erythroid and hepatic cells showed different molecular size (estimated as 73 kDa or 55kDa, for each protein translated from liver polysomes or erythroid polysomes, respectively) based on SDS-PAGE analysis[12]. Interestingly, these authors also found that molecular size of each mitochondrial enzymes purified from chicken erythrocyte or liver was different from those of polysome derived ALAS protein. Moreover, Ouchterlony double-diffusion study demonstrated that ALAS purified from chicken erythroid cells was only partially cross-reactive with the antibody against ALAS that was purified from chicken liver mitochondria[12]. These results strongly suggested that ALAS expressed in hematopoietic tissues was not identical to that expressed in liver, and each ALAS was modified post-translationally during or after mitochondrial translocation.

Finally, molecular cloning of distinct cDNA was required to distinguish ALAS isozymes. ALAS cDNA for chicken[13], mouse[14] and human[15] were cloned from liver cDNA library. Independently, screening of chicken erythroid cell cDNA library resulted in the isolation of erythroid-specific transcript[16]. However, some researchers still believed that single ALAS mRNA species was transcribed from unique gene in all tissues based on ubiquitous expression of hepatic ALAS cDNA[14, 17, 18]. In 1989, Riddle et al. reported the molecular cloning of two independent chick ALAS cDNAs which encoded erythroid specific isozyme (named erythroid-specific ALAS; ALAS-E) or ubiquitously expressed isozyme (named non-specific ALAS; ALAS-N)[19]. Although each isozyme was encoded by distinct gene, predicted amino acid sequence of chicken ALAS-E protein was 57% identical to that of chicken ALAS-N in over all protein, and it was increased to more than 75% when their internal core region were compared[19]. Moreover, predicted molecular size of chick ALAS-E protein based on open reading frame (ORF) of erythroid ALAS cDNA was approximate 55 kDa[19], which showed excellent agreement to the estimated molecular size of in vitro translated protein using polysome derived from chicken erythroid cells[12]. Following this report, the expression of erythroid-specific ALAS mRNA in erythroid cells was established in mouse[14, 20], rat[21] and human[22]. Mapping of genes for ALAS-N and ALAS-E was also performed, and the results

confirmed that ALAS-N and ALAS-E were encoded by distinct gene. ALAS-N protein and ALAS-E protein was encoded by ALAS1 gene and ALAS2 gene, respectively. ALAS1 and ALAS2 was mapped on chromosome 3 (3p21) and X chromosome (Xp11.21) for human[22-24], or chromosome 9 (mouse genome informatics ID:87989) and X chromosome[25] for mouse, respectively.

V. Regulation of ALAS-N (ALAS1) expression

A. Heme is a powerful suppressor for ALAS-N expression

After the initial proposal by Granick in 1966[26], it has been demonstrated that heme strongly suppressed ALAS-N expression at several steps, including transcription, translation and post-translational modification (Figure 2). Hence, depletion of free heme pool (alternatively, regulatory heme pool), which may be caused by decreased de novo heme synthesis or unexpected consumption of excess amount of heme, is able to induce ALAS-N expression in non-erythroid cells.

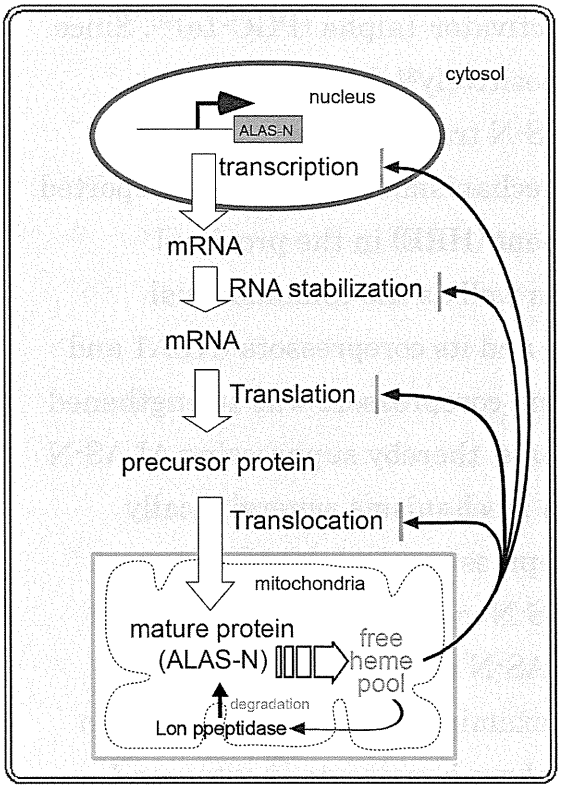

Figure 2.
Negative feedback regulation of ALAS-N expression by heme
The figure summarizes the negative feedback regulation of ALAS1 expression by heme. Red bar with arrow indicates the suppression of each step. (Adopted from ref. 1)

1. Negative regulation of ALAS-N transcription and mRNA stability by heme

Yamamoto et al. found that intravenous administration of hemin to rats dose-dependently suppressed the in vitro translational ability of ALAS-N by polysomes isolated from liver, in which ALAS-N expression was induced by allylisopropylacetamide (AIA) pretreatment[27]. Authors also found that the half-life of mRNA for ALAS-N in the polysome fraction was not shortened after hemin administration, suggesting that hemin could suppress ALAS-N expression at transcriptional level. Hemin-mediated transcriptional suppression of ALAS-N was also confirmed in vivo by measuring ALAS-N mRNA level and its half-life in rat liver, which was treated with AIA and inhibitors of transcription[28].

At least two independent mechanisms might involve in this heme-mediated transcriptional repression of ALAS-N. It has been reported that orphan nuclear receptor Rev-arbα act as a heme sensor[29], and heme binding to it recruited NCoR/histone deacetylase 3 (HDAC3) corepressor complex, which repressed the transcription of peroxisome proliferator-activated receptor gamma coactivator 1alpha (PGC-1α)[30]. Since PGC-1α regulates ALAS-N transcription positively[31], heme binding to Rev-arbα resulted in the reduction of ALAS-N transcription[32].

Gotoh et al.[33] proposed another mechanism. These authors reported the identification of heme-responsive element (HRE) in the proximal promoter region of mouse ALAS-N gene, as well as the combinatorial binding of early growth response 1 (Egr-1) and its corepressors (NBA1 and NBA2) to HRE. The interaction of Egr-1 and corepressors was strengthened by higher concentration of intracellular heme, thereby suppressing ALAS-N transcription. It is thus possible that both mechanisms synergistically regulate heme-mediated transcriptional repression of ALAS-N gene.

As for the effect of hemin on ALAS-N mRNA stability, Drew and Ades reported that hemin destabilized ALAS-N mRNA in chick embryo hepatocyte in vitro[34]. Hamilton et al. also examined the effect of hemin on the half-life of ALAS-N mRNA in check embryo hepatocyte in vitro, and found that heme decreased ALAS-N mRNA stability, while such effect was

prevented by the treatment with certain inhibitor for translation, such as cycloheximide[35]. These results suggested that hemin involved in the destabilization of ALAS-N mRNA by enhancing the synthesis of a labile protein. Similar negative effect of heme on ALAS-1 mRNA stability was also observed in rat cell line[36]. Moreover, Roberts et al. found that alternative splicing of human ALAS-N gene produced longer transcript at its 5'UTR, and such longer transcript was resistant to heme-mediated ALAS-N mRNA decay[37]. These authors also suggested that de novo translated proteins from this extended 5'-UTR of ALAS-N mRNA may be involved in the stabilization of this longer transcript[37]. However, such protein that involves in the stabilization of ALAS-N mRNA has not yet been identified to date.

2. Heme-mediated inhibition of ALAS-N translation

Effect of heme on the translation of ALAS-N protein has been reported by Sassa and Granick using in vitro culture of chick embryo liver cells[38]. In this paper, authors found that the induction of ALAS activity in these cells by AIA or γ-hexachloro-cyclohexane was primarily mediated at translational level, and it was suppressed by the treatment with hemin. Furthermore, Yamamoto et al. examined the effect of hemin on the translation of ALAS-N using polysomes isolated from rat liver in combination with in vitro translation system[39]. Proteins were labeled with radioisotope during in vitro translation, and synthetized ALAS-N protein was specifically immune precipitated using anti-ALAS-N antibody to determine the translational efficiency. As a result, authors found that synthesis of ALAS-N protein was reduced to about 40% of the control by 30μM hemin, whereas efficiency of total protein synthesis was not affected[39]. However, molecular mechanism for this regulation still remains unclear.

3. Inhibition of mitochondrial translocation of ALAS-N by heme

It has been confirmed that ALAS-N was localized at mitochondrial matrix[40, 41], although ALAS-N is a nuclear encoded mitochondrial enzyme[22]. ALAS-N protein thus must be translated in cytosol as a precursor protein, and then transported into mitochondrial matrix to exhibit its function. As

discuss below, it has been well known that heme inhibits the translocation of ALAS-N protein into mitochondria, thereby suppresses ALAS activity. Since succinyl CoA, an essential substrate for ALAS, is exclusively available in mitochondrial matrix, ALAS is not able to produce ALA in cytosol. However, mechanisms for mitochondrial targeting of ALAS and its regulation by heme were unknown for a long time.

Sholnick et al. have first reported a partial purification of ALAS from soluble cytosol fraction of normal or AIA-treated rat liver[42]. Either enzyme purified from normal or AIA-treated rat was enzymatically active in vitro, while ALAS activity in soluble fraction increased approximate ten times after AIA-treatment. Since it was found that AIA-treatment induced the accumulation of ALAS in cytosol, as well as in mitochondria, authors hypothesized that ALAS might translocate from cytosol into mitochondria. Interestingly, using partially purified ALAS, these authors demonstrated that heme could directly inhibit ALAS activity in vitro in dose dependent manner, although the mechanism of such direct effect of heme on ALAS activity was unclear.

Hayashi et al. also found that the AIA-treatment on rat induced the accumulation of ALAS in soluble fraction of the liver, as well as in mitochondria[43]. They have successfully purified ALAS from soluble fraction of the liver, and confirmed that such enzyme was active in vitro. It was also found that AIA-treatment mediated accumulation of ALAS protein in cytosol and mitochondrial fraction was abolished by the concurrent treatment with cycloheximide, mitomycin C, actinomycin D or hemin. Based on these results, authors hypothesized that ALAS was synthesized in cytosol, then subsequently translocated into mitochondria. Authors also found that the half-life of each protein and the suitable condition for ammonium sulfate fractionation for purification was different between cytosolic enzyme and mitochondrial enzyme, suggesting that mitochondrial ALAS was modified before or after entering the mitochondria. These authors also demonstrated that the administration of hemin into AIA-pretreated rat induced the accumulation of ALAS in cytosolic fraction of the liver, where as mitochondrial ALAS activity was greatly suppressed[44]. Immunochemical

analysis revealed that specific antibody against the cytosolic enzyme similarly reacted to the mitochondrial one[45]. Thus, the line of evidence suggested that cytosolic ALAS in liver should be a precursor form of mitochondrial ALAS.

Yamauchi et al. finally demonstrated that newly synthesized ALAS protein in cytosol was rapidly incorporated into mitochondria, and hemin could inhibit this step in the liver of AIA-treated rat[46]. Interestingly, it was found that molecular size of newly synthesized ALAS subunit in vitro using polysomes isolated from rat liver was similar to that of ALAS purified from rat cytosol fraction, but significantly larger than that of ALAS purified from rat mitochondrial fraction[47]. Since the difference in the subunit molecular size between cytosolic ALAS and mitochondrial ALAS was reproducibly observed in chicken liver[12], authors hypothesized that ALAS was translated as a precursor form in cytosol, then was processed in the course of translocation into mitochondrial matrix to become mature form. Hayashi et al. confirmed it using in vitro translation and translocation system[48]. Briefly, radio-labeled precursor form of chicken ALAS-N was translated in vitro from chicken liver ribosomes, then translated proteins were incubated with purified chicken liver mitochondria. After the incubation, samples were centrifuged for separating mitochondrial fraction and supernatant to purify ALAS-N protein from each fraction by immunoprecipitation using anti ALAS-N antibody. After the purification of ALAS-N protein from each fraction, samples were analyzed by SDS-PAGE followed by autoradiography. As a result, in the mitochondrial fraction, precursor form and the mature form of ALAS-N protein was observed, latter of which was not detected in the supernatant. Authors also found that hemin could inhibit the mitochondrial translocation even in this cell-free system, suggesting that hemin might directly regulate this process.

Hemin-mediated accumulation of ALAS in liver cytosol was observed in several species, including rat (see above) and chicken[49], however, details of the mechanism for this regulation remained unknown until the excellent report by Lathrop and Timko[50]. In this report, authors identified that amino (N-) terminal region of mouse ALAS-E worked as a presequence

for mitochondrial targeting and translocation into matrix. Although amino acid sequence of presequence was only 24% identical between ALAS-N and ALAS-E, authors found that a part of presequence were conserved between ALAS-E and ALAS-N presequence in human, chicken and mouse, and termed heme regulatory motif (HRM) (Figure 3). Consensus amino acid sequence of HRM consists of four peptides (Arg, Lys or Asn-Cys-Pro-Lys, Leu, Met or hydrophobic residue), whereas Cys-Pro dipeptides motif seemed to be a core sequence of HRM. Importantly, in vitro transcription, translation and mitochondrial import experiment revealed that these motives are responsible for the hemin-mediated inhibition of mitochondrial translocation of mouse ALAS-E protein in vitro. Briefly, authors identified two distinct HRMs within the presequence of mouse ALAS-E, and disruption of both HRMs by replacing cycteine residue with serine residue released mouse ALAS-E protein from the heme-mediated inhibition of translocation. Of note, mitochondrial translocation of human ornithine transcarbamoylase (OTC), which was originally unregulated mitochondrial protein, was clearly inhibited by hemin in vitro, when single HRM was inserted into OTC presequence. Thus, authors concluded that not only ALAS-E but also ANAS-N should be regulated their translocation from cytosol to mitochondrial matrix through HRM by hemin.

In fact, Munakata et al. expressed the precursor protein for rat ALAS-N in Quail fibroblast, and observed that the mitochondrial translocation of rat ALAS-N was clearly inhibited by exogenous hemin, although such heme-mediated inhibition was reversed by the introduction of the mutations, which replaced cysteine residue with alanine, into all three HRMs. These results indicated that HRMs of rat ALAS-N were responsible for the heme-mediated inhibition of mitochondrial translocation in vivo[51]. These authors also demonstrated that the mitochondrial translocation of ALAS-E was not inhibited by exogenous hemin, suggesting that the expression of ALAS-E is only roughly regulated at this step by heme, compared to ALAS-N.

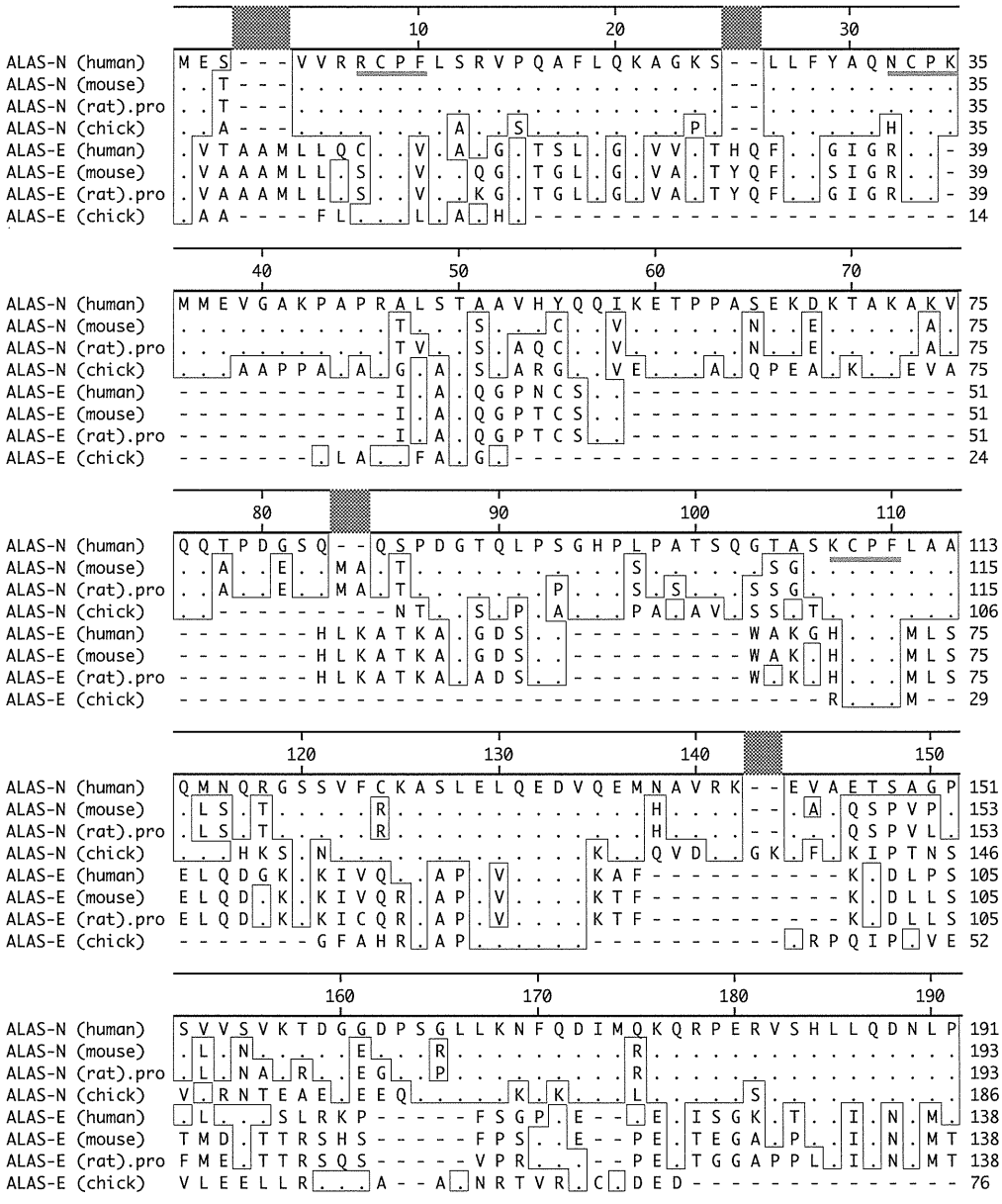

Figure 3. Amino acid sequence of human, mouse, rat and chicken ALAS at their N-terminal end.

Identical amino acid to that of human ALAS-N was shown as dot, and they were boxed. Lack of identical or similar amino acid to corresponding sequence of the other proteins was shown as "- ". Red horizontal bar indicates heme regulatory motif (HRM).

Dailey et al. also confirmed the biological consequence of HRMs in the presequence of human ALAS-N[52]. Similar to mouse and rat ALAS-N, there are three HRMs were identified in human ALAS-N, one of which was located at the N-terminal end of mature protein, and the other two were located within the presequence. Authors expressed N-terminal portion of human ALAS-N, which contained all putative HRMs, as a fusion protein with green fluorescent protein (GFP) in murine hepatoma cell line. Under the basal condition, targeting of GFP to mitochondria was not observed, suggesting that human ALAS-N presequence was sensitive to intracellular heme level in these cells, which might allow minimum amount of ALAS-N for targeting to mitochondria.

4. Heme-mediated degradation of ALAS-N protein in mitochondria

Heme-mediated protein degradation of ALAS-N in mitochondrial matrix was reported in rat hepatocyte[53], although its precise mechanism remained unknown. Using human liver cells, Tian et al. showed the evidences that lon peptidase 1 (LONP1), which was a nuclear encoded mitochondrial protease, involved in the degradation of mature ALAS-N protein in mitochondria. Moreover, authors demonstrated that the degradation of mature ALAS-N was accelerated by the increase of intracellular heme level, while suppression of LONP1 expression by specific siRNA diminished heme-dependent acceleration of ALAS-N degradation[54]. However, the detail is still unclear how heme regulates the LONP1 protein expression.

B. Xenobiotic-mediated induction of ALAS-N expression

During an acute attack, a large amount of ALA and PBG was excreted in urine from patients with acute porphyria[55], and the induction of ALAS in the liver was responsible to this disease[56]. Since a lot of medical

reagents induced ALAS expression in patient's liver[2], several chemical reagents, such as Sedormid[57], DDC[26, 58], hexachlorobenzene[59] or AIA[26, 60, 61] were examined to induce the accumulation of ALA in animal body, to establish an animal model for acute porphyria. Especially, ALA was found to be greatly accumulated along with strong hepatic ALAS induction in guinea pig liver [6] or rat liver [7] by DDC or AIA treatment, respectively, these reagents were thus widely used to examined the mechanisms for ALAS1 induction in animal liver. Mechanisms for an induction of ALAS1 by these reagents have long been discussed, and it was found that their function was related to the interaction-base inactivation of cytochrome P-450 followed by the decrease of free heme pool[62, 63]. Briefly, DDC interact with an active site of cytochrome P-450, resulting in an alkylation of pyrrole nitrogen atom of the heme moiety of cytochrome P-450. An alkylated heme loses its iron atom, thereby forming N-methyl protoporphyrin (N-methylPP). Since N-methylPP is a potent inhibitor of ferrochelatase, the last enzyme of heme biosynthetic pathway, formation of N-methylPP reduces heme supply to the free heme pool. AIA also alkylate nitrogen atom of heme moiety of cytochrome P-450, however, AIA covalently bind to the alkylated heme, and dissociate it from cytochrome P-450. Then, apo-P-450 combines with free heme to reconstitute P-450, resulting in the reduction of free heme pool. Thus, famous porphyrinogenic reagents, such as DDC and AIA, may indirectly induce ALAS-N expression in chicken, mouse or rat, through the feedback regulation triggered by the depletion of free heme pool.

As discussed above, proximal region of rat ANAS-N promoter plays a critical role for transcriptional regulation by hemin. However, Podvinec et al. pointed out that proximal promoter region of human ALAS-N gene was not responsible to the induction of ALAS-N promoter activity in Leghorn male hepatoma (LMH) cells by xenobiotics, such as phenobarbital, rifampicin or propylisopropylacetamide[64]. These authors performed in silico analysis and identified putative enhancer elements for xenobiotic-mediated induction at 20 kbp and 16 kbp upstream of the transcription initiation site. It was shown that these enhancer regions directly responded to prototypic inducers, and interacted with human pregnane X receptor (PXR) and human

constitutive androstane receptor (CAR), both of which were known as an orphan nuclear receptor involved in drug-induced expression of cytochrome P450 (CYP). Thus, coordinated regulation of ALAS1 and CYPs in liver by several inducers could be explained well by this mechanism. These authors also proposed similar mechanism for drug-induced induction of ALAS-N expression in chicken[65] and mouse[66]. Knockout study of PXR or CAR provided further evidences of the involvement of these molecules in the regulation of ALAS1 expression. Namely, PXR-null mice showed decreased response of ALAS1 mRNA induction in liver against xenobiotics treatment, whereas ablation of CAR gene resulted in decreased basal expression level of ALAS1 mRNA, but retained its inducibility against stimulation by xenobiotics[67]. These results suggested that several mechanisms are involved in the drug-induced upregulation of ALAS1 gene.

C. Other regulators for ALAS-N expression

1. Glucose metabolism and heme biosynthesis

Blood sugar was maintained at certain level in healthy person by a consequence of insulin and glucagon action. Food intake increases blood sugar level that stimulates insulin secretion, and the fasting induced glucagon secretion. It has been reported that fasting is a precipitating factor for acute attack of acute intermittent porphyria (AIP), and urinary excretion of PBG of patient with AIP was reciprocally related to the intake of carbonhydrate[68]. Thus, large amount of glucose administration has been recognized as a useful treatment for acute attack of AIP[2]. Tschudy et al. found that carbonhydrate administration suppressed ALAS activity, which was induced by AIA treatment, in rat liver[69]. Giger and Meyer presented the evidences that ALAS expression in the liver was induced by glucagon and cyclic AMP (cAMP), suggesting that expression level of ALAS was at least partially regulated by intracellular cAMP level in rat liver[70]. Since molecular cloning of rat ALAS-N gene revealed the presence of several

consensus cis elements for transcription factors, such as NRF-1, NF-kB and GATA, in rat ALAS-N proximal promoter region[71, 72], several researchers have tried to identify the responsible region for this "glucose effect" in the promoter. Scassa et al. revealed that the regulatory region for response to insulin was located between -459 and -354 in rat ALAS-N promoter, which contained cis elements for nuclear factor 1 (NF-1) and hepatic nuclear factor 3 (HNF-3)[73]. Interestingly, these authors found that the region highly homologous to insulin responsive element (IRE), which was identified in the promoter of several genes repressed by insulin[74], was located between two distinct HNF-3 sites of rat ALAS-N promoter.

It has been known that peroxisome proliferator-activated receptor gamma coactivator 1alpha (PGC-1α) induces the expression of many gluconeogenic key molecules, such as phosphoenolpyruvate carboxykinase and glucose-6-phosphatase, under fasting condition[75]. It was also reported that the transcription of PGC-1α was upregulated by binding of cyclic AMP response element (CRE) binding protein (CREB) to PGC-1α promoter under glucose deprivation[76]. Handschin et al. [31] examined a role of PGC-1α on ALAS-N expression, and found that the expression of ALAS-N and PGC-1α in mouse liver was concomitantly regulated in fasting and feeding, and the forced expression of PGC-1α induced ALAS-N expression in hepatocyte by recruiting FOXO1 and NRF-1 to IRE and NRF-1 binding site, respectively. PGC-1α related induction of ALAS-N mRNA was repressed by insulin, which disrupting the interaction of PGC-1α and FOXO1 by phosphorylation of FOXO1[31]. Finally, these authors also found that ALAS-N induction by fasting was not observed in liver-specific PGC-1α knockout mice, suggesting the essential role of PGC-1α in the regulation of ALAS-N expression in fasting condition. Thus, it was speculated that PGC-1α expression was upregulated in the liver as a consequence of glucagon action in fasting condition, and PGC-1α induced the ALAS-N transcription, thereby causing acute attack in patients with AIP. In contrary, administration of glucose increased the secretion of insulin, which inhibited the fasting-mediated induction of ALAS-N expression by reducing PGC-1α expression, as well as

inhibiting the interaction of FOXO1 with PGC-1α at IRE in ALAS-N promoter.

2. Circadian rhythm of heme biosynthesis

Zheng et al. first documented a circadian oscillation of ALAS-N mRNA expression in mice, which was regulated by mPer clock genes[77]. Moreover, Yin et al reported the involvement of Rev-erbα in the circadian regulation of ALAS-N expression[29]. As discussed above, Rev-erbα is able to bind to heme, resulting in the reduction of ALAS-N expression[29]. It has been reported that Rev-erbß, which was structurally related to Rev-erbα, was also heme ligand[78]. Since both Rev-erbα and Rev-erbß are critical components of mammalian clock, ALAS-N expression oscillates in a circadian manner[79]. It was reported that binding of heme to Rev-erbs recruited corepressor NcoR to its binding site, thereby suppressed the target genes[78], including several key molecules for glucose metabolism, lipid metabolism, myogenesis and adipogenesis[79]. Thus, heme should be widely involved in the regulation of metabolism and circadian clock.

3. Other transcription factors regulating ALAS-N expression

Giono et al. reported that there are two CRE like sites in the proximal promoter region of rat ALAS-N gene, and provided the evidences for direct binding of CREB to these CRE like sites[80]. These CRE like sites were essential for cAMP dependent protein kinase (PKA)-induced regulation of ALAS-N promoter activity, authors thus concluded that CREB mediated the PKA based regulation of ALAS-N expression. Since these CRE like sites were located at -38 (proximal) and -142 bp (distal) of rat ALAS-N promoter, CREB might directly regulate ALAS-N expression, in addition to the indirect activation of ALAS-N gene through the induction of PGC-1α, which was discussed above.

Guberman et al. reported that AP-1 family proteins, which expression was induced by 12-*O*-tetradecanoylphorbol-13-acetate (TPA), could bind to rat ALAS-N promoter region at -261 and -255 bp from transcription initiation site, thereby suppressed rat ALAS-N promoter

activity[81]. Moreover these authors provided evidences that this region played an important role for several signaling pathways mediated by PKC, phosphatidylinositol 3-kinase (PI3K), extracellular-signal regulated kinase (ERK1/2), and c-Jun N-terminal kinase (JNK)[82].

Furthermore, two nuclear respiratory factor 1 (NRF-1) binding site has been identified at -58 and -88 bp of rat ALAS-N promoter, and functional consequence of these cis elements was determined[71]. As a result, disruption of one of each NRF-1 binding site decreased ALAS-N promoter activity and disruption of both sites diminished promoter activity completely. Thus, these NRF-1 binding sites play an essential role for maintaining the basal transcriptional level of ANAS-N gene.

VI. Expression and its regulation of ALAS-N in vivo

Although ALAS-N ubiquitously expresses in all tissues including erythroid tissues, its expression level is somewhat vary. For example, Srivastava et al. found that the highest expression of ALAS-N mRNA was observed in adrenal grand, and an approximate 50% of adrenal grand expression level was observed in small intestine, lung, heart and testis at the steady state level. As for in liver and kidney, only quarter of ALAS-N mRNA level in adrenal grand was detected[83]. Although a crucial role of ALAS-N expression has been expected in the context of an essential role of heme in several organisms, it has never been examined in vivo until establishment of ALAS-N targeting mouse by Okano et al[84]. This mice line was created by replacing the second exon of mouse Alas1 gene with green fluorescent protein (GFP) expression cassette. Although authors found that heterozygous mice were viable and fertile, homozygous knock out mice (ANAS-N-null mice) were severely retarded in utero at embryonic day 6.5 (E6.5) and were completely reabsorbed by E8.5, demonstrating that ALAS-N is indispensable for the early embryogenesis of mouse[84].

Since second exon of Alas1 gene contained translation initiation site for ALAS-N, and GFP expression cassette carried an original translation initiation site and poly adenylation signal for expression of GFP, GFP

should expressed from targeted allele instead of ALAS-N protein. Since targeted allele derived GFP mRNA does not fused to Alas1 mRNA, which stability was regulated by intracellular heme level (see above), expression level of GFP should mimic the transcriptional level of Alas1. In this context, authors have tried to determine the cell specific or tissue specific transcriptional regulation of Alas1 gene using heterozygous ($Alas1^{+/GFP}$) mice.

In adult $Alas1^{+/GFP}$ mice, observation using fluorescent microscopy revealed that GFP fluorescence was observed in all tissues examined, and the strong GFP fluorescence was observed in liver, Harderian glands, testes (Leydig cells and spermatid), exocrine glands (submandibular and parotid glands) and endocrine glands (adrenal and thyroid glands), hematopoietic tissues (myeloid cells) and brain (meninges and choroid plexus). Remarkable GFP fluorescence was also observed in mucous epithelium cells in oral cavity, pharynx, larynx, nasal cavity and respiratory system (trachea and bronchi). These results suggested that intensity of GFP fluorescence seemed to correlate to demand for heme supply for drug metabolism (liver and lung) and steroid hormone production (Leydig cells and adrenal gland). Interestingly, strongest GFP fluorescence among hematopoietic cells was observed in granulocytes, in which myeloperoxidase was expressed as a hemoprotein[85].

Using $Alas1^{+/GFP}$ mice, authors also examined whether ALAS-N expression in liver oscillated or not, in a 12 hour light and 12 hour dark cycle. As a result, it was confirmed ALAS-N mRNA expression, as well as GFP mRNA expression, displayed similar circadian oscillation[84], suggesting that basic circadian control for ALAS-N mRNA expression might be regulated at transcriptional level.

VII. Regulation of ALAS-E (ALAS2) expression

Expression of ALAS-E is restricted in erythroid cells, in which large amount of heme is produced for hemoglobin formation. Therefore apparent negative feedback regulation for ALAS-E expression by heme was not

observed. Instead, erythroid cells are able to export excess amount of heme from cytosol to the outside through feline leukemia virus subgroup C receptor (FLVCR), and suppression of FLVCR in K562 cells impaired erythroid maturation and leades to apoptosis[86]. Moreover, genetic ablation of FLVCR gene causes lack of definitive erythropoiesis and FLVCR-null mice die in midgestation[87], suggesting that erythroid progenitor cells hardly regulate heme production precisely during its terminal differentiation.

A. Transcriptional regulation of ALAS-E gene (ALAS2)

1. Identification of several erythroid specific cis acting elements in the proximal promoter region of mouse Alas2 gene

Cloning of mouse ALAS-E gene was first reported by Schoenhaut and Curtis in 1989[20]. Mouse Alas2 gene consists of eleven exons, and spans about 24 kbp. These authors identified five independent erythroid specific DNAse I hypersensitive sites, one of which located at promoter region. Moreover, putative SP-1 binding site was recognized at proximal promoter region, although functional consequence of this cis element remained unknown[20].

Functional analysis of mouse Alas2 promoter was intensively performed by Kramer et al.[88]. These authors first cloned genome DNA fragment of Alas2 gene, which contained promoter region including up to 1.4 kbp upstream of transcription initiation site. Several deletion mutants were prepared to determine the essential region for transcriptional activity. As a result, they found that proximal -714 bp promoter region required for full promoter activity in erythroid cells, as well as in non-erythroid cells. Within this region, authors identified several putative cis-acting elements important for erythroid differentiation, including EKLF/Sp1, two GATA-1 and NF-E2 and HIF-1. Further deletion of promoter region revealed that a region located between -538 to -315 bp was required for transcriptional activation of Alas2 during erythroid differentiation, however, no known erythroid-specific cis element was identified in this region except for HIF-1 binding site. Authors thus proposed that unknown erythroid-specific

regulatory element(s) might be located within this region. Furthermore, authors confirmed that GATA-1 protein could bind to both GATA-1 binding sites, and disruption of either GATA-1 binding site in -714 bp promoter fragment decreased its promoter activity in erythroid cells to about 50%, while such modification of promoter sequence did not affect its promoter activity in non-erythroid cell. These results suggest that both GATA-1 binding sites in proximal promoter region are critical to confer erythroid-specific expression of mouse Alas2 gene[88].

2. Transcriptional regulation of human ALAS2 gene by its promoter

Cox et al. first reported the proximal promoter sequence of human ALAS2 gene, and identified several erythroid-specific transcription factor binding site, such as GATA, CCAAT and NF-E2, as well as TATA sequence within 100bp upstream of transcription initiation site[89].

Surinya et al. also cloned human ALAS2 promoter region including 10.3 kbp upstream of transcription initiation site, and tried to determine an important region for erythroid-specific expression of ALAS2[90]. Based on promoter deletion assay, authors found that proximal 293 bp of ALAS2 promoter exhibit the strongest promoter activity in erythroid cells. Within this region, authors identified several erythroid specific cis-elements, including GATA, Ets, CCAAT, CACCC and NF-E2, and they tried to examine biological significance of GATA, CACCC and NF-E2 consensus sequence. There were three putative GATA binding sites within 140 bp from transcription initiation site, two of which were relatively distal region (located at -124 and -100 bp from transcription initiation site, named as distal GATAs) and the other one was located at proximal region (named as proximal GATA). It was confirmed that GATA-1 could bind to these three GATA sequence by gel-retardation assay, and promoter assay experiments revealed that all these GATA sequence were functional in erythroid cells. Interestingly, proximal GATA sequence was partially overlapped with TATA-like sequence, at which association of TATA-binding protein (TBP) was confirmed. Since the conversion of this overlapped sequence to canonical

GATA binding sequence or canonical TATA sequence resulted in the reduction of promoter activity, authors suggested that functional interaction of GATA-1 and TBP at this element might be important for maintaining appropriate promoter function. Authors also demonstrated that CACCC sequence was functional in erythroid cells by binding erythroid Krüppel-like factor. On the other hand, these authors concluded that NF-E2 sequence was not functional on this promoter, and it is consistent with the result shown by Andrews et al[91], in which NF-E2 element in human ALAS2 proxymal promoter did not bind NF-E2 transcription factor.

Bekri et al.[92] identified a mutation at 206 bp upstream of transcription initiation site in patient with X-linked sideroblastic anemia, which was caused by the loss of function mutation of ALAS2 gene[93]. Authors found that ALAS2 mRNA level in patient's erythroid precursors was decreased to 87% of normal subject, and confirmed this promoter mutation reduced the promoter activity in K562 erythroid cells, whereas it did not affect promoter activity in non-erythroid cells. Barton et al. also reported that this mutation might be related to the severe iron overload observed in patients with sickle cell anemia[94]. However, biological impact of this promoter mutation remained to be elusive, since this mutation was also reported as SNP[95].

3. Erythroid-specific enhancer in intron 8 for transcriptional regulation of ALAS2 gene

Surinya et al.[96] searched the erythroid-specific enhancer throughout human ALAS2 gene by sequencing analysis of whole gene, and compared it with the previously reported DNAse I hyper sensitive sites in mouse Alas2 gene[20]. As a result, authors identified three independent putative enhancer regions at intron 1 (4.9 kbp), intron 3 (850 bp) and intron 8 (480 bp). Using transient transfection of reporter plasmids into erythroid and non-erythroid cells, authors found that intron 1 and intron 8 could enhance ALAS2 proxymal promoter activity, whereas intron 3 showed rather inhibitory effect. Further analysis using intron 8, in which two CACCC elements and four GATA binding elements were identified, revealed that this region

enhanced ALAS2 proxymal promoter activity to twelve times higher level in K562 human erythroid cell lines. Importantly, such enhancing effect of this region was not observed in non-erythroid cell line, suggesting that this enhancer functioned in erythroid-specific manner. Deletion analysis of intron 8 further revealed that both CACCC elements and two GATA binding elements (adjacent to CACCC elements) played a critical role in K562 cells. These two CACCC elements (designated CACC-A and CACC-B) and two GATA elements (designated GATA-A and GATA-B) were lined from 5' to 3' of coding strand, and the disruption of any cis element, except for GATA-A disruption, diminished the enhancing activity of this region. Using nuclear extract from erythroid cell, authors confirmed that GATA-1 protein bound to GATA elements, and SP-1 protein and other unknown protein bound to CACCC elements. However, ectopic expression of GATA-1 alone or in combination of GATA-1 and SP-1 could not accelerate enhancing activity of this region, suggesting that binding of unknown protein on CACCC elements might critical for erythroid-specific enhancing activity of this region[96].

4. Hypoxic upregulation of ALAS2 mRNA expression

Hypoxia induces several proteins involved in erythropoiesis, such as erythropoietin[97], transferrin[98] and transferrin receptor[99], by activating hypoxia inducible factor 1α (HIF-1α), which bind to hypoxia responsive element (HRE) as a heterodimer with HIF-1β[100]. Since putative HRE was identified in the proximal promoter region of murine Alas2 gene[88], Hofer et al. examined the effect of hypoxia on the expression level of ALAS2 mRNA in murine erythroid cells[101]. As a result, hypoxic upregulation of Alas2 mRNA was confirmed, and responsible promoter region of ALAS2 gene for this hypoxic upregulation was identified. Although this promoter region contained putative HRE, further experiments revealed that hypoxic induction of murine ALAS2 was independent of this putative HRE. Thus, responsible mechanism for hypoxic upregulation of ALAS2 gene still remains elusive.

In human, Narayan et al. reported that hypoxia induced erythroid differentiation of hematopoietic stem cell (CD34+/Lin-) in vitro, which was related to the induction of ALAS2 mRNA[102]. Kaneko et al. also reported increased hemoglobinization of human erythroleukemia cells along with the induction of ALAS2 mRNA in these cells[103]. Authors tried to determine the involvement of HIF-1α in ALAS2 upregulation, however, a line of evidence indicated that HIF-1α did not directly regulate ALAS2 transcription in these cells.

On the other hand, Zhang et al identified three putative HRE, which were clustered at the 3' flanking region of human ALAS2 gene[104]. Authors showed that hypoxia induced ALAS2 mRNA in normal erythroid progenitor cells derived from $CD34^+$ stem cells, as well as in K562 human erythroid cells. Then it was confirmed that the DNA fragment included these putative HREs (located between +610 and +750 from the 3' end of the last exon of ALAS2 gene) enhanced SV40 promoter activity in non-erythroid cells under hypoxic condition. Moreover, direct binding of HIF-1α to this region in vivo was demonstrated using chromatin immunoprecipitation assay (ChIP), suggesting that these HREs were functional[104]. However, it should be noticed that the 3' flanking region of ALAS2 gene is overlapped with apurinic/apyrimidinic endonuclease 2 gene (APEX2), and indicated region for putative HREs is located at the last exon of APEX2 gene (accession: NG_008983). Thus, it should be carefully examined whether this HRE was involved in the hypoxic regulation of human ALAS2 gene.

B. Translational regulation of ALAS-E

1. Intracellular iron regulates ALAS-E translation

It has been established that iron regulatory protein (IRP) bind to the stem loop structure of mRNA (referred as iron responsive element, IRE) to regulate the translation of candidate mRNA[105]. IRE has been identified at 5'- or 3'- untranslated region (UTR) of several mRNAs, which encode proteins involving iron metabolism, such as ferritin[106], transferrin receptor 1 (TFR1)[107], divalent metal transporter 1 (DMT1)[108], mitochondrial