GAPDH itself delivers heme to sGCβ1 and can regulate these processes. GAPDH binds heme and may transfer its bound heme directly to the globins or transfer its heme to hsp90/AHSP to enable heme-insertion into the corresponding client globins, followed by chaperon assisted folding of these globins, resulting in heme-containing Hb dimers, tetramers and Mb monomers. GAPDH mutants H53A and K227A downregulate or inhibit Hb/Mb heme-maturation and this inhibition

can be completely rescued by overexpressing GAPDH or partly by sGCβ1 overexpression. While addition of D-Ala and ferric citrate can enhance heme-insertion into Hb and Mb, a combination of overexpressed GAPDH, FLVCR1b, and supplementation of D-ALa plus ferric citrate gives the maximum heme-insertion into target Mb, suggesting that these factors act in a concerted way to enhance heme-insertion into Hb or Mb and are important for cellular heme homeostasis