Original

Erythroid-specific 5-aminolevulinate synthase is stabilized by HSPA9 in mitochondrial matrix

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Abstract

In vertebrates, the rate-limiting step of heme biosynthesis is catalyzed by two isoforms of 5'-aminolevulinate synthase (ALAS) in a tissue-specific manner. The nonspecific isoform ALAS1 is expressed in all cells and undergoes heme-dependent degradation by mitochondrial matrix proteases, whereas ALAS2 is the erythroid-specific isoform that is strictly and stably expressed in erythroid cells to supply excess heme for hemoglobin production. Unlike that of ALAS1, the regulation of ALAS2 protein turnover in mitochondria has yet to be unequivocally elucidated. In this study, we found that FLAG-tagged ALAS2 (ALAS2F) expressed in a nonerythroid human cell line has a longer half-life than FLAG-tagged ALAS1, although it similarly forms complexes with mitochondrial matrix proteases under conditions of excess heme. To identify the possible proteins stabilizing ALAS2, we analyzed ALAS2F protein immunoprecipitates using mass spectrometry and identified several mitochondrial chaperone proteins, including HSPA9. Knockdown or chemical inhibition of HSPA9 caused a decrease in the ALAS2F protein level, which was assumed to result from increased degradation of ALAS2F, implying that HSPA9 plays a vital role in stabilizing the ALAS2 protein to resist degradation in the mitochondrial matrix.

I. Introduction

In nonplant higher eukaryotes and α-proteobacteria, the first and rate-limiting reaction in the heme biosynthesis pathway is catalyzed by 5-aminolevulinate synthase (ALAS) [1].

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In vertebrates, two ALAS isoforms exist that are differentially expressed and regulated in a tissue-specific manner. ALAS2 is an erythroid-specific isoform that catalyzes the synthesis of heme for incorporation into hemoglobin, whereas the nonspecific isoform (ALAS1) is a ubiquitous housekeeping isoform that catalyzes the synthesis of heme in all tissues including

erythroid cells [2-4]. ALAS2 is of greater clinical significance since its loss-of-function mutations cause congenital sideroblastic anemia (CSA) inherited in an X-linked manner (XLSA/ SIDBA[1]), which is characterized by microcytic hypochromic anemia and the presence of ring sideroblasts in patients' bone marrow [5], while its gain-of-function mutations cause X-linked dominant protoporphyria (XLDP) [6]. Owing to its clinical relevance, extensive research on ALAS2 regulation is ongoing, and thus far it has been reported that the transcription of the *ALAS2* gene is strongly induced during erythroid differentiation, and that the translation of the corresponding protein is regulated by iron through the iron-responsive element (IRE)-iron regulatory protein (IRP) system in the 5'-untranslated region (5'-UTR) of its mRNA [4,7]. The posttranslational regulation of ALAS2 protein turnover remains unclear, while ALAS1 undergoes heme-induced degradation by the mitochondrial matrix proteases CLPXP [8] and LONP1 [8,9].

HSPA9 is a mitochondrial 70 kDa heat shock protein encoded by the *HSPA9* gene on chromosome 5 in humans [10]. HSPA9 is an indispensable protein for hematopoiesis and erythroid maturation in mice and humans [11,12]. Low expression and/or loss-of-function mutations of HSPA9 cause an autosomal recessive type of CSA (SIDBA4) [13]. As a chaperone protein, HSPA9 has many functions, including iron sulfur cluster biogenesis in mitochondria, that are involved in the IRE-IRP system regulating ALAS2 expression [14].

In this study, we established a fibroblast cell line with doxycycline-inducible expression of an ALAS2 protein with a FLAG tag at its c-terminus (ALAS2F). Using this cell line, we found that the ALAS2F protein was more stable than FLAG-tagged ALAS1 (ALAS1F) when cells were incubated with hemin, which is a heme analogue and increases the intracellular heme concentration [15-17]. Moreover, immunoprecipitation of the ALAS2F protein followed by tandem mass spectrometry revealed that the ALAS2F protein forms a protein complex with several proteins, such as mitochondrial matrix proteases (CLPXP and LONP1) and chaperone proteins (HSPD1 and HSPA9) (manuscript in preparation). Since these matrix proteases were also identified in the ALAS1F immunoprecipitates in our previous report [8], we hypothesized that there may be additional factors or interacting proteins that protect ALAS2F from the rapid heme-induced degradation observed for ALAS1F. Among these proteins coimmunoprecipitated with ALAS2F, we focused on HSPA9, whose loss-of-function mutations cause CSA, to clarify the roles of the HSPA9 protein in the stabilization of the ALAS2F protein.

II. Materials and Methods

1. Reagents

Chemical reagents were purchased from Sigma–Aldrich (St.Louis, MO, USA), Nacalai Tesque (Kyoto, Japan) or FUJIFILM-Wako Pure Chemical Corporation (Osaka, Japan) unless otherwise noted. Complete EDTA-free protease inhibitor cocktail was purchased from Roche Diagnostics GmbH (Mannheim, Germany). The antibody vendors and the dilutions used for Western blotting were as follows: anti-FLAG M2 monoclonal antibody-HRP Conjugated (#A8592, 1:3,000) and the anti-LONP1 antibody (#HPA002192, 1:2,000) were obtained from Sigma–Aldrich; the anti-CLPX (#ab168338,

1:2,000) and anti-CLPP (#ab124822, 1: 3,000) antibodies were obtained from Abcam (Cambridge, UK); anti-GAPDH mAb-HRP DirecT (#M171-7, 1:10,000), anti- β -Actin pAb - HRP DirecT (#PM053-7, 1:10,000), and anti- α Tubulin pAb-HRP-DirecT (#PM054-7, 1:10,000) were obtained from Medical and Biological Laboratories Co., Ltd. (MBL, Nagoya, Japan); and the anti-Grp75/Mortalin (HSPA9) rabbit pAb (#ADI-SPS-827-F, 1:5,000) was obtained from Enzo Life Sciences (Farmingdale, NY, USA). Anti-DDDDK antibody-conjugated agarose (anti-DDDDK-agarose) and DDDDK peptides for the purification of FLAG-tagged proteins were purchased from MBL. Restriction enzymes were purchased from New England Biolabs (Ipswich, MA, USA).

2. Cell Culture

Flp-In T-Rex 293 (FT293) cells (Thermo Fisher Scientific, Waltham, MA, USA) were maintained in high glucose Dulbecco's modified Eagle's medium (FUJIFILM-Wako Pure Chemical Corporation) supplemented with 10% fetal bovine serum, 15 μg/ml Blasticidin S, 100 μg/ml Zeocin, 50 units/ml Penicillin and 50 μg/ml Streptomycin. Expression of FLAG-tagged proteins was induced by the addition of doxycycline (final concentration, 1 μg/ml) to the culture medium, and the cells were incubated for 48-72 hours. To deplete intracellular heme, cells were treated with 1 mM succinylacetone (SA) for 24 hours, while 0.1 mM hemin was used to simulate an excess of heme.

3. Construction of plasmids and establishment of FLAG-tagged protein-expressing cells

The plasmid containing ALAS2F cDNA was described previously as pGEM-AET [18]. The cDNA of ALAS2FΔIRE, which does not contain an IRE in its 5'untranslated region, was synthesized by polymerase chain reaction (PCR) using a specific primer set (5-TTCAAGATGGTGACTGCAGCCATGC-3', 5'-TCACTTGTCGTCATCGTCCTT-3') and pGEM-AET as a template. The amplified fragment was cloned into the pGEM-Teasy vector (Promega Corporation, Madison, WI, USA), which was designated pGEM-AEfΔIRE. Then, pGEM-AET and pGEM-AEfΔIRE were digested by the Not I restriction enzyme, and each cDNA was cloned into the Not I-digested pcDNA5/FRT/TO plasmid (Thermo Fisher Scientific) to construct pFRT-AEf and pFRT-AEfΔIRE. As a control, the OTC-LucF expression plasmid was constructed as follows. Human ornithine transcarbamoylase (OTC) cDNA (NM_000531.5), encoding an OTC precursor protein, was amplified by PCR with the following primers: 5'-GAATTCAAGATGCTGTTTAATCTGAGG-3', 5'-GTCGACAAATTTAGGCTTCTGGAGCTG-3'. The amplified products were cloned into the pMD20-T vector (Takara Bio, Shiga, Japan). The resulting plasmid was used as a template for PCR using the primers 5-GAATTCAAGATGCTGTTTAATCTGAGG-3' and 5'-CCCGGGTTGTAGTGGTTGTCCACACCGA-3' to amplify the cDNA sequence corresponding to the mitochondrial targeting signal of OTC (amino acids 1-32) [19]. Then, the PCR product was digested with EcoRI and XmaI and cloned into the pEGFP-N2 vector (BD Bioscience, Franklin Lakes, NJ, USA) with the luciferase-FLAG gene, which was derived from a plasmid described previously [20]. The resulting plasmid was digested with EcoRI, and the cDNA fragment was ligated to an EcoRI-NotI adaptor oligonucleotide. The OTC-Luciferase-FLAG fragment, which was obtained by NotI digestion of the ligation reaction product, was
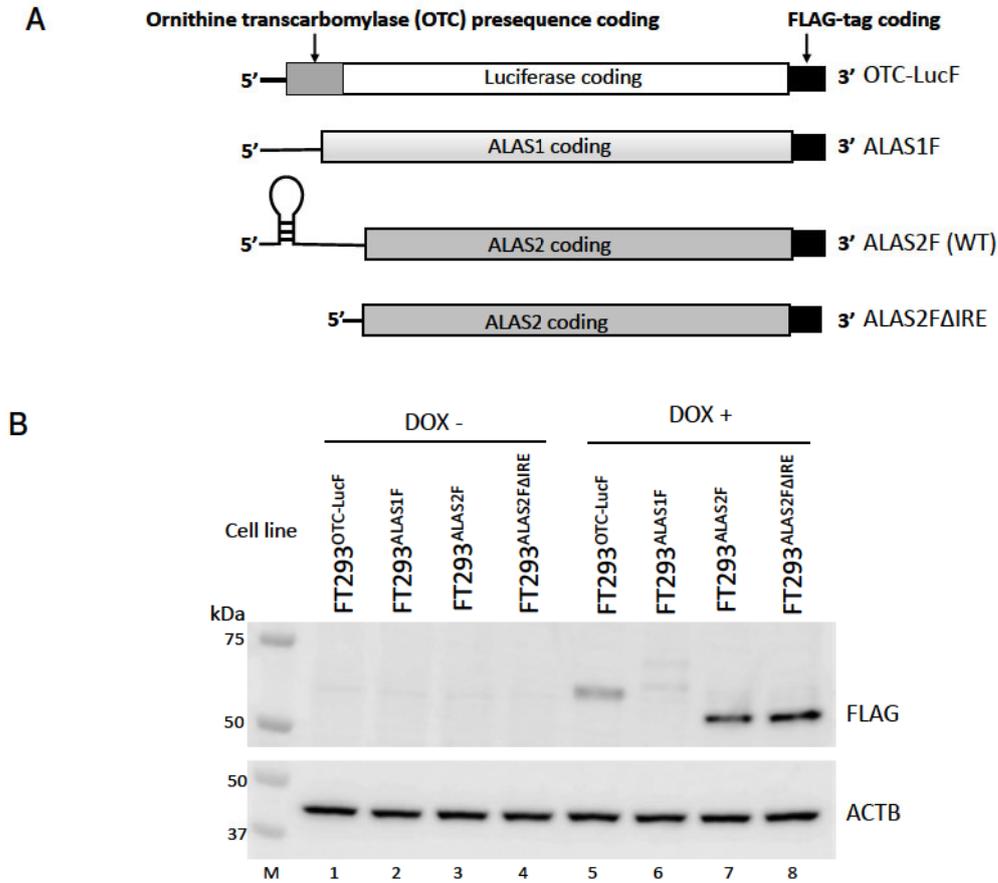
Fig. 1. Doxycycline-inducible expression of FLAG-tagged proteins in FT293 cells.
  A) Schematic representation of the mRNA structures encoding the FLAG-tagged proteins expressed in FT293 cells.
  B) Western blot analysis of proteins prepared from cells treated with doxycycline (DOX+, Lanes 5-8)) and untreated cells (DOX-, Lanes 1-4). The same concentration of DOX was added to the culture medium, while DOX was not added to untreated cells. Even though the expression cassettes had the same promoter, the abundances of ALAS2F proteins expressed from ALAS2F WT (Lane 7) and ALAS2F Δ IRE mRNA (Lane 8) were higher than those expressed from ALAS1F (Lane 6) and OTC-LucFmRNA (Lane 5).

cloned into the pcDNA5/FRT/TO vector, and the resulting plasmid was referred to as the OTC-LucF plasmid. To establish FT293 cells with doxycycline-inducible expression of FLAG-tagged ALAS2, the pFRT-AEf, pFRT-AEf ΔIRE or OTC-LucF plasmid was introduced into FT293 cells with the pOG44 plasmid according to the manufacturer's instructions, and the selected hygromycin-resistant clones were named FT293^{ALAS2F}, FT293^{ALAS2F Δ IRE} or FT293^{OTC-LucF}, respectively. The establishment of

ALAS1F-expressing FT293 cells (FT293^{ALAS1F}) was described previously [8]. A schematic representation of each mRNA structure expressed in these cells is shown in Fig. 1A.

4. Preparation of cell lysates and Western blot analysis

Harvested cells were lysed in lysis buffer (20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% Triton X-100, 10% glycerol, cOmplete EDTA-free protease inhibitor cocktail, 1 mM EDTA (pH 8.0), 1 mM NaF and 0.4 mM Na_3VO_4) by

vigorous pipetting. The lysed cells were then incubated on ice for 10 minutes and centrifuged at $16,100 \times g$ for 15 minutes at $4\,°C$, and the supernatant was used for further experiments. The protein concentration in each lysate was determined using Pierce 660 nm protein assay reagent (Thermo Fisher Scientific) using bovine serum albumin as a standard. The final protein concentration in the samples used for SDS–PAGE was adjusted to $1\,\mu g/\mu l$ using lysis buffer (or ice-cold phosphate buffered saline) and 6x SDS–PAGE sample buffer with reducing reagent (#09499-14, Nacalai Tesque), and the samples were then incubated at $95\,°C$, for 10 minutes. Samples containing 5 to 10 μg of protein were loaded onto a 7.5 or 10% TGX acrylamide gel (Bio-Rad Laboratories Inc., Hercules, CA, USA) for electrophoresis, and after acrylamide gel electrophoresis, proteins were transferred to a PVDF membrane with a Trans-Blot Turbo system (Bio-Rad Laboratories) according to the manufacturer's protocol. After protein transfer, the PVDF membrane was incubated with 5% skim milk in Tris-buffered saline with 0.05% Tween 20 (TBS-T) for 1 hour for blocking. Then, each membrane was incubated with a diluted primary antibody for 1-2 hours at room temperature or overnight at $4\,°C$ and was then washed with TBS-T three times for 7 minutes each. Then, the washed membrane was incubated with an HRP-conjugated secondary antibody for 1 hour at room temperature prior to washing with TBS-T three times for 7 minutes each before the detection step. For the detection of FLAG-tagged proteins, GAPDH, β-actin or α-tubulin, HRP-conjugated primary antibodies were used, and the incubation step with the secondary antibody was skipped. Protein signals were detected using Clarity ECL

Western Substrate (Bio-Rad Laboratories) and visualized using an ImageQuant LAS500 image analyzer (GE Healthcare, Uppsala, Sweden). Signal quantification was performed using NIH ImageJ software.

5. Knockdown of endogenous proteins

Two HSPA9-specific siRNAs (Silencer Select Validated siRNA #1, s6988; #2, s6989), Silencer Select GAPDH Positive Control siRNA (#4390849, siGAPDH) and Silencer Select Negative Control siRNA No. 1 (#4390843, siNC) were purchased from Thermo Fisher Scientific. Each siRNA was transfected into FT293 cells using Lipofectamine RNAiMAX reagent (Thermo Fisher Scientific) according to the manufacturer's protocols. Seventy-two hours after transfection, cells were harvested for the preparation of total cell lysates as described above.

6. Immunoprecipitation and mass spectrometry analysis

An aliquot of the total cell lysate was set aside to prepare the sample for SDS–PAGE, while the majority of the lysate (approximately 2 mg) was subjected to immunoprecipitation using anti-DDDDK-agarose beads. The methods for immunoprecipitation, elution using DDDDK peptides and preparation of the samples for SDS–PAGE were described previously [8]. These immunoprecipitates were also used for mass spectrometry analysis as described previously [8]. Briefly, immunoprecipitated FLAG-tagged ALAS2 protein was digested with trypsin and the dried peptide extract was dissolved in sample solution [5% acetonitrile and 0.1% trifluoroacetic acid]. Each sample was injected into an EasynLC-1000 system (Thermo Fisher Scientific, Waltham, MA,USA), which was connected to an EASY-Spray column (Thermo

Fisher Scientific). Eluted peptides were then ionized and analyzed using a Fusion mass spectrometer (Thermo Fisher Scientific) coupled to a nano-spray source. High-resolution full scan MS spectra were acquired in the Orbitrap followed by MS/MS in the linear ion trap. The MS/MS data were analyzed by sequence alignment with variable and static modifications

7. Protein stability assays

To evaluate ALAS1F stability, cells were seeded into 12-well culture plates at 1×10^5 cells/well, inclubated at 37 ℃ in a humidified incubator with 5% CO_2 for 24 hours, and then treated with 1 μg/mL doxycycline for 48 hours, with 1 mM succinylacetone (SA) added for the last 24 hours to inhibit heme biosynthesis. Thereafter, cycloheximide (CHX) chase experiments were carried out by treating cells with 20 μM CHX for 30 minutes prior to treatment with or without 0.1 mM hemin (referred to as "CHX+H" and "CHX", respectively, in the figures). As a control for CHX or hemin treatment, cells were incubated with an appropriate volume of dimethyl sulfoxide (DMSO), which was used as the solvent for CHX and hemin (referred to as "DMSO" in the figures). Then, 0, 10, 30, 60, and 120 minutes after the initiation of hemin treatment, cells were harvested and used for Western blot analysis. A similar protocol was employed for the ALAS2F stability assay under basal HSPA9 expression conditions, with modifications to the time intervals, where cells were harvested after 0, 1, 2, 4, and 8 hours. To study ALAS2F stability under HSPA9 knockdown conditions, slight modifications to the protocol were made, as shown in the "Results" section.

8. Quantitative real-time polymerase chain reaction

Total RNA was extracted from cells using Isogen II reagent (Nippon Gene, Tokyo, Japan). The concentration of the purified RNA sample was quantified using a UV-1800 spectrophotometer (Shimadzu, Kyoto, Japan), and 0.5 μg of total RNA was used for the reverse transcriptase reaction using the PrimeScript® RT Reagent Kit with gDNA Eraser (Takara Bio, Shiga, Japan). Quantitative real-time PCR of ALAS2 mRNA was performed according to the Premix ExTaq™ (Takara Bio) protocol using predesigned primer-probe assay mixtures (ALAS2: Hs.PT. 56a.1433425.g, HPRT1: Hs.PT.58v.45621572; Integrated DNA Technologies, Inc., Coralville, IA, USA) in a Roche LightCycler96 System (Roche Diagnostic GmbH) according to the manufacturer's instructions.

9. Statistical analysis

For Western blot analysis, all experiments were independently repeated at least 3 times, and the quantitative data are expressed as the means ± standard deviations (SDs).

For real-time PCR, three biological and two technical replicates of each experiment were used. Statistical analysis was performed using the Tukey HSD test in R statistical software for comparison of means among more than two groups. Differences were considered significant at $p < 0.05$.

III. Results

1. ALAS2F is more stable than ALAS1F against heme-induced degradation

First, we compared the expression levels of FLAG-tagged proteins at steady-state. As shown in Fig.1B, a high abundance of the ALAS2F (Lanes 7,8) protein compared to the ALAS1F (Lane 6) or OTC-LucF (Lane 5) protein was

observed in both FT293^{ALAS2F} and FT293^{ALAS2FΔIRE} cells when these cells were treated with the same concentration of DOX (1 μg/mL) for 48 hours. This higher abundance of ALAS2F suggests a higher stability of ALAS2F compared to ALAS1F or OTC-LucF in FT293 cells, since the transcription of these genes was regulated by the same promoter in the expression cassette. Moreover, the marginally lower expression of the ALAS2F protein in FT293^{ALAS2F} cells (Lane 7) compared to FT293^{ALAS2FΔIRE} cells (Lane 8) might reflect the suppressive effect of the IRE in the 5'UTR of ALAS2 mRNA. Importantly, induction of ALAS2F protein expression induces heme biosynthesis in cells[20], therefore, the intracellular heme level should be increased in ALAS2F-expressing cells. Thus, these results also suggest that the ALAS2F protein is resistant to heme-induced degradation relative to the ALAS1F protein, which is susceptible to rapid heme-induced degradation mediated by the CLPXP matrix protease[8].

To study the role of the intracellular heme level on the stability of the ALAS2F and ALAS1F proteins, FLAG-tagged protein-expressing cells were treated with succinylacetone (SA, a specific inhibitor of heme biosynthesis) alone or in combination with the heme analog hemin. As shown in Fig.2A (Lanes 4-6), SA treatment caused the accumulation of ALAS1F, which was reversed by the addition of hemin to the culture medium, as previously reported by Kubota et al.[8]. This phenomenon was also observed for ALAS2F, albeit at a lower magnitude than observed for ALAS1F (Fig.2A, Lanes 7-9). The SA treatment resulted in slight accumulation of the ALAS2F protein, and the addition of hemin marginally decreased the abundance of ALAS2F proteins that accumulated after SA treatment. Neither SA nor hemin affected the protein level of OTC-LucF(Fig.2A, Lanes 1-3).

Then, we proceeded to examine the stability of these two proteins under treatment with the same concentration of hemin. Since SA treatment stabilized both proteins (Fig.2A), the half-lives of these proteins were not well determined during SA treatment without hemin (CHX-treated cells in Fig.2C, E). As shown in Fig.2B and C, the calculated half-life of the ALAS1F protein in hemin-treated FT293 cells was 13 minutes, whereas ALAS2F has a half-life of approximately 3.5 hours in hemin-treated FT293 cells (Fig.2D, E). These results suggest that the ALAS2F protein is more stable than the ALAS1F protein under excess heme conditions.

2. ALAS2F associates with CLPXP, LONP1 and HSPA9

It has been reported that ALAS1 is negatively regulated by heme, while ALAS2 expression is induced during erythroid differentiation[3]. Although the details of the difference between ALAS1 and ALAS2 in the regulation of gene expression remain largely unknown, our results suggest that the susceptibility of these isozymes to heme-induced degradation seems to be different. Thus, we hypothesized that some proteins associate with the ALAS2 protein to protect it from heme-dependent degradation. To identify binding partners of the ALAS2 protein, ALAS2F immunoprecipitates were analyzed by tandem mass spectrometry, and many proteins were identified (manuscript in submission). We speculate that some of these proteins may interact with the ALAS2 protein and regulate its turnover in the mitochondrial matrix. Among the proteins identified as
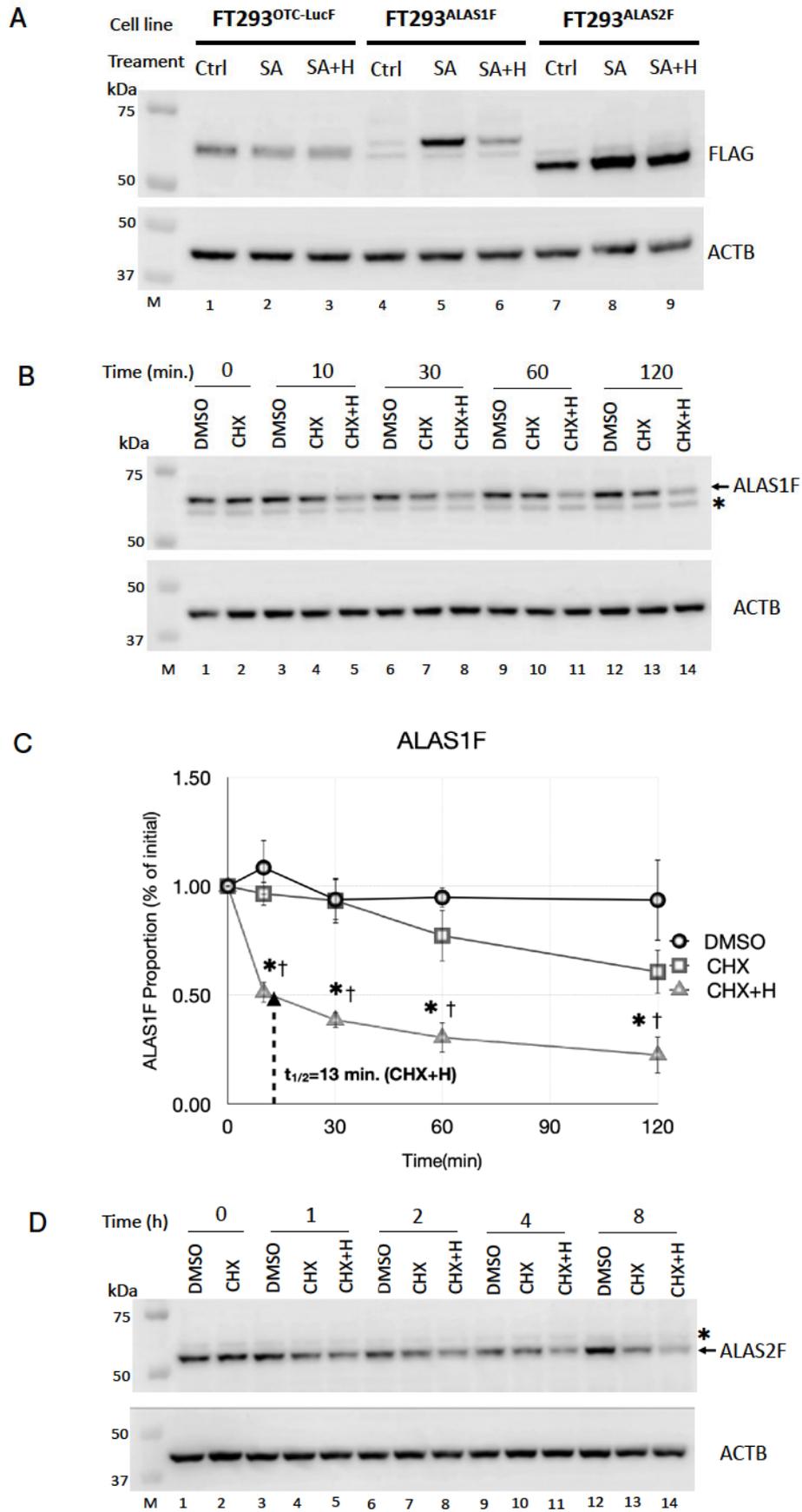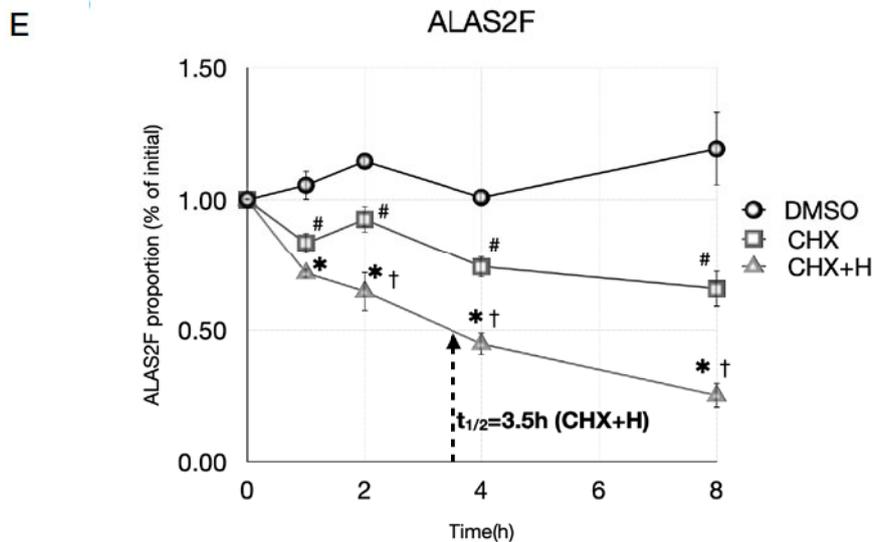
Fig. 2. The ALAS2F protein is more stable than the ALAS1F protein under hemin treatment
   conditions in FT293 cells.
   A) Effect of succinylacetone (SA) and hemin on the steady-state protein levels of ALAS2F
      and ALAS1F. FT293^OTC-LucF, FT293^ALAS1F, and FT293^ALAS2F cells were treated with 1 mM SA
      (Lanes 2, 5, 8) followed by treatment with hemin (Lanes 3, 6, 9). Untreated cells were used as
      a control (Lanes 1, 4, 7).
   B) Total lysates of ALAS1F-expressing cells pretreated with SA for 24 hours were prepared
      0, 10, 30, 60 and 120 minutes after the addition of cycloheximide with or without hemin to
      the culture medium. The representative result of three independent experiments is shown.
      The black arrow indicates the signal of mature ALAS1F, whereas the asterisk indicates the
      nonspecific signal detected by the anti-FLAG monoclonal antibody.
   C) The intensity of the signals produced by the ALAS1F protein was normalized to that
      produced by actin. The ALAS1F expression level at each time point relative to that in the
      control cells at 0 minutes was used to determine the half-life of the ALAS1F protein. The
      data are expressed as the mean and standard deviation of three independent experiments.
      The asterisk (*) and the dagger (†) denote significant differences in the "DMSO vs. CHX+H"
      or "CHX vs. CHX+H" comparisons, respectively. The open circles, open squares and open
      triangles indicate the mean ALAS1F expression level in DMSO-treated, cycloheximide-treated
      and cycloheximide plus hemin-treated cells, respectively.
   D) Total lysates of FT293^ALAS2F cells pretreated with SA for 24 hours were prepared 0, 1, 2, 4
      and 8 hours after the addition of cycloheximide with or without hemin to the culture medium.
      The representative result of three independent experiments is shown. The black arrow
      indicates the band for mature mitochondrial ALAS2F, whereas the asterisk indicates the
      nonspecific band detected by the anti-FLAG monoclonal antibody.
   E) The half-life of the ALAS2F protein was determined using a similar method used for
      determination of the ALAS1F half-life. The asterisk (*), hashmark (#) and dagger (†) signs
      represent significant differences in the "DMSO vs. CHX+H", "DMSO vs. CHX" and "CHX
      vs. CHX+H" comparisons, respectively. The open circles, open squares and open triangles
      indicate the mean ALAS2F expression level in DMSO-treated, cycloheximide-treated and
      cycloheximide plus hemin-treated cells, respectively.

ALAS2F-associated proteins, we selected CLPX, CLPP and LONP1, which have been previously identified in ALAS1F protein immunoprecipitates[8], as well as HSPA9, which has been reported to be a causative gene for congenital sideroblastic anemia. As a first step, we confirmed the associations between ALAS2F and these selected proteins using

A

B
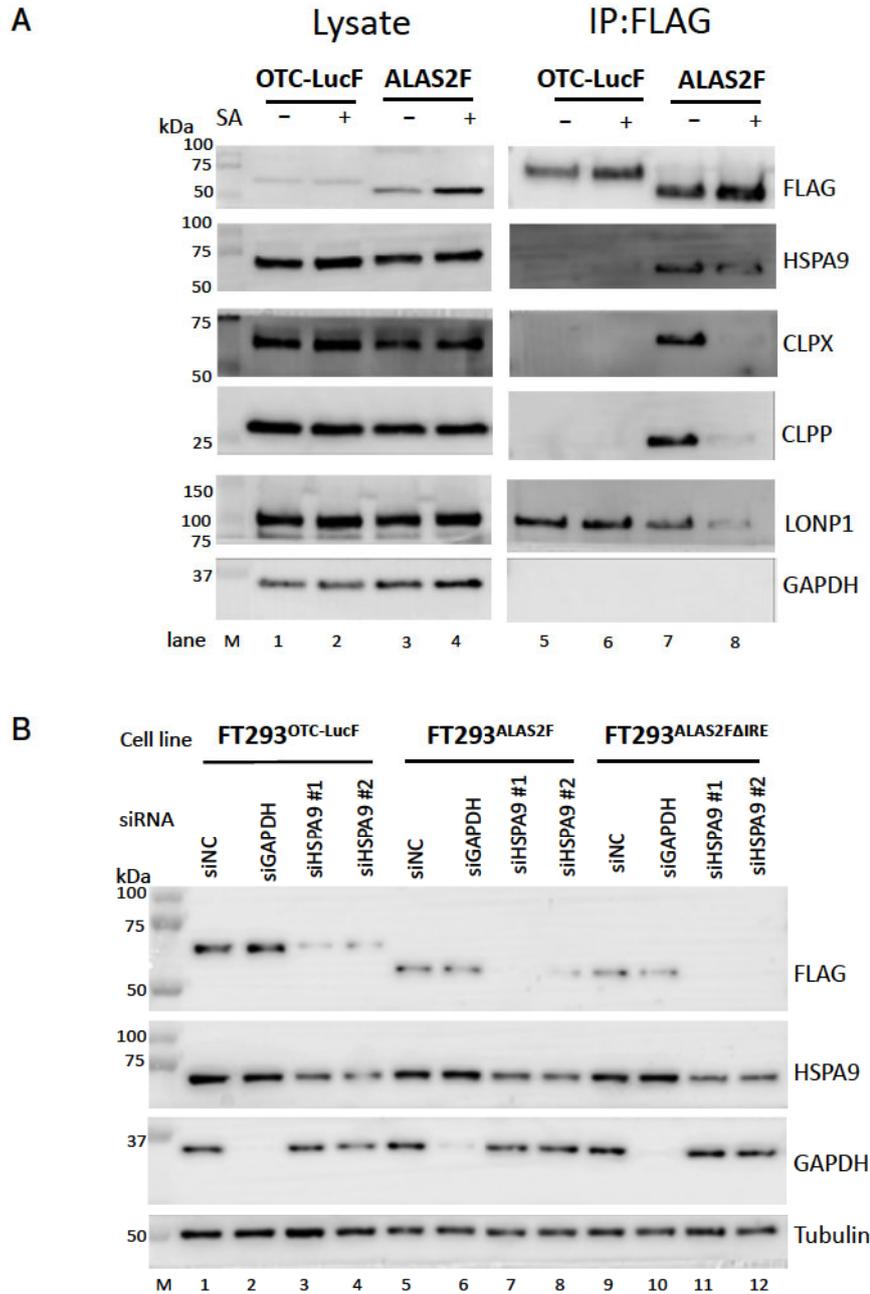
immunoprecipitation followed by Western blot analysis. As shown in Fig.3A, the CLPX, CLPP and LONP1 proteins were enriched in the ALAS2F protein immunoprecipitates from cells without SA treatment, while these proteins were barely detected after SA treatment. These results suggest that CLPXP (CLPX and CLPP) and LONP1 associate with ALAS2F in

a heme-inducible manner and could be involved in the degradation of ALAS2 under excess heme conditions, as observed for ALAS1F degradation[8]. Interestingly, the chaperone protein HSPA9 was also detected in the ALAS2F immunoprecipitates under both SA-untreated and treated conditions, suggesting that the HSPA9 protein may modulate the ALAS2F
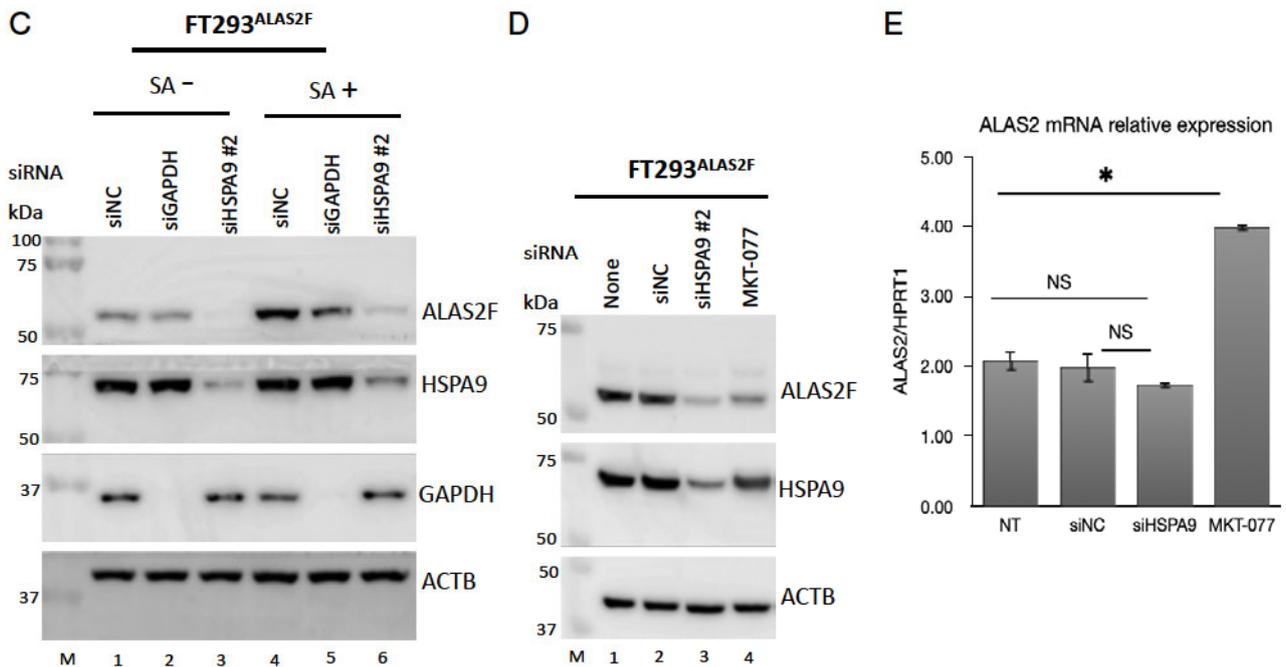
Fig. 3. HSPA9 regulates the protein stability of ALAS2F expressed in FT293 cells.
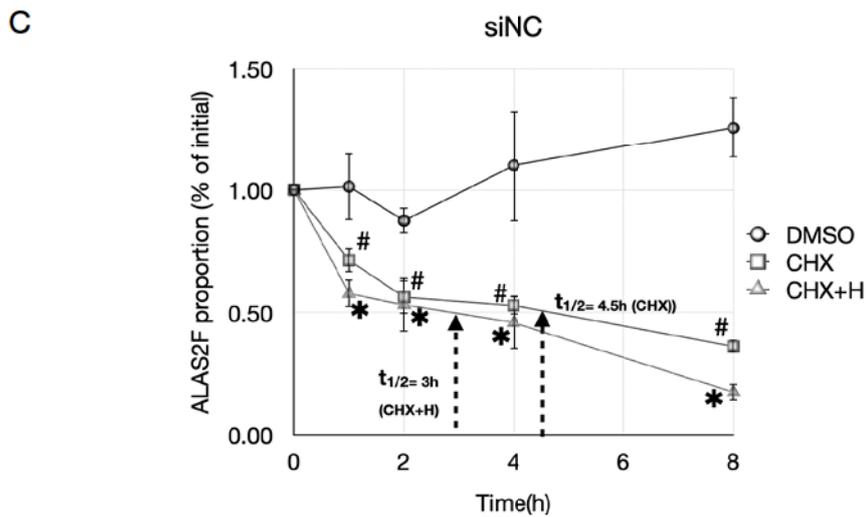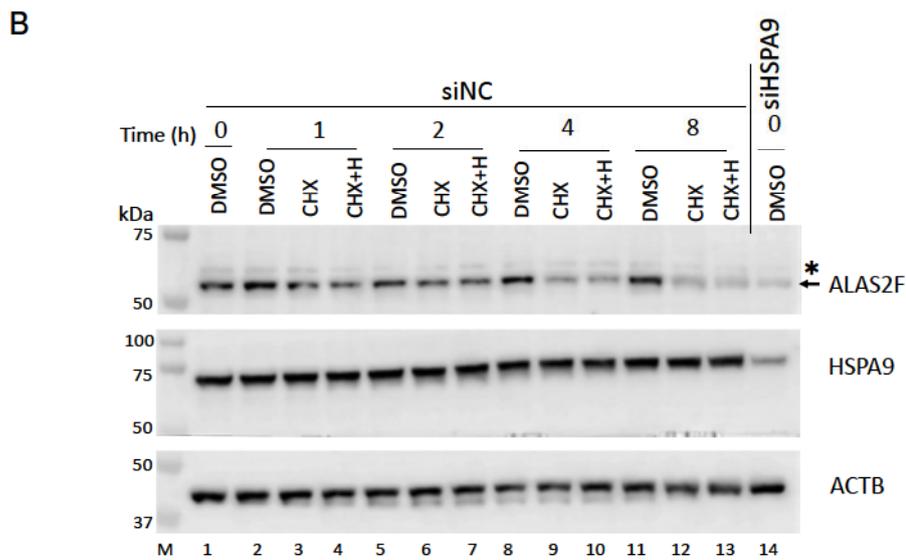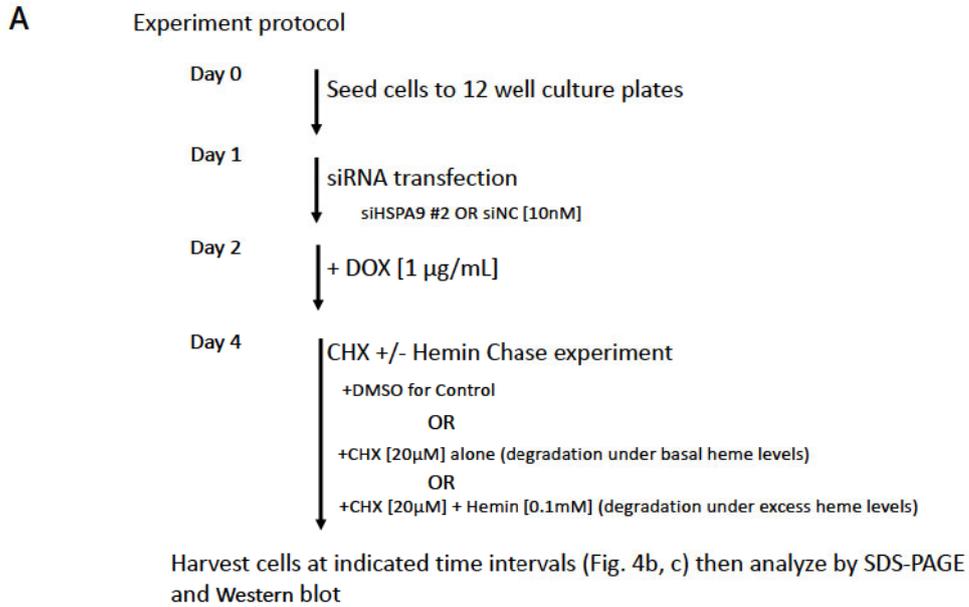
A) ALAS2F forms a complex with HSPA9, CLPX, CLPP and LONP1; FT293[OTC-LucF] and FT293[ALAS2F] cells were incubated with (Lanes 2, 4, 6, and 8) or without (Lanes 1, 3, 5, 7) SA for 24 hours and were then immunoprecipitated using anti-DDDDK agarose. FLAG-tagged HSPA9, CLPX, CLPP, and LONP1 proteins in the immunoprecipitates and total cell lysates were detected by Western blotting using specific antibodies. GAPDH was used as a loading control for the total cell lysate. M, Molecular weight marker.

B) FT293[OTC-LucF] cells (Lanes 1-4), FT293[ALAS2F] cells (Lanes 5-8) and FT293[ALAS2FΔIRE] cells (Lanes 9-12) were transfected with two independent siRNAs against HSPA9 (siHSPA9 # 1 and siHSPA9 #2) for 72 hours, followed by induction of the expression of FLAG-tagged by treatment with 1 μg/mL doxycycline for 48 hours, and Western blot analysis was then performed. A nontargeted siRNA (siNC, Lanes 1, 5, 9) was used as a negative control and siGAPDH (Lanes 2, 6, 10) was used as a positive control for siRNA transfection.

C) The effect of HSPA9 KD on ALAS2F expression was also evaluated in FT293[ALAS2F] cells treated with (Lanes 4-6) or without (Lanes 1-3) SA.

D) The effects of HSPA9 KD using siHSPA9 #2 (Lane 3) and the inhibitor of HSPA9 function MKT-077 (Lane 4) on ALAS2F expression were determined.

E) ALAS2F mRNA expression was measured using real-time PCR. n = 3, the error bars denote the SDs, *$p < 0.05$, NS, not statistically significant.

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protein level through either direct or indirect association under both basal and heme-depleted conditions. Thus, we further analyzed the roles of HSPA9 in the expression of the ALAS2F protein.

3. Knockdown or chemical inhibition of HSPA9 decreases the ALAS2F protein level

To investigate the role of HSPA9 in ALAS2F protein expression, we used two independent siRNAs (siHSPA9#1 and siHSPA9#2) to knock down endogenous HSPA9 expression. Each siRNA targeted a different sequence of the HSPA9 mRNA, and both siRNAs reproducibly suppressed HSPA9 expression in transfected cells (Fig.3B, Lanes 3, 4, 7, 8, 11, 12). It should be noted that the ALAS2F protein level was
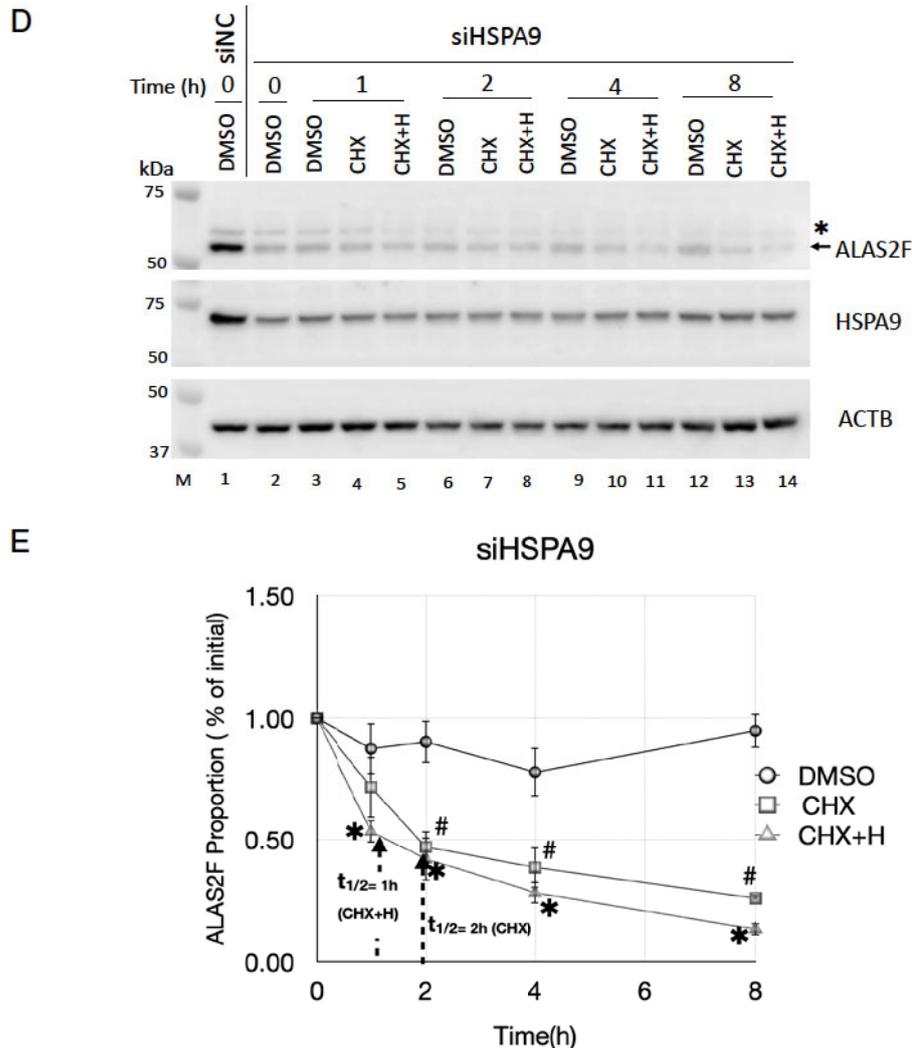
A   Experiment protocol

Day 0 | Seed cells to 12 well culture plates

Day 1 | siRNA transfection
         | siHSPA9 #2 OR siNC [10nM]

Day 2 | + DOX [1 µg/mL]

Day 4 | CHX +/- Hemin Chase experiment
         | +DMSO for Control
         | OR
         | +CHX [20µM] alone (degradation under basal heme levels)
         | OR
         | +CHX [20µM] + Hemin [0.1mM] (degradation under excess heme levels)

Harvest cells at indicated time intervals (Fig. 4b, c) then analyze by SDS-PAGE and Western blot

B

C

Fig. 4. Knockdown of HSPA9 accelerates the degradation of ALAS2F in FT293$^{ALAS2F\Delta IRE}$ cells.
A) Schematic representation of the experimental protocol for determining the ALAS2F half-life in siNC-and siHSPA9-transfected cells. B) Representative immunoblot results of the ALAS2F protein stability assay in siNC-transfected FT293$^{ALAS2F\Delta IRE}$ cells. The small black arrow denotes the signal for the mature mitochondrial ALAS2F protein, while the asterisk indicates the nonspecific signal detected by the anti-FLAG monoclonal antibody. C) Graphical presentation of the ALAS2F protein stability assay in siNC-transfected cells. The half-life of ALAS2F is denoted by the black dotted arrows at 4.5 hours (CHX only) and at 3 hours (CHX+H). The asterisk (*) represents a significant difference between DMSO (DMSO)- and CHX plus hemin (CHX+H)-treated cells, while the hashmark (#) represents a significant difference between DMSO- and CHX-treated cells. The open circles, open squares, and open triangles indicate DMSO-treated cells (DMSO), CHX-treated cells (CHX) and CHX plus hemin-treated cells (CHX+H), respectively. The mean of 3 independent experiments (n = 3) was plotted on graphs to extrapolate the protein half-life. The error bars indicate the SDs; *p < 0.05 (DMSO vs. CHX+H); #p < 0.05 (DMSO vs. CHX). D) Representative immunoblot results of the ALAS2F protein stability assay in siHSPA9-transfected FT293$^{ALAS2F\Delta IRE}$ cells. The small black arrow denotes the signal of the mature mitochondrial ALAS2F protein, while the asterisk indicates the nonspecific signal detected by the anti-FLAG monoclonal antibody. E). Graphical presentation of the ALAS2F protein stability assay in siHSPA9-transfected cells. The black dotted arrows denote the half-life of the ALAS2F protein at 2 hours and 1 hour in CHX-treated cells and CHX+H-treated cells, respectively. The asterisk (*) represents a significant difference between DMSO (DMSO)- and CHX plus hemin (CHX+H)-treated cells, while the hashmark (#) represents a significant difference between DMSO- and CHX-treated cells. The open circles, open squares and open triangles indicate DMSO-treated cells (DMSO), CHX-treated cells (CHX) and CHX plus hemin-treated cells (CHX+H), respectively. The mean of 3 independent experiments (n = 3) was plotted on graphs to extrapolate the protein half-life. The error bars indicate the SDs; *p < 0.05 (DMSO vs. CHX+H); #p < 0.05 (DMSO vs. CHX).

decreased in siHSPA9-transfected cells (Fig.3B, Lanes 7, 8). Interestingly, these decreases in the ALAS2F level were also observed in FT293$^{ALAS2F \Delta IRE}$ cells (Lanes 11,12). While it has been reported that HSPA9 regulates ALAS2 protein expression through its function in iron sulfur cluster biogenesis, which affects the translation of ALAS2 mRNA [14], our results suggest that an independent mechanism, such as a direct association between ALAS2 and HSPA9, might be important for the protein expression of ALAS2F. On the other hand, we unexpectedly found that suppression of HSPA9 using siRNAs resulted in a decrease in the OTC-LucF protein level (Fig.3A, Lanes 3,4). While the association between the OTC-LucF and HSPA9 proteins was not well confirmed using immunoprecipitation followed by Western blot analysis (Fig.3A), the interaction was detected by LC–MS/MS using the OTC-LucF protein as a bait (data not shown). These results suggest that HSPA9 also associates with the LucF protein for stabilization in the mitochondrial matrix, although the binding affinity of HSPA9 for OTC-LucF might be weaker than that for ALAS2F. Then, we also examined the role of HSPA9 in ALAS2F protein expression in the presence of SA, since the HSPA9 protein associated with the ALAS2F protein after treatment with SA (Fig.3A, Lane 8). For this purpose, we induced ALAS2F expression after the introduction of each siRNA and then treated these cells with SA. As shown in Fig.3C, ALAS2F protein expression was downregulated in HSPA9 knockdown cells even after SA treatment (Fig.3C, Lane 6), suggesting that HSPA9 has an essential role in stabilizing ALAS2F regardless of the intracellular heme concentration. Although siHSPA9 reduced the expression of the endogenous HSPA9 protein to approximately less than half (Fig.3C, Lanes 3,6) of its level in control cells (Fig.3C, Lanes 1,4), a certain amount of HSPA9 was still expressed in these cells; therefore, as a next step, we tried to use MKT-077, a specific HSPA9 inhibitor [21-23], to suppress the function of the HSPA9 protein. As shown in Fig.3D, the expression level of the HSPA9 protein was not changed by treatment with MKT-0 77 compared to those in control cells (Lanes 1,2 and 4), whereas ALAS2F protein expression was reduced in MKT-077-treated cells (Lane 4) to a level comparable to that in siHSPA9-treated cells (Lane 3). Since the ALAS2F protein level in MKT-077-treated cells (Fig.3D, Lane 4) was slightly higher than that in siHSPA9-transfected cells (Fig.3D, Lane 3), we determined the ALAS2F mRNA expression level in these cells. The ALAS2F mRNA level was not significantly different among control cells (NT), siNC-transfected cells and siHSPA9-transfected cells, whereas it was significantly higher in MKT-077-treated cells than in these other cells (Fig.3E). Although the mechanism underlying the induction of ALAS2F mRNA expression by MKT-077 remains unclear, these results suggest that the suppression of HSPA9 protein expression by siRNA, as well as the inhibition of HSPA9 function by the specific inhibitor, similarly resulted in decreased expression of the ALAS2F protein. Thus, we successfully confirmed the essential role of HSPA9 in maintaining the protein level of ALAS2F in mitochondria.

4. Knockdown of HSPA9 accelerates the degradation of ALAS2F

As a last step, we tried to determine whether HSPA9 plays a role in protecting the ALAS2F protein under conditions of excess heme. Thus,

we tried to determine the effect of hemin on the half-life of the ALAS2F protein in HSPA9-suppressed cells and compared it to that in control cells according to the protocol shown in Fig. 4A and in the "Materials and Methods" section. As shown in Fig. 4B and C, the half-life of the ALAS2F protein in siNC-transfected cells was approximately 3 hours and 4.5 hours in the presence and absence of hemin, respectively. However, in siHSPA9-transfected cells, the half-life of the ALAS2F protein was decreased to approximately 1 hour and 2 hours in the presence and absence of hemin, respectively (Fig. 4D, E). These results suggest that HSPA9 protein protects ALAS2F from degradation in the mitochondrial matrix not only under steady state but also under excess heme conditions. It should be noted that the ALAS2F protein level in siHSPA9-transfected cells (Fig. 4D, Lane 2) was already decreased compared to that in siNC-transfected cells (Fig. 4D, Lane 1) before treatment with CHX or hemin. Thus, the HSPA9 protein plays an essential role in maintaining the ALAS2F protein level under steady-state (Figs. 3B, 4D,E), heme-depleted (Fig. 3C) and excess heme (Fig. 4D,E) conditions.

IV. Discussion

In this study, we compared the stability of recombinant c-terminal FLAG-tagged ALAS proteins expressed in fibroblast (FT293) cells under excess heme conditions and found that the ALAS2F protein had a longer half-life (approximately 3.5 hours) than the ALAS1F protein (approximately 13 minutes). In addition, Rondelli et al.[24] recently investigated the protein level of endogenous ALAS2 in cultured murine erythroleukemia (MEL) cells, and the half-life of the ALAS2 protein can be extrapolated from a graph in their paper to be 4 hours in differentiated (hemoglobinized) wild-type (WT) cells. Even though the intracellular condition in differentiated MEL cells may have been different from the condition in the hemin-treated FT293 cells in our study, the close approximation of the calculated half-life of ALAS2F in our study to that of endogenous ALAS2 protein in the referenced work[24] supports the usefulness of experimentation with recombinant proteins to study the behavior of native proteins.

Given the high similarity in the amino acid sequence (~60% identity) between ALAS1 and ALAS2[25], the presence of heme regulatory motifs[8, 26, 27], and the heme-dependent formation of complexes with mitochondrial matrix proteases (CLPXP and LONP1) observed for ALAS2F, as shown in Fig. 3A, the large difference in the half-lives of ALAS2F and ALAS1F is quite an intriguing phenomenon. On the one hand, the difference could be due to the slight sequence and structural dissimilarity of these proteins, especially in the N-terminal and C-terminal extensions[25], but on the other hand, there might be additional factors and/or interacting proteins that specifically regulate the stability of ALAS2.

In the ALAS2F immunoprecipitates, many other mitochondrial proteins, including important chaperone proteins, were identified by tandem mass spectrometry, and these proteins will be described in a separate article by our group (manuscript in submission). In the present paper, we focused on HSPA9, of which we confirmed an association with ALAS2F (Fig. 3A), and together with loss-of-function mutations in its cochaperone HSCB, loss-of-function mutations in HSPA9 have been reported to cause CSA[13,28], similar to loss-of-function mutations in ALAS2[5].

Indeed, when we investigated the role of HSPA9 in ALAS2F protein stability, we found that knockdown of HSPA9 by siRNA or functional inhibition of HSPA9 by MKT-077 resulted in a decrease in the ALAS2F protein level even when ALAS2F was expressed from ALAS2F ΔIRE mRNA. Moreover, when cells were incubated with hemin, HSPA9 knockdown resulted in rapid degradation of the ALAS2F protein, with a half-life of approximately 1 hour (Fig. 4D,E), compared to the 3 hours in siNC-treated cells (Fig. 4B,C). This means that HSPA9, in addition to regulating ALAS2 mRNA translation through the IRE-IRP system, as postulated previously [14], also regulates the stability of the ALAS2 protein in the mitochondrial matrix.

Previously, it was reasoned that ALAS2 proteins in erythroid cells are protected from heme-mediated regulation because most heme molecules are bound to globin, making the labile regulatory heme concentration low [27]; however, our analysis using ALAS2F subjected to excess heme in nonerythroid cells supports the hypothesis that interacting partner proteins such as HSPA9 confer stability against heme-induced degradation on the ALAS2 protein. In the present study, we used FT293 fibroblast cells, instead of human erythroid cell lines such as K562 cells [29], for studying the stability of the ALAS2 protein. Although we have tried to establish a K562 cell line that stably expresses ALAS2F protein, we have never succeeded in obtaining such cell lines (data not shown). We wondered whether stably expressing ALAS2F might stimulate the production of excess heme, causing serious oxidative stress. Thus, we decided to introduce the ALAS2F expression cassette into commercially available FT293 cells,

in which the expression of ALAS2F protein is regulated in a DOX-dependent manner. We are now trying to establish a Flp-In T-Rex K562 (FTK562) cell line to confirm the results observed using FT293 cells.

Currently, it remains unclear whether ALAS2 and HSPA9 directly bind or are part of a large protein complex. To answer this question, it may be useful to perform an *in vitro* pull-down assay using recombinant ALAS2 protein [20] and recombinant HSPA9 protein [30,31] to confirm the direct association between ALAS2 and HSPA9. Additionally, the involvement of CLPXP in the heme-dependent degradation of the ALAS2 protein could be determined using an *in vitro* degradation assay.

In summary, our study reveals that recombinant ALAS2 protein (ALAS2F) expressed in a nonerythroid cell line (FT293) is more stable than ALAS1F when exposed to an excess amount of heme. This higher stability of ALAS2F is conferred by interacting proteins such as HSPA9, which protects ALAS2 from degradation by mitochondrial matrix proteases. Although this analysis of recombinant ALAS2 protein expressed in nonerythroid cells may be representative for the native/wild-type protein, future analysis using human erythroid cells, such as K562 cells, is warranted to clarify the biochemical relationship between ALAS2 and HSPA9.

Conflicts of Interest: The authors have no conflicts of interest to declare.

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赤芽球特異的 5- アミノレブリン酸合成酵素はミトコンドリア内で HSPA9 により安定化される

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要旨

　脊椎動物におけるヘム生合成は 5- アミノレブリン酸合成酵素 (ALAS) の 2 つのアイソフォームにより組織特異的に制御される．全細胞で発現する非特異的アイソフォームの ALAS1 はミトコンドリアマトリクスのタンパク質分解酵素によりヘム依存性に分解されるが，赤芽球特異的なアイソフォームの ALAS2 は赤芽球で安定に発現しヘモグロビンにヘムを供給する．このような ALAS1 とは異なる ALAS2 のタンパク質の安定性の詳細を明らかにするため，FLAG タグを付与した ALAS1（ALAS1）と ALAS2（ALAS2F）の安定性を比較した．その結果，ヘム過剰状態で同じ分解酵素と複合体を形成するにも関わらず ALAS2F の半減期の方が長いことを見出した．さらに，ALAS2F 複合体を免疫沈降法により精製した後に質量分析法を用いて解析し，ALAS2F と結合するタンパク質として HSPA9 を同定した．HSPA9 の発現や機能の抑制により ALAS2F のタンパク質が減少したことから，HSPA9 は ALAS2 タンパク質の分解を抑制し安定化する役割を持つものと考えられた．