THE MITOCHONDRIAL HEME METABOLON AND PROGESTERONE RECEPTOR MEMBRANE COMPONENT 1: REGULATION OF MAMMALIAN HEME BIOSYNTHESIS VIA PROTEIN-PROTEIN INTERACTIONS

by

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(Under the Direction of Amy Medlock)

ABSTRACT

Heme is an iron-containing tetrapyrrole cofactor that is essential for metazoan life. Heme is synthesized in a highly conserved enzymatic pathway in the mitochondria and cytosol. We have identified a complex of interacting proteins which contains several members of the heme biosynthetic pathway, mitochondrial transport proteins, and proteins thought to mediate mitochondrial membrane morphology. We have termed this complex the mitochondrial heme metabolon. Progesterone receptor membrane component 1 (Pgrmc1), a sparsely characterized heme-binding protein with diverse putative functions, was identified as part of this complex. My work has focused on dissecting the role of Pgrmc1 in heme synthesis. Our data suggest that Pgrmc1 regulates heme synthesis via interactions with the heme biosynthetic enzyme Ferrochelatase and other metabolon components. We have utilized in vitro and in vivo models, including human erythroid cell culture and zebrafish, to define the function of Pgrmc1 in heme synthesis and erythroid development. We have also begun work on PGRMC2, a paralogue of

Pgrmc1, which may have an overlapping function. Our work supports a model in which the mitochondrial heme metabolon, including Pgrmc1, serves as an essential hub for the synthesis of heme, the regulation thereof, and for the intracellular distribution of heme post-synthesis.

INDEX WORDS: Heme, Pgrmc1, Pgrmc2, Ferrochelatase, Mitochondria, Metabolon

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TABLE OF CONTENTS

CHAPTER 1: INTRODUCTION AND LITERATURE REVIEW	**1**
Heme	1
ALA synthesis	4
The Cytosolic Heme Synthesis Enzymes	6
Return to the Mitochondria	12
Mitochondrial Iron Acquisition and Trafficking	17
Mitochondrial Heme Export and Intracellular Distribution	21
Figures	26
References	29
Chapter 2: THE MITOCHONDRIAL HEME METABOLON: INSIGHTS INTO THE COMPLEX(ITY) OF HEME SYNTHESIS AND DISTRIBUTION	**44**
Abstract	45
Introduction	45
Metabolons and the role of protein interactions in Heme synthesis	47
The (Mitochondrial) Heme Synthesis Metabolon	49
Novel Heme Synthesis Metabolon Proteins	53
Dynamic and Transient Nature of the Complex	55
Conclusion	57
Figures and Tables	58
References	62

CHAPTER 3: A NOVEL ROLE FOR PROGESTERONE RECEPTOR MEMBRANE COMPONENT 1 (PGRMC1): A PARTNER AND REGULATOR OF FERROCHELATASE **68**

Abstract 69

Introduction 70

Materials and Methods 73

Results 80

Discussion 88

Figures and Tables 92

Supporting Information 105

References 112

CHAPTER 4: CREATION AND PRELIMINARY CHARACTERIZATION OF PGRMC1/2 NULL CELL LINES AND ZEBRAFISH **124**

Abstract 125

Introduction 125

Results 128

Discussion and Future Directions 131

Conclusion 136

Methods 137

Figures and Tables 140

References 146

CHAPTER 5: CONCLUSIONS 150

The Mitochondrial Heme Metabolon 150

Pgrmc1 152

Pgrmc2 154

References 156

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

Heme

Heme, a tetrapyrrole cofactor composed of a single iron ion coordinated to a protoporphyrin IX macrocycle, is essential for metazoan life. The multiple available oxidation states of the iron ion, as well as the ability to distribute electron density over the porphyrin macrocycle make heme a versatile cofactor in many processes throughout the body. All metazoans and the majority of organisms utilize heme, and the ability to synthesize this cofactor has been remarkably well conserved. Of the metazoans, only a few species, including the model organism *Caenorhabditis elegans* and a few other helminths [1] and the cattle tick *Boophilus microplus[2]*, are unable to synthesize heme *de novo*. However, these organisms still require heme for life and must acquire it via diet. In addition to heme, other related tetrapyrrole cofactors, including cobalamin (Vitamin B12) and chlorophyll, are also widely used in diverse organisms, further demonstrating the utility and necessity of this family of molecules.

Heme is utilized in all human tissues and cell types and is found in diverse subcellular compartments where it functions not only as an enzyme cofactor, but also as a versatile signaling molecule[3]. Heme is most well-known for its role in oxygen transport via binding in hemoglobin and myoglobin. However, even the lesser-known functions of heme are just as essential. The presence of heme as a cofactor in cytochromes of the electron transport chain is

crucial for cellular respiration. Heme is also necessary for nitric oxide (NO) production as a cofactor in the enzyme NO synthase[4]. Heme is also found in catalase and various peroxidases, which protect cells from oxidative damage. The metabolism of drugs and other xenobiotic compounds requires heme as a cofactor in cytochromes P450. Heme also helps to regulate gene expression through interactions with various transcription factors[3], as well as post transcriptionally through interactions with mRNA regulatory motifs[5] and in microRNA processing[6]. Additionally, heme is involved in protein degradation through the N-end rule pathway[7]. Even the regulation of circadian rhythm[8] has found a place in the ever-expanding list of functions for heme.

Decreased production or availability of heme usually manifests as a group of diseases called anemias[9-12]. Anemias are characterized by a decrease in the number of circulating red blood cells or in the amount of hemoglobin present in those cells. The reason anemia is the condition most often seen in cases of decreased organismal heme levels is two-fold. First, developing erythroid cells represent by far the largest demand for heme in the body, with ~85% of total body heme present in hemoglobin[13]. As such, these cells would be the first affected by relatively mild decreases in heme levels. The second reason is that severe decreases in heme synthesis or availability are incompatible with life since these organisms would not be able to acquire or transport oxygen.

While undoubtedly essential for life, the production of heme can be a double-edged sword. The same unique reactivity that allows heme to fulfill its functional niche can prove harmful to the cell if not properly controlled. Free heme, heme not bound to proteins or otherwise sequestered, can be toxic to the cell through the generation of harmful free radical species[14, 15]. Many of the intermediates of heme synthesis, porphyrins and porphyrin

precursors, are toxic as well. Accumulation of these compounds, many of which are photoreactive, results in a class of diseases called the porphyrias[10, 12, 16, 17]. Deficiency in any one of the enzymes in the heme synthesis pathway results in a distinct type of porphyria, characterized by accumulation of different porphyrin intermediates and/or precursor molecules. Each of these diseases in genetically heritable through mutations in heme synthesis enzymes; however, several can also be acquired sporadically through the inhibition of these enzymes. The most common precipitating/exacerbating factors for the porphyrias include disruption of iron homeostasis, drug or alcohol use, fasting, and concurrent disease. Individual porphyrias vary in clinical presentation, though most show either cutaneous symptoms, due to porphyrin accumulation in circulating erythrocytes, or neurological symptoms, due to interaction of the early pathway intermediates with receptors for endogenous neurotransmitters[12, 18]. A common complication seen in porphyric individuals is liver damage, which occurs as porphyrins which originate from erythrocytes are trafficked to and accumulate in the liver hepatocytes and bile canaliculi[19]. This effect is exacerbated by the fact that the liver itself represents a major site of heme synthesis in the body, second only to developing erythrocytes, due to production of the cofactor for cytochromes P450[20]. The specific causes and characteristics of each of the porphyrias will be further described below along with their corresponding heme synthesis enzymes.

Heme synthesis in mammals is carried out via a highly conserved pathway consisting of eight enzymes (Figure 1). Though direct evidence is not forthcoming, it is currently thought that every cell synthesizes its own heme *de novo*. Therefore, the complete pathway must be present in each cell in the body. The heme biosynthesis pathway spans two cellular compartments, with

enzymes located in both the mitochondria and cytosol. Each enzyme as well as relevant transport mechanisms for their intermediates are reviewed below.

ALA synthesis

The first committed step in heme synthesis is the condensation of succinyl-CoA and glycine to form δ-aminolevulinic acid (ALA) and CO_2. Eight molecules of ALA must be synthesized to form each molecule of heme. This reaction occurs in the mitochondrial matrix and is catalyzed by ALA synthase (Alas). ALA synthesis is generally considered to be the rate limiting step of the heme biosynthetic pathway[10]. The crystal structure of Alas from *Rhodobacter capsulatus* was solved in 2005, revealing a homodimeric enzyme that utilizes pyridoxal phosphate (PLP) as a cofactor[21]. However, the bacterial enzyme lacks a C-terminal domain that is present in eukaryotes. In 2018, the crystal structure of a eukaryotic Alas from *Saccharomyces cerevisiae* was solved, revealing that the aforementioned C-terminal residues contact the active site of the enzyme in the crystalized structure and impact enzyme activity[22]. Additionally, PLP binding was shown to impart structural changes to the enzyme that are important for substrate binding. These observations point to the importance of enzyme structure and stabilizing interactions for the catalytic activity of Alas and regulation thereof.

In mammals, there are two distinct forms of Alas transcribed from separate genes[23]. *Alas-1* is expressed in all cell types and is responsible for non-erythroid heme synthesis. This includes in the liver, where it can be dramatically upregulated to supply heme for cytochrome P450 synthesis in detoxification of drugs and other xenobiotics[24, 25]. *Alas-2* is expressed in

developing erythroid cells and is responsible for the synthesis of heme destined for hemoglobin during erythropoiesis[23].

Alas-1, the housekeeping form of the enzyme, is found on chromosome 3 in humans and is subject to feedback inhibition by heme at several levels. Transcription of *Alas-1* is repressed in a heme dependent manner by Rev-erbα, which recruits HDAC3 to inhibit transcription of the *Alas-1* activator PGC-1α [26]. It is also repressed by heme-bound Egr-1, which binds and represses Alas-1 directly[27]. Additionally, there exists evidence that *Alas-1* mRNA is destabilized in the presence of heme[28]. Alas-1 activity is also repressed by heme at the post-translational level via the presence of an N-terminal heme regulatory motif that, upon binding heme, recruits ClpXP, an unfoldase/peptidase complex, to degrade the protein[5, 29-31].

During erythropoiesis, ALA production shifts from Alas-1 to the erythroid specific form, Alas-2. *Alas-2*, encoded on the human X chromosome, is not subject to the same level of feedback inhibition as Alas-1; this is logical due to the high levels of heme required for hemoglobin production during erythroid differentiation. In addition to transcriptional regulation by erythropoietic transcription factors such as GATA-1, Alas-2 translation is coupled to iron availability via an iron responsive element (IRE) found in its 5' untranslated region. Under iron deficient conditions, iron responsive protein 1 (IRP1) binds to the *Alas-2* IRE, inhibiting translation. When sufficient iron is present, the IRP1 binds a [4Fe-4s] cluster and dissociates from *Alas-2* mRNA, allowing translation to proceed[32-36]`. This iron dependent regulation prevents the accumulation of harmful un/mismetalated porphyrins during erythroid differentiation.

There are two disease states associated with improper function of Alas-2, X-linked sideroblastic anemia (XLSA) and X-linked protoporphyria (XLPP). XLSA is caused by loss of

function mutations in ALAS-2. The impaired synthesis of ALA leads to decreased heme synthesis and anemia, accompanied by accumulation of iron in the mitochondria of developing erythrocytes. Cells exhibiting this iron accumulation are called ringed sideroblasts for the ring of iron loaded perinuclear mitochondria that become visible in these cells after staining with Prussian blue. XLSA can be caused by a variety of mutations, many of which are thought to decrease the affinity of ALAS-2 for its cofactor PLP. More than half of XLSA patients are responsive to treatment with pyridoxine (vitamin B6), which is metabolized into PLP, compensating for the decreased affinity of the mutant ALAS-2[10, 37]. Alternatively, XLPP is caused by mutations in the C-terminal region of ALAS-2 which result in a hyperactive enzyme. In XLPP, ALA is overproduced to the point that it out-paces iron insertion by ferrochelatase (FECH) in the terminal step of heme synthesis. This leads to accumulation of photoreactive protoporphyrin IX (PPIX) and Zn-PPIX resulting in severe photosensitivity [38]. In some patients, PPIX accumulation can also lead to liver damage via biliary occlusion. XLPP is the most recently described of the porphyrias and shows symptoms similar to those seen in erythropoietic protoporphyria (EPP), which results from FECH deficiency rather than ALA overproduction. Both diseases show accumulation of PPIX, though ZnPPIX levels are elevated in XLPP compared to EPP. Treatment of XLPP by iron supplementation has been attempted with limited success in alleviation of photosensitivity.[10, 16, 38, 39]

The Cytosolic Heme Synthesis Enzymes

Following its synthesis in the mitochondrial matrix, ALA must be transported to the cytosol where the next four pathway enzymes reside. While the mechanism of this transport is not currently fully defined, it has been suggested that the mitochondrial solute carrier proteins

SLC25A38[40] and SLC25A39[41] may play a role. However, SLC25A38 has also been shown to transport glycine into the mitochondria[32, 40] and SLC25A39 has been implicated in mitochondrial iron and/or heme transport[41, 42], though the capacity to transport multiple substrates has not been ruled out for either enzyme.

The first of the cytosolic enzymes and second in the heme biosynthetic pathway, porphobilinogen synthase (Pbgs, previously called aminolevulinate dehydratase: Alad), catalyzes the condensation of two molecules of ALA to form one molecule of porphobilinigen (PBG), a monopyrrole. The crystal structure of human PBGS has been solved multiple times to date[43-45]. These structures have shown that the protein forms homo-multimers composed of homodimeric subunits. The first of these to be described was the homo-octomeric form[43], the high activity form of the enzyme. Subsequent studies revealed the existence of a lower activity hexameric form, suggesting the activity of this enzyme is modulated by differential multimerization. The formation of these variable homomultimers, also known as morpheeins, is likely regulated by allosteric effectors[44, 45]. Each monomer also contains a zinc ion cofactor, where within each homodimeric subunit one zinc ion is essential for catalytic activity and the other for stabilization of the active octomeric form[45].

In contrast to *Alas*, there exists a single gene for *Pbgs* that is responsible for both housekeeping and erythroid synthesis of PBG; this is also the case for the remaining enzymes in the heme synthesis pathway. While *Pbgs* is transcribed from a single gene on chromosome 9, promotor elements and splice variants differ between erythroid and non-erythroid expression. Specifically, the housekeeping variant possesses an additional 5’ untranslated region in its mRNA[46]. The translated proteins, however, are identical.

Deficiency in PBGS results in a rare, recessively inherited porphyria called aminolevulinate dehydratase porphyria. This disease is characterized by accumulation of ALA and acute neurological symptoms as well as excretion of ALA and coproporphyrinogen III in the urine[10, 12, 16]. Neurological symptoms associated with this disease are thought to result from interaction of excess ALA with the receptor for the neurotransmitter γ-aminobutyric acid [18]. Interestingly, PBGS is strongly inhibited by exposure to Pb^{2+}, which is able to displace the Zn^{2+} cofactor, resulting in low PBGS activity and accumulation of ALA similar to that seen in Alad porphyria[47]. This accounts for some of the neurological symptoms associated with lead poisoning.

The next pathway enzyme, hydroxymethylbilane synthase (Hmbs, previously called porphobilinogen deaminase: Pbgd) joins together four molecules of the monopyrrole PBG head-to-tail to form the linear tetrapyrrole hydroxymethylbilane (HMB) and four ammonium molecules. Hmbs is an interesting enzyme in that it synthesizes its own cofactor. Following translation, apo-Hmbs will use six molecules of PBG to synthesize a hexapyrrole, which is then cleaved to release the linear tetrapyrrole HMB. Following this initial catalytic cycle, the remaining dipyrromethene will stay covalently bound to the enzyme and serve as a cofactor[48]. In subsequent cycles, PBG monomers will be extended from the cofactor and then cleaved to release HMB. Crystal structures for both bacterial[49] and human[50] Hmbs have been solved. The human structure shows only a small interface for possible dimerization relative to other functionally dimeric proteins[50], however, recent affinity purification studies in an erythroid model cell line indicate that the protein does indeed exist in cells as a dimer[51].

Like *Pbgs*, *Hmbs* is transcribed from a single gene with erythroid and housekeeping splice variants. In humans, *Hmbs* is encoded on chromosome 11. The housekeeping variant is

transcribed and translated from sites in exon 1, while the erythroid specific form is transcribed from exon 2 and translated from a site in exon 3. Exon 2 is noncoding and is not translated in either form. Thus, the housekeeping form of Hmbs possesses an additional 17 amino terminal residues compared to the erythroid form.[52]

A deficiency in HMBS result in acute intermittent porphyria (AIP). AIP is a disease characterized by acute neurological symptoms and abdominal pain resulting from accumulation of ALA and PBG. AIP is an autosomal dominant disorder and the most common acute porphyria[10, 16]. As the name implies, AIP attacks are sporadic in nature and can be caused by a number of aggravating factors such as consumption of alcohol, various drugs, hormone-based contraceptives, fasting, and concurrent disease[53]. Administration of exogenous hemin can sometimes alleviate attacks through its suppression of ALA synthesis. Carbohydrate loading via glucose supplementation has also been shown to decrease recovery time following an attack via PGC1α mediated suppression of *Alas-1*.[54]

Following the synthesis of HMB, the linear tetrapyrrole is cyclized by the enzyme uroporphyrinogen synthase (Uros) to form uroporphyrinogen III (Uro'gen III). Crystal structures of the human[55] and bacterial[56] forms of the enzyme reveal a monomer that does not utilize a cofactor. Uros possesses an intriguing structure consisting of two separate domains connected by a flexible linker region. The enzyme is highly conformationally dynamic in solution, with apoprotein crystallization exhibiting a variety of relative orientations of the two domains and the product-bound form assuming a "closed" conformation stabilized by hydrogen bonding[56]. It is likely that the flexible nature of this protein allows for stabilization of the HMB intermediate in conformations favorable for cyclization to Uro'gen III rather than uroporphyrinogen I (Uro'gen I).

The human *Uros* gene is found on chromosome 10 where expression is driven by tissue specific promotor elements. Transcription of the housekeeping form initiates in exon 1 and transcription of the erythroid form initiates in exon 2[57]. Exon 1, however, is noncoding, and thus erythroid and nonerythroid expression results in identical mature proteins.

Deficiency in Uros results in accumulation of the spontaneously cyclized tetrapyrrole, Uro'gen I. In the absence of the enzyme, HMB will spontaneously cyclize to form Uro'gen I. Though Uro'gen I is a viable substrate for the next pathway enzyme, uroporphyrinogen decarboxylase, the resulting product, coproporphyrinogen I (Copro'gen I), is a dead-end intermediate which cannot be utilized by the following enzyme, coproporphyrinogen oxidase[10]. The accumulation of Uro'gen I and Copro'gen I due to UROS deficiency results in congenital erythropoietic porphyria (CEP). CEP is an autosomal recessive disease in which afflicted individuals suffer from anemia, as well as severe cutaneous photosensitivity[10]. Other symptoms include excessive hair growth, skin pigmentation abnormalities, and the accumulation of porphyrins in teeth causing reddish discoloration. Progressive photomutilitaion is also observed in CEP patients, resulting from the cutaneous accumulation of reactive Uro'gen I and Copro'gen I and their subsequent oxidation to form porphyrins[16, 58]. Blood transfusion can be used to treat the anemia, with a portion of patients being transfusion-dependent. Additionally, bone marrow transplant has been used successfully as a more comprehensive treatment in some cases of CEP. [17]

The last of the cytoplasmic pathway enzymes is uroporphyrinogen decarboxylase (Urod). This enzyme is responsible for the decarboxylation of the four acetate groups of Uro'gen III to form coproporphyrinogen III (Copro'gen III), also yielding four molecules of CO_2[10, 59]. Urod functions as a homodimer and does not utilize a cofactor[60]. Though Urod is capable of

decarboxylating uroporphyrinogen groups in any order, it appears to function in a preferred order physiologically, starting with decarboxylation of the D ring acetate and proceeding in a clockwise fashion through the A, B, and finally C ring acetate groups[59]. The gene encoding Urod is located on chromosome 1 in humans and does not appear to possess any tissue specific splice variants.

Dysfunction of UROD results in porphyrias known as porphyria cutanea tarda (PCT) and hepatoerythropoietic porphyria (HEP). These diseases result from respective heterozygotic and homozygotic defects in UROD[61]. Both manifest as accumulation of photoreactive uroporphyrinogen intermediates which result in cutaneous photosensitivity as well as porphyrin accumulation in the liver[39, 61]. The less severe of the two, PCT, can manifest in two forms. Familial PCT (F-PCT, also called type 2) is an autosomal dominant disease resulting from loss of function mutations in *Urod*. F-PCT accounts for approximately 20% of PCT cases and is marked by ~50% UROD activity in all tissues[61, 62]. Sporadic PCT (S-PCT- also called type I) accounts for the majority of PCT cases. Interestingly, in S-PCT, no mutations are present in *Urod* and enzyme activity is reduced only in the liver. This reduction in activity is mediated by the Urod inhibitor uroporphomethene, which is generated in the liver through an iron dependent process[62]. Both forms of PCT are exacerbated by hemochromatosis or other iron overload, alcohol abuse, estrogen treatment, or concurrent disease, particularly hepatitis C. Treatments for PCT include repeated phlebotomy to reduce iron levels, and administration of chloroquine to stimulate porphyrin excretion[61].

Return to the Mitochondria

Following the cytoplasmic portion of the heme synthesis pathway, Copro'gen III is delivered to the mitochondrial intermembrane space, though the mechanism by which this is accomplished remains poorly defined at present. While there exists some evidence that the ATP-binding cassette transporter Abcb6 may be involved in mitochondrial Copro'gen III import[63-65], these results are far from conclusive. To date, Abcb6 has only been demonstrated to transport planar porphyrin molecules and not the non-planar Copro'gen III[63, 65]. Abcb6 is an ATP-dependent integral membrane transporter found on the mitochondrial outer membrane as well as on the plasma membrane[66]. Abcb6 is upregulated during erythroid differentiation, however, Abcb6 null mice do not show heme synthesis defects under normal conditions. Interestingly, these mice did exhibit apparent compensatory increases in other heme synthesis related enzymes, showed accumulation of PPIX, and suffered higher rates of mortality when challenged with phenylhydrazine, a chemical that necessitates greatly increased heme synthesis by causing the destruction of mature erythrocytes[64]. These data suggest that Abcb6 does have some effect on heme production and/or erythropoiesis, however, it is unlikely to be the primary importer of Copro'gen III.

Upon delivery to the mitochondrial intermembrane space (IMS), Copro'gen III is converted to protoporphyrinogen IX (Proto'gen IX) by coproporphyrinogen oxidase (Cpox) via the oxidative decarboxylation of the A and B ring propionate groups to form vinyl groups. This process requires two molecules of oxygen and produces two molecules of CO_2[10]. The protein is synthesized with an exceptionally long, 120 amino acid mitochondrial leader sequence. This leader sequence is responsible for targeting to the IMS and is proteolytically removed upon localization[5, 67] While a recent study of the sub-mitochondrial localization of several enzymes

confirmed that the majority of the Cpox enzyme does indeed localize to the IMS, it showed that a segment of the N-terminus of the protein resides in the matrix, suggesting the protein may span the mitochondrial inner membrane (IMM)[68]. Human CPOX has been crystalized[69] and shown to function as a homodimer with no cofactor[70].

The *Cpox* gene is found on chromosome 3, where there is evidence for differential regulation by housekeeping and erythroid specific promotor elements. Specifically, the erythroid promoter includes a GATA site not required in non-erythroid cells[71]. However, these mechanisms have not been as thoroughly defined as those of other heme synthesis genes. Interestingly, overexpression of Cpox in K-562 human myelogenous leukemia (K562) cells, an erythroid model, was accompanied by both increased heme synthesis and expression of Fech upon differentiation, suggesting a possible role for Cpox in regulation and/or induction of heme synthesis[72].

Deficiency in CPOX activity results in accumulation of Copro'gen III, and leads to neurological symptoms similar to those in AIP, though usually less severe. A minority of cases also exhibit cutaneous sensitivity. This disease is autosomal dominant in inheritance and termed hereditary coproporphyria (HCP). A variety of CPOX mutations can cause HCP and symptoms can vary in severity depending on the specific mutation, relative activity of the mutated protein, and whether mutations are found in one or both alleles[12]. Treatments are similar to those employed for AIP, including mitigation of any potential exacerbating factors including alcohol consumption, drug use, or female hormone administration; coupled with supplementation of carbohydrates and hemin. Liver transplant has also been used as a treatment in severe, recurring HCP attacks[61].

Following its synthesis in the IMS, Proto'gen IX must be delivered to the mitochondrial matrix, where the final two enzymes of the heme biosynthetic pathway reside[51, 68]. This is likely accomplished via transmembrane protein 14C (Tmem14c), a transporter localized to the mitochondrial inner membrane[73]. Tmem14c is enriched in erythropoietic tissues and was shown to be upregulated during terminal erythropoiesis[41, 73]. Knockout of Tmem14c results in arrest of heme synthesis and erythropoiesis accompanied by accumulation of porphyrin precursors and profound anemia. The heme synthesis defect can be rescued by supplementation of deuteroporphyrin IX, an analog of protoporphyrin IX (PPIX)[73]. These data suggest the involvement of Tmem14C in transport of Proto'gen IX from the mitochondrial IMS to the matrix during heme synthesis.

Once inside the mitochondrial matrix, Proto'gen IX is oxidized by protoporphyrinogen oxidase (Ppox) to form protoporphyrin IX (PPIX). The reaction requires three O_2 molecules and has been shown to produce three molecules of H_2O_2 *in vitro*, though it is possible other molecules and/or proteins participate in this electron transfer *in vivo*. The crystal structure for human PPOX has been solved and shows the protein to function as a homodimer with each subunit containing one FAD cofactor[74]. Interestingly, the catalytic site is located in a tunnel which passes through the protein, implying a possible route for substrate/product flux through the enzyme. Ppox is translocated to the mitochondria via the presence of two mitochondrial targeting sequences located internally and on the N-terminus. Both of which are required for effective localization[5, 75-77].

Human *Ppox* is encoded on chromosome 1. Both erythroid and housekeeping specific promotor elements have been documented for the *Ppox* gene[78, 79]. Splice variants have also been reported in the 5' UTR of the transcript; these have been suggested as possible mediators of

transcript stability or regulators of translation[80]. A single mature form of the protein is synthesized from all promoters or splice variants.

Defects in PPOX result in the disease variegate porphyria (VP) which can be caused by a variety of mutations and is inherited in an autosomal dominant fashion. VP is a relatively rare porphyria in most of the world, though the disease is unusually common in South Africa due to a founder effect from Dutch settlers in 1688 [81, 82].This porphyria is one of two, along with HCP, that manifests with both neurological and cutaneous symptoms resulting from accumulation of ALA, PBG, Copro'gen III, and PPIX. Symptoms of VP can vary between individuals and mimic those of other porpyrias due to the variety of porphyrin intermediates which accumulate in this disease. Afflicted individuals are often asymptomatic outside of relatively rare acute attacks, which can be brought on by exacerbating factors such as drug or alcohol use, concurrent illness, or iron overload.[12, 39]

The final step of the heme synthesis pathway is catalyzed by ferrochelatase (Fech). This enzyme catalyzes the insertion of ferrous iron into the center of the PPIX macrocycle to produce heme and two protons. The enzyme is localized to the matrix side of the inner mitochondrial membrane via an N-terminal leader sequence, which is proteolytically cleaved following localization[83, 84]. The mature protein functions as a homodimer with each subunit containing a [2Fe-2S] cluster[83]. Though the cluster does not participate directly in the catalytic mechanism of the enzyme, it is required for activity[85]. It has been proposed that this cluster serves as either an intrinsic regulatory element for ensuring adequate iron levels[86], as a site for regulation of the enzyme via nitric oxide signaling[87], or as a sensor of other cellular conditions such as redox potential[88]. Crystal structures have been solved for wild type human FECH as well as numerous structural variants. These studies have revealed a highly dynamic enzyme that

undergoes considerable conformational changes around the active site which facilitate substrate binding and product release[89-91].

The *Fech* gene is located on chromosome 18 and is regulated by erythroid and housekeeping promotor elements[92-94]. Splice variants have been reported, with one group showing alternative polyadenylation initiation sites in the 3' UTR where transcript abundance varied between erythroid and nonerythroid cells [95]. A second group demonstrated splice variants attributed to skipping of the second exon. These transcripts possess an alternative translation start site and result in the expression of a truncated, inactive protein. The relative amount of the truncated enzyme was shown to increase following treatment with hemin and decrease upon treatment with succinylacetone, an inhibitor of heme synthesis via Pbgs, suggesting a potential role in feedback regulation of Fech activity[96].

Deficiency in Fech activity results in accumulation of Fech's substrate, unmetalated PPIX. This condition is known as erythropoietic proroporphyria (EPP). Affected individuals suffer from cutaneous photosensitivity and variable, mild anemia. In rare cases, patients manifest liver disease, which can be severe. This liver damage is caused by diffusion of excess PPIX from circulating erythroid cells, which then accumulates in the liver and bile. Rising PPIX concentrations can eventually cause porphyrin insolubility and impaired bile circulation, which in turn impairs PPIX excretion from the liver. This results in a compounding cycle where PPIX accumulation causes oxidative damage and hemolysis, which results in upregulation of erythropoiesis, further exacerbating PPIX accumulation[10]. For manifestation of clinical symptoms, FECH activity must be reduced to approximately 30%. EPP inheritance follows a somewhat unique pattern. The disease is nominally autosomal recessive, as one non-functional allele will not normally reduce FECH activity below the threshold required for manifestation of

symptoms. However, a large majority of EPP cases result from a primary loss of function mutation on one allele in combination with a low expression allele (IVS3-48C) that causes aberrant splicing which results in most of that allele's transcript being degraded via nonsense mediated decay[97, 98]. This combination of loss of function and reduced expression alleles results in low enough Fech activity to cause disease. A minority of cases result from true homozygous loss of function mutation, whereas individuals possessing a single mutated Fech allele generally remain asymptomatic[10]. Concomitant liver and bone marrow transplant has been used to treat EPP, however treatment is normally limited to reducing exposure to sunlight.

Mitochondrial Iron Acquisition and Trafficking

In order to complete the pathway and transform cytotoxic, photoreactive protoporphyrin to heme, ferrous iron must be delivered to Fech in the mitochondrial matrix. These processes must be efficiently carried out and tightly regulated both due to the myriad of essential functions of heme and iron in the cell and the fact that incomplete porphyrins as well as unchaperoned iron can cause harmful oxidative damage if allowed to accumulate improperly[61, 99].

Under normal physiological conditions, almost all imported iron enters the cell in the form of diferric transferrin (Tf), which is bound by transferrin receptor 1 (Tfrc) and subsequently endocytosed. The endosome is then acidified via proton pumps where the low pH allows for release of Tf bound iron[100, 101]. Ferric iron is then reduced to physiologically useful ferrous iron by the metalloreductase Steap3[102]. Following reduction, iron can then be delivered from the endosome to the rest of the cell.

There exist two predominant models for trafficking of iron from the endosome to the mitochondria (Figure 2). In the first of these, iron is exported to the cytosol by divalent metal transporter 1 (DMT1) where it forms a "labile iron pool" from which iron can be further distributed for incorporation into proteins or cofactors, various regulatory functions, storage bound to ferritin, or exported from the cell via ferroportin[32, 42, 103]. Due to the reactive nature of iron, especially in the easily oxidized ferrous state, it has been hypothesized that iron must be constantly bound to some form of chaperone molecule, compartment, or protein in order to prevent extraneous redox chemistry. Unchaperoned iron can result in the formation of insoluble ferric hydroxides or catalyze the formation of harmful reactive oxygen species[42].

The second and more recent of the two models is commonly referred to as the "kiss and run" model. In this model, iron-containing endosomes interact directly with the mitochondria to deliver iron independent of a cytosolic pool[13, 104]. This model is supported by several distinct pieces of evidence. Radiolabeling studies using ^{59}Fe demonstrated the dependence of iron chelation in reticulocytes on active endosome trafficking[105]. Additionally, inhibition of myosins, motor proteins responsible for movement of endosomes along the cytoskeleton, resulted in decreased heme synthesis in these cells[105]. Transmission electron microscopy also confirmed direct interaction between iron-containing endosomes and mitochondria[106]. The exact mechanisms by which endosomes localize to and dock with the mitochondria are not currently understood. However, there does exist evidence of other metabolite transport pathways which are reliant on the close interaction and/or fusion of membrane bound organelles. In one example, calcium ions have been shown to be delivered to the mitochondria through contact sites with the endoplasmic reticulum (ER)[107, 108]. It is possible that the endosome-mitochondria contacts in the kiss and run model could share cellular machinery with these processes.

Additionally, ER-mitochondria contacts and mitochondrial fusion/fission[109] may also provide avenues for non-cytoplasmic, intracellular iron trafficking.

Though they have been described separately here, it is possible, if not probable, that both of the models presented above have a role in intracellular iron trafficking. One model may predominate iron transport depending on cell type, temporal variability in iron demand, or the ultimate destination of the iron being trafficked. Transport mechanisms may also change with stages in differentiation, particularly in erythroid cells, as iron demand for the synthesis of heme increases drastically in the later stages of erythropoiesis. A recent study by Ryu et al. suggests that, during early erythropoiesis, iron is delivered to ferritin by PCBP1 and subsequently distributed via the autophagic turnover of ferritin, mediated by NCOA4[110]. However, as erythropoiesis progresses, data is consistent with a transition to direct endosomal delivery of iron to the mitochondria[110]. It should be noted that a majority of data regarding intracellular iron trafficking was obtained using erythroid models. Further studies in non-erythroid cells may offer additional insight on the variable utilization of iron transport pathways.

Irrespective of transport mechanism, there exists evidence that mitochondrial and cytoplasmic iron levels are tied to one another in some form of equilibrium[42]. For example, overexpression of the mitochondrial form of the iron storage protein ferritin (Ftmt) results in mitochondrial iron loading and a decrease in cytoplasmic iron levels[111]. However, it remains unclear whether these effects are due to mitochondrial iron acquisition from a cytosolic labile iron pool, or from re-routing of iron containing endosomes that would otherwise deliver iron to cytosolic proteins/reservoirs. It is also possible this effect results from a combination of these mechanisms.

Once iron has reached the mitochondria, it must be imported to the matrix for incorporation into heme, iron sulfur clusters, or other destinations including Ftmt mediated storage. It is unclear how iron traverses the outer mitochondrial membrane (OMM), though endosome-mitochondrial fusion or additional/non-specific functionality of known OMM transporters are possibilities. Once in the IMS, iron is transported across the IMM by the protein mitoferrin (Mfrn)[112]. There exist two homologues of Mfrn; Mfrn1 is expressed in erythroid cells and Mfrn2 is expressed ubiquitously[112]. Mfrn1 expression is tightly controlled during erythropoiesis; it is both upregulated transcriptionally and governed by a posttranslational mechanism whereby the protein half-life of Mfrn1 increases from approximately 7 hours in erythroid precursors to over 24 hours following differentiation[113]. This dramatic increase in protein half-life is at least partially mediated by Abcb10[114], an ATP-binding cassette protein under the control of the erythroid differentiation factor GATA-1[115]. Abcb10 has been demonstrated to stabilize Mfrn1 via its presence in a complex with both Mfrn1 and Fech[114, 116]. Knockout of either *Mfrn1*[117] or *Abcb10*[118] results in heme synthesis defects. Interestingly, *Mfrn1* knockout only results in porphyrin accumulation when the IRE-IRP regulation of Alas-2 has been bypassed, either via expression of Alas-2 lacking its IRE or by supplementation with exogenous ALA[117]. These data demonstrate coupling of iron import and insertion with porphyrin production to ensure adequate mitochondrial iron availability for the synthesis of heme during erythropoiesis.

Second to the heme biosynthetic pathway, another major destination for mitochondrial iron is the synthesis of iron sulfur clusters[119]. Iron sulfur clusters serve as cofactors or regulatory elements for a variety of enzymes including metazoan Fech[85]. Additionally, iron sulfur cluster insertion into IRP1 is responsible for the dissociation of the protein from IREs in

the mRNA of iron regulated genes including *Alas-2*[33, 34]. This communication between iron sulfur cluster and heme synthesis is demonstrated by the observation that deficiencies in glutarerdoxin5 (Glrx5), a mitochondrial enzyme involved in iron sulfur cluster synthesis, result in sideroblastic anemia due to IRP1 mediated repression of *Alas-2*[35, 120]. Further demonstrating this crosstalk is the fact that loss of Abcb7, an ATP binding cassette protein implicated in the synthesis and/or mitochondrial export of cytosolic iron sulfur clusters[121], also results in sideroblastic anemia[122, 123]. Though the exact mechanisms remain elusive, it is clear that mitochondrial iron import, iron sulfur cluster metabolism, and heme synthesis co-exist in an interconnected web of regulatory pathways to ensure efficient and well controlled acquisition, distribution, and synthesis of these essential metabolites.

Mitochondrial Heme Export and Intracellular Distribution

Once synthesis is complete, heme must be distributed from the mitochondrial matrix to the rest of the cell (Figure 3). However, the mechanisms governing mitochondrial heme export and subsequent distribution remain poorly understood. As described previously, free heme is cytotoxic; it is therefore likely that heme is either bound to a protein or other chaperone molecule or sequestered from the cellular milieu in a lipid bound compartment at all times to prevent cellular damage. It is interesting to note that in *in vitro* assays, product release of completed heme from Fech is the rate limiting step[124]. This is thought to be due to the lack of an acceptor molecule/protein in the *in vitro* system[125]. The relevance of this data to living organisms remains unclear, though it does hint at the possibility of a regulatory mechanism for heme synthesis through modulation of product release from Fech. At present, a dedicated mitochondrial matrix or IMM heme acceptor/chaperone has not been identified.

Following release from Fech, whether chaperone mediated or not, the next step in intracellular heme distribution is export from the mitochondria. The transmembrane transporter feline leukemia virus subgroup C receptor 1b (Flvcr1b) has been suggested to perform this function. Knockdown of Flvcr1b causes mitochondrial heme accumulation and blocks erythroid differentiation[126]. In addition, overexpression of Flvcr1b was shown to increase cytosolic heme levels[126]. Flvcr1b is an interesting enzyme in that it is one of two heme transporters transcribed from a single gene. Flvcr1b is the short variant of the Flvcr1 transcript, resulting from an alternative initiation site in the first intron. The full-length enzyme, termed Flvcr1a, localizes to the plasma membrane where it exports heme from the cell in concert with the extracellular heme binding protein hemopexin[127]. At present, it remains unclear whether Flvcr1b localizes to the inner or outer mitochondrial membrane and therefore to what compartment, IMS or cytoplasm, it delivers heme. In any case, it is unlikely that Flvcr1b is the only mode of mitochondrial heme export, as argued for by the lack of any Flvcr homologues in yeast[128].

It has been suggested that diffusion in lipid membranes could be a possible mechanism for mitochondrial heme export and/or intracellular trafficking. Heme has been described as phospholipid-like, where the charged propionate groups mimic the phosphate head group and the aromatic porphyrin core behaves similar to the lipid tails[128]. This has led to the hypothesis that heme may be trafficked in a manner similar to mitochondrially-derived lipids such as phosphatidylethanolamine or cardiolipin. However, this model encounters difficulty in that heme can cause oxidative damage to lipids when unchaperoned, and that the hydrophilic propionate groups would limit heme to travelling along rather than actually passing through a

lipid membrane[128, 129]. It is therefore more likely that heme is exported via a transport protein or other mechanism.

Once heme has reached the cytoplasm, it can be bound by a number of yet-to-be-identified proteins and/or chaperones, which collectively make up an "exchange-labile heme pool"[128]. This collection of heme binding molecules serves to buffer cytosolic heme, preventing the cytotoxicity inherent to unbound heme, as well as providing a reservoir of available heme that can be incorporated to various cytoplasmic hemoproteins or trafficked elsewhere in the cell[125, 128-130]. Some of the proteins proposed to buffer/chaperone the cytosolic heme pool include heme binding proteins (HBP) HBP22[131, 132] and HBP23[133], Glutathione S-transferases[134], SOUL[131, 132], and fatty acid binding proteins[135]. These proteins seem to bind heme in a non-specific fashion and it is unclear if they possess any heme related function other than buffering/sequestration. Interestingly, the glycolytic enzyme glyceraldehyde phosphate dehydrogenase (GAPDH) has also been included in this group, where it is required for delivery of heme to nitric oxide synthase(NOS)[136]. The heme binding activity of GAPDH itself, as well as that of many other hemoproteins, is dependent on nitric oxide (NO)[136, 137]. This interconnected regulation hints at a potential mechanism to control mobilization of heme from cytosolic chaperones via NO-mediated signaling[138], though further characterization is required.

Another potential mechanism for intracellular heme distribution has been proposed wherein heme is mobilized from the mitochondria via a combination of direct contact sites between organelles and vesicle mediated transport[128, 129]. This model would shield heme from any unwanted reactions in the cytosol in much the same way the kiss-and-run model is proposed to in iron trafficking[104]. Several studies have previously described interactions

between the mitochondria and ER[107, 139]. Mitochondria-associated membranes (MAMs) are areas of direct membrane contact between the mitochondrial outer membrane (OMM) and ER, which were originally implicated in lipid exchange[139]. These contact sites have been further defined with the characterization of the ER-mitochondrion encounter structure (ERMES), a protein complex responsible for mitochondrial-ER tethering[107]. MAMs and the ERMES have been shown to facilitate transport of both ionic species such as calcium, as well as lipids[107]. Since the structure of heme possesses a mixed hydrophilic/hydrophobic nature as previously described[128], it seems reasonable that heme could also be trafficked in this manner. In further support of this hypothesis is the fact the membrane associated heme synthesis enzymes Cpox and Fech were found to associate with MAMs[140, 141]. The heme binding protein HBP1, as well as heme oxygenase 2 were also identified in these experiments[140, 141], providing further evidence that heme may be associated with these sites. While fusion of the OMM and ER for the mitochondrial export and intracellular distribution of heme appears to be a promising model, MAMs and the ERMES alone do not address the issue of heme trafficking from the mitochondrial matrix to the IMS. It is possible that a previously described transporter such as Flvcr1b[126] or Adenine nucleotide translocases 2 or 3[142, 143] could mediate export to a "pool" in the IMS, which would then be distributed through OMM-ER fusion.

However, an alternative mechanism for mitochondrial heme export has been proposed that is mediated by the mitochondrial contact site and cristae organizing system (MICOS, also called MINOS). The MICOS is a protein complex centered around the membrane protein mitofilin (Mic60), which governs mitochondrial cristae morphology as well as contact sites between the inner and outer mitochondrial membranes[144]. These IMM and OMM junction points, coupled with MAM and ERMES regulated ER-OMM contacts represent a possible

mechanism for direct communication and metabolite transfer between the mitochondrial matrix and ER lumen. In this model, heme synthesized in the matrix would be moved directly to the ER and subsequently distributed to the rest of the cell, including to proteins in the mitochondria. Due to the interconnected nature of the ER, nucleus, Golgi apparatus, and secretory pathway, it is conceivable that heme may be delivered via this route to hemoproteins or regulatory elements in any of these organelles, or to more distal destinations via subsequent vesicular transport from the Golgi or secretory pathway. In addition to vesicular transport from these organelles, mitochondrially derived vesicles (MDVs) have also been characterized and shown to travel to peroxisomes and lysosomes[145, 146]. It is tempting to speculate that heme may also be transported via this route.

While several seemingly distinct transport mechanisms have been described above, it is highly likely that there is some degree of simultaneous function or overlap between them. Heme may be delivered to different destinations via different mechanisms, or some of the pathways described may be redundant to one another to ensure adequate mobilization of this essential, reactive metabolite. It is also likely that each of these mechanisms may be differentially utilized depending on tissue or cell type or in response to cellular demand for heme, which can exhibit extreme temporal variation, especially during oxidative stress response, drug detoxification, or erythropoiesis.

This literature review is intended to provide a summary of current knowledge regarding enzymes and processes relevant to the following chapters, which will discuss the identification of the mitochondrial heme synthesis metabolon and the characterization of novel, associated proteins.

Figures and Tables

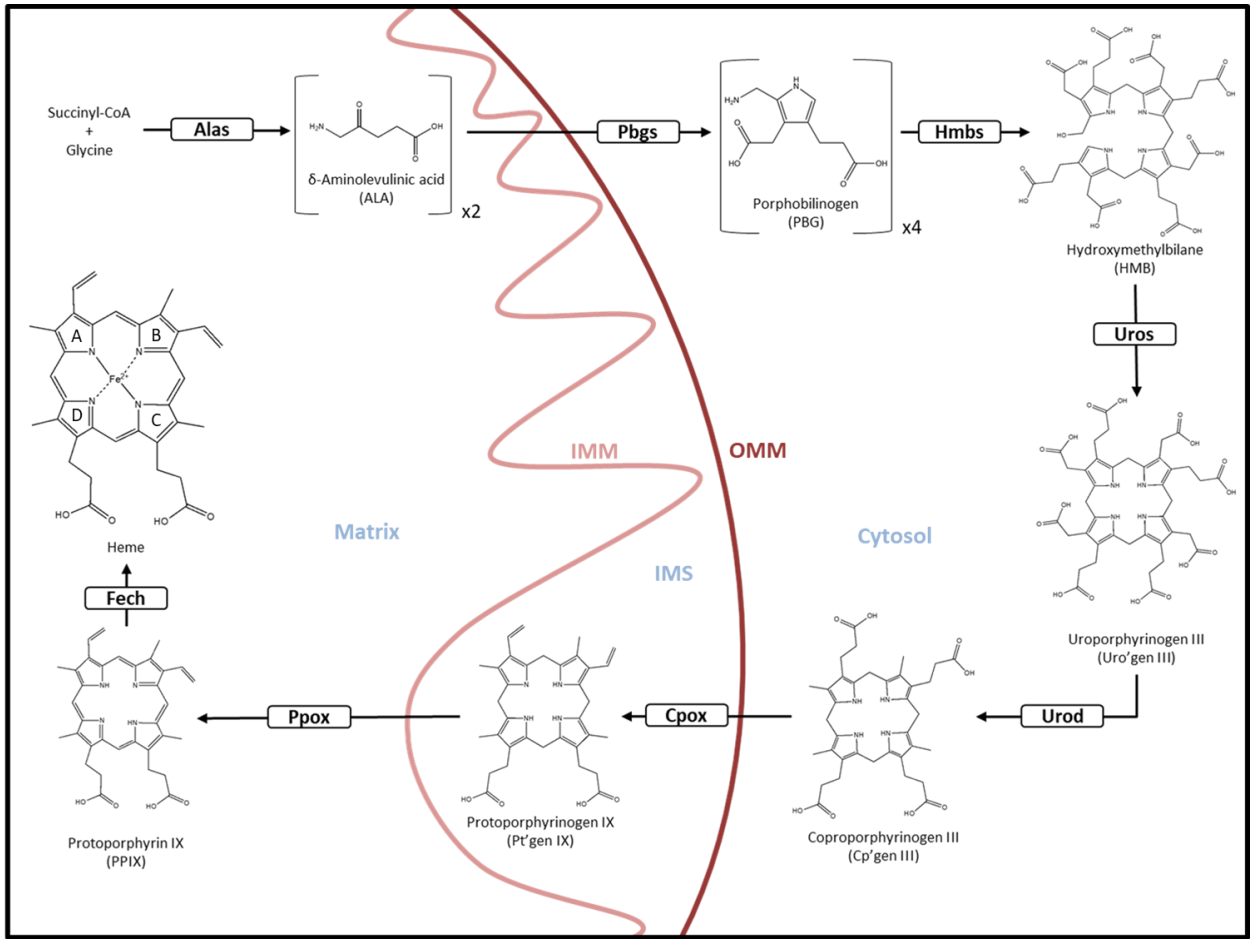

Figure 1. The mammalian heme synthesis pathway.

This diagram depicts the enzymes, substrates, and intermediates of the mammalian heme biosynthetic pathway as localized in the mitochondria and cytosol.

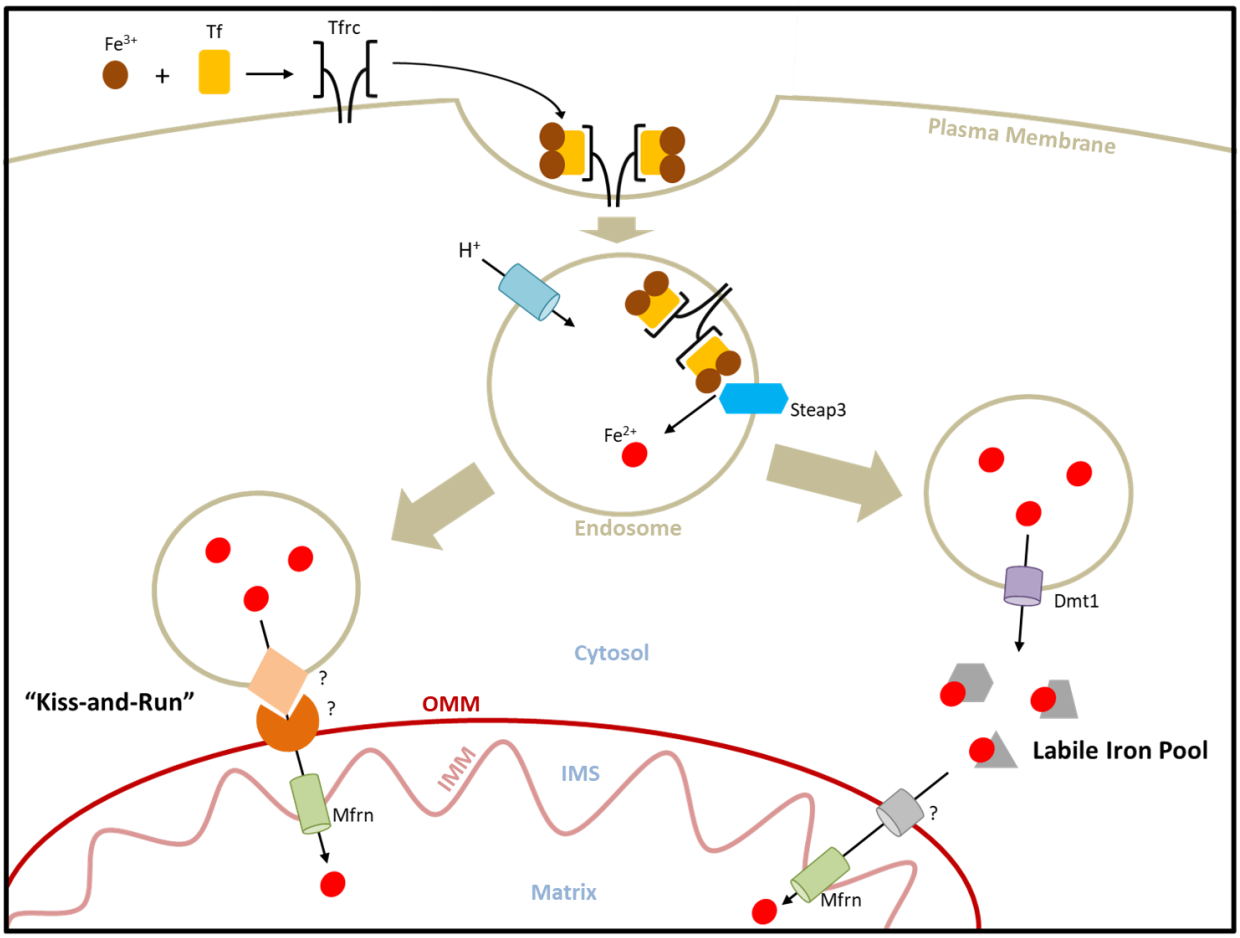

Figure 2. Models of iron trafficking to the mitochondria.

This schematic depicts models of mitochondrial iron import, including Dmt1 mediated export to a cytosolic labile iron pool and subsequent mitochondrial import, as well as direct endosomal delivery via the "kiss-and-run" model.

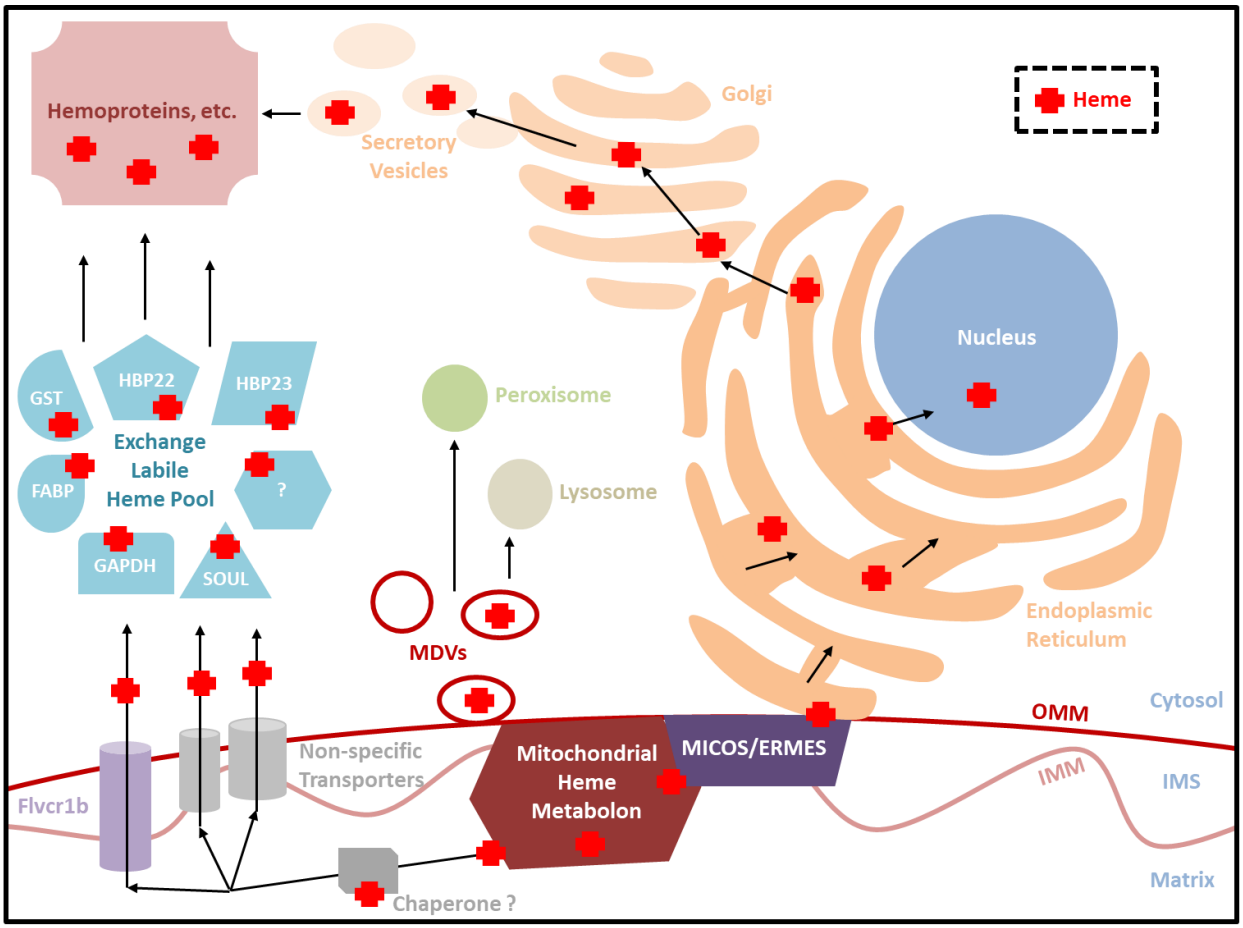

Figure 3. Models of intracellular heme trafficking.

This diagram shows potential mechanisms of intracellular heme trafficking, including export to a cytosolic exchange-labile heme pool, packaging of heme in mitochondrially derived vesicles, and direct inter-organelle trafficking through contact sites between the mitochondria and other membrane-bound organelles.

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CHAPTER 2

THE MITOCHONDRIAL HEME METABOLON:

INSIGHTS INTO THE COMPLEX(ITY) OF HEME SYNTHESIS AND DISTRIBUTION

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Abstract

Heme is an essential cofactor in metazoans that is also toxic in its free state. Heme is synthesized by most metazoans and must be delivered to all cellular compartments for incorporation into a variety of hemoproteins. The heme biosynthesis enzymes have been proposed to exist in a metabolon, a protein complex consisting of interacting enzymes in a metabolic pathway. Metabolons enhance the function of enzymatic pathways by creating favorable microenvironments for pathway enzymes and intermediates, facilitating substrate transport, and by providing a scaffold for interactions with other pathways, signaling molecules, or organelles. Herein we detail growing evidence for a mitochondrial heme metabolon and discuss its implications for the study of heme biosynthesis and cellular heme homeostasis.

Keywords: Heme, Metabolon, Heme Biosynthesis, Porphyria, Anemia

Introduction

Heme is an indispensable cofactor for metazoan life. It consists of a protoporphyrin IX macrocycle with a centrally coordinated iron ion. Heme is utilized in a diverse array of cellular processes including oxygen binding, redox reactions, drug detoxification, and management of reactive oxygen species, as well as regulation of transcription and translation for a variety of genes. The necessity of heme represents an interesting challenge for organisms since not only is heme an essential prosthetic group required in all cellular compartments, but heme, as well as its biosynthetic intermediates, are highly reactive molecules that can be toxic to the cell[1, 2]. Thus, both the production and distribution of heme must be tightly regulated.

In metazoans, heme is synthesized by a highly conserved pathway. The canonical metazoan heme synthesis pathway consists of eight enzymes and begins in the mitochondrial matrix with the synthesis of 5-aminolevulinic acid (ALA) from succinyl-CoA and glycine catalyzed by the enzyme 5-aminolevulinate synthase (Alas). ALA is then transported to the cytosol where two ALA molecules are combined to form the monopyrrole porphobilinogen (PBG) by porphobilinogen synthase (Pbgs), four PBGs are then cyclized, and modified by the next three enzymes in the pathway, hydroxymethylbilane synthase (Hmbs), uroporphyrinogen III synthase (Uros), and uroporphyrinogen III decarboxylase (Urod), to form the tetrapyrrole coproporphyrinogen III. Coproporphyrinogen III is then transported back into the mitochondria for further modification by coproporphyrinogen oxidase (Cpox) and protoporphyrinogen oxidase (Ppox), and finally iron is inserted by ferrochelatase (Fech)(Figure 1). Following its synthesis, heme must be distributed to all cellular compartments, yet details regarding this trafficking as well as incorporation into hemoproteins are greatly lacking. Additionally, mechanisms governing the regulation of heme synthesis remain relatively poorly understood. Disorders impacting heme biosynthesis can have a wide array of downstream consequences due to the requirement for heme in numerous cellular processes. This, coupled with the fact that heme synthesis lies at the convergence of diverse metabolic pathways including overall carbon metabolism, iron acquisition and transport, as well as the aforementioned variety of destinations for heme post production, argues for the necessity of well controlled regulation and facilitation of heme synthesis.

In metazoans with blood, developing erythrocytes represent the highest demand for heme anywhere in the body due to hemoglobin production[2]. The large amount of heme required, as well as the necessity for rapid production, results in a uniquely high level of heme synthesis in

these cells. This presents a distinct set of regulatory challenges relative to other cell types which require and synthesize heme at much lower levels. One means by which this level of regulation occurs is via the first enzyme in the pathway. In mammalian developing erythroid cells, the first step in the pathway is catalyzed by Alas-2, which is encoded on the X-chromosome. Other cells utilize the housekeeping form of Alas, Alas-1. Second to developing erythroid cells, the liver requires high levels of heme synthesis for cytochrome P450 function. It is from these tissues with high levels of heme production that symptoms associated with the misregulation of heme synthesis arise, which include anemias and porphyrias[2] (Table 1).

Recently, we presented evidence for a mitochondrial heme synthesis protein complex[3], or metabolon. In this metabolon, several heme synthesis enzymes, mitochondrial transporters, adapter and structural proteins, and intermediary metabolic enzymes involved in upstream substrate synthesis, interact with one another in a complex spanning both mitochondrial membranes (Figure 2). It is clear that regulation of both the synthesis of heme and its trafficking throughout the cell are controlled via proteins within this complex. Herein we discuss the role of the metabolon with regard to heme synthesis and transport and consider the direction of future studies in the field.

Metabolons and the role of protein interactions in Heme synthesis

A metabolon is defined as a complex of interacting enzymes within a metabolic pathway[4]. Metabolons have been described for a wide range of processes including the TCA cycle[5], glycolysis[6], the urea cycle[7], fatty acid synthesis[8], amino acid metabolism[9], and transmembrane transport[10]. Additionally, there is evidence to support the existence of

metabolons in various cofactor biosynthesis pathways including those for vitamin B12[11] and coenzyme F430[12], demonstrating a precedent for such complex formation in tetrapyrrole metabolism. The close interactions of pathway proteins can provide a number of advantages for the synthesis of a given product, often through the creation of favorable micro-environments where conditions such as pH, hydrophobicity, reactant and product concentrations, or even the availability of various binding partners and cofactors can be modulated independently from the overall cellular environment. Metabolons can aid in acquisition, movement, and/or delivery of pathway metabolites, thereby increasing flux through the pathway, as well as shielding reactive or unstable intermediates from contact with the cellular environment at large. Metabolons allow for modulation of pathway activity via interactions with regulatory factors/proteins or by providing a means to sense overall cell status. These protein complexes can aid in positioning of pathway components with respect to both proximity to other proteins as well as positioning either within or between organelles.

For heme synthesis, a metabolon offers several advantages. First, many of the pathway intermediates as well as the ultimate product, heme, are chemically reactive when free in the cell[1, 2]. Thus, sequestration of these compounds from the cellular milieu is essential to reduce cytotoxicity of the molecules in question, as well as to protect intermediates from loss to extraneous reactions. Substrate channels or close associations between proteins where intermediates could be channeled directly circumvent this problem. Second, as the pathway progresses, intermediates must cross both mitochondrial membranes and the end product, heme, must be exported from the mitochondrial matrix for delivery to diverse cellular compartments. This movement argues for the presence of proteins and/or channels to facilitate transport. Third, the demand for heme synthesis is dynamic between cell types and dependent on cell status. For

example, in liver cells, heme production must be upregulated in response to cytochrome P450 synthesis for xenobiotic detoxification[13]. Likewise, erythroid heme synthesis presents several unique challenges, foremost of which is the dramatically increased demand for heme production[2]. The potential for increased speed and efficiency of heme production conferred by a metabolon further add to the attractiveness of the hypothesis.

The idea of a metabolon for the mitochondrially localized enzymes was first proposed by Grandchamp et al. in 1978[14] and later expanded by others[15, 16]. This idea received support from *in silico* modeling based upon the crystal structures of Ppox[17] and Fech[18]. Published kinetic data are consistent with some level of substrate channeling, although these studies did not demonstrate the existence of a stable complex of the mitochondrial enzymes[15, 19]. However, available data clearly are consistent with the existence of a dynamic complex involving the terminal enzymes of the pathway. Similar proposals have been made for the formation of a complex involving at least some of the cytosolic enzymes, particularly Hmbs and Uros to prevent the non-productive cyclization of tetrapyrrole isomers which occurs in the absence of Uros [1]. Although complexes of heme synthesis enzymes seem likely in both the cytosol and mitochondria, no direct evidence in metazoa had demonstrated physical interactions between any of the enzymes.

The (Mitochondrial) Heme Synthesis Metabolon

As part of a comprehensive study, Medlock et al. conducted a series of experiments designed to elucidate proposed protein-protein interactions among heme biosynthesis enzymes and other peripheral enzymes such as mitochondrial transporters and potential regulatory/adapter

proteins[3]. In these experiments, FLAG-tagged human heme synthesis enzymes were expressed in a murine erythroid model cell line. Cells were fractionated and affinity purification was carried out with the cytosolic and mitochondrial fractions. Proteins interacting with the tagged enzyme were identified by Multidimensional Protein Identification Technology mass spectrometry (MudPIT-MS)[20]. Surprisingly there were no interactions detected between the cytosolic enzymes; Pbgs, Hmbs, Uros, and Urod; nor was a common protein with which all of these enzymes might interact identified. The lack of demonstrated interactions in these experiments does not preclude the existence of a cytosolic complex; such a complex may be extremely transient or indirectly linked by cytoskeletal structures. Additional experiments such as proximity labeling may help to shed light on these likely transient interactions.

In contrast to the cytosolic enzymes, interactions were detected between several of the mitochondrial heme synthesis enzymes. Affinity purification of Fech and reciprocal experiments with Ppox were consistent with each protein interacting with the other. In addition, both Fech and Ppox were found to be part of larger molecular weight complexes present in mitochondrial preparations[3]. A surprising finding was that the erythroid-specific form of the first enzyme in the pathway, Alas-2, interacts with the both Fech and Ppox[3]. It is interesting to speculate about the function this interaction may have in regulating production of ALA and subsequent intermediates. In the context of the most recently identified porphyria (Table 1), X-linked protoporphyria (XLP), which results from increased activity of Alas-2[21], determining whether XLP variants of the enzyme retain this interaction will be important in understanding the pathophysiology of this disease.

As well as enzymes with a known role in heme biosynthesis, many additional proteins were found to interact with the mitochondrial heme metabolon, including proteins involved in

succinyl-CoA synthesis, iron metabolism, and mitochondrial metabolite transport (Table 2). The presences of proteins involved in iron metabolism in affinity purification data[3] indicates that the heme synthesis metabolon may have a role in regulating cellular iron homeostasis or vice versa. Metabolon interaction partners involved in iron transport include Abcb7, a mitochondrial transporter which facilitates cytosolic Fe-S cluster synthesis[22]; Abcb10, an ATP-binding cassette transporter shown to stabilize the iron importer mitoferrin(Mfrn1)[23, 24]; and transferrin receptor, a protein important for cellular iron import and potentially direct delivery of iron to the mitochondria via a kiss and run mechanism[25-27]. Also found in the metabolon is the protoporphyrinogen transporter Tmem14c. This protein is responsible for the transport of protoporphyrinogen IX into the mitochondrial matrix following conversion of coproporphyrinogen III to protoporphyrinogen IX by Cpox in the mitochondrial inner membrane space. Disruption of Tmem14c in mice results in the accumulation of porphyrins in the fetal liver, arrest of erythroid maturation and profound anemia[28]. Proper facilitation and coordination of iron delivery and porphyrin transport within the heme synthesis pathway is necessary for not only heme synthesis itself, but also for preventing detrimental accumulation of un/mis-metallated porphyrins.

The interaction between Alas-2 and Ppox/Fech suggest a wider level of communication/coordination among all mitochondrially-located heme synthesis enzymes. Additionally relevant to the integrated regulation of both ends of the pathway is the interaction found between the metabolon enzymes and enzymes responsible for the synthesis of the ALA precursor succinyl-CoA. The interaction between Sucla2, the ATP-utilizing subunit of succinyl CoA synthetase (SCS), with Alas-2[29], Fech, and Ppox[3] provides an intriguing link between the TCA cycle and the heme synthesis metabolon. It had long been presumed that succinyl-CoA

used in heme synthesis is provided by the TCA cycle via the ATP-driven reverse Sucla2-SCS reaction. Recent work by Burch et. al[30] demonstrated that the main carbon source for ALA in developing erythroid cells is instead glutamine. Glutamine destined for ALA synthesis must first be converted to α-ketoglutarate, then to succinyl-CoA by the α-ketoglutarate dehydrogenase complex (KDH). They proposed that the observed interaction between the Sucla2 subunit of SCS and heme synthesis proteins may function to stabilize the apoproteins of Alas-2 and Fech prior to cofactor assembly or serve to sequester Sucla2 to prevent the ATP-utilizing reverse SCS reaction. Interestingly, subunits of KDH were also found to interact with the metabolon in developing erythroid cells[30].

It is becoming evident that one function of the heme synthesis metabolon is to mediate carbon flux in concert with the TCA cycle for the purpose of initial substrate synthesis. It should be noted that the interactions described for Sucla2 and KDH are with Alas-2, the erythroid specific form of ALA synthase. Additionally, the interaction with Sucla2 has been previously shown to only occur with Alas-2 and not Alas-1[29, 31]; however, it is unclear whether any interaction for KDH occurs in non-erythroid cells with Alas-1. It is tempting to speculate that mechanisms of regulation and possibly carbon source may be distinct between erythroid and non-erythroid heme synthesis. It stands to reason that, given the enormous demand for heme production during erythroid differentiation, it would be inefficient and energetically expensive to produce succinyl-CoA through the ATP dependent SCS path. Furthermore, withdrawing the large amount of succinate required for heme synthesis in developing erythroid cells from the TCA cycle could have detrimental effects on cell metabolism as a whole. The use of glutamine as a substrate avoids these problems as it appears that KDH may serve a moonlighting role in its interactions with Alas-2 during late erythropoiesis to facilitate succinyl-CoA synthesis for highly

upregulated heme synthesis independent of TCA cycle intermediate pools. This circumvention may not be necessary for the drastically lower levels of heme synthesis present in non-erythroid cells. Continued characterization of the heme synthesis metabolon and its interplay with the TCA cycle in non-erythroid cells will be necessary to answer these intriguing questions.

Novel Heme Synthesis Metabolon Proteins

As noted, one role of the mitochondrial heme metabolon is the downstream distribution of heme post synthesis. Heme is both essential in all compartments and can be highly cytotoxic if not properly contained[1, 2]; thus, well regulated, timely, and efficient trafficking is a necessity. Intriguingly, Opa1, a GTPase required for mitochondrial fusion[32], was present in the mitochondrial co-immunoprecipitation studies performed by Piel et al.[33]. In mammalian cells, Opa1 is not only required for fusion but has also been identified as part of the mitochondrial contact site and cristae organizing system (MICOS)[34, 35], a protein complex responsible for anchoring junction points between the inner and outer mitochondrial membranes and stabilizing cristae structure[36]. There have been several proposed mechanisms for heme trafficking that involve mitochondrial fusion/fission, direct inter-organelle contacts, or mitochondrially derived vesicle transport[37]. Such mechanisms would allow for heme to be distributed in bulk throughout the cell while remaining sequestered from the larger cellular environment. Both Mic60 (mitofilin, Immt) and Mic27 (Apool), also members of the MICOS complex[38-40], were likewise found to interact with the heme synthesis metabolon[33]. These data suggest a direct interaction of the heme synthesis metabolon and the MICOS complex to localize the metabolon for efficient import of porphyrinogens and iron and export of heme. Further studies to understand

the role of mitochondrial structure and dynamics in porphyrin and heme trafficking will shed light on to the role of these in heme synthesis.

In addition to enzymes with well-characterized functions, several proteins with no previously described role in heme synthesis were found to be present in the metabolon. One such protein was progesterone receptor membrane component 1 (Pgrmc1)[33], a heme binding protein that has been reported in a variety of cellular compartments and cell types. Pgrmc1 has many reported functions including binding and activation of cytochromes P450[41-44], promotion of autophagy[45] and endocytosis[46], and regulation of iron metabolism in concert with hepcidin[47]. Pgrmc1 was also found to be enriched at ER-mitochondria contact sites[48]. In the context of heme synthesis, recent studies have demonstrated that Pgrmc1 can inhibit Fech activity in vitro and that treatment of a mammalian erythroid cell model with AG-205, a small molecule which perturbs heme binding in Pgrmc1, results in decreased hemoglobin production during differentiation[33]. At present, the mechanism by which Pgrmc1 influences heme synthesis is unclear. One possibility is that Pgrmc1 functions as a heme chaperone and influences flux through the synthesis pathway by facilitating release of heme from its interaction partner, Fech. Adding to the credibility of this model is the fact that, when tested against several conformation-specific variants of Fech, Pgrmc1 interacts most strongly with the variant in the release/heme bound conformation[33]. An alternative interpretation of this finding is that Pgrmc1 interacts differently with different conformations of Fech for the purpose of regulating enzyme activity through stabilization of particular conformations. Recently, Pgrmc1 has been shown to exhibit heme dependent dimerization, with dimerization altering binding affinity for other proteins[49]. It is conceivable that the effect of Pgrmc1 on Fech activity may be further mediated by cellular heme status, as detected by Pgrmc1's ability to bind heme with moderate

affinity[33, 50]. Notably, Pgrmc1 was also shown to interact with Mic60, Mic27, and Opa1[33], which are involved in mitochondrial structure and dynamics as discussed previously. The presence of these interactions hint at a function for Pgrmc1 beyond its interaction with Fech alone. A homologue of Pgrmc1, Pgrmc2 was also found in the heme synthesis metabolon[33], though its role has been less well described. While the exact functions and mechanisms of these proteins remain elusive at present, they serve as intriguing evidence that examining heme synthesis and regulation from a metabolon-centric point of view has the potential to reveal previously undescribed mechanisms of regulation for heme synthesis and its coordination with other aspects of cell metabolism.

Dynamic and Transient Nature of the Complex

The mitochondrial heme synthesis metabolon is almost certainly a highly dynamic entity, with protein-protein interactions forming and dissociating depending on a number of factors including cell type, stage in development, induction of heme synthesis, response to various cell states and signaling pathways, as well as conformational changes in the enzymes themselves as they progress through their catalytic cycles. The most immediately apparent evidence in support of a dynamic metabolon is the sheer number of interaction partners documented for each protein examined[3]. For some protein types such as transporters and adapters, this finding could be rationalized by claiming that a protein species may fill several distinct niches simultaneously, each with its own set of interactions, resulting in diverse binding partners in coimmunoprecipitation assays. However, this argument is less compelling for enzymes with a more singular function such as Fech or Ppox. A more generally plausible explanation is that a given protein will interact with many different partners depending on the current demands of the

cell or progress through the enzyme's own catalytic cycle. Many of the relevant assays, including coimmunoprecipitation experiments, are, by necessity, conducted on heterogeneous populations of cells and mitochondria. Though they are of the same cell type, individual cells will exist at varying stages of differentiation. Additionally, individual mitochondria will exist in at least slightly disparate conditions, and the individual enzymes will certainly not be synchronized in their activity. This results in data representing interactions from various cellular and enzymatic states and stages. The relative amounts of a particular protein-protein interaction found will thus represent not only the strength of the interaction, but also relative abundance or temporal persistence of any given state. This view of a dynamic metabolon is further supported by the fact that the interaction between Fech and Pgrmc1 has been shown to be conformation dependent[33]. Since Fech assumes several distinct conformations over the course of its catalytic cycle[51-53], this suggests that the Fech-Pgrmc1 interaction is broken and reformed repeatedly during heme synthesis. This is likely the case for other interactions as well. Given the variety of downstream destinations for heme and the variety of cellular signals heme synthesis must respond to, a dynamic heme synthesis metabolon exhibiting many transient interactions seems likely.

As noted with respect to the cytosolic heme synthesis enzymes, the lack of interaction partners in co-immunoprecipitation assays is not sufficient to rule out all types of interactions or complexes. It is likely that a portion of interactions in a complex such as this will be either very transient, or too low affinity to persist through immunoprecipitation conditions. It is also likely that some interactions will be indirect; mediated by either adapter proteins, membrane lipids, or cytoskeletal components. To further investigate weak, transient, or indirect interactions, conditions within the immunoprecipitation assays can be further modulated, i.e. affinity tag type,

detergent concentrations, crosslinking, etc. or alternative methods can be attempted. Attractive alternate methods include those in which interactions are characterized by proximity of the components rather than direct interaction, including proximity labeling, proximity ligation assays or bi-molecular fluorescence assays, though the later require a putative interaction to be identified prior to testing.

Conclusion

While originally proposed four decades ago[14], it is only recently that substantial data have been presented that are consistent with the existence of a transient complex centered around the mitochondrial heme biosynthesis enzymes. This metabolon appears to play a role in the facilitation and regulation of heme synthesis via modulation of enzyme activity, metabolite transport, and pathway response to cell status and other signals. Further studies to elucidate the nature and individual functions of these interactions will help to broaden our understanding of the porphyrias, anemias, and other heme related disease states. The identification and characterization of the mitochondrial heme synthesis metabolon provides a new platform from which to examine the essential and multifaceted processes of heme synthesis and distribution.

Figures and Tables

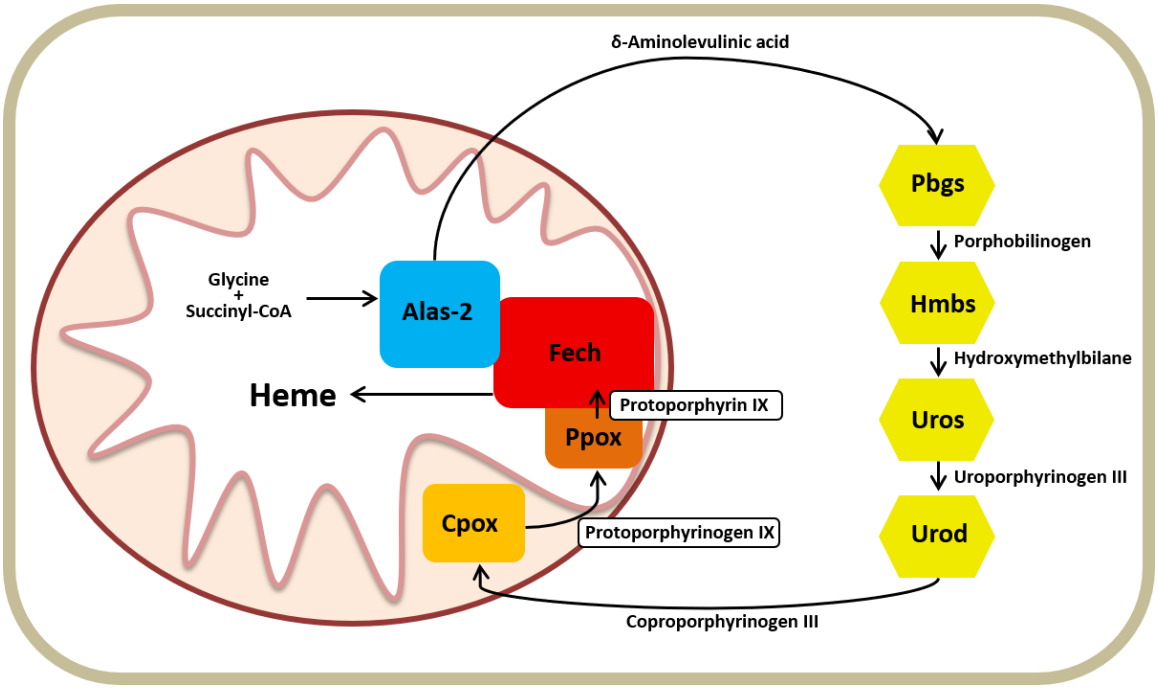

Figure 1. The Mammalian Heme Synthesis Pathway

This diagram depicts the eight enzymes of the heme biosynthetic pathway and their intermediates as localized in the mitochondria and cytosol.

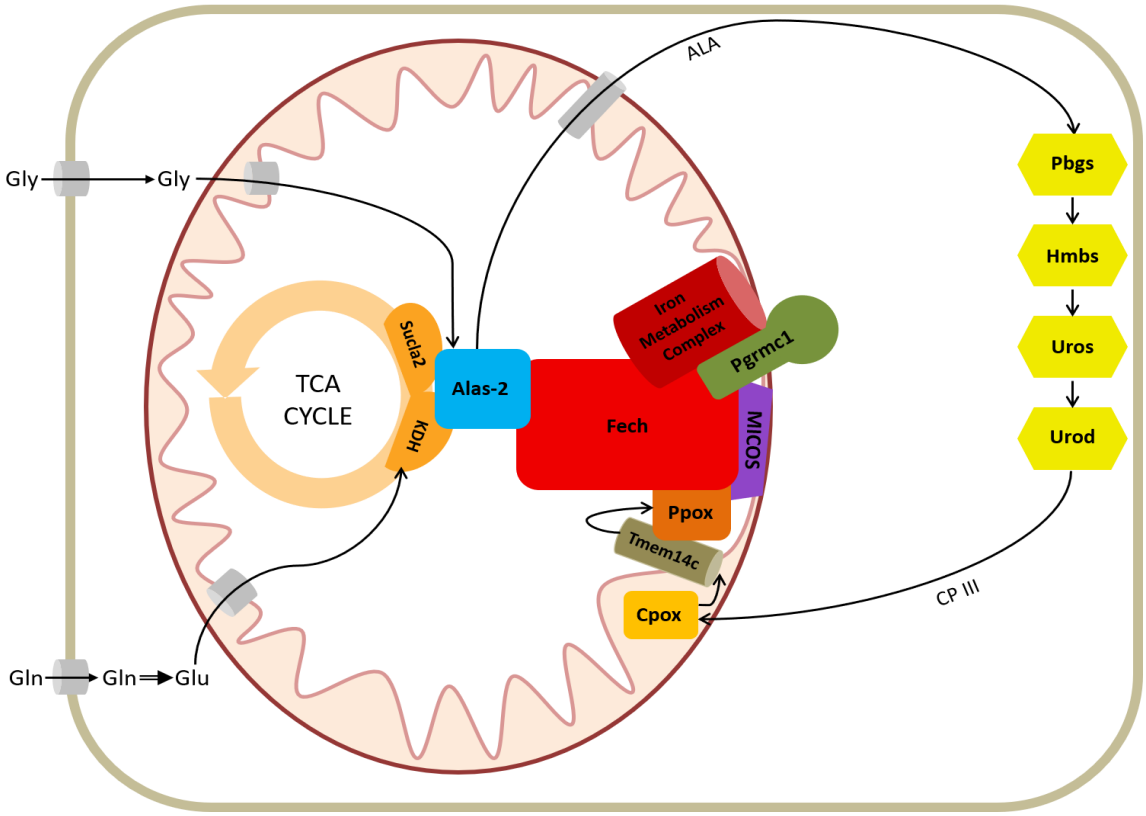

Figure 2. The Heme Synthesis Metabolon

Representation of heme synthesis metabolon as localized in the mitochondria. Iron metabolism proteins found to interact with the metabolon include Abcb7, Abcb10, Mfrn1, and Tfrc. Interacting members of the MICOS include Mic60, Mic27, and Opa1. The cytoplasmic enzymes of the heme synthesis pathway are also shown.

Table 1. Heme Synthesis Enzymes and Associated Disorders

Enzyme	Mutation (Gain/Loss of Function)	Disorder
Alas-2	Gain	X-Linked Protoporphyria
	Loss	X-Linked sideroblastic anemia
Pbgs	Loss	Aminolevulinate dehydratase porphyria
Hmbs	Loss	Acute intermittent porphyria
Uros	Loss	Congenital erythropoietic porphyria
Urod	Loss	Porphyria cutanea tarda
Cpox	Loss	Hereditary coproporphyria
Ppox	Loss	Variegate porphyria
Fech	Loss	Erythropoietic protoporphyria

Table 2. Components of the Mitochondrial Heme Synthesis Metabolon

Protein	Function
Fech	Conversion of Protoporphyrin IX to Heme (Iron insertion)
Ppox	Conversion of Protoporphyrinogen IX to Protoporphyrin IX
Cpox	Conversion of Coproporphyrinogen III to Protoporphyrinogen IX
Alas-2	5-aminolevulinate synthesis
Sucla2	Subunit of succinyl-CoA synthetase
Ogdh	Subunit of α-ketoglutarate dehydrogenase complex
Pgrmc1	Heme binding
Pgrmc2	Heme binding
Mic60	MICOS complex subunit
Mic27	MICOS complex subunit
Opa1	Mitochondrial fusion and MICOS complex
Ymel1	Opa1 cleavage
Ant1	Membrane transport
Ant2	Membrane transport
Abcb7	Fe-S cluster synthesis
Abcb10	Membrane transport and stabilizes mitoferrin
Glrx5	Fe-S cluster synthesis
Tfrc	Iron import
Tmem14c	Mitochondrial Protoporphyrinogen IX import
Aralar1	Mitochondrial membrane transport
Mfrn1	Mitochondrial iron import

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CHAPTER 3

A NOVEL ROLE FOR PROGESTERONE RECEPTOR MEMBRANE COMPONENT 1 (PGRMC1): A PARTNER AND REGULATOR OF FERROCHELATASE

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Abstract

Heme is an iron containing cofactor essential for multiple cellular processes and fundamental activities such as oxygen transport. To better understand the means by which heme synthesis is regulated during erythropoiesis, affinity purification coupled with mass spectrometry (MS) was carried out to identify putative protein partners interacting with ferrochelatase (FECH), the terminal enzyme in the heme biosynthetic pathway. Both Progesterone Receptor Membrane Component 1 (PGRMC1) and Progesterone Receptor Membrane Component 2 (PGRMC2) were identified in these experiments. These interactions were validated by reciprocal affinity purification followed by MS analysis and immunoblotting. The interaction between PGRMC1 and FECH was confirmed in vitro and in HEK293T cells, a non-erythroid cell line. When cells that are recognized models for erythroid differentiation were treated with a small molecule inhibitor of PGRMC1, AG-205, there was an observed decrease in hemoglobinization relative to untreated cells. In vitro heme transfer experiments showed that purified PGRMC1 was able to donate heme to apo-cytochrome b_5. In the presence of PGRMC1 in vitro measured FECH activity decreased in a dose dependent manner. Interactions between FECH and PGRMC1 were strongest for the conformation of FECH associated with product release suggesting that PGRMC1 may regulate FECH activity by controlling heme release. Overall, the data illustrate a role for PGRMC1 in regulating heme synthesis via interactions with FECH and suggest that PGRMC1 may be a heme chaperone or sensor.

Introduction

Heme is an essential cofactor in metazoa for many important cellular processes. The ability of heme to bind to small molecules such as gases (O_2 and CO) and to participate in redox reactions are some of its more commonly known roles. However, in the past decade additional essential roles for heme as a regulator of processes including the circadian rhythm[1], microRNA processing[2], protein degradation[3], the cell cycle[4], and ion transport[5-7] have been identified. Hemoproteins are involved in a variety of cellular reactions and are distributed throughout the cell in multiple cellular compartments. For example, the respiratory cytochromes are found in the inner mitochondrial membrane, some cytochrome P_{450}s and cytochrome b_5 are associated with the endoplasmic reticulum (ER) membrane, catalases are localized to the peroxisome and peroxidases to the lysosomes, and heme-binding transcription factors are found in the nucleus[8]. However, despite the biological necessity for heme as a cofactor, heme in its free state is cytotoxic since it can generate harmful free radicals in the presence of oxygen[9, 10].

Heme is synthesized in metazoa by a pathway composed of eight enzymes which starts and ends in the mitochondrial matrix. In erythroid cells, studies show that several components of the heme synthesis pathway exist in a complex to provide feedback regulation on the process of heme synthesis[11]. These components include aminolevulinic acid synthase-2 (ALAS-2), protoporphyrinogen, oxidase (PPOX) and ferrochelatase (FECH). This mitochondrial heme metabolon physically links the first step catalyzed by ALAS-2 with the terminal step catalyzed by FECH, supporting previous studies that showed both enzymes to be important regulatory points in heme synthesis[12-14]. Both ALAS-2 and FECH are regulated at different levels by cellular iron[15-17]. In addition, other proteins involved in mitochondrial cellular iron metabolism were

also found in the heme metabolon suggesting an additional level of coordination for heme and iron availability for optimal heme production during erythropoiesis[11].

The convergence of iron and porphyrin metabolism occurs at the terminal step of heme synthesis, which is catalyzed by FECH. FECH inserts ferrous iron into protoporphyrin IX to form protoheme and two protons. Structural studies of human FECH have shown it to be a conformationally dynamic enzyme that undergoes several changes in its structure over the course of the catalytic cycle[18-20]. These conformational changes have been proposed to allow interaction with different protein partners facilitating substrate delivery and product release. Besides its use of iron as a substrate, FECH from animals and some lower organisms possesses a [2Fe-2S] cluster that has been shown to be required for enzyme activity[16] and important in regulating enzyme activity[21]. Release of heme from FECH is the rate-limiting step in the reaction[22] and likely controlled in vivo by interactions with transporters or chaperones.

The protein Progesterone Receptor Membrane Component 1 (PGRMC1) has been proposed to be a heme chaperone[23, 24]. PGRMC1 is a 25 kDa protein which belongs to the Membrane-Associated Progesterone Receptor (MAPR) family. The MAPR family is a subset of the cytochrome b_5 family consisting in mice and humans of PGRMC1 and Progesterone Receptor Membrane Component 2 (PGRMC2), as well as the more recently discovered proteins Neudesin and Neuferrin[25]. Of the MAPR proteins, PGRMC1 and PGRMC2 show the closest homology. Neudesin and Neuferrin are secreted proteins that bind heme and are thought to possess neurotrophic and neurogenic activity[26, 27]. Yeast have only a single MAPR family member, DAP1, which has been studied in both *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*. The precise cellular roles of PGRMC1 and DAP1 are not clear although it has been implicated in a variety of cellular functions including stimulation of P_{450}s[28, 29] and cholesterol

metabolism[30, 31], autophagy[32], endocytosis[33], intracellular signal transduction[34], and iron metabolism[35, 36]. Additionally, PGRMC1 has been reported to play a role in DNA damage protection and response to oxidative stress[33], likely through an iron dependent process[37]. While PGRMC1 has been shown to bind progesterone[24], its involvement in progesterone signaling remains uncertain. However, it has clearly been demonstrated that PGRMC1 binds heme[23, 24, 38] though not with the avidity of a typical stable hemoprotein such as myoglobin or cytochrome b_5.

The PGRMC1 protein consists of an N-terminal region that is proposed to be a transmembrane domain and C-terminal region that contains a cytochrome b_5-like motif heme binding domain[33, 39]. In most cytochrome b_5-like proteins the heme cofactor is coordinated via two histidine residues[40, 41]. However, spectroscopic and mutagenesis studies are consistent with PGRMC1 binding heme via tyrosine ligands rather than histidine[23, 24, 42]. This coordination motif is a feature shared with purported heme transporters such as ShuT[43] and PhuT[44] as well as the heme binding protein HasA[45, 46].

Herein we identify and characterize an interaction between FECH and PGRMC1 as well as PGRMC2. We identify the cellular and subcellular localization of PGRMC1 in murine erythroleukemia cells (MEL) and show that the small molecule inhibitor of PGRMC1, AG-205, decreases heme synthesis in differentiated MEL cells in a dose dependent manner. We show that PGRMC1 interaction with FECH is dependent on the molecular conformation of FECH and that PGRMC1 decreases FECH activity in in vitro assays. These findings along with previous reports suggest PGRMC1 may play a role in regulating heme synthesis.

Materials and methods

Vectors, Cell Lines and Reagents

Human *PGRMC1* and *PGRMC2* were cloned from bacterial expression vectors (gift of Dr. Peter Espenshade) into pEF1alpha FLAG biotag vector (gift of Alan Cantor)[47]. To produce N-terminal FLAG tagged proteins, cloned cDNA encoding full length *PGRMC1* and *PGRMC2* were amplified and cloned into pEF1alpha using the XmaI and BamHI and XmaI and XbaI sites, respectively. An N-terminal FLAG tagged human *FECH* expression vector was produced as previously described[11].

Cell lines utilized for tissue culture experiments were DS19 murine erythroleukemia (MEL) cells[48, 49], human embryonic kidney 293T (HEK 293T) cells (ATCC – CRL3216) and K-562 human myelogenous leukemia (K562) cells (ATCC – CCL243)[50, 51]. To create DS19 MEL and HEK 293T cell lines expressing human FLAG tagged *FECH*, *PGRMC1* and *PGRMC2*, cells were transfected with expression vectors by electroporation and stably expressing cell lines were selected for puromycin resistance. 5 μg/mL of puromycin (Cellgro, Manassas, VA) was included in media for the selection step. Expression of tagged proteins was confirmed by immunoblot analysis using anti-FLAG antibody (Sigma, St. Louis, MO). All mammalian cells were cultured in DMEM with 25 mM glucose, 1 mM sodium pyruvate and 4 mM glutamine (Cellgro) plus 10% FBS (Atlanta Biologicals, Flowery Branch, GA) and 1% penicillin/streptomycin (Cellgro). For induction of MEL cells 1.5% dimethylsulfoxide (DMSO) (Sigma, St. Louis, MO) was included in the growth media and cells were grown for 72 hours for maximal expression of heme biosynthetic enzymes[52]. For addition to MEL cells, hemin (Sigma) was prepared in DMSO and added at a final concentration of 12.5 μM. For induction of K562 cells 1 mM sodium butyrate was included in growth media and cells were grown 6 days[53, 54].

For recombinant production in *Escherichia coli*, wild-type and variant *FECH* were expressed and purified as previously described[55]. Full length human *PGRMC1* was cloned into pTrcHisA (Life Technologies, Grand Island, NY) using the NheI and HindIII sites for production of N-terminal his-tagged proteins. For the non-tagged form *PGRMC1* was cloned with NcoI and HindIII. Wild-type *PGRMC1* was expressed and purified as previously described for human FECH[55].

The PGRMC1 inhibitor AG-205[56, 57](Sigma) was prepared as a 1 mM stock in DMSO.

Affinity Purification and Mass Spectrometry

Affinity purification and MS experiments were carried out in MEL cell lines stably expressing FLAG tagged *FECH, PGRMC1*, and *PGRMC2*. In addition, affinity purification of HEK 293T cells expressing FLAG tagged *FECH* followed by immunoblots was carried out as previously described[11, 58]. For PGRMC1 and PGRMC2, which both form homodimers or homomultimers, we found an equivalent amount of tagged exogenous and endogenous protein with the average ratio being 2.1$\pm$0.1 and 1.1$\pm$0.1, respectively. This suggests that in the differentiated state there were comparable amounts of the tagged exogenous and the endogenous protein orthologues. From all experiments an average of ~300 proteins were observed with normalized spectral abundance factor values over that of the control experiments. Pull downs using FLAG tagged PGRMC1 and PGRMC2 resulted in a large number of identified mitochondrial proteins in the recovered pool. From the MS results the criteria used to confirm interactions were based on the number of spectral counts, the number of unique peptides recovered and the percent sequence coverage which occurred over that of the background in two biological replicates and in reciprocal pull down experiments in two biological replicates. The protein interactions presented here have been

submitted to the IMEx (http://www.imexconsortium.org) consortium through IntAct[59] and assigned the identifier IM-25485.

Additional experiments with higher stringency washes, specifically 1% Nonidet P-40 in the wash buffer, and harsher elution from the agarose using 6M urea were carried out. Eluted protein samples were then subjected to tryptic digestion and shotgun proteomics performed on a Thermo Fisher Orbitrap XL (Thermo Fisher Scientific, Grand Island, NY) according to a previously described protocol[60]. Data were searched in Proteome Discoverer 1.4 using Sequest HT (Thermo Fisher Scientific) with the percolator node set at a 1% peptide false-discovery rate.

In vitro Interaction Experiments

Expression of his-tagged wild-type FECH, FECH variants, and non-tagged PGRMC1 in *E. coli* was carried out by growth in Circlegrow media (MP Biomedicals, Santa Ana, CA) for 18-20 hours at 30°C. Cells were harvested by centrifugation at 5,000 x g for 10 min, resuspended in solubilization buffer (50 mM Tris-MOPS, pH 8.0, 100 mM KCl, 1% sodium cholate) and sonicated three times on ice for 30 seconds. The resulting lysate was then centrifuged at 100,000xg for 20 minutes and the supernatant reserved. Supernatant from non-tagged PGRMC1 was then mixed with the his-tagged wild-type and variant FECH supernatant and loaded onto HisPur Cobalt Resin (Thermo Fisher Scientific). The column was then washed with wash buffer (50 mM Tris-MOPS pH 8.1, 100 mM KCl, 1% sodium cholate, 15 mM imidazole) and subsequently eluted using elution buffer (50 mM Tris-MOPS pH 8.1, 100 mM KCl, 1% sodium cholate, 250 mM imidazole). Presence of PGRMC1 and relative amounts of PGRMC1 and FECH, both wild-type and variants, were analyzed by SDS-PAGE and immunoblots.

Transcript analysis from in vitro erythroid expansion of peripheral blood CD34$^+$ mononuclear cells

In vitro erythroid expansion of peripheral blood CD34$^+$ mononuclear cells and gene expression analysis at multiple days of differentiation was carried as previously described [61]. Probes used were Hs00998344_m1 for *PGRMC1* and Hs01128672_m1 for *PGRMC2*, (Applied Biosystems, Foster City, CA). Levels of mRNA were normalized to an endogenous control human GUSB (beta glucuronidase) (Applied Biosystems) and gene expression is expressed in arbitrary units.

Immunoblots

For immunoblots, eluted protein from affinity purification, column chromatography and cellular lysates was separated on Mini-PROTEAN TGX Stain-Free gels (BioRad, Hercules, CA) and then transferred by Transblot semi-dry blotting (BioRad). Antibodies used included Anti-FECH (generated in house by H.A.D. at U.G.A.) at a dilution of 1:50,000-100,000, Anti-PGRMC1 (Sigma) at a dilution of 1:2,000, Anti-PGRMC2 (Sigma) at a dilution of 1:500, Anti-cytochrome c (BD Biosciences, San Jose, CA) at a dilution of 1:50,000, Anti-Mitofilin (Gene-Tex, Irvine, CA) at a dilution of 1:500, Anti-SUCLA2 (Gene-Tex) at a dilution of 1:2,500, Anti-ABCB10 (Gene-Tex) at a dilution of 1:1,000, Anti-ABCB7 (Gene-Tex) at a dilution of 1:500, and Anti-α-tubulin (Gene-Tex) at a dilution of 1:1000. Secondary antibodies used included Anti-Rabbit IgG (H+L) HRP conjugate (Promega, Madison, WI) and Anti-mouse IgG (H+L) HRP conjugate (Promega) at dilutions of 1:30,000-60,000. For detection, SuperSignal West Pico Chemiluminescent substrate (Thermo Fisher Scientific) and X-ray film or ChemiDoc imaging system (BioRad) were used.

Hemoglobin Measurements

Hemoglobin (Hb) content of intact MEL cells was determined using an Olis CLARiTY Spectrophotometer (Olis, Bogart, GA) as previously described[62]. Cell counts were taken using Scepter handheld automated cell counter (Millipore, Billerica, MA) using the 40 μm tip. % WT hemoglobin/cell was reported to normalize for variation in differentiation as cultures are passaged.

Cellular Fractionation

MEL cells were fractionated using the Mitochondria Isolation kit (Thermo Fisher Scientific). Mitoplasts were prepared from the mitochondrial fraction and protease protection was carried out as previously described[11, 63, 64].

Immunocytochemistry

Preparation of cells for immunofluorescence was carried as previously described[65] with several modifications. Briefly, MEL cells were attached to poly-L-lysine coated coverslips (BD Bioscience) by incubating undifferentiated cells on coverslips for 24 hours at 37°C in 5% CO_2. Cells were fixed in 4% paraformaldehyde for 20 minutes, permeabilized with 0.1% Triton X-100 for 20 minutes and blocked with 5% bovine serum albumin for 1 hour. Cells were incubated with rabbit anti-PGRMC1 (Sigma) diluted 1:100 and mouse anti-TIM23 (BD Biosciences) at a dilution of 1:200 overnight. Incubation with AlexaFluor 488 goat anti-rabbit IgG (H+L) (Life Technologies, Carlsbad, CA) and AlexaFluor 633 goat anti-mouse IgG (H+L) (Life Technologies) was carried out for 1 hour. Finally cells were counterstained with 300 mM DAPI nucleic acid stain (Thermo Fisher Scientific) for 5 minutes prior to mounting with ProLong Gold antifade (Life Technologies). Cells were washed with phosphate buffered saline (PBS) three times between each

step in the process, reagents were diluted in PBS and all incubations were carried out at room temperature. Cells were imaged using a Zeiss LSM 710 Inverted Confocal microscope (Zeiss, Thornwood, NY) using a 100X oil immersion objective and images processed using Zen (Zeiss) software.

Structural Model

Interactive modeling was carried out using the full length amino acid sequence of human PGRMC1 via the Protein Model Portal (http://www.proteinmodelportal.org/)[66]. In silico tools employed to produce predictive structures for the full length protein were RaptorX[67], IntFOLD2[68, 69], Phyre2[70] and ITASSER[71-74]. Images of structures were generated using PyMol[75].

Heme and FECH Activity Measurements

The heme content of purified PGRMC1 was measured as its pyridine hemochromogen as previously described[76]. FECH activity alone and in the presence of PGRMC1 was assayed with mesoporphyrin IX (Frontier Scientific, Logan, UT) and ferrous ammonium sulfate using the continuous direct spectroscopic method[77]. Assays were performed in triplicate on at least two independent protein preparations.

Heme Transfer and Gel Staining

Interprotein transfer of heme was determined by mixing an equivalent amount of PGRMC1, FECH or HasA with apo-cytochrome b_5 protein and incubating 15 minutes at 4°C. Apo-cytochrome b_5 was prepared as previously described[78] except the extraction was carried out at

room temperature for 6 hours. For native-PAGE, loading buffer minus SDS and without reductant was added and running buffer without SDS was used. Gels were stained as previously described[79] excluding the trichloroacetic acid wash.

Statistical Analysis

Statistical analysis was carried out using one-way ANOVA followed by Tukey HSD test. A P value of 0.05 was set as the cutoff for statistical significance.

Results

Identification of protein partners of PGRMC1 *and* PGRMC2

To identify novel protein partners for FECH an affinity purification of FLAG tagged human FECH in induced MEL cells was conducted as previously described[11]. Two of the proteins identified with the largest number of spectral counts, unique peptides and % coverage were PGRMC1 and PGRMC2 (Fig. 1 and Table 1). To validate these interactions we performed the reciprocal pull down experiments using N-terminal FLAG tagged human PGRMC1 and PGRMC2 and recovered murine FECH at levels above that of non-specific interactions (Fig. 1 and Table 1). Results from tagged human forms of PGRMC1 and PGRMC2 showed that these proteins formed multimers with their mouse counterparts (Fig. 1 and Table 1). This is consistent with previous demonstrations showing that PGRMC1 forms multimers[23, 42]. The interactions with the endogenous mouse protein serve as a control for protein folding of the exogenous tagged protein as well as quality control for affinity purification and MS. Additionally, affinity purification of the tagged PGRMC1 resulted in the recovery of murine PGRMC2, and likewise PGRMC2 in the recovery of murine PGRMC1.

A number of additional putative protein partners for PGRMC1 and PGRMC2 were identified in the affinity purification experiments. Of note, many of the protein partners for FECH were recovered in the PGRMC1 and PGRMC2 affinity purification experiments (Table S1). Several of these interactions were confirmed for PGRMC1 by immunoblot (Fig. 2A). Many of these proteins are involved in iron metabolism, including iron transport from the cell surface[80, 81], iron trafficking[64, 82] and iron-sulfur cluster biogenesis[83], or are putative heme or porphyrin transporters[84, 85].

An additional novel protein partner found in common with FECH, PGRMC1 and PGRMC2 was IMMT (Fig. 1 and Table 1). This interaction was confirmed via immunoblot analysis (Fig. 2A). IMMT, also known as Mitofilin or Mic60, is a protein found at junction points between the inner and outer mitochondrial membranes and is thought to stabilize mitochondrial structure[86]. In addition to IMMT, several other protein components of the mitochondrial inner membrane organizing system (MINOS) were also identified and include OPA1[87, 88] and APOOL (i.e MIC27)[89] (Table 1).

Further characterization of interactions

To confirm that the interactions between PGRMC1 or PGRMC2 and FECH were not an artifact of the cell line utilized or the purification process, we performed several additional experiments. First, we utilized a non erythroid human cell line, human embryonic kidney (HEK) 293T cells, to validate the in vivo interaction. HEK293T cells lines stably expressing FLAG-FECH were created and used for affinity purification and western blot analysis to detect the interaction. Immunoprecipitation of FLAG-FECH from HEK293T cells resulted in the recovery of endogenous PGRMC1 (Fig. 2*B*). This interaction was further confirmed independently in HEK293T cells by high throughput affinity purification studies which used C-terminus HA-tagged FECH as bait and recovered PGRMC1[90]. These data which are available via the Biological General Repository for Interaction Datasets (BioGRID - http://thebiogrid.org/) confirm the FECH/PGRMC1 interaction in a distinct cell line.

Second, we performed a higher stringency purification of FLAG-FECH and associated proteins from induced MEL cells by increasing the detergent concentration in the wash buffer during the affinity purification procedure. MS of the affinity purified tagged proteins resulted in the recovery

of PGRMC1 and PGRMC2 with FLAG-FECH and FECH with FLAG-PGRMC1 and FLAG-PGRMC2 (Table S2).

Third, we investigated the interaction between FECH and PGRMC1 proteins in vitro using affinity chromatography of his-tagged FECH and non-tagged PGRMC1. Purification of his-tagged FECH resulted in the recovery of non-tagged PGRMC1 (Fig. 3). Reciprocal affinity chromatography experiments were not possible due to the non-specific interaction of FECH with anti-his resin. Experiments with PGRMC2 were not carried out due to the low level of PGRMC2 expression. Together the result from MEL cells, HEK293T cells and in vitro experiments demonstrate the interaction between FECH and PGRMC1 or PGRMC2 in a relatively stable protein complex.

Expression of PGRMC1 *and* PGRMC2 *during erythropoiesis*

In order to understand the roles of PGRMC1 and PGRMC2 in erythropoiesis, we investigated the expression of each during erythroid differentiation. To investigate gene expression of *Pgrmc1* and *Pgrmc2* during murine erythropoiesis, we first queried the ErythronDB database[91, 92]. Transcript levels for *Pgrmc1* decrease throughout adult definitive differentiation, while those for *Pgrmc2* changed less. We further investigated expression levels in peripheral blood $CD34^+$ mononuclear cells in an in vitro erythroid expansion system[61]. Relative expression levels of *PGRMC1* decreased, while *PGRMC2* expression increased when cells were cultured until day 16 (Fig. 4*A*). To determine if the protein levels correlated to expression data, we differentiated MEL cells for 4 days and performed Western blot analysis on PGRMC1 and FECH. Protein levels of FECH increase as previously described[52]. Levels of PGRMC1 showed significant variation over the time followed (Fig. 4*B*).

Cellular and subcellular localization of PGRMC1 *in MEL cells and* PGRMC1 *modeling*

Initial affinity purification and MS experiments were conducted using fractionated MEL cells, specifically the mitochondrial cell fraction. The identification of PGRMC1 and PGRMC2 as a partner of FECH in these experiments is consistent with the mitochondrial co-fractionation and possible localization of these proteins. This localization was further investigated using immunohistochemistry and confocal microscopy in both differentiated and undifferentiated MEL cells (Fig. 5*A* and Fig. S1). While fractionation via differential centrifugation may contain contaminates of other membranes including ER and vesicles, data is consistent with PGRMC1 being at least associated with the mitochondrial membrane either directly or via other membrane interactions such as ER-mitochondrial junctions.

In order to further examine submitochondrial localization of PGRMC1, proteinase protection assays were performed on both whole mitochondria and mitoplasts from MEL cells. Results from these studies are consistent with PGRMC1 being associated with the outer face of the outer mitochondrial membrane (Fig. 5*B*). The antibody used to detect PGRMC1 recognizes an antigen on the C-terminal end of the protein. Thus the C-terminus and the identified heme binding domain of PGRMC1 appears to reside on the outside of the mitochondria. This finding is significant since FECH is localized to the inner face of the inner mitochondrial membrane[93-95] and suggests that a portion of the N-terminal end of PGRMC1 interacts with FECH.

To understand how PGRMC1 might interact with FECH yet be associated with the outside of the mitochondria, we carried out modeling studies of full length PGRMC1. Recently, the structure of an N-terminal truncated form of human PGRMC1 (PDB ID 4X8Y) was determined with heme bound[42]. Submission of the human, full length, amino acid sequence of PGRMC1 to the Protein Model Portal[66] produced models that used the structure of the *Arabidopsis thaliana* protein

At2g24940 or the truncated PGRMC1 as a backbone. The structure of At2g24940 (PDB ID 1T0G and 1J03) was solved by the NMR spectroscopy by two independent structural genomics groups[96, 97] and shown to possess a cytochrome b_5-like heme binding domain. Amino acid sequence comparison of At2g24940 with PGRMC1 shows these proteins are 25.2% homologous with an identity of 16.1%[98]. From all proposed structures, only those modeled by the four programs, RaptorX[67], ITASSER[71-74], IntFold2[68, 69], and Phyre2[70], were of the full length protein. These models along with the structure of the truncated PGRMC1 with heme bound are shown in Fig. 6. Of note is the long N-terminal, putative helical transmembrane domain which may span the mitochondrial membranes at inner and outer mitochondrial membrane junction points. This would allow FECH and a portion of PGRMC1 to reside in different compartments yet physically interact.

Decreased heme synthesis in the presence of PGRMC1 *inhibitor*

A structure-based screen identified several small molecule ligands to the *A. thaliana* protein At2g24940[57]. One of these small molecule ligands AG-205 has a reported K_d of 64 μM for At2g24940[57] and spectroscopic studies suggest that AG-205 alters heme binding to human PGRMC1[56]. In two different cancer cell lines, AG-205 has been shown to increase PGRMC1 protein levels and result in decreased viability in serum free media[56]. To investigate if AG-205 altered the differentiation profile of model erythropoietic cell lines undifferentiated and differentiated MEL and K562 cells grown in serum containing media were treated with AG-205. The inhibitor was added at time 0 at concentrations ranging from 0 to 10 μM and the level of hemoglobin per cell (pg/cell) were measured. While AG-205 had no effect on the undifferentiated cells, it resulted in a dose dependent decrease in the hemoglobin per cell in differentiated MEL

cells most pronounced at 72 hours (Fig. 7*A*). This decrease was partially rescued in induced MEL cells by overexpression of PGRMC1 (Fig. 7*A*). K562 cells exhibited the same sensitivity to AG-205 (Fig. S2).

Because heme synthesis during MEL cell differentiation is a biphasic process that requires heme[99], we investigated the effects of AG-205 when added after the onset of differentiation. AG-205 at 5 and 10 μM concentrations was added at time 0, 24 and 48 hours (Fig. 7*B*). The decrease in hemoglobin per cell was less when AG-205 was added at later time points. Additionally, we investigated the ability of hemin to rescue hemoglobinization in AG-205 treated cells. Hemin was able to rescue hemoglobinization at 5 μM concentrations of AG-205 (Figure *7C*).

Characterization of PGRMC1

To further characterize PGRMC1 and its interaction with FECH, we cloned, expressed and purified a his-tagged version of the full length wild-type human PGRMC1. Heme content of the purified wild-type PGRMC1 was determined by pyridine hemochromogen assay[76]. The extinction coefficient for the Soret band (~410 nm) of heme bound to PGRMC1 was calculated to be 144 mM^{-1} cm^{-1}. This Soret extinction coefficient is similar to that of DAP1 and the truncated mouse PGRMC1 previously described[23]. The average heme content of the as purified wild-type PGRMC1 was determined to be 18.5%$\pm$0.6, consistent with reported values[23, 24].

Heme transfer

PGRMC1 has been shown to bind both heme and progesterone and it has been suggested that PGRMC1 may function as a heme chaperone[24]. This function of PGRMC1 was initially

proposed from studies with DAP1[23], but later refuted based on the low of affinity of PGRMC1 for heme[38]. In light of the new findings we investigated the ability of PGRMC1 to transfer heme to an apo-hemoprotein. We incubated PGRMC1 with apo-cytochrome b_5 and then separated the protein on native PAGE and stained for heme as well as detected for protein. As controls we also included the bacterial heme-binding protein HasA in both the native and SDS-PAGE and FECH in the SDS-PAGE. Comparison of protein level and heme showed that heme can be transferred from PGRMC1 to apo-cytochrome b_5, while no transfer from HasA or FECH to apo-cytochrome b_5 was observed (Fig. 8 and Fig. S3). Our findings are consistent with recent data[24] and add support to the function of PGRMC1 as a heme donor for at least some hemoproteins.

In vitro activity of FECH *with* PGRMC1

To determine the effect of PGRMC1 on the *in vitro* enzyme activity of FECH, we performed assays of wild-type FECH in the presence of PGRMC1. The assay utilized is a direct assay and measures the disappearance of porphyrin substrate to determine heme production[77]. PGRMC1 alone did not bind porphyrin and showed no activity (Table 2). Inclusion of PGRMC1 in assays with FECH showed a dose dependent inhibition of FECH up to equimolar amounts. Beyond equimolar amounts, which resulted in a 40% decrease in FECH activity, no change in inhibition was observed (Fig. 9). In addition to wild-type FECH, we also assayed the F110A FECH variant in the presence of PGRMC1 at equimolar amounts. The activity of the F110A variant showed slightly higher inhibition than the WT enzyme. To rule out non-specific interactions between FECH and PGRMC1, we utilized several other proteins including uroporphyrinogen decarboxylase (UROD)[100] or augmenter of liver regeneration protein (ALR)[101] in the assay in equimolar amounts in the FECH assay. These proteins did not result in any significant loss of

activity (Table 2). These data suggest that PGRMC1 and FECH specifically interact and this interaction results in decreased FECH activity in vitro, possibly in a conformationally dependent manner.

Conformation of FECH *affects* PGRMC1 *interaction*

The structure of FECH has been well studied and the enzyme has been shown to exist in several distinct conformational states. These conformations are proposed to represent discrete steps in the catalytic cycle of the enzyme and would thus present distinct surfaces for protein-protein interactions[18-20]. Conformational states include the open conformation, the closed conformation with porphyrin bound, and the release conformation with heme bound[18, 20]. In order to determine if PGRMC1 interaction is specific to any one of these conformations, *in vitro* purification and affinity chromatography experiments using his-tagged FECH enzymes, both wild-type and variants, and the non-tagged PGRMC1 protein were carried out. Variants used represent two of the distinct conformation of FECH. The F110A variant is most stable in the product bound or release conformation in which the enzyme has heme bound and a partially unwound π helix[20]. The E343K variant adopts the closed conformation with the porphyrin substrate bound[18]. Results from these experiments showed that PGRMC1 interacts more tightly with the F110A variant of FECH than either the wild-type enzyme or the E343K variant (Figure 3*A*). Quantitation of multiple blots showed over twice as much PGRMC1 was present when the F110A variant was used (Figure 3*B*). This finding is consistent with PGRMC1 interacting with FECH in the release or product bound conformation, suggesting that PGRMC1 may regulate FECH activity by stabilizing a specific conformational state and regulating heme release from FECH.

Discussion

PGRMC1 and PGRMC2 are small hemoproteins whose cellular functions are not well defined. Both proteins are members of the membrane associated progesterone receptor family and are highly homologous[25]. Of the two proteins, PGRMC1 has been studied in greater detail and reported to be involved in a variety of cellular pathways, including cell proliferation, cholesterol synthesis and autophagy. Several published observations have likely contributed to the unclear function of PGRMC1 in cells, including its upregulation in some cancers, characterization of truncated, tagged and heterogeneous protein, and the lack of functional assays for many of its reported roles. Despite the smaller number of studies carried out on PGRMC2, it is also thought to play a role in cancer[102]. It is not known if PGRMC1 and PGRMC2 have overlapping functions in the cell; however we and others[103] have shown that PGRMC1 and PGRMC2 interact and form heterodimers or multimers.

One property of both PGRMC1 and PGRMC2 which has not been questioned is their ability to bind heme. Heme binding has been characterized through spectroscopic[23, 24, 104] and crystallographic studies[42] for mammalian PGRMC1. Multiple studies also characterized the yeast homolog, DAP1[23, 30, 38] and have shown that several of the proposed functions of PGRMC1 and DAP1 are heme dependent[24, 30, 31, 38, 104]. Spectroscopic studies have been carried out to characterize the heme binding pocket of human PGRMC1 and are consistent with heme binding via a conserved tyrosine residue in a high spin, five coordinate environment[24]. This type of environment is similar to several bacterial heme transport proteins including ShuT and HasA[43, 105]. Recent crystallographic data showed that the dimerization of a truncated form of PGRMC1, which lacks the transmembrane domain, occurs via their heme molecules which interact with each monomer on its surface[42]. This unique and weak binding of heme by

PGRMC1 has reinvigorated the proposal that PGRMC1 may function as a heme chaperone in cells[23, 31]. Our data that demonstrate a clear interaction between PGRMC1, PGRMC2, FECH and the mitochondrial heme metabolon add additional evidence to this proposed function.

Consistent with the proposal that PGRMC1, and possibly PGRMC2, function as heme chaperones are the findings that PGRMC1 can be observed in multiple cellular locations; something that would be expected for a heme chaperone that obtains heme from the mitochondrion and transports it to a variety of cellular locations for assembly into holo-hemoproteins. Previous studies have localized PGRMC1 to a variety of cellular compartments including the ER, nucleus, cytoplasm, cell membrane, and the mitochondria[106]. Several of these locations correspond to the proposed functions of PGRMC1. For example, PGRMC1 has been shown to stimulate several cytochrome P_{450}s which are found in the ER membrane[28, 29]. Another role reported for PGRMC1 is vesicle transport of the epidermal growth factor receptor. In these studies, PGRMC1 was cofractionated with EGFR to cytoplasmic vesicles[34]. In our studies, we have shown that PGRMC1 and PGRMC2 cofractionate and colocalize with the mitochondrial heme biosynthesis metabolon[11]. This pattern was observed in both undifferentiated and differentiated MEL cells. Further experiments to characterize the sub-mitochondrial localization were consistent with the majority of PGRMC1 being on the outside of the outer mitochondrial membrane. This localization would make it difficult if not impossible for the heme binding domain of PGRMC1 to interact with FECH to obtain heme and suggests interactions with a transmembrane protein for heme transport, such as ABCB10. Thus FECH and PGRMC1 interaction would take place at or near the N-terminus of PGRMC1 and upon heme binding PGRMC1 would either undergo proteolytic processing or significant conformational rearrangement for heme transport from the mitochondria

(Figure 10A). More detailed studies examining different regions of PGRMC1 are necessary to validate this model of interaction with FECH.

In addition to the possible role of PGRMC1 as a heme chaperone, several equally feasible roles for PGRMC1 in heme production exist that are based on previous data as well as those presented herein. First, as mentioned above, PGRMC1 has been implicated in vesicle transport[34, 107]. Our affinity purification data of PGRMC1 showed that transferrin receptor 1 was recovered from both the PGRMC1 and PGRMC2 experiments similar to what was found with other mitochondrial heme metabolon components. Previous work has proposed a novel delivery system for iron to the mitochondria during erythropoiesis via a kiss and run mechanism[108-110]. Considering the reported vesicle transport function of PGRMC1, it is possible that PGRMC1 could play a role in iron transport to the mitochondria (Figure 10C). If it is shown that PGRMC1 with heme bound is involved in iron transport, heme may serve as an intracellular second messenger to regulate iron homeostasis, which supports the work of Li et al.[36].

Second is the possible function of PGRMC1 as a heme sensor that regulates heme production via either i) stabilizing or destabilizing the mitochondrial heme metabolon (Figure 10D) or ii) directly regulating the activity of FECH (Figure 10B). Our findings clearly support a role for PGRMC1 in regulating heme biosynthesis and/or transport. Inclusion of PGRMC1 in FECH assays resulted in a dose dependent decrease in enzyme activity that saturates at about 60% of maximal FECH activity. We also demonstrated that this interaction is dependent on the conformation of FECH and is strongest when FECH is in the product bound/release conformation. This conformation specific interaction of FECH with a partner was previously hypothesized due to the distinct surfaces of FECH presented during its catalytic cycle[20]. Additionally in an erythroid cell culture model, we were also able to demonstrate that targeting of PGRMC1 with a

small molecule inhibitor decreases hemoglobinization in differentiated MEL cells. These data are consistent with PGRMC1 regulating heme synthesis via its interactions with FECH.

The models proposed herein for PGRMC1 function in heme synthesis are not exclusive and likely overlap in some fashion. For example, our findings demonstrating the ability of PGRMC1 to transfer heme to an apo-hemoprotein and to interact with the "release conformation" of FECH support a model in which PGRMC1 serves as both a heme chaperone and regulator of FECH. Overall our data are consistent with PGRMC1 being an essential component of the mitochondrial heme metabolon and playing a role in regulating heme synthesis in erythroid differentiation. While the precise roles of PGRMC1 and PGRMC2 need additional clarification, planned studies, including the production of transgenic animals and knock-out cell lines, will define the roles of these proteins in heme biosynthesis.

Figures and Tables

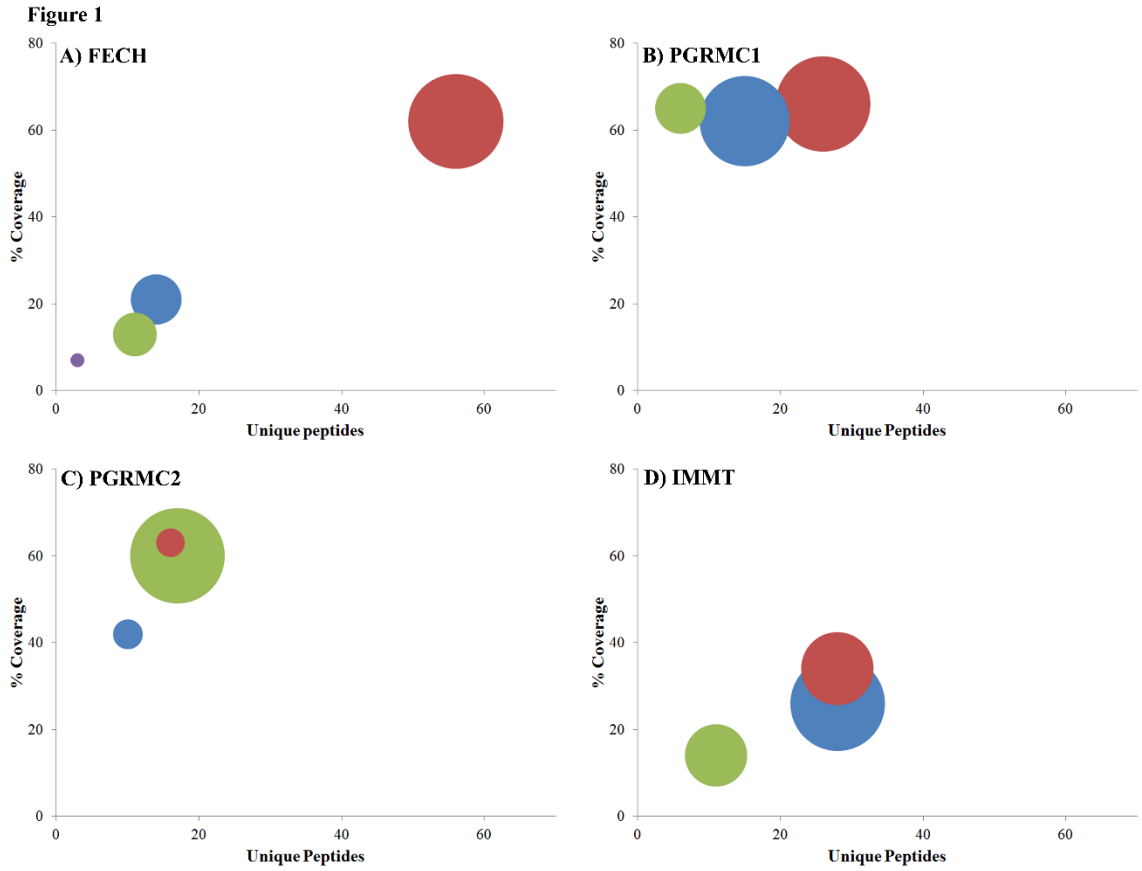

Figure 1. Graphical representation of affinity purification and MS analysis of FLAG-FECH (red), FLAG-PGRMC1 (blue) and FLAG- PGRMC2 (green) and FLAG-CPOX (purple, negative control). Each panel represents an identified mouse protein recovered with the bait human protein listed in the legend of panel A. Panels are as follows: A - FECH, B - PGRMC1, C - PGRMC2 and D - IMMT. Number of unique peptides (x axis), % coverage (y axis) and spectral counts (bubble size) for each was calculated using the maximal values obtained minus the maximal values observed in the control samples (empty vector). Size of bubbles represents the % of the total spectral counts identified for each mouse protein. The maximal spectral counts of each of the proteins was FECH=417, PGRMC1 = 115, PGRMC2 = 491 and IMMT = 59.

Figure 2

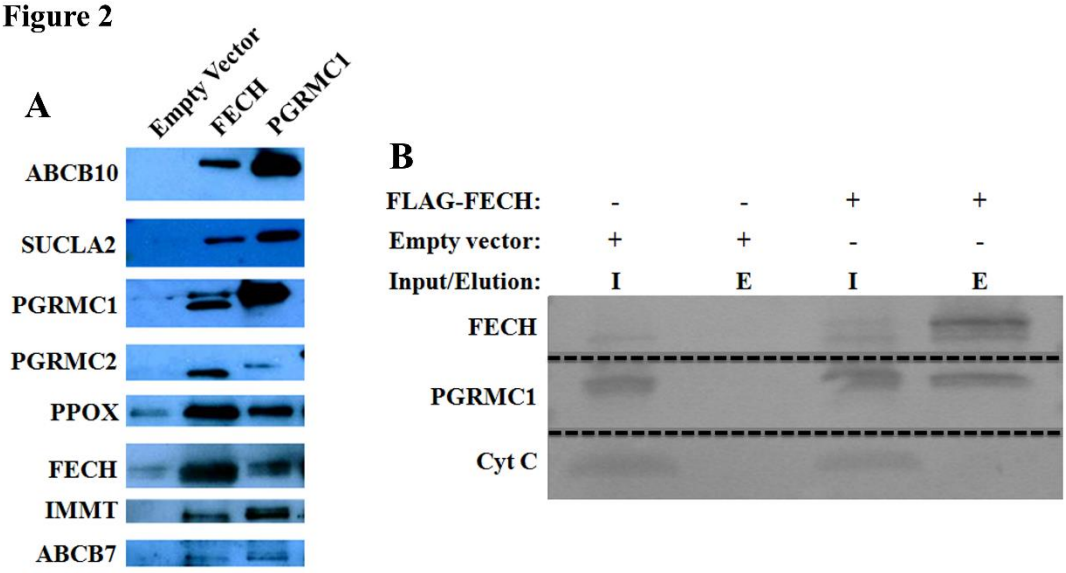

Figure 2. Immunoblot from AP of FLAG elutions. A, Each lane represents a representative blot from a FLAG elution from an affinity purification experiment from differentiated MEL cells using empty vector (lane 1), FLAG-FECH (lane 2) and FLAG-PGRMC1 (lane 3). Blots were probed for ABCB10, SUCLA2, PGRMC1, PGRMC2, PPOX, FECH, IMMT and ABCB7. B, AP from HEK293T cells using empty vector (lanes 1 and 2) and FLAG FECH (lanes 3 and 4). Blot was probed for PGRMC1. I is input and E is FLAG elution.

Figure 3

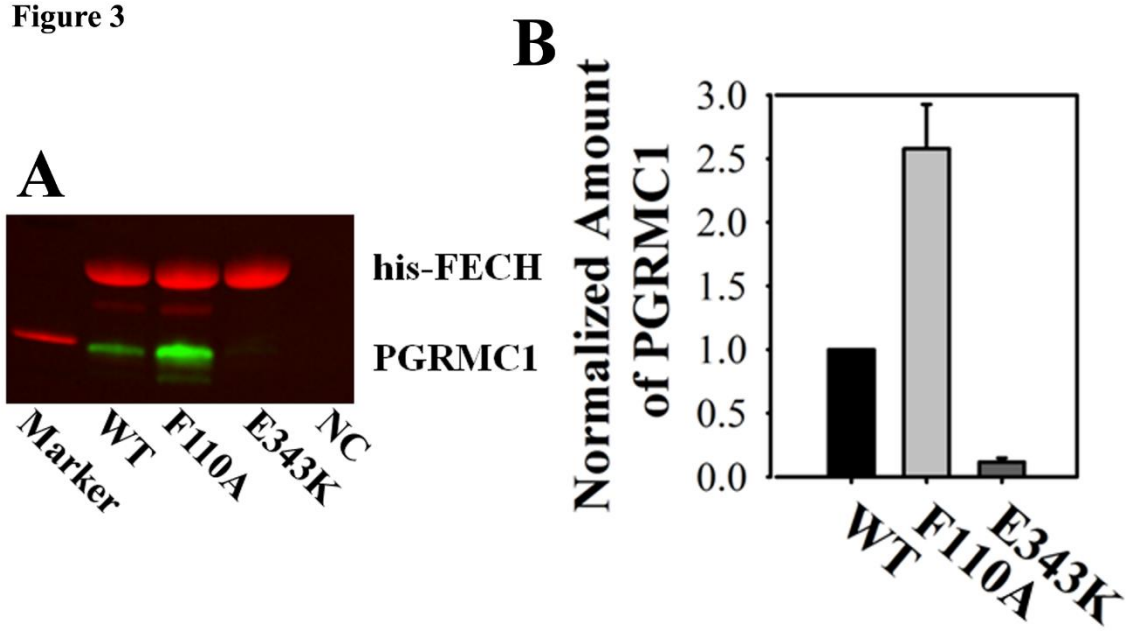

Figure 3. Analysis of WT FECH and FECH variant interactions with PGRMC1. A, Representative SDS-PAGE stain free gel visualization of FECH (red) and immunoblot analysis of non-tagged PGRMC1 (green) recovered from in vitro experiments. Protein standard is in lane 1, wild-type (WT) FECH is in lane 2, the F110A FECH variant in lane 3, the E343K FECH variant in lane 4 and the negative control (NC), only non-tagged PGRMC1 in lane 5. Experimental conditions are described in *Materials and Methods* section. FECH variants are described in Results section *Conformation of* FECH *affects* PGRMC1 *interaction.* B, Quantitation of PGRMC1 with wild-type (WT), F110A and E343K FECH from four biological replicates. P values for each variant with the wild-type and each other are < 0.0001.

Figure 4

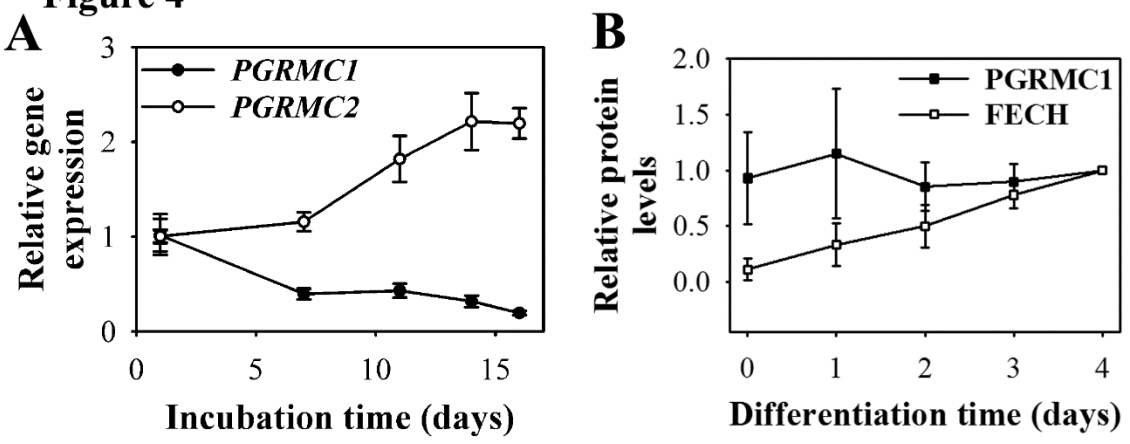

Figure 4. Gene expression of *Pgrmc1* and *Pgrmc2* and protein levels of PGRMC1 and FECH during differentiation in two model systems. A, Expression levels of PGRMC1 and PGRMC2 in an erythroid expansion model system relative to the controls GUSB. B, Protein levels of PGRMC1 and FECH in differentiating MEL cells normalized to day 4 levels.

Figure 5

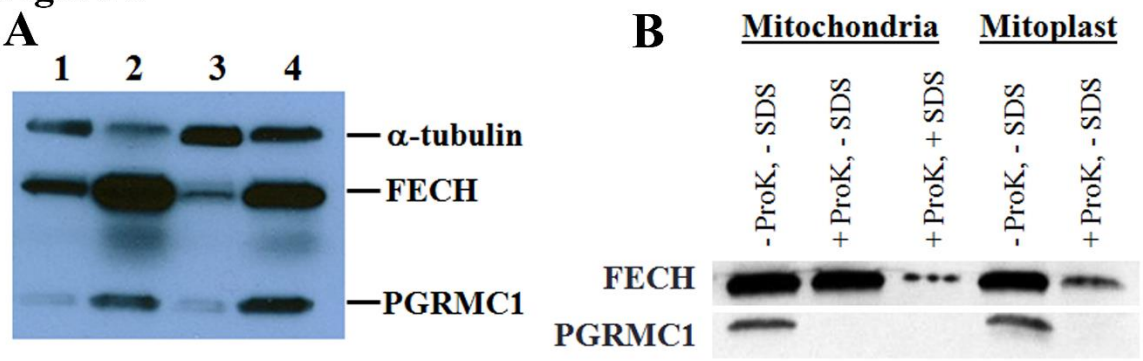

Figure 5. Cellular and subcellular localization of PGRMC1 and FECH in MEL cells. A, Cellular localization of PGRMC1 and FECH in undifferentiated and differentiated MEL cells. Representative immunoblot of FECH, PGRMC1 and α-Tubulin detected from MEL cellular fractions. Lane 1 is the cytosolic fractions and lane 2 mitochondrial fraction from differentiated MEL cells, while lane 3 is the cytosolic fractions and lane 4 mitochondrial fraction from undifferentiated MEL cells. 12.5 μg total protein was loaded in each lane. B, Submitochondrial localization of PGRMC1 was determined by mitoplast preparation and protease protection assay. Representative immunoblot of FECH and PGRMC1 from induced fractionated MEL cells.

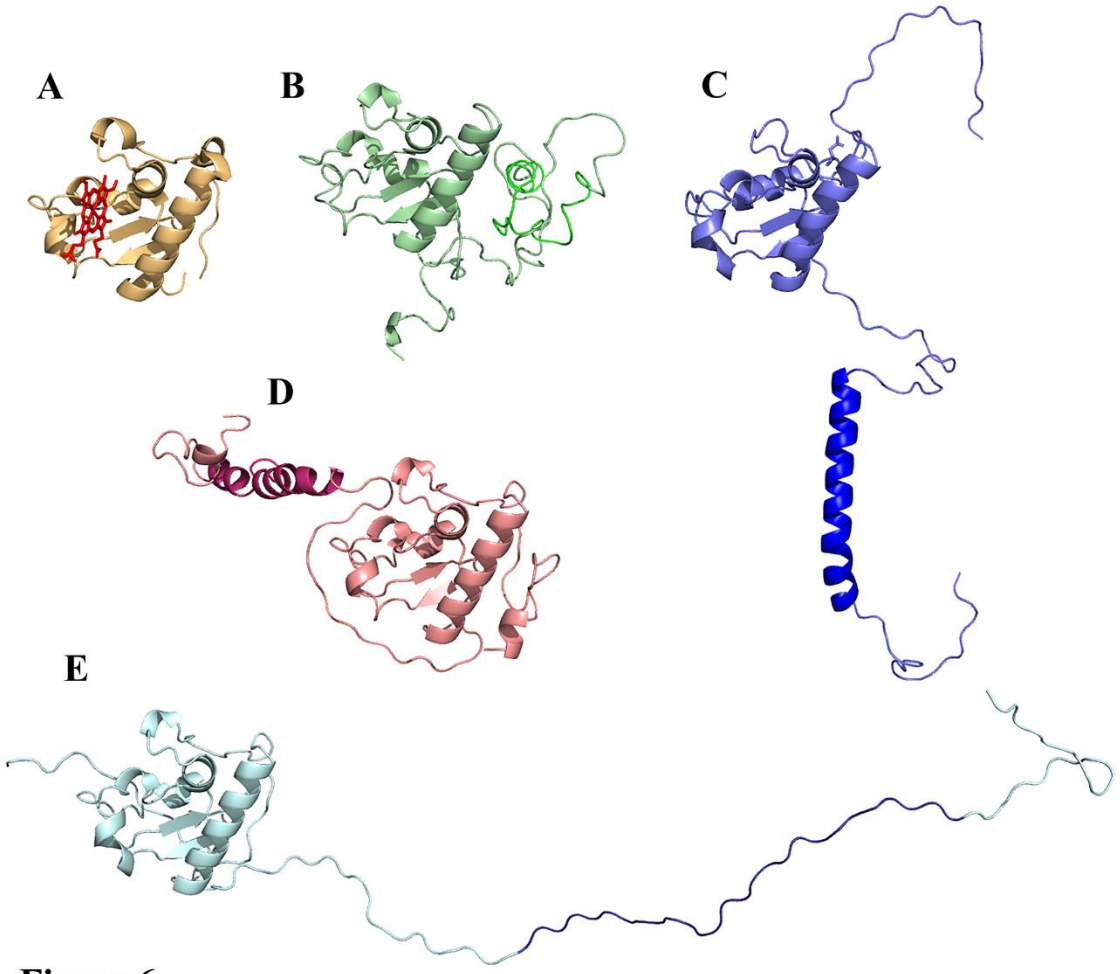

Figure 6

Figure 6. Structure of truncated PGRMC1 and models of full length PGRMC1. A, PGRMC1 (PDB ID 4X8Y) structure and models of PGRMC1 as generated by B, ITASSER[71-74], C, RaptorX[67], D, Phyre2[70] and E, IntFOLD2[68, 69]. Putative transmembrane domains in B thru E are shown with darker color and heme bound in PGRMC1 is shown as red sticks. All structure rendered using PyMol[75].

Figure 7

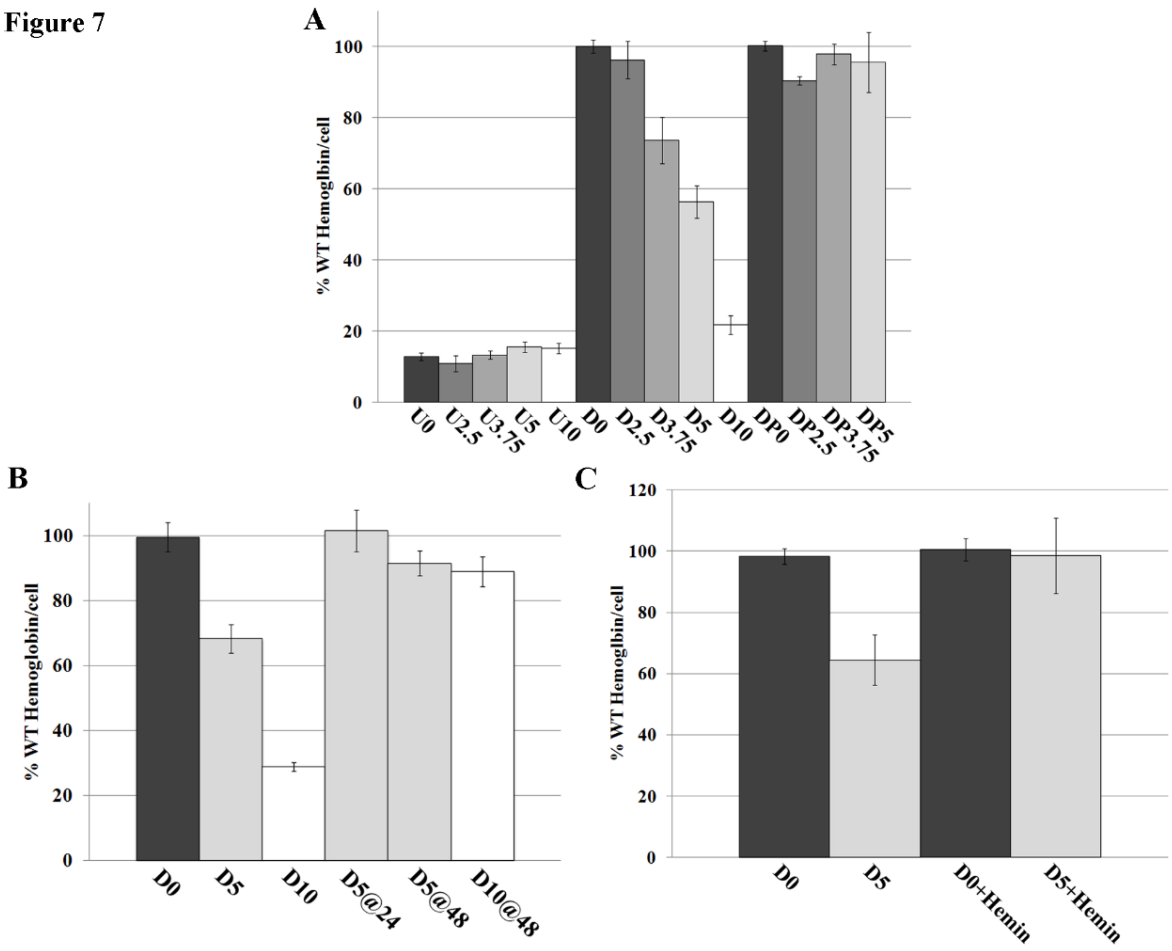

Figure 7. Inhibition of heme synthesis in MEL cells by AG-205. A, AG-205 inhibits heme synthesis in differentiating (D) MEL cells, but not undifferentiated (U) cells. Concentration of AG-205 were 0, 2.5, 3.75, 5 and 10 μM. Differentiated cell overexpressing PGRMC1 (DP) were treated with AG-205 at 2.5, 3.75, and 5 μM. For undifferentiated cells, $P< 0.01$ for U2.5 vs U5 and U2.5 vs U10. For differentiated cells, $P<0.01$ was found for all comparisons except D0 vs D2.5. For differentiated cells expressing PGRMC1, no statistically significant difference were found. B, AG-205 added after initiation of differentiation at either 24 (@24) or 48 (@48) hours has less of an effect at 5 and 10 μM than when added at the initiation of differentiation. $P< 0.01$ was found for D0 vs D5, D0 vs D10, D5 vs D5@24, D5 vs D5@48, D5 vs D10@48, D5 vs D10,

D10 vs D5@24, D10 vs D5@48 and D10 vs D10@48. $P<0.05$ was found for D0 vs D10@48 and D5@24 vs D10@48. C. Addition of 12.5 μM hemin results in rescue of hemoglobinization at 5 μM AG-205. % WT Hemoglobin (pg) per cell for cultures with hemin are normalized to D0 control with hemin added. $P< 0.01$ for D0 vs D5, D5 vs D0+Hemin and D5 vs D5+ Hemin.

Figure 8

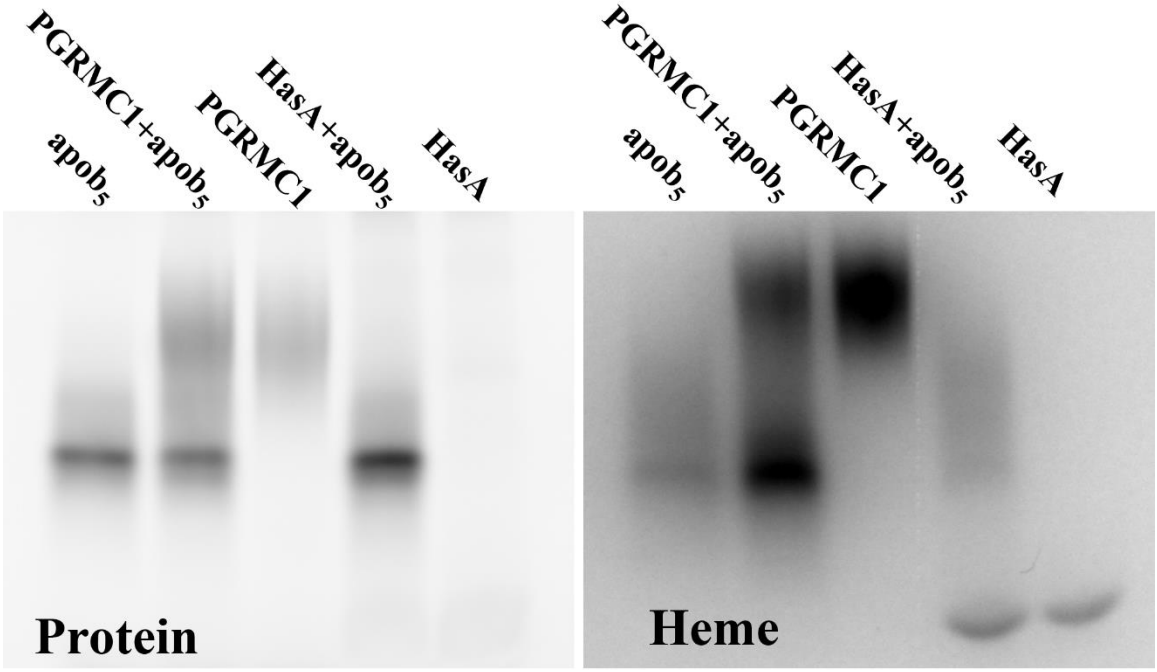

Figure 8. Heme Transfer to Apo cytochrome b_5. A, Native PAGE of proteins alone or in combination of apo-cytochrome b_5 ($apob_5$), PGRMC1 and HasA detected for protein (left) and heme (right).

Figure 9

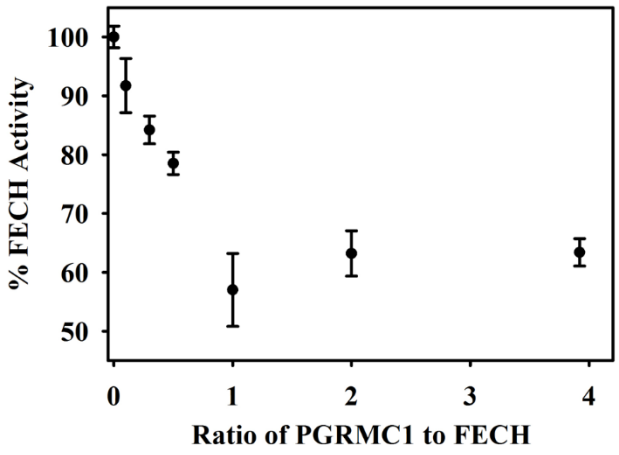

Figure 9. Effect of PGRMC1 on FECH activity. % FECH activity with different molar amounts of PGRMC1 is shown.

Figure 10

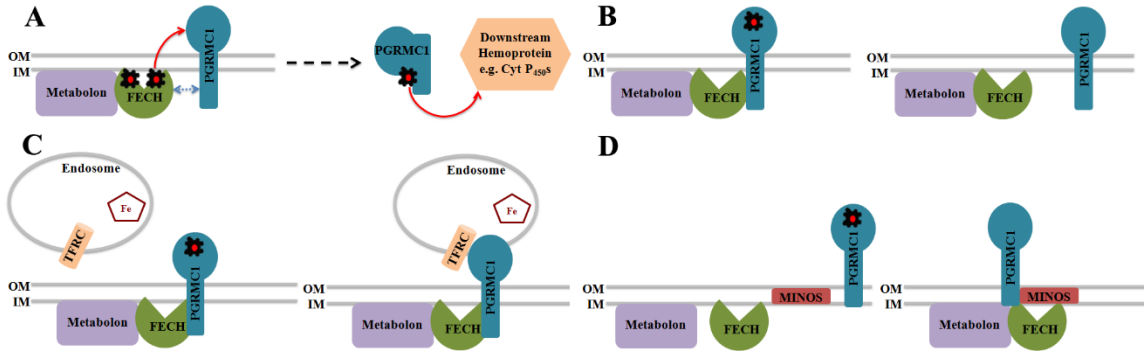

Figure 10. Models for Role of PGRMC1 in Heme Synthesis. A, PGRMC1 functions as a heme chaperone to deliver newly synthesized heme to hemoproteins in different cellular locations, B, PGRMC1 serves as a heme sensor which directly interacts with FECH and decreases FECH activity, C, PGRMC1 serves as a heme sensor to regulate endosomal trafficking of iron to the mitochondria for heme synthesis, D, PGRMC1 serves as a heme sensor to regulate the localization of the mitochondrial heme biosynthesis complex to inner and outer membrane junction points.

Table 1. Affinity Purification and MS Results of FECH, PGRMC1 and PGRMC2.

Murine Protein	Human Input			
	FECH (n=2)	**PGRMC1** (n=2)	**PGRMC2** (n=2)	**Empty Vector** (n=2)
FECH	**297-443[a]** **26-63** **46-79%**	**81-142** **12-21** **22-38%**	**72-115** **16-18** **26-30%**	**9-26[a]** **4-7** **13-17%**
PGRMC1	**39-115** **16-26** **52-66%**	**93-104** **11-15** **36-62%**	**57-65** **5-6** **27-33%**	**0** **0** **0%**
PGRMC2	**22-43** **10-16** **54-63%**	**34-49** **3-10** **16-42%**	**241-491** **15-17** **42-61%**	**0** **0** **0%**
IMMT	**12-40** **8-34** **13-45%**	**63-65** **34** **37%**	**30-31** **16-17** **21-25%**	**3-6** **2-6** **4-11%**
APOOL	**0-9**	**13-29**	**7**	**0**

	0-9 **0-43%**	**6-7** **25-29%**	**5** **21-22%**	**0** **0%**
OPA1	**0-28** **0-28** **0-37%**	**9-25** **6-17** **8-21%**	**3-9** **3-6** **5-9%**	**0** **0** **0%**

Data shown from top value to bottom as: spectral counts/unique peptides, percent sequence coverage.

[a]data from FECH-FECH and Empty vector-FECH interactions as previously reported[11].

Table 2. FECH Enzyme Activity. Equimolar amounts of WT and Variant FECH as well as control proteins were used.

Sample	**Activity (% + SD)**
FECH	**99 + 2.4**
FECH + PGRMC1	**57 + 6.2**
F110A FECH	**90 + 6.8**
F110A FECH + PGRMC1	**41 + 3.3**
FECH + UROD	**89 + 1.9**
FECH + ALR	**106 + 8.6**
PGRMC1	**3 + 1.3**

Supporting Information

TABLE S1. Affinity Purification and MS Results of PGRMC1 and PGRMC2.

Murine Protein	Human Input		
	PGRMC1 (n=2)	PGRMC2 (n=2)	Empty Vector [a] (n=2)
PPOX	11-28 4-9 11-24%	7-12 2-6 4-16%	2-7 2-3 7-9%
CPOX	0-9 0-6 0-14.2%	10-15 5-8 15-21%	5-10 4-7 12-23%
ALAS-2	0-2 0-2 0-4%	0-2 0-2 0-3.7%	0 0 0%
SUCLA2	11-44 6-21 13-40%	9 7-8 18-19%	0-2 0-2 0-7%
ABCB10	80-97 17-28 21-35%	35-36 14-15 19-21%	3-10 2-6 4-11%
ABCB7	106-190 16-23 12-21%	22-36 10 13-14%	0-4 0-3 0-6%

ADP/ATP translocase 1 slc25a4 (ANT1)	140-179 15-18	26-42 13-16	3-13 2-4
	24-37%	27-30%	6-7%
ADP/ATP translocase 2 slc25a5 (ANT2)	126-399 17-22 32-36%	56-92 15-16 27-30%	9-13 4-7 12-13%
voltage dependent anion selective protein channel 2 (VDAC2)	27-37 7-12 25-40%	21-25 9 30-33%	0 0 0%
2-oxoglutarate/malate carrier slc25a11 (OCG)	29-55 9 22-25%	23-32 5-5 15-18%	0-2 0-2 0-8%
transferrin receptor protein 1 (TFRC)	11-16 7-12 10-13%	19-26 11-12 13%	0-2 0-2 0-3%
Metalloreductase steap3 (STEAP3)	0-6 0-6 0-13%	0-3 0-3 0-6%	0 0 0%

Data shown from top value to bottom as: spectral counts/unique peptides, percent sequence coverage. [a] data Empty vector interactions as previously reported [1]

Table S2. Affinity purification and MS results of FECH, PGMRC1 and PGRMC2 with higher stringency wash.

Murine Output	Human Input		
	FECH	PGRMC1	PGRMC2
FECH	867 19 83%	113 24 70%	193 25 70%
PGRMC1	62 8 57%	150 17 59%	0 0 0%
PGRMC2	20 7 54%	0 0 0%	0 0 0%

Data shown from top value to bottom as: spectral counts/unique peptides, percent sequence coverage.

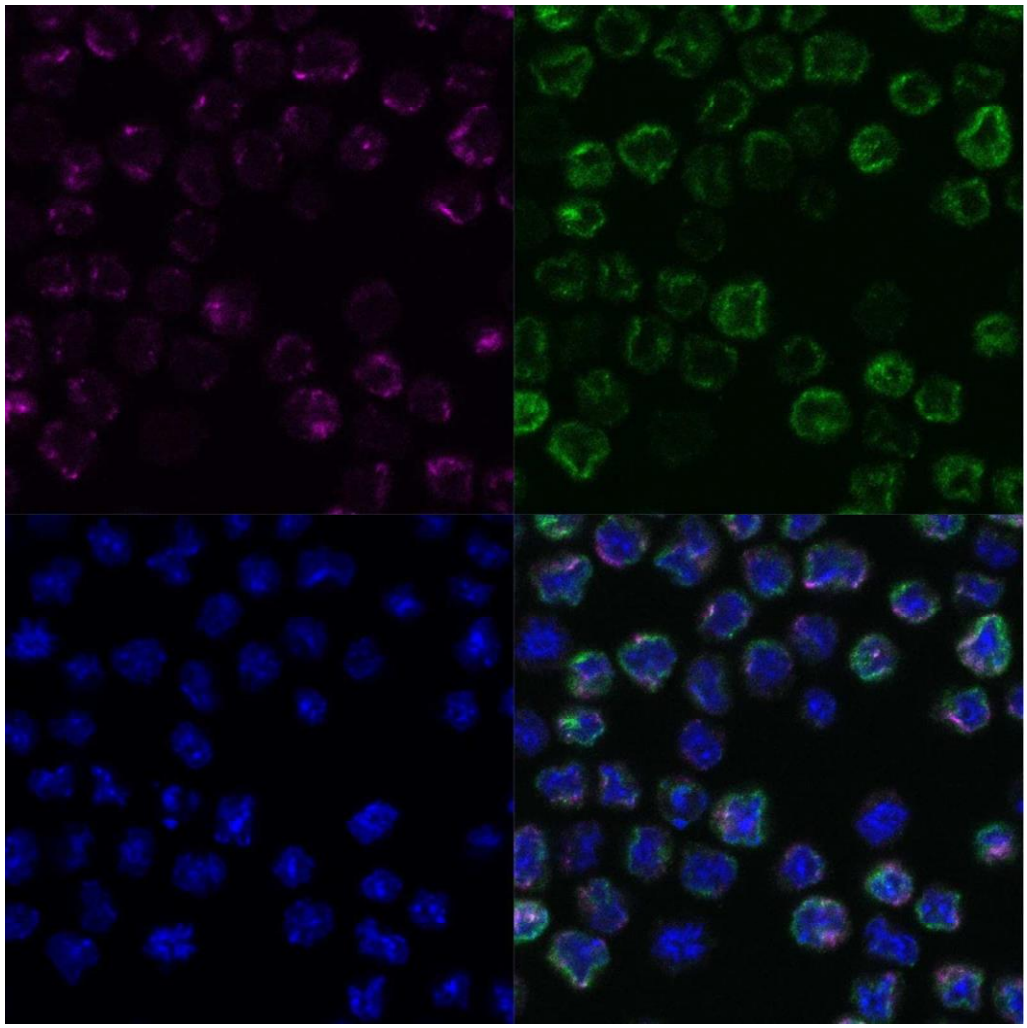

Figure S1. Mitochondrial localization of PGRMC1 in undifferentiated MEL cells. Top left, Detection of TIM23 with mouse Anti-TIM23 and Alexa Fluor 633 Anti-mouse, Top right, Detection of PGMRC1 with rabbit Anti-PGRMC1 and Alexa Fluor 488 Anti-rabbit, Bottom left, DAPI staining of cells, Bottom right, merged image showing some colocalization of TIM23 and PGRMC1 around cell nuclei. Magnification of cells 100x.

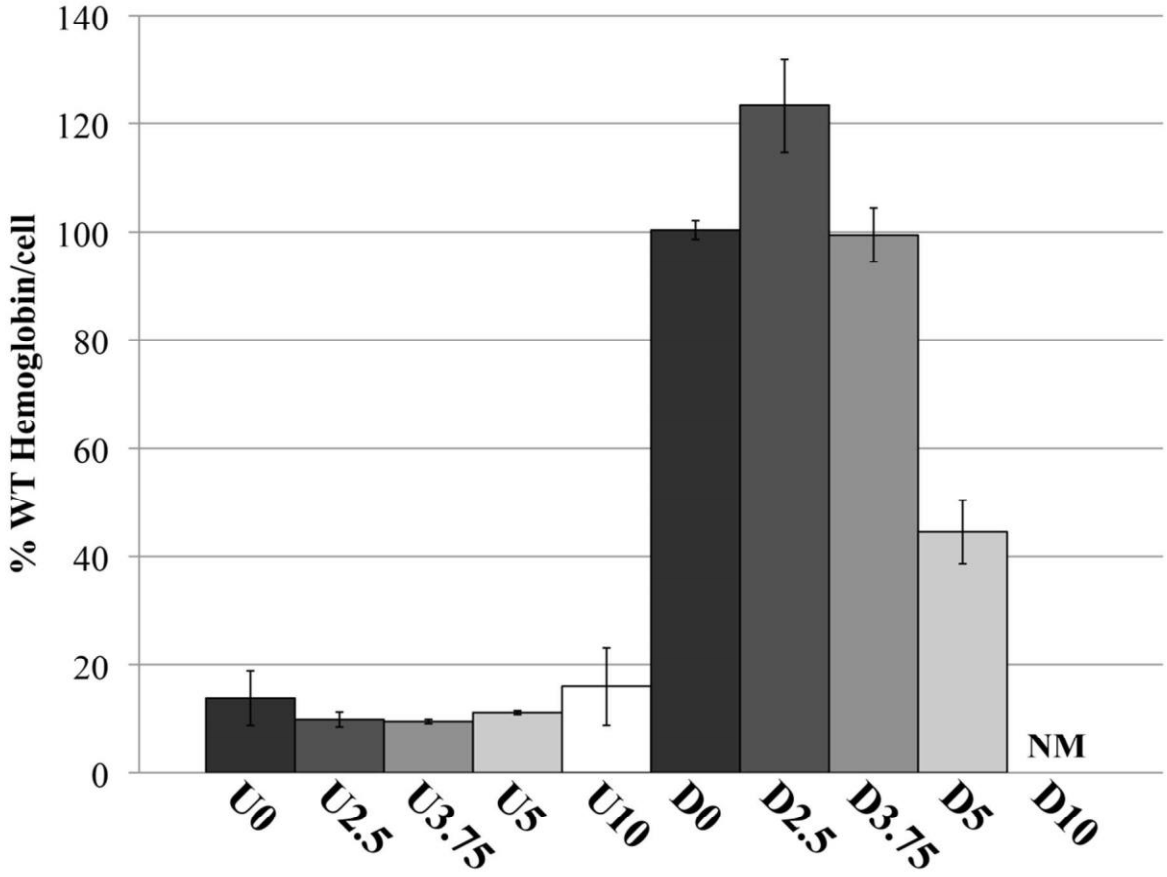

Figure S2. Inhibition of heme synthesis in K562 cells by AG-205. AG-205 inhibits heme synthesis in differentiating (D) K562 cells, but not undifferentiated (U) cells. Concentrations of AG-205 were 0, 2.5, 3.75, 5 and 10 μM. NM is not measurable.

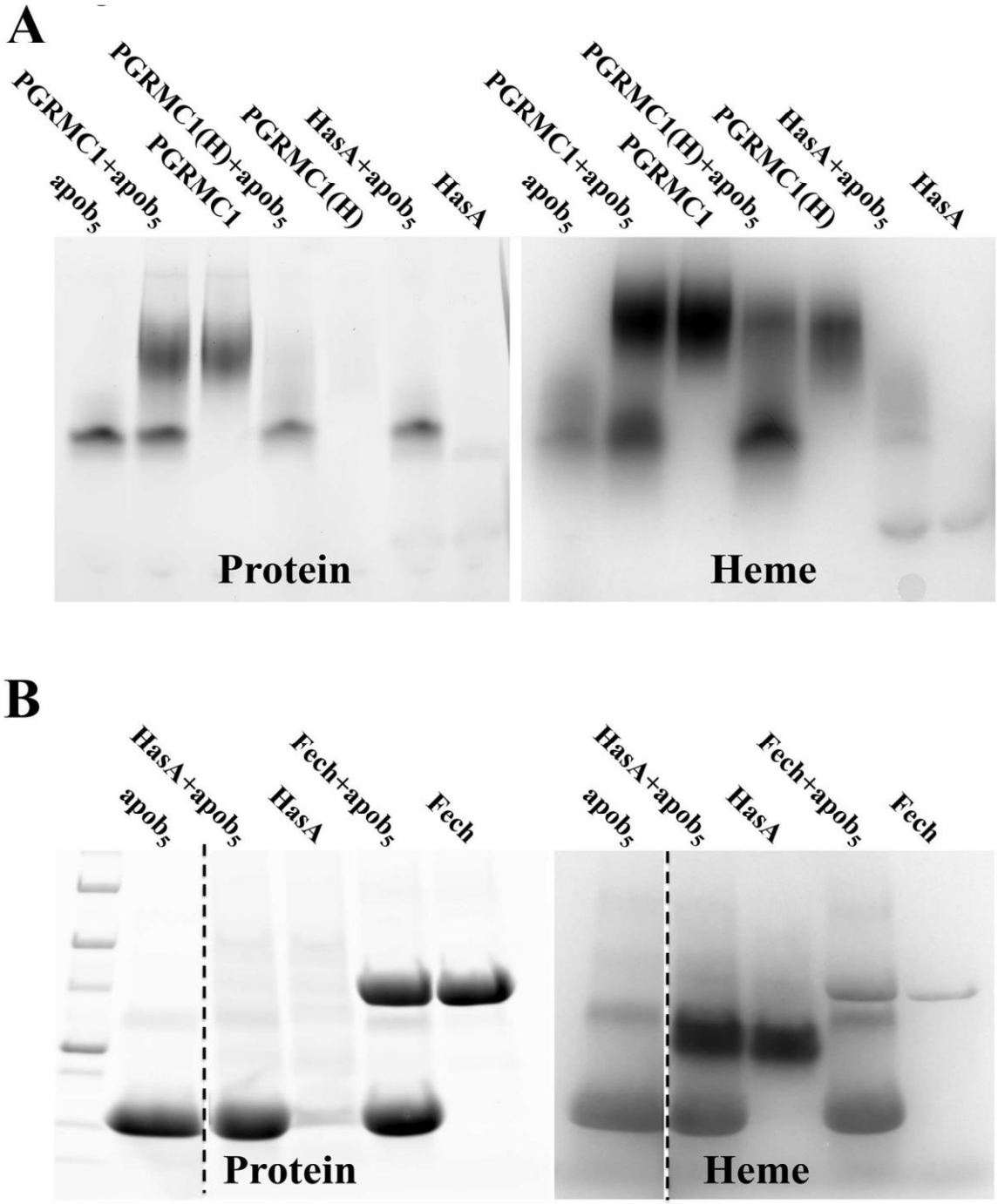

Figure S3. Heme transfer to apo-cytochrome b_5. A, Native PAGE of proteins alone or in combination of apo-cytochrome b_5 ($apob_5$), PGRMC1, PGRMC1 with heme added (PGRMC1H) and HasA detected for protein (left) and heme (right). B, SDS PAGE of protein combinations of apo-cytochrome b_5 ($apob_5$), HasA and FECH detected for protein (left) and heme (right).

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Abbreviations

FECH, ferrochelatase; PGRMC1, progesterone receptor membrane component 1; PGRMC2, progesterone receptor membrane component 2; MAPR, Membrane-Associated Progesterone Receptor; MudPIT, multidimensional protein identification technology; MS, mass spectrometry.

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CHAPTER 4

CREATION AND CHARACTERIZATION OF PGRMC1/2 NULL CELL LINES AND ZEBRAFISH

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Abstract

Progesterone receptor membrane component 1 (Pgrmc1) is a 25 kDa heme-binding protein with a plethora of putative functions. We have previously shown that Pgrmc1 interacts with the mitochondrial heme metabolon and that small molecule inhibition of the protein results in decreased heme synthesis in erythroid cell culture[1]. In this study, we use CRISPR-Cas9 mutagenesis to generate knockout models for Pgrmc1 and its homologue, Pgrmc2, in both human erythroid cell culture and zebrafish. We show that knockout of Pgrmc1 results in increased heme synthesis in cell culture. We also generate several non-heme-binding mutants of Pgrmc1 in order to determine the relevance of heme binding to the protein's function. Data presented herein support our previous proposal that Pgrmc1 functions as a regulator of heme synthesis. We also outline additional experiments to further elucidate the mechanism and function of Pgrmc1.

Introduction

Progesterone receptor membrane component 1 (Pgrmc1) is a 25 kDa protein consisting of an N-terminal transmembrane domain and a globular C-terminal domain which contains a cytochrome b5-like heme binding motif[2-4]. Mammalian Pgrmc1 and its *Saccharomyces cerevisiae* homologue, Dap1, have been linked to a myriad of cellular functions including progesterone binding[5, 6], intracellular signal transduction[7, 8], autophagy[9], endocytosis[10], gas sensing[11], and DNA damage protection from oxidative stress[10]. Both have also been proposed as regulators of iron metabolism[12], with Pgrmc1 influencing organismal iron homeostasis in mammals through regulation of hepcidin synthesis[13]. Pgrmc1 has also been ascribed a function in mediation of steroidogenesis and xenobiotic detoxification via interactions

with cytochromes P450, possibly through delivery of the heme cofactor[3, 4]. Our group recently showed that Pgrmc1 interacts with a number of enzymes in the heme synthesis metabolon[1]. The heme synthesis metabolon is a mitochondrial protein complex consisting of several heme synthesis pathway enzymes, various metabolite transporters and stabilizing proteins, as well as regulators of mitochondrial membrane structure[14]. Of particular note was the interaction of Pgrmc1 with ferrochelatase (Fech), the terminal enzyme of the heme synthesis pathway. The interaction between Fech and Pgrmc1 was shown to be dependent on the catalytic conformation of Fech, and to regulate Fech activity[1].

With the diverse proposed functions for Pgrmc1, perhaps the only one to be universally agreed upon is its ability to bind heme[4, 6, 8, 15]. Recently, crystal structures were solved for the cytosolic domain of human PGRMC1, revealing that the protein forms a homodimer, where dimerization occurred through the direct stacking of heme bound to each monomer via a tyrosine residue at position 113. Kabe et al.[4] also showed that several interactions between PGRMC1 and other proteins, as well as the downstream functions of the interacting proteins, were dependent on this heme-mediated dimerization of Pgrmc1. These studies centered around PGRMC1's interactions with several cytochromes P450 and the signaling protein EGFR[4]. However, the precise role of heme binding in Pgrmc1 and the mechanism by which it influences protein interaction partners and their functions remains unclear at present. One proposed role of Pgrmc1 is as a heme chaperone[6, 8], where Pgrmc1 could accept heme from Fech and deliver it to other hemoproteins in the cell. This function is supported by 1) the interaction of Pgrmc1 and Fech 2) the many reported subcellular localizations of Pgrmc1 and 3) Pgrmc1's relatively moderate affinity for heme[8, 15] when compared to other hemoproteins. Another potential function of Pgrmc1 is that of a heme sensor, where the moderate affinity of the protein for heme

would allow heme binding and subsequent signal transduction to occur once heme concentrations reach a certain threshold. The multiple subcellular localizations and interaction partners of Pgrmc1 are consistent with this model.

Pgrmc2, a paralogue of Pgrmc1, was also found to interact with members of the heme synthesis metabolon, including Pgrmc1[1]. Pgrmc2 and Pgrmc1 share 49% homology[16]. Like Pgrmc1, Pgrmc2 has been implicated in both heme and progesterone binding[2, 17, 18]. Pgrmc2 has also been suggested to promote adipogenesis through heme mediated signaling with the transcription factor Rev-erbα[16]. Currently, it is unclear to what degree the function of Pgrmc2 overlaps with that of Pgrmc1.

Herein we further characterize the function of Pgrmc1 and its ability to bind heme with respect to regulation of heme synthesis and intracellular trafficking. We report CRISPR-Cas9 mediated knockout of Pgrmc1 in both mammalian tissue culture cells and zebrafish models. These models were analyzed for perturbations in growth, heme synthesis, and accumulation of porphyrins. We also present work on studies centered around knockout of Pgrmc2. These studies should help to elucidate the roles of Pgrmc1 and Pgrmc2, as well as any potential functional overlap or cooperation between the two paralogues. Additionally, non-heme-binding mutants of PGRMC1 were created and characterized. These will be used in future studies designed to query the importance of heme binding in the physiological function of Pgrmc1 and for its interactions with other proteins.

Results

Knockout of Pgrmc1 in an erythroid cell culture model results in increased hemoglobinization

In a previous study, our group showed that treatment of mammalian erythroid cell culture models with AG-205, a small molecule inhibitor of Pgrmc1[19, 20], resulted in decreased hemoglobinization in differentiated cells in a dose dependent manner[1]. This decrease in hemoglobin in differentiated cells could be partially rescued by overexpression of exogenous Pgrmc1. The hemoglobinization defect was not observed in AG-205 treated undifferentiated cells.[1]. While a phenotype was observed in differentiated cells, the exact nature of the interaction between AG-205 and Pgrmc1 remains unclear. AG-205 has been suggested to displace heme from Pgrmc1[19, 20]. However, the direct effect of AG-205 on Pgrmc1 function has not been demonstrated. It is also unclear to what degree AG-205 influences Pgrmc2 and what contribution this may have to the observed hemoglobinization phenotype. In an effort to continue characterization of Pgrmc1 function, we created a knockout of *Pgrmc1* in K-562 human myelogenous leukemia (K562) cells using CRISPR-Cas9 genome editing. This strategy allows for specific perturbation of Pgrmc1, without the potential pleotropic effects of AG-205 treatment. The knockout was performed using two target sites simultaneously (Figure 1a) and consists of an approximately 110 bp deletion resulting in frame shift and ablation of protein expression (Figure 1b,c). Pgrmc1 null cells showed approximately 5-fold increased hemoglobinization, relative to wild type, in undifferentiated cells and an approximately 2-fold increase in differentiated cells (Figure 1d). K562 cells lacking Pgrmc1 also exhibit slowed growth compared to wild type cells (Figure 1e).

Hemoglobinization in these cells is determined via spectroscopy through an increase in absorbance at 410-415 nm. In differentiated wild type erythroid models, the bulk of absorbance

in this wavelength range is assumed to correspond to heme bound to hemoglobin. However, free heme, heme bound to other proteins, and unmetallated porphyrins also absorb in this range. Knockout cells were further examined to determine more precisely what molecular species were altered in these cells. Preliminary studies measuring heme, protoporphyrin IX (PPIX), and total porphyrins in undifferentiated Pgrmc1 null cells were performed via ultra-performance liquid chromatography. In these studies, heme levels increased by 59%, PPIX increased 24%, and total porphyrins increased 44% compared to wild type cells (Table 1). Further measurements will be performed to validate these initial findings and to determine changes in differentiated cells.

Knockout of Pgrmc1 in Zebrafish

In parallel to knockout studies in mammalian cell culture, we have generated a zebrafish line lacking Pgrmc1. This knockout was generated with the use of CRISPR-Cas9 and two simultaneous target sites. Targeting of the gene with both guides results in a ~1200 bp deletion spanning a region containing the end of exon 1, the entirety of intron 1, and the start of exon 2 (Figure 2a,b). In initial screens, several injected fish were found to contain the deletion. These fish were outcrossed to wild type fish in order to create stable knockout lines. At present, two female injected fish containing the excision mutation, as identified by PCR, have been successfully outcrossed and offspring are being raised to establish stable mutant lines. Each mutant line will be validated via DNA sequencing. Data from preliminary in-crosses of injected (F0 generation) fish containing a disruption in a single allele of *Pgrmc1* show morphological and behavioral defects in approximately 24% of offspring (Figure 2c,d). These defects include abnormal spinal curvature, craniofacial defects, and decreased response to physical stimuli.

However, further characterization in stable, out-crossed lines is required as it is possible these phenotypes could arise from off-target effects.

Knockout of Pgrmc2 in human cell culture and zebrafish

In an attempt to determine Pgrmc2 function with respect to heme metabolism, CRISPR-Cas9 mutagenesis constructs targeting *Pgrmc2* have been designed and cloned for use in both human cell culture and zebrafish. For human cells, the *Pgrmc2* targeted CRISPR-Cas9 mutagenesis constructs correspond to two sites found in exon 1, spanning 304 bp between them (Figure 3a). These constructs are complete and have been verified via DNA sequencing. In zebrafish, CRISPR-Cas9 mutagenesis was performed, targeting sites in exon 1 and 2. This strategy was designed with the intent to excise a 6720 bp section containing part of exon 1, all of intron 2, and part of exon2 (Figure 3b). Screening of embryos from batch mating of injected fish has shown evidence of mutations (Figure 3c), however, thus far no founders have been isolated. Screening in these fish has proved difficult due to an increased mortality rate in fish injected with *Pgrmc2* targeted constructs compared to wild type or those injected with constructs targeting *Pgrmc1*.

Mutations at Tyrosine 113 of human Pgrmc1 result in altered heme affinity

Heme binding in human PGRMC1 occurs via a tyrosine residue at position 113[4]. A Y113F mutant has been previously characterized as unable to bind heme. The same group performed shRNA knockdown of PGRMC1, which resulted in dysfunction of Pgrmc1 interaction partners including EGFR and several cytochromes P450. While addition of wild type PGRMC1 was able to rescue these phenotypes, addition with the Y113F mutant was not able to do so[4].

We have expressed and purified several PGRMC1 mutants with substitutions of alanine, cysteine, histidine, and phenylalanine at the Y113 position (Figure 4a). In our own characterization of these mutants, Y113F retained minimal heme binding activity as measured by UV-vis spectroscopy and *o*-dianisidine staining (Figure 4b,c). The Y113A, C, and H mutants showed no ability to bind heme, though these mutants showed reduced expression and/or stability compared to the previously characterized Y113F (Figure 4b-d). Of these mutants, Y113A did not show degradation and was expressed at a level most similar to Y113F (Figure 4a). Therefore, in future experiments, we will utilize Y113A as a non-heme binding mutant, as well as Y113F as a reduced affinity mutant.

Discussion and Future Directions

In a previous study, we presented evidence for the interaction of the heme-binding protein Pgrmc1 with the mitochondrial heme metabolon[1]. This membrane-associated complex consists of proteins ranging from heme synthesis pathway enzymes and associated transporters to proteins involved in regulation of mitochondrial cristae morphology and mitochondrial dynamics[1, 21]. Pgrmc1 has been proposed to perform several, possibly overlapping functions with regard to heme metabolism, including chaperone, sensor, and regulator of heme synthesis through contacts with the mitochondrial heme metabolon[1, 14]. Pgrmc1 has also been shown to interact with other proteins outside the metabolon such as cytochromes P450, where it has been proposed to deliver heme to these proteins for use as a cofactor[3, 4]. In support of Pgrmc1's putative delivery of heme from Fech in the matrix to cytochromes P450 in the ER, Pgrmc1 was recently identified in a screen for proteins which are enriched at ER-mitochondrial contact sites[22].

In an attempt to clarify the role of Pgrmc1 in heme metabolism, we initially treated mammalian erythroid cell models with a purported inhibitor of Pgrmc1, AG-205[19, 20]. Administration of AG-205 resulted in decreased hemoglobinization of these cells upon differentiation[1]. This was an intriguing result, as this was the first time Pgrmc1 had been directly implicated in the regulation of heme synthesis. However, this method of characterizing Pgrmc1 is not ideal for several reasons. First, the function of Pgrmc1 is currently unknown; therefore, it may be premature to claim AG-205 is an inhibitor. What is known is that AG-205 appears to alter binding of heme with Pgrmc1, as evidenced by a shift in the protein's absorbance spectrum at ~400 nm following AG-205 exposure[20]. Additionally, it is reasonable to suggest that AG-205 may also affect Pgrmc2, as the paralogue shares the ability to bind heme[2, 17, 18]. Finally, the specificity of AG-205 with respect to off-target effects on other cellular components has not been fully characterized.

In order to more directly examine the function of Pgrmc1, we created a knockout of the gene in erythroid cell culture. In *Pgrmc1*$^{-/-}$ K562 cells, hemoglobinization is increased compared to wild type in both undifferentiated and differentiated cells. This phenotype stands in contrast to the decreased hemoglobinization observed in differentiated cells alone following AG-205 treatment. This suggests either a wider range of targets for AG-205, or a more complex mechanism for Pgrmc1 function where the apparent perturbation of heme binding results in a different effect on the cell compared to the absence of the protein. A recent study showed that interactions between Pgrmc1 and some of its protein partners, including several cytochromes P450, were dependent on dimerization of Pgrmc1. Additionally, this dimerization was found to be heme-dependent and to occur through heme-heme stacking between monomers. Furthermore, the group also presented evidence that interaction with dimerized holo-Pgrmc1 was necessary for

proper function of these interacting proteins[4]. The group also showed that this dimerization, as well as the downstream function of the described interaction partners, is disrupted in the presence of CO, which binds to heme in the holo-monomer and prevents stacking[4]. Thus it is possible AG-205 affects dimerization and results in decreased heme synthesis, while lack of the protein causes increased heme synthesis.

To explore the role of Pgrmc1 *in vivo,* we have also generated *Pgrmc1* knockouts in zebrafish. In preliminary examinations, roughly one-in-four embryos from an in-cross of individual *Pgrmc1*$^{+/-}$ (F0 generation) fish show morphological and behavioral defects. No gross erythropoietic phenotypes were observed, however explicit testing for these phenotypes has yet to be conducted. We caution that this is a preliminary result that requires further investigation. Fish in the F0 generation contain *de novo* mutations and therefore individual fish are likely to have slightly different mutations from one another, both at the target site or elsewhere in the genome, which can complicate phenotype characterization. It is necessary to out-cross injected lines for several generations in order to control for any potential off-target effects arising from mutagenesis. Once stable *Pgrmc1* knockout lines have been established containing a single excision mutation, ablation of protein expression will be confirmed via western blot and characterization can begin in earnest. *Pgrmc1* null zebrafish will be examined for perturbations of erythropoiesis or heme synthesis, as well as for morphological and developmental defects.

In continuation of the work presented herein, we will perform a series of experiments utilizing a combination of *Pgrmc1* knockout, non-heme-binding mutants, CO exposure, and AG-205 treatment to further elucidate the role of Pgrmc1 in the regulation of heme synthesis. The first of these experiments will be the attempted rescue of *Pgrmc1*$^{-/-}$ K562 cells and zebrafish via expression of exogenous Pgrmc1 in order to confirm the specificity of the observed knockout

phenotypes. As mentioned previously, heme binding is the one universally agreed upon activity for Pgrmc1. In an attempt to determine its effect on heme metabolism, we will also attempt rescue of *Pgrmc1*$^{-/-}$ K562 cells and zebrafish with non-heme-binding (Y113A, Y113F) PGRMC1 variants. Based on the reported necessity of heme binding for dimerization and protein-protein interactions in Pgrmc1, we expect to see phenotypic differences between models rescued with wild type and variant PGRMC1. Currently, it remains unclear whether mediation of dimerization and subsequent protein-protein interaction is the only function of heme binding in Pgrmc1. In an attempt to disentangle heme binding alone from heme mediated dimerization, we will treat wild type K562 cells with CO via addition of CO-releasing molecules (CORMs) to the growth media. Since CO inhibits dimerization, but not heme binding, if CORM treatment results in phenotypes distinct from those in cells expressing Y113 mutant PGRMC1, it will suggest a role for heme binding in Pgrmc1 independent of dimerization. To control for off-target effects of CO treatment, we will also treat cells expressing Y113 mutant PGRMC1 with CO. Since the mutant PGRMC1 cannot bind heme, CO should not affect Pgrmc1 function in these cells. We will also attempt similar studies using AG-205 in place of CO.

As discussed above, Pgrmc2 is a sparsely characterized homologue of Pgrmc1. Both proteins bind heme and were found to interact with members of the heme synthesis metabolon, including one another[1]. Given the homology of the proteins and the similarities of the few known characteristics of Pgrmc2 to those of Pgrmc1, it seems likely that the two proteins may have related functions. In an attempt to better understand the protein, Pgrmc2 knockout and rescue experiments, as well as characterization of potential downstream phenotypes, will be carried out mirroring experiments for Pgrmc1. While CRISPR-Cas9 injections targeting *Pgrmc2* have been carried out in zebrafish, and batch matings of injected fish show evidence of

mutagenesis, isolation of founders has proved difficult due to high mortality rates. It is unclear at present whether the increased mortality is due to loss of Pgrmc2 function. These constructs target a large section of the gene for excision, measuring 6720 bp and including the entirety of the 6372 bp intron 1. It is possible that the loss of untranslated elements in this intron is responsible for the increased mortality observed in these fish. Screening efforts of previously injected fish are ongoing and alternative CRISPR gRNA targets will be designed with the aim of creating an excision that lies within a single exon, in order to mitigate any potential off target effects resulting from the loss of intron 1.

Given the homology between the two proteins[2, 17, 18], it is reasonable to suggest that Pgrmc1 and Pgrmc2 may overlap to some degree in their functions. It is possible the proteins carry out similar functions but in different cell types, with unique protein partners, in separate subcellular compartments, or at differing stages of differentiation. Pending successful CRISPR-Cas9 mediated knockout of *Pgrmc2*, cell culture and zebrafish models will be created in which both *Pgrmc1* and *Pgrmc2* have been knocked out. These tandem knockout models will be analyzed as described above for Pgrmc1 and monitored for any phenotypes that do not arise from the knockout of either of the individual proteins. Additionally, experiments will be carried out where exogenous addition of Pgrmc2 will be used in an attempt to rescue phenotypes resulting from knockout of *Pgrmc1* and vice versa. These experiments should elucidate whether shared functionality exists between the two homologues.

Conclusion

Herein we describe experiments intended to decipher the functions of Pgrmc1 and Pgrmc2 with respect to heme synthesis. Both proteins are widely expressed, relatively poorly characterized, and implicated in a wide array of phenotypes in both normal and disease states. It is our hope that the creation of *Pgrmc1* and *Pgrmc2* null cell lines and organisms will foster greater understanding of these proteins, their role in regulating heme synthesis, and the heme synthesis metabolon in general. As mentioned previously, Pgrmc1 and Pgrmc2 have each been assigned a large number of putative functions in the literature, these range from regulation of drug sensitivity in cancer[23, 24] to roles in diseases of the female reproductive system[25]. We seek to unravel which, if any, of the various putative functions of these proteins may in fact be downstream consequences of their roles in the regulation of heme synthesis and/or distribution.

Methods

Tissue culture

Human erythroid tissue culture experiments were conducted in K-562 human myelogenous leukemia (K562) cells (ATCC – CCL243)[26, 27]. Cells were cultured in DMEM with 25 mM glucose, 1 mM sodium pyruvate and 4 mM glutamine (Cellgro) plus 10% FBS (Atlanta Biologicals,Flowery Branch, GA) and 1% penicillin/streptomycin (Cellgro). For induction of K562 cells 1 mM sodium butyrate was included in growth media and cells were grown 6 days[28, 29].

CRISPR-Cas9 mutagenesis in K562 cells utilized PX458 (Addgene, Plasmid #48138) a vector expressing all parts of CRISPR-Cas9 machinery and a GFP reporter gene[30]. A pair of oligonucleotides (Sigma) corresponding to each gRNA target site (Figure 1a, 4a) and its reverse complement were annealed and cloned into PX458 using BbsI(NEB)[30]. A single completed plasmid was transfected into K562 cells for each desired target site (Figure 1a, 4a), with two plasmids being transfected simultaneously for generation of excision mutants. Transfection of plasmids into K562 cells was accomplished using X-tremeGENE HP DNA Transfection Reagent (Sigma) according to manufacturer's instructions at a ratio of 1 μg plasmid DNA to 1 μL reagent. Single cells expressing PX458 constructs were identified and isolated at the University of Georgia CTEGD Cytometry Shared Resource Lab using the MoFlo XDP (Beckman Coulter, Hialeah, Florida) cell sorter screening for GFP fluorescence.

Successful excision mutation of human *Pgrmc1* in K562 cells was verified using PCR (Figure 1a,b). Knockout was further verified via Sanger sequencing (Eton Bioscience Inc, San

Diego CA)(Figure 1a). Ablation of PGRMC1 protein expression was verified via immunoblot of cell lysate as previously described[1].

Hemoglobin (Hb) content of intact K562 cells was determined using an Olis CLARiTY Spectrophotometer (Olis, Bogart, GA) as previously described[31]. Cell counts were taken using Scepter handheld automated cell counter (Millipore, Billerica, MA) using the 60 μm tip. Heme, protoporphyrin IX, and total porphyrin levels in K562 cells were measured via ultra-performance liquid chromatography as previously described[14, 32].

Zebrafish

Experiments utilizing zebrafish were performed in accordance with an University of Georgia IACUC approved protocol. TL zebrafish (Zebrafish International Resource Center, Eugene, OR) were utilized as wild type in these experiments. CRISPR knockouts in zebrafish (Figure 2a, 3b) were performed by the University of Utah Health Sciences Mutation Generation and Detection core facility. Injected fish were screened by PCR (Figure 2a,b, 3b,c) using genomic DNA extracted from F1 generation embryos from either batch mating of injected fish or out-cross of individual injected fish to wild type fish. Genomic DNA template for PCR was prepared by overnight digestion of zebrafish tissue in a buffer containing 10 mM Tris HCl pH 8, 1 mM EDTA, 50 mM KCl, 0.3 %(v/v) Tween 20, and 2 mg/mL Proteinase K followed by 1:20 dilution in ultra-pure water.

In vitro protein expression

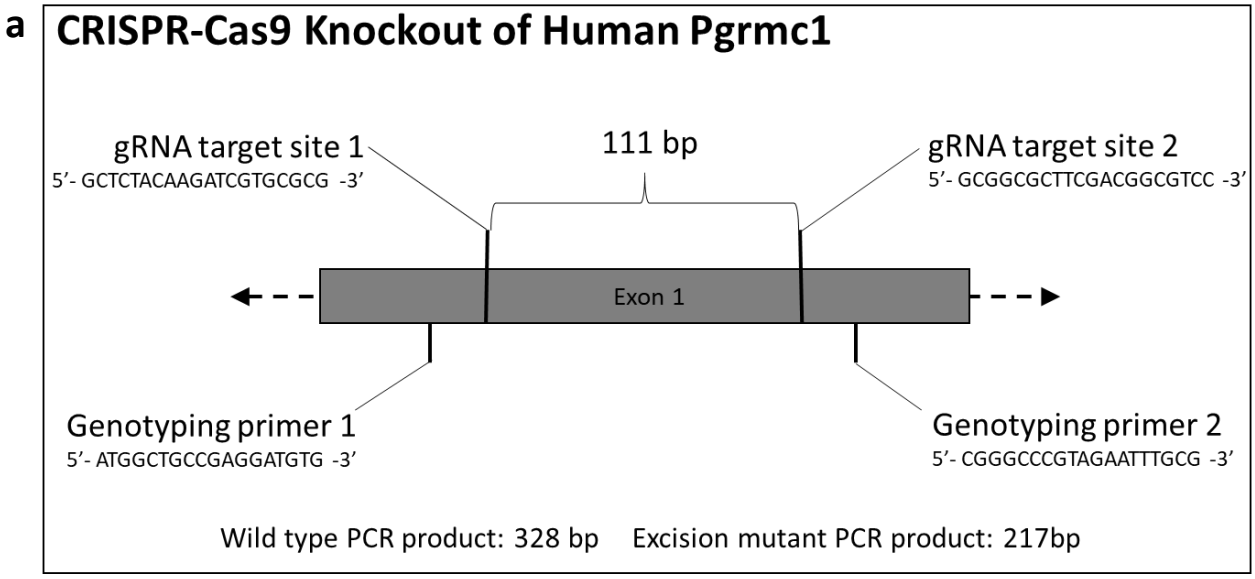
For recombinant production in *Escherichia coli*, wild-type and Y113 mutant PGRMC1 were expressed and purified as previously described[1, 33]. Y113 A, C, H, and F mutants of

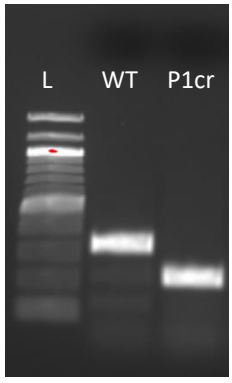
Pgrmc1 were constructed via QiukChange (Agilent) mutagenesis of pTrcHisA (Life Technologies, Grand Island, NY) containing human Pgrmc1.

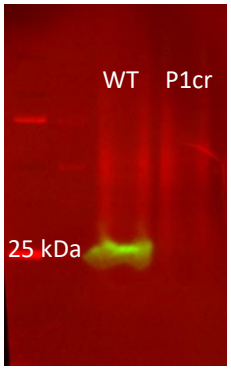
For SDS PAGE and immunoblot analysis, eluted protein from column chromatography and cellular lysates was separated on Mini-PROTEAN TGX Stain-Free gels (BioRad, Hercules,CA). SDS PAGE gels were stained directly for heme by submersion in a buffer containing 0.1 %(w/v) *o*-dianisidine, 10 %(v/v) glacial acetic acid, 50 mM sodium citrate, and 0.06 %(v/v) H_2O_2. Immunoblots and SDS PAGE gels were visualized using a ChemiDoc imaging system (BioRad).

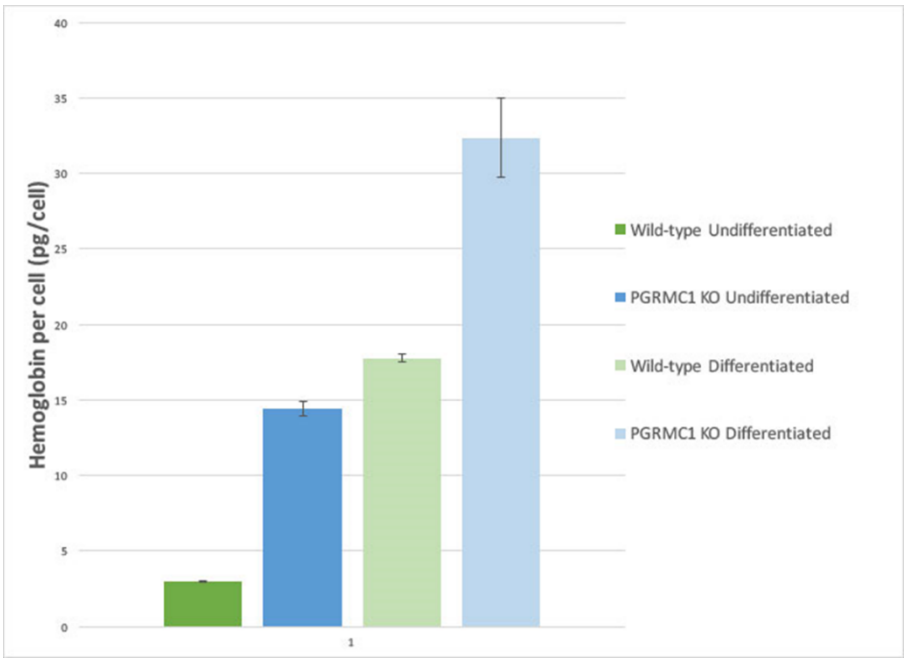
UV-vis spectra of purified wild type and mutant PGRMC1 were measured using a CARY 1G UV-Visible Spectrophotometer (Agilent, Santa Clara, CA).

Figures and Tables









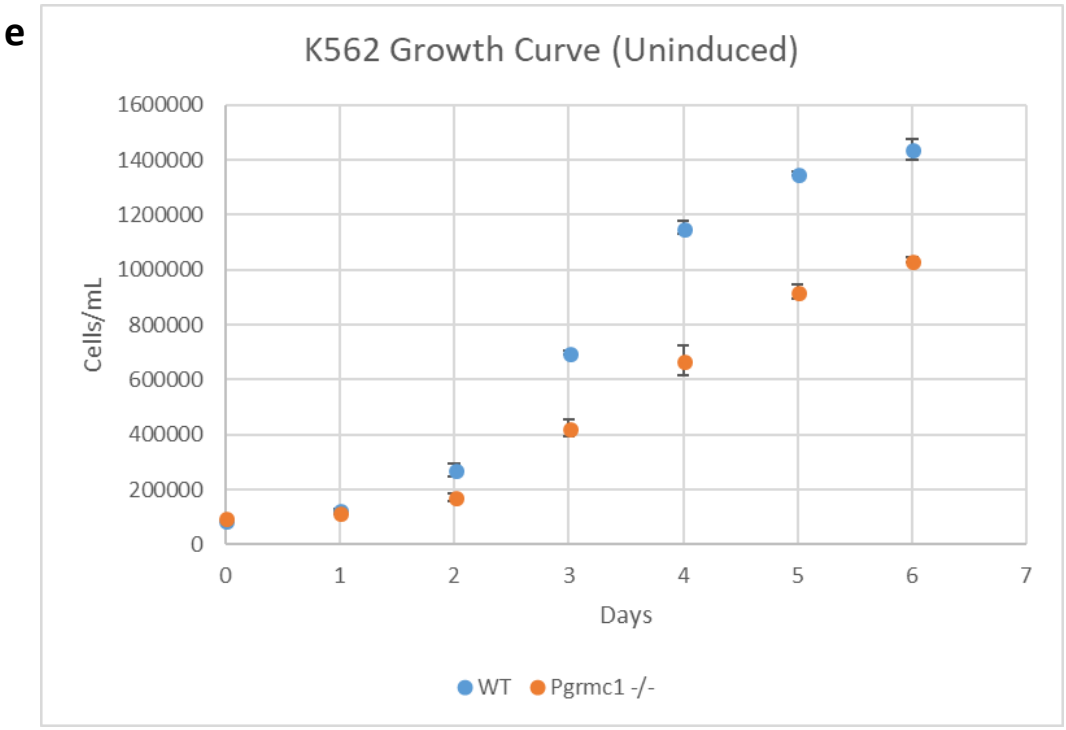


Figure 1. Knockout of Pgrmc1 in K562 cells. (a) Diagram of CRISPR-Cas9 gRNA target sequences and genotyping strategy for human Pgrmc1. (**b**) Agarose gel showing PCR based genotyping of wild type (WT) and *Pgrmc1* knockout (P1cr) K562 cells with 100 bp DNA ladder (L) (**c**) Western blot of WT and P1cr K562 cells showing ablation of PGRMC1 expression with total protein in red and αPgrmc1 antibody in green. (**d**) Hemoglobinization in K562 cells. n=3. (**e**) Growth curves of uninduced WT and P1cr K562 cells. n=3. Graphs show the mean with the error bars indicating the standard deviation.

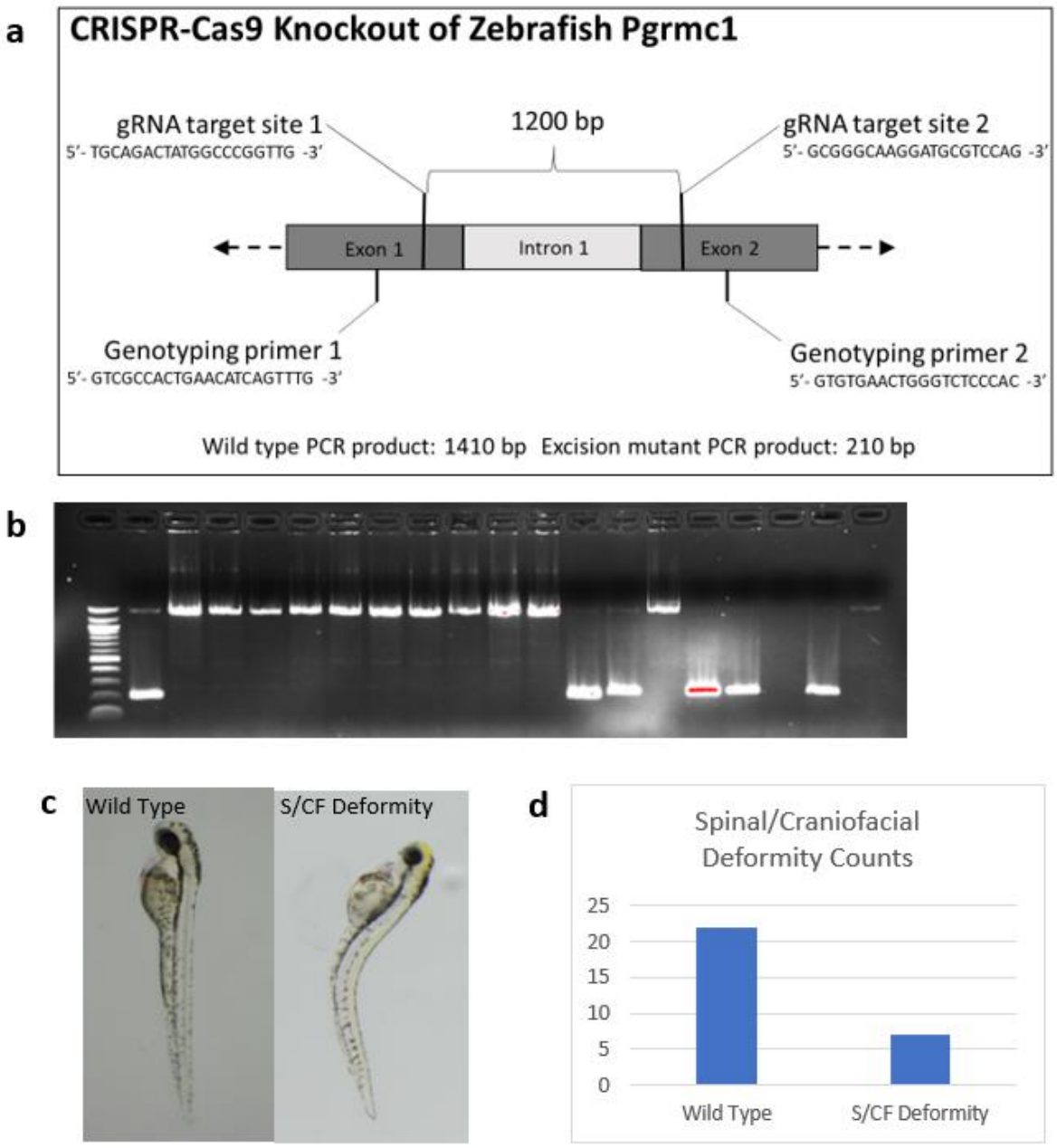
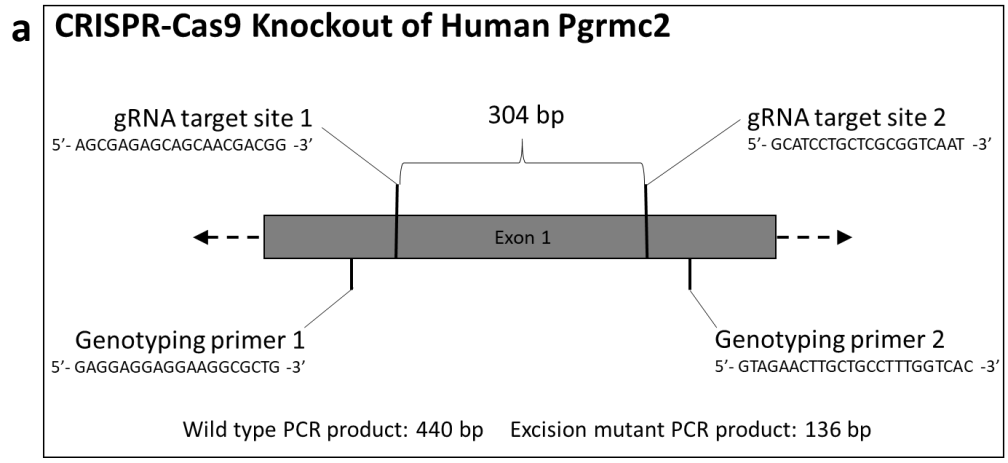
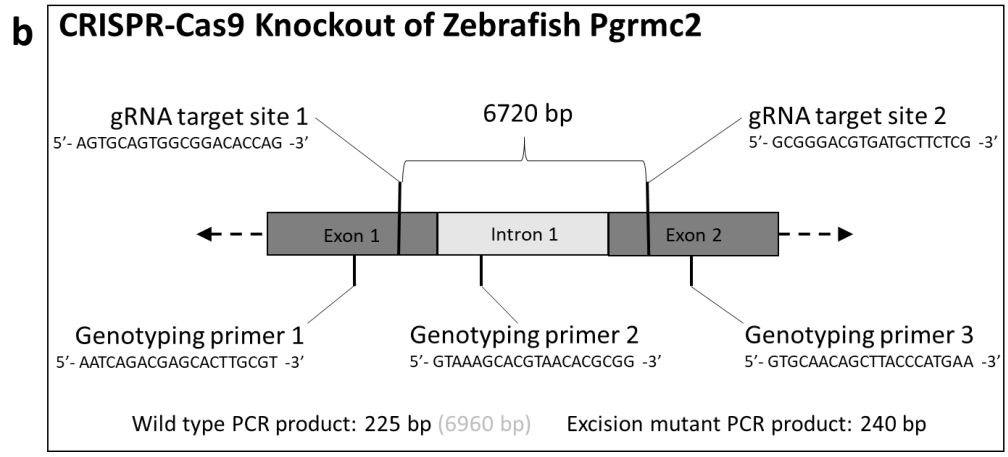


Figure 2. Knockout of Pgrmc1 in Zebrafish. (**a**) Diagram of CRISPR-Cas9 gRNA target sequences and genotyping strategy for zebrafish *Pgrmc1*. (**b**) Agarose gel showing PCR based genotype screening for *Pgrmc1* knockout zebrafish embryos with 100 bp DNA ladder (L) (**c**) Representative image of spinal/craniofacial deformity observed in zebrafish embryos from *Pgrmc1*$^{+/-}$ in-cross at ~2 dpf. (**d**) Number of embryos displaying wild type and spinal/craniofacial deformity phenotypes from a batch-mating of *Pgrmc1*$^{+/-}$ zebrafish.





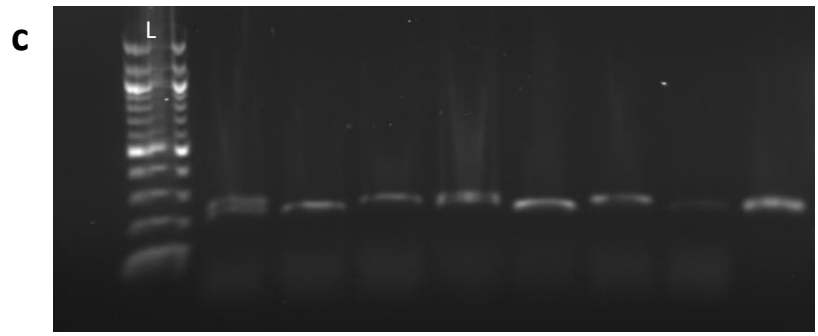


Figure 3. Knockout of Pgrmc2 in K562 cells and Zebrafish. (**a**) Diagram of CRISPR-Cas9 gRNA target sequences and genotyping strategy for human Pgrmc2. (**b**) Diagram of CRISPR-Cas9 gRNA target sequences and genotyping strategy for zebrafish *Pgrmc2*. (**c**) Agarose gel showing PCR based genotype screening for *Pgrmc2* knockout zebrafish embryos with 100 bp DNA ladder (L)

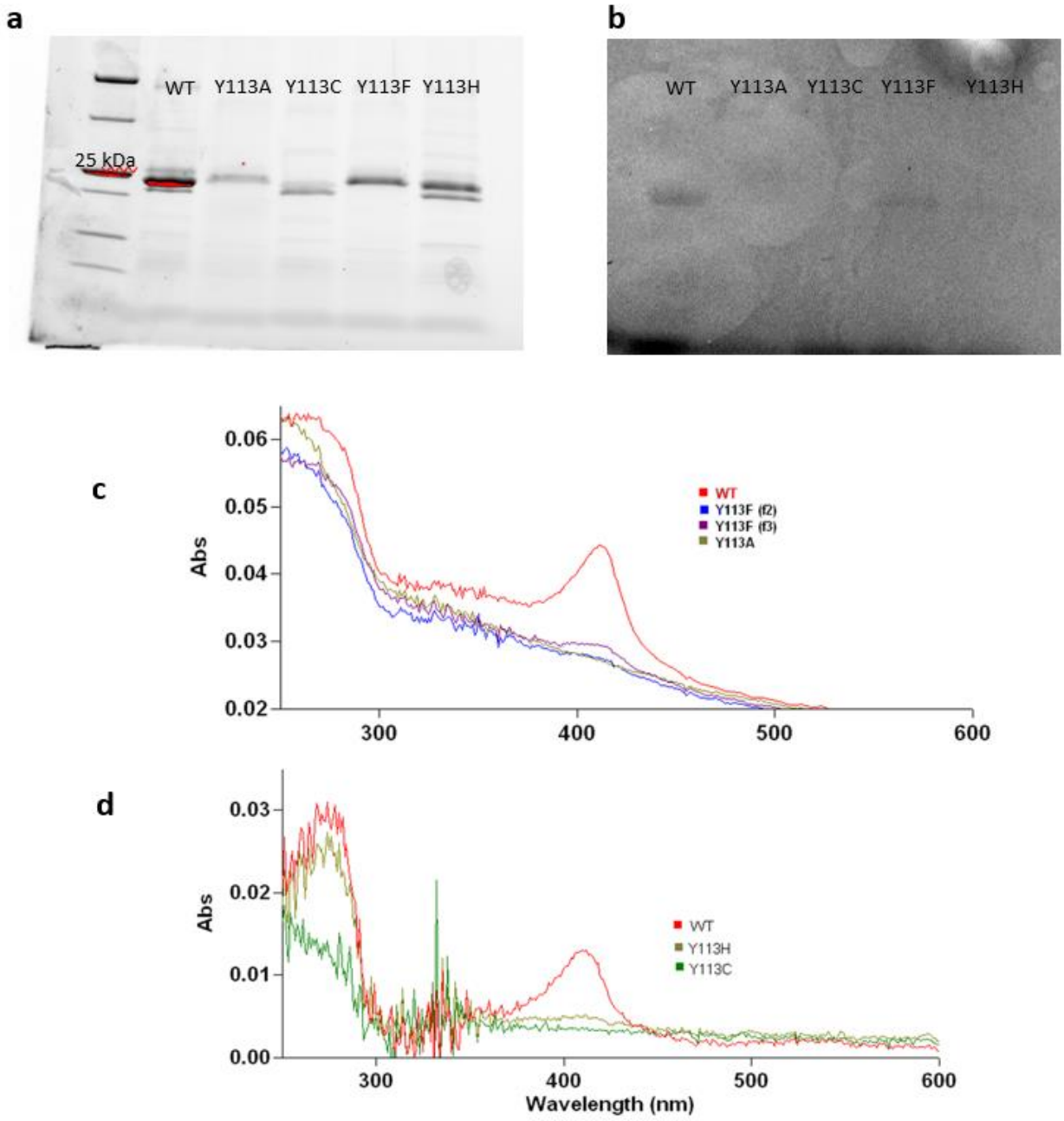


Figure 4. Characterization of Y113 Mutants of Recombinant Human Pgrmc1. (**a**) SDS-PAGE showing protein expression of wild type (WT) and Y113 mutant Pgrmc1. (**b**) *o*-dianisidine staining of SDS-PAGE for heme bound to WT and Y113 mutant PGRMC1. (**c/d**) UV-vis absorbance spectra of Y113 mutant Pgrmc1, showing heme peak at ~410 nm.

Table 1. Heme and Porphyrin Levels in Wild Type and *Pgrmc1*$^{-/-}$ undifferentiated K562 cells.

Sample	**Hemin nmol/mg Avg ± SD**	**PPIX pmol/mg Average ± SD**	**Porphyrins nmol/mg Average ± SD**
Wild Type	0.181 ± 0.016	0.730 ± 0.150	0.764 ± 0.041
Pgrmc1$^{-/-}$	0.287 ± 0.017	0.903 ± 0.150	1.099 ± 0.123

Table 2. Proteins to be screened for expression changes in cells lacking Pgrmc1 or expressing Y113 variants.

Protein	**Function**
Pgrmc2	Heme binding, unknown
Fech	Heme synthesis/iron insertion
PPOX	Protoporphyrin IX synthesis
Alas-2	ALA synthesis
HO-1	Heme degradation
HO-2	Heme degradation
Flvcr1a	Cytoplasmic heme export
Flvcr1b	Mitochondrial heme export

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CHAPTER 5

CONCLUSIONS

The Mitochondrial Heme Metabolon

We have described the identification of the mitochondrial heme metabolon, a complex of interacting proteins spanning both mitochondrial membranes[1]. This complex contains several members of the heme biosynthetic pathway, enzymes involved in iron sulfur cluster metabolism, various mitochondrial transport proteins, and structural proteins thought to mediate mitochondrial membrane morphology. Metabolon components were also found to interact with enzymes in the TCA cycle[2], and several sparsely characterized proteins with unknown functions[1, 3]. We propose that the mitochondrial heme metabolon serves as an essential hub for the synthesis of heme, the regulation thereof, and for intracellular distribution of heme post-synthesis. In particular, there are two emerging areas of study in which the metabolon is likely involved, related to porphyrin synthesis and heme trafficking.

The first of these deals with the production of one of the initial substrates of the heme synthesis pathway, succinyl-CoA, and the regulation of the first enzyme in the pathway, Alas. The identification of the mitochondrial heme metabolon confirmed the previously described interaction between the erythroid specific form of the first enzyme in the heme synthesis pathway, Alas-2, and Sucla2[1, 4], a subunit of the TCA cycle enzyme succinyl-CoA synthetase (SCS). A second member of the TCA cycle, the α-ketoglutarate dehydrogenase complex (KDH),

was also found to interact with the metabolon[2]. Both of these enzymes are involved in the synthesis of succinyl-CoA. The significance of the interactions between these TCA cycle enzymes and Alas-2 is currently unknown, however it is tempting to speculate that the interaction may provide a mechanism for coordination of heme synthesis and the production of its initial substrates. Furthermore, study of the metabolon also uncovered a novel interaction between Alas-2 and the terminal enzyme of the heme synthesis pathway, Fech[1], suggesting additional coordination between opposite ends of the pathway. These interactions suggest a previously undescribed level of co-regulation between distal ends of the heme synthesis pathway and the initial substrate source. These mechanisms could have interesting implications for the study of heme synthesis regulation in general, as well as disease states such as XLPP.

A second interesting finding to emerge from study of the mitochondrial heme metabolon was the interactions of heme synthesis enzymes and other metabolon components with members of the MICOS complex[1, 3]. The MICOS complex is a group of proteins centered around Mic60 (mitofilin, Immt), that is responsible for maintenance of mitochondrial cristae morphology and mediation of contact sites between the mitochondrial inner and outer membranes[5]. Recent models of intracellular heme trafficking suggest that heme may be mobilized throughout the cell via direct inter-organelle contacts and membrane bound vesicular transport[6]. The interaction between heme synthesis enzymes and mediators of mitochondrial membrane dynamics provides support for such a model.

Each of these potential roles of the mitochondrial heme metabolon provide intriguing support for emerging models for heme synthesis regulation and heme trafficking. However, to date, the metabolon has only been characterized in erythroid cell models[1, 3]. Heme synthesis is dramatically upregulated in erythroid cells relative to other cell types and many levels of

differential regulation are known to exist between erythroid and non-erythroid heme synthesis[7]. It is currently unknown to what extent the metabolon functions in non-erythroid cells, or how it may differ in composition in these cell types. Examination of the mitochondrial heme metabolon in non-erythroid cell types presents an intriguing area of study that is necessary for a complete understanding of heme synthesis and its regulation. Better definition of erythroid specific mediators of heme synthesis will allow for more precise targeting of these factors in the study and treatment of disease states such as the anemias and porphyrias, which result from defects in erythroid heme synthesis.

Pgrmc1

Through study of the mitochondrial heme metabolon, a novel interaction between Pgrmc1 and several heme synthesis enzymes was discovered. Pgrmc1 is a small, heme binding protein with many reported functions throughout the cell in both normal and disease states[8]. Relevant to heme synthesis, Pgrmc1 has been previously proposed as a potential heme chaperone, due to its heme binding activity and association with a number of other proteins, including several cytochromes P450[9]. We have demonstrated that Pgrmc1 interacts with the terminal enzyme in the heme synthesis pathway, Fech, directly and is able to repress its activity *in vitro*[3]. These experiments represent the first time Pgrmc1 had been directly linked to the regulation of heme synthesis. In further studies, we both attempted inhibition of Pgrmc1 with a small molecule inhibitor thought to disrupt heme binding in the protein and performed CRISPR-Cas9 mediated knockout of the gene. In erythroid cell models, these techniques produced opposite results, with the small molecule inhibitor leading to decreased heme production, and the knockout resulting in increased heme levels[3][Chapter 4]. Providing a possible explanation of

this counterintuitive result, another group has recently demonstrated that Pgrmc1 interaction with other protein binding partners is dependent on dimerization of Pgrmc1, which is mediated by heme-heme stacking between holo-monomers[10].

We propose that Pgrmc1 is involved in the regulation of heme synthesis and/or the trafficking of heme through a mechanism where the apo-monomer and holo-dimer perform differing functions. This is likely mediated by dimerization-dependent interaction with members of the mitochondrial heme metabolon and other associated proteins. To further test this hypothesis, we have generated and characterized mutants of Pgrmc1 that are unable to bind heme [Chapter 4]. We intend to use these mutants in a series of studies to determine the role of heme binding in Pgrmc1. In addition to attempted rescue of knockout phenotypes as described in Chapter 4, we intend to perform co-immunoprecipitation studies similar to those originally used to identify Pgrmc1 as a member of the metabolon[3]. We speculate that pull-down studies using FLAG-tagged Pgrmc1 mutants will result in a different set of interaction partners as compared to the wild type enzyme due to the disruption of dimerization mediated interaction. We will also perform these experiments with wild type Pgrmc1 in the presence of CO. CO has been demonstrated to disrupt the dimerization of Pgrmc1 without displacing its heme cofactor[10]. It is our hope that by uncovering differing sets of interaction partners for apo, heme-bound monomeric, and dimerized Pgrmc1 we will further decipher the mechanisms by which Pgrmc1 is able to influence heme production and transport.

Similar to the characterization of the mitochondrial heme metabolon, most of the data regarding Pgrmc1's effects on heme metabolism has been acquired in studies of erythroid cell models[3][Chapter 4]. In an attempt to expand our understanding of this protein, we propose to study its role in heme synthesis and trafficking in other model organisms as well. Studies in non-

erythroid cell culture models will be carried out similar to those in erythroid models. Furthermore, we have already begun characterization of zebrafish lacking Pgrmc1 and plan to continue this work through mRNA injection rescue experiments with wild type and non-heme-binding mutant Pgrmc1. In addition to work in cell culture and zebrafish, the generation/acquisition of a Pgrmc1 knockout mouse would provide a powerful tool for study of Pgrmc1 in a system more analogous to human physiology.

Pgrmc2

Pgrmc1's paralogue, Pgrmc2, has seen significantly less study to date. Pgrmc2 expression has been shown to affect several disease states, including cancers[11] and uterine[12] disease. The protein has also recently been implicated in heme mediated regulation of lipogenesis[13]. Pgrmc2 has been shown to bind heme, similar to Pgrmc1[8]. Additionally, we have demonstrated interactions between Pgrmc2 and the mitochondrial heme metabolon, including Pgrmc1[3]. We hypothesize that Pgrmc2 may share some degree of functionality with Pgrmc1. It is possible these proteins perform similar functions, but at different stages in cell development/differentiation, or in different cell types. This idea is supported by the observation that Pgrmc2 expression increased during differentiation of an erythroid cell culture model, while Pgrmc1 expression decreased over the same time[3]. We plan to study Pgrmc2 function in a manner similar to that described for Pgrmc1 in hopes to discover the role of this elusive protein as well as any function it may share with its paralogue.

The work presented in this dissertation describes the identification and characterization of the mitochondrial heme metabolon, including two proteins with previously undescribed roles in heme metabolism, Pgrmc1 and Pgrmc2. The elucidation of this protein complex has exciting implications for the study of mechanisms involved in the regulation of heme synthesis and transport. A more complete understanding of these mechanisms may yield promising targets for study and treatment of the anemias and porphyrias. In addition to their potential roles in diseases of heme synthesis, Pgrmc1 and Pgrmc2 have also been proposed as mediators of female reproductive disease and various cancers. Continued study of these proteins and their roles in heme metabolism could help to further the understanding of these disease states as well.

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