DEVELOPMENT OF NEW HEME SENSORS AND NOVEL INSIGHTS INTO HEME HOMEOSTASIS

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DEVELOPMENT OF NEW HEME SENSORS AND NOVEL INSIGHTS INTO HEME HOMEOSTASIS

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To the memory of

Albert Adepoju Dominic (August 19, 1936 – January 15, 2006)

Comfort Dola Dominic (July 4, 1944 – August 24, 2024)

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all of our relationship up till now has existed alongside this PhD journey. The road has not been easy, and we have weathered much, and a lot of the progress that we have made has been due to her intentionality. I do not have enough words to fully express all that she means to me, and I only pray for time and grace to be able to show her how much I love her.

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LIST OF SYMBOLS AND ABBREVIATIONS

5-ALA	5-aminolevulinic acid
ABC	ATP-binding cassette
ABCC	ATP-binding cassette subfamily C
ABCG2	ATP-binding cassette subfamily G member 2
AD	Alzheimer’s disease
AMPK	AMP-activated protein kinase
APX	Ascorbate peroxidase
ATP	Adenosine triphosphate
Aβ	Amyloid-β
BACH1	BTB and CNC homology 1
BCRP	Breast cancer resistance protein
BVR	Biliverdin reductase
CO	Carbon monoxide
COX1	Cyclooxygenase 1
COX2	Cyclooxygenase 2
CP	Cys-Pro motif
DAB	3,3′-Diaminobenzidine
DMEM	Dulbecco’s Modified Eagle Medium
DNA	Deoxyribonucleic acid
EBFP	Enhanced blue fluorescent protein
ECFP	Enhanced cyan fluorescent protein
EGFP	Enhanced green fluorescent protein

ER	Endoplasmic reticulum
ERMES	Endoplasmic Reticulum and Mitochondria Encounter Structure
ESCRT	Endosomal Sorting Complex Required for Transport
EV	Empty vector
EYFP	Enhanced yellow fluorescent protein
FABP	Fatty acid binding protein
FECH	Ferrochelatase
FLIM	Fluorescence lifetime imaging
FLVCR	Feline leukemia virus subtype C receptor
FRET	Fluorescence resonance energy transfer
G4	Guanine quadruplex
GAAC	General amino acid control
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GFP	Green fluorescent protein
GST	Glutathione S-transferase
HAS	*Heme a* synthase
HD	Heme-depleted
HDL	High density lipoprotein
HEBP1	Heme Binding Protein 1
HO-1	Heme oxygenase-1
HO-2	Heme oxygenase-2
HOS	*Heme o* synthase
HPLC	High performance liquid chromatography
HRG	Heme responsive gene
HRP	Horse radish peroxidase

HS1	Heme sensor 1
ICAM	Integrating cavity absorption meter
IMM	Inner mitochondrial membrane
LB	Lysogeny broth
LDL	Low density lipoprotein
MAM	Mitochondria associated membranes
MAPK	Mitogen-activated protein kinase
MAPR	Membrane-associated progesterone receptor
MCS	Membrane contact site
MDV	Mitochondria derived vesicle
MEF	Mouse embryonic fibroblast
MICOS	Mitochondrial contact site and cristae organizing system
miRNA	Micro RNA
mKATE2	Monomeric Katushka 2
MRP	Multidrug resistance protein
MVB	Multivesicular body
NADPH	Nicotinamide adenine dinucleotide phosphate
NEAT	Near transporter
NO	Nitric oxide
NOS	Nitric oxide synthase
OMM	Outer mitochondrial membrane
PANTHER	Protein ANalysis THrough Evolutionary Relationships
PBS	Phosphate-buffered saline
PCARP	Posterior column ataxia and retinitis pigmentosa
PD	Parkinson’s disease

PGRMC	Progesterone receptor membrane component
PKA	Protein kinase A
PPIX	Protoporhyrin IX
PRDX1	Peroxiredoxin 1
RES	Reticulo-endothelial system
RNA	Ribonucleic acid
SA	Succinylacteone
SC	Synthetic complete
SDS-PAGE	Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis
sGCβ	Soluble guanylyl cyclase β
TBS	Tris-buffered saline
TOR	Target of rapamycin
TORC1	Target of rapamycin complex 1
UV	Ultraviolet
WT	Wildtype
YPD	Yeast-peptone-dextrose

SUMMARY

Heme is an essential iron-containing nutrient important for processes such as gas transport, electron transfer, and signaling.[1, 2] Defects in heme homeostasis have been implicated in a variety of disorders, including anemias, some cancers, cardiovascular diseases, and neurological diseases, which highlight its broad importance.[3-5] On the flipside, the iron center of heme makes it potentially cytotoxic, suggesting that the cell must have evolved mechanisms to limit the deleterious effects heme may have on membranes and proteins. While heme biosynthesis and degradation are generally well characterized processes, many aspects of heme trafficking and mobilization after synthesis remain unclear.[6,7] To enable the development of better medical solutions for heme-related conditions, there is a need to improve our understanding of heme trafficking and regulation mechanisms.

To probe labile heme availability, previous work in our lab had developed genetically encoded, tri-domain, ratiometric heme sensors consisting of cytochrome b562 as the heme binding module, GFP as the heme reporter, and mKATE2 as the sensor expression control domain.[8] These sensors have been targeted to the cytoplasm, mitochondria and nucleus, and have vastly increased our understanding of heme homeostasis across several different organisms.

To expand the ability of these tools to probe heme in cell biology in multiple contexts, we embarked on a program to **a.** target the heme sensors to organelles like the ER and vacuole and **b.** diversify the color pallet of heme sensors for multi–

compartmental and –cellular imaging. Additionally, a serendipitous observation led us to **c.** characterize intercellular heme transfer in Baker's yeast, determine its genetic components and probe its physiological significance.

To develop an ER-targeted heme sensor, we tethered a well characterized ER-targeting sequence to different sensor scaffolds generated via iterative protein engineering (amino acid substitutions, domain rearrangement, domain linker length and oxidation sensitivity) and evaluated their functionality in Baker's yeast and HEK293 cells (Chapter 2).

To make the CFP variant of HS1, we applied mutations to the GFP module that are well established to blue-shift the chromophore fluorescence and generate a stable CFP molecule. We also manipulated domain linker length to achieve a functional molecule which was characterized in Baker's yeast and HEK293 cells (Chapter 3).

For studying the novel phenomenon of intercellular heme exchange in Baker's yeast, we investigated nutrient and environmental effects, probed the genetic determinants of transfer using selected knockout strains from the yeast non-essential gene deletion library, and assessed physiological significance on parameters such as growth and respiration (Chapter 4).

CHAPTER 1. INTRODUCTION

Heme *b* (iron protoporphyrin IX; hereafter referred to as heme) is an essential but potentially harmful cofactor and signaling molecule, required by nearly all aerobic organisms.[3] Due to its unique properties, including Lewis acidity, redox activity, and hydrophobicity, heme plays a vital role in various biochemical processes such as electron transfer and catalysis, and is thus important in crucial cellular functions including gas transport, mitochondrial respiration and drug detoxification.[4] Interestingly, these same properties that make heme indispensable are also responsible for its potential toxicity. For instance, the hydrophobicity of heme enables it to adhere to and intercalate within membranes leading to their disruption, while its strong redox activity can drive harmful redox reactions, leading to the destruction of cellular macromolecules.[5] Indeed, dysregulation in heme homeostasis has been linked to numerous diseases, including cancers, neurodegenerative conditions, cardiovascular diseases, and various anemias.[6–8] Additionally, heme has been found to be a virulence factor in many pathogenic diseases.[9,10]

As a result of this dichotomous nature of heme – essentiality and potential toxicity, – cells have developed strategies to manage harmful heme precursors and safely control heme movement and trafficking. However, the mechanisms and factors involved in regulating heme availability across different cellular compartments remain poorly understood.[4] Obtaining this knowledge would be advantageous for the development of therapeutic and diagnostic solutions against heme-related

conditions[11] and would more broadly complete our understanding of the overall homeostasis of this ancient and ubiquitous molecule in biology. This chapter, therefore, will offer a brief overview of the current state of knowledge of heme's role in biology and disease, heme trafficking pathways within and between cells, as well as tools for studying heme bioavailability.

1.1 Heme in Biology

As mentioned earlier, heme is a metallo-nutrient essential for all aerobic life, where it is found in almost every subcellular compartment. For instance, soluble guanylyl cyclase β-subunit (sGCβ) is in the cytosol, prostaglandin synthases COX1 and COX2 in the ER, catalase in peroxisomes, and BACH1 in the nucleus.[12,13] In these proteins, heme's unique properties enable it function as a cofactor and signaling molecule. While its roles as a cofactor have been studied to much depth, the signaling functions of heme are less well-known and is an emerging area of research. For example, heme binding can regulate the expression and activity of transcription factors, cell surface receptors, kinases, and ion channels.[7]

The synthesis of heme and its degradation are well studied processes, and both are important for the regulation of heme homeostasis, offering several checkpoints at which heme concentrations can be controlled.[1] Animal and fungal eukaryotes synthesize heme via the C_4 (Shemin) pathway, and this takes place in eight enzymatic steps between the mitochondria and cytosol.[14,15] The pathway begins with the condensation of glycine with succinyl-CoA to produce 5-aminolevulinic

acid (5-ALA) which is then exported to the cytosol.

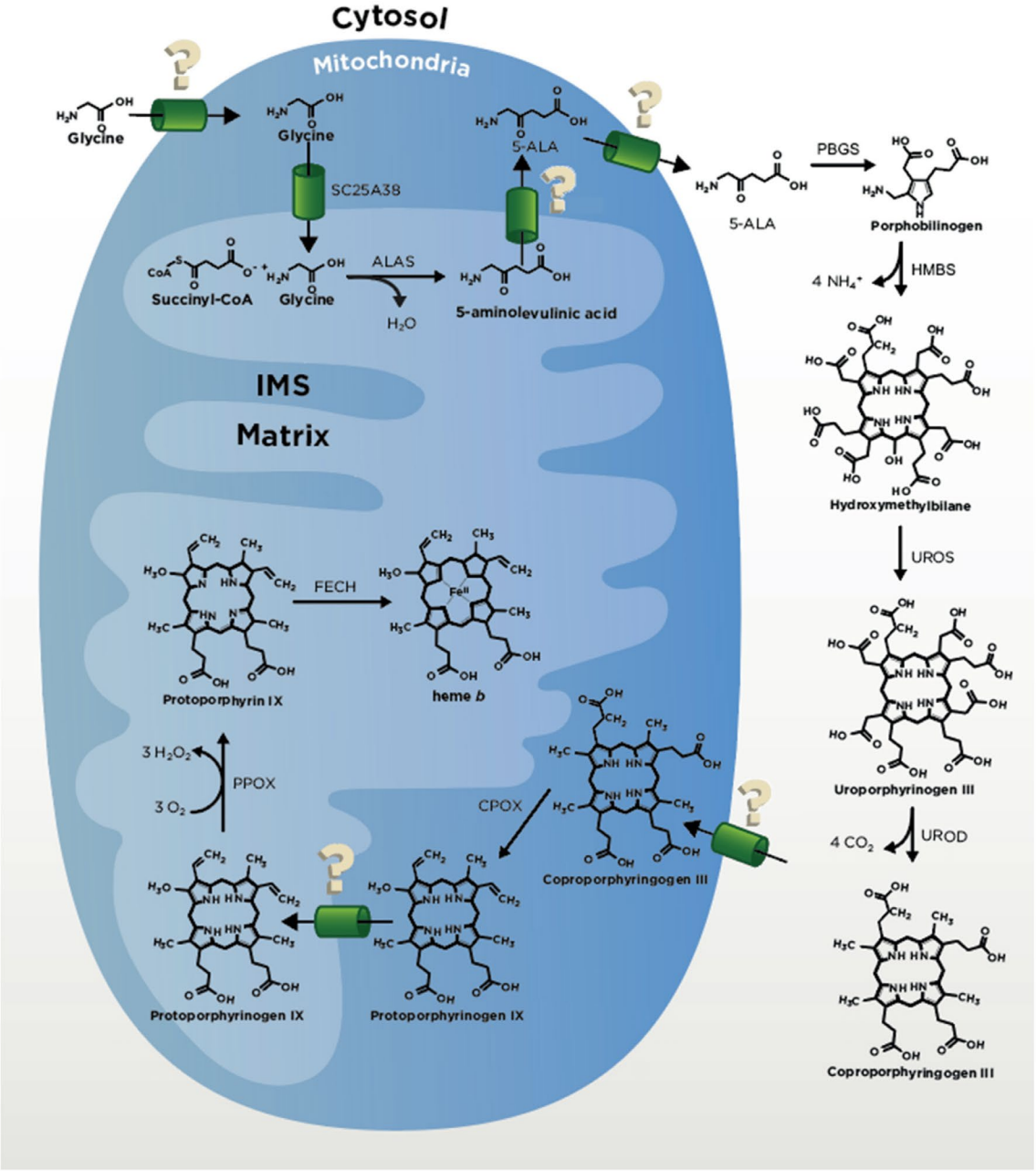

Figure 1. Heme biosynthesis in eukaryotes. Heme is made in eukaryotes via a highly conserved eight-step enzymatic pathway which occurs between the cytosol and mitochondria. Figure reproduced with permission from Swenson et al, Copyright © 2020 Cells.[1]

In the cytosol, the next four steps of synthesis take place, with 5-ALA as the initial substrate and coproporphyrinogen III as the final cytosolic product. Coproporphyrinogen III is then transported back into the mitochondria for the remaining biosynthetic steps, culminating with the insertion of Fe^{2+} into protoporphyrin IX (PPIX) by ferrochelatase (FECH) to produce heme *b* on the matrix side of the inner mitochondrial membrane (Figure 1).[14,15]

Heme degradation is effected by heme oxygenases (HO) which catalyze the breakdown of heme into biliverdin, carbon monoxide (CO) and free iron. Higher eukaryotes possess two HO isoforms: the inducible HO-1 which responds to several inducers and stressors such as heme and oxidative stress, and the constitutive HO-2 which is highly expressed in specific tissues such as the testis and brain.[16,17] As the name of the enzyme implies, the reaction requires oxygen and reducing equivalent is supplied from NADPH via cytochrome P450 reductase. The biliverdin product is rapidly converted to bilirubin by biliverdin reductase (BVR) which is localized in proximity to HO-1 to prevent feedback inhibition of the latter. Interestingly, the products of heme catabolism – CO, biliverdin and bilirubin – can themselves act in signaling capacities. For example, CO may regulate circadian rhythm and ion channels,[18] biliverdin has effects on the inflammatory response,[19] and bilirubin possesses endocrine, anti-inflammatory and immune effects.[20]

While heme *b* is the most abundant form of heme, there are other heme types, all of which are derived from heme *b* via enzymatic modification of the porphyrin ring side chains (Figure 2). Heme *c*, found in cytochromes *c* and *c1*, is unique as it is covalently bonded to its protein hosts via thioether linkages of the vinyl side chains

to Cys residues.[1] Heme *o* is made by the addition of a farnesyl group to the C_2 vinyl of the porphyrin ring, and heme *a* by oxidation of a C_8 methyl of heme *o* to an aldehyde.[1] Heme *a* is a cofactor in cytochrome c oxidase while heme *o* serves only as an intermediate to heme *a* synthesis in eukaryotes. In *E. coli*, however, heme *o* is found in one of the terminal ubiquinol oxidases.[21]

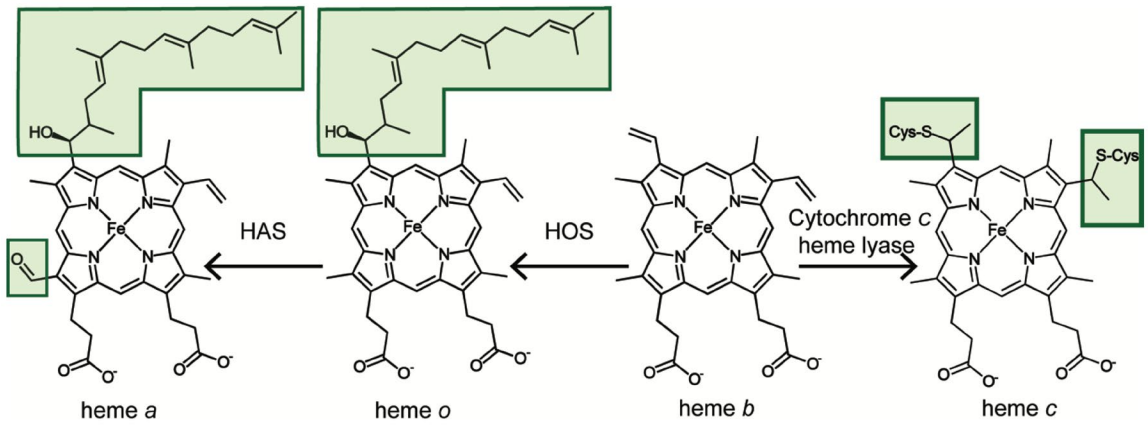

Figure 2. Structures of different types of heme. Heme *b* is the most prevalent and biologically relevant heme type, and the precursor for hemes *c*, *o* and *a*. Green boxes highlight where structural features are different from heme *b*. HOS, heme *o* synthase; HAS, heme *a* synthase. Figure reproduced with permission from Swenson et al, Copyright © 2020 Cells.[1]

1.2 Heme in Health and Disease

The ubiquity of heme derives from its versatility which in turn is a consequence of its unique chemical properties that have made it such an essential molecule. The same properties that make heme so useful, however, can also make it deleterious. As such, when heme homeostasis becomes unbalanced, it can lead to or exacerbate disease states.

Conditions such as sickle cell anemia, β-thalassemia and malaria that cause massive hemolysis produce heme-related pathologies because the defensive

ability of hemopexin is overwhelmed. In the absence of hemopexin, the vascular endothelium becomes compromised.[6] In sickle cell anemia and β-thalassemia, there is vascular inflammation and damage that leads to cardiovascular dysfunction.[22] In malaria, unchecked increases in free vascular hemoglobin and heme can additionally lead to cerebral malaria which is a severe complication that is typically refractory to treatment.[23] Hemopexin therapy has been suggested for such hemolytic conditions as it has been shown to be effective in mouse models.[22] In addition to these, heme has been implicated in several other cardiovascular pathologies including atherosclerosis, myocardial damage, degenerative aortic valve stenosis, cardiac iron overload and ferroptosis secondary to heart disease.[8]

Porphyrias are conditions characterized by an impairment of heme biosynthesis, and eight distinct types of porphyria have been described, each resulting from a partial deficiency of a specific enzyme of the heme biosynthetic pathway.[6,24] In porphyria, as the name suggests, there is accumulation of specific porphyrin intermediates (depending on the defective enzyme) in various tissues, leading to several complications including hepatic and hematopoietic alterations, as well as neurological and cutaneous symptoms.[6,24] X-linked sideroblastic anemia is one that results from impaired or total loss of function of the *ALAS2* gene, leading to iron accumulation in the mitochondria of erythroid precursors that presents as hypochromic, microcytic anemia.[25]

In addition to the more commonly encountered conditions mentioned above, several rare diseases related to proteins directly or indirectly involved in heme homeostasis have been identified. A deficiency of HO-1 function presents as

hemolytic anemia, asplenia, renal and hepatic iron deposition, and endothelial dysfunction.[6] The *FLVCR1* gene is responsible for plasma membrane and mitochondria integral membrane proteins FLVCR1a and FLVCR1b, respectively (via splice variant transcription) suggested to be involved in heme transport.[1] Mutations in the *FLVCR1* gene are causative for posterior column ataxia and retinitis pigmentosa (PCARP) which is a childhood-onset, autosomal-recessive, neurodegenerative syndrome with the clinical features of sensory ataxia and retinitis pigmentosa.[6] A related gene, *FLVCR2*, encodes another putative plasma membrane heme transporter which when mutated causes Fowler Syndrome, characterized by cerebral glomeruloid vasculopathy and limb deformities.[26] The ATP-binding cassette transporter ABCG2 has been shown to transport heme and porphyrin in addition to a variety of drugs.[1] Additionally, urate has been identified as a physiological substrate of ABCG2 which explains why defects in its function lead to hyperuricemia and gout.[6,27]

Both heme deficiency and excess are deleterious to neuronal cells, suggesting that heme concentrations must be finely controlled at the systemic and cellular levels.[28] Evidence abound that indicate a role for heme in the pathophysiology of some neurodegenerative disorders. In Alzheimer's Disease (AD), heme and hemoglobin have been shown to bind to amyloid-β (Aβ),[29,30] preventing uptake of the latter into primary mouse astrocytes and suppressing their immune activity.[30] Heme-Aβ complexes also exhibit peroxidase activity, resulting in oxidative damage to macromolecules.[31] Heme synthesis has also been found to be dysregulated in AD.[28,32] In Parkinson's Disease (PD), heme binding to α-synuclein has been

reported, along with altered heme metabolism.[28] Heme's role in AD and PD is still unclear, however, and more research is needed in this area.

Still emerging is the relationship between heme and cancer. Studies appear to support the idea that heme contained in food can sustain cancer by different mechanisms, particularly those of the upper gastrointestinal tract and colon.[33] Additionally, hemoprotein enzymes like myeloperoxidase may enhance tumor angiogenesis and metastasis, and heme could potentially play a role in cancer epigenetics, miRNAs and tumor innervation.[33] The frequent upregulation of heme synthesis and transport in certain cancers, along with downregulation of heme degradation, might suggest promotion of tumorigenesis by heme, but such connections are still unclear.[33] Nevertheless, the accumulation of porphyrin in tumor cells following 5-ALA administration has been exploited for photodynamic therapy[34] and heme binding to the tumor suppressor p53 is responsible for the successful application of iron chelation therapy.[35]

Another area of importance with respect to heme pathology is in the context of infectious diseases. Pathogens require heme for their survival and to cause disease. Heme is a key component of proteins which are needed for several important processes in the pathogens such as energy production, evasion of host immune effectors, and other processes that propagate pathogen virulence. In this sense, heme must either be synthesized *de novo* or acquired from the host, and pathogenic organisms have evolved sophisticated means to satisfy their heme requirements despite the host deploying nutritional immunity strategies to prevent such acquisition. Conversely, excess heme is toxic to pathogens and detoxification

pathways are also important for their survival. Some of the pathways for heme acquisition, synthesis and degradation are unique to these pathogens and can be studied for the development of therapeutics against these infections of global relevance.[9,36–43]

1.3 Heme Trafficking and Transport

As already mentioned, heme biosynthesis and degradation are biochemical processes that have received extensive attention. Much less understood, however, is what happens between these extremes of birth and death. How does heme, with its final synthesis step taking place in the mitochondrial matrix, safely traverse both mitochondrial membranes and access its client proteins found in virtually every intracellular locale?[7]

Traditionally, heme had been viewed as a static cofactor only found tightly bound to these hemoprotein clients, but emerging findings have challenged this notion. The current paradigm suggests that the totality of heme exists as inert and labile pools, wherein the former are those bound tightly to proteins (as just described) and not readily exchangeable, and the latter represent heme weakly associated with molecules to the extent that they are readily exchangeable and bioavailable within the cell.[4] The existence of this labile heme pool, therefore, raises more questions, such as its concentration across the cell, the identities of the players with which it associates, and how all of these – including heme synthesis and catabolism – are regulated to mitigate against heme toxicity and control overall heme homeostasis.[7]

The dual nature of heme as a transition metal and lipid can be helpful in guiding research that explores the landscape of labile heme trafficking mechanisms. As a metal, heme may require factors such as chaperones, buffers, and transporters, while its lipid characteristics would suggest the involvement of lipid transfer molecules, vesicles, membrane tethering complexes and organelle contact sites. In fact, it is likely that combinations of these mechanisms are employed based on considerations such as specific organelle characteristics and the physicochemical nature of the local environment.[2,11]

1.3.1 Intracellular Heme Trafficking

After heme is synthesized in the mitochondria, a complete picture of how it reaches all its downstream hemoprotein targets – within the cytosol and traversing organellar membranes – and stays buffered against potential toxicity is still lacking. Additionally, in certain relevant cases, it is not clear how imported heme is incorporated into the labile pool, as well as how it may be prepared for extracellular export. Following, the known and putative factors and mechanisms are briefly overviewed.

Mechanisms of heme efflux post-mitochondrial synthesis have been the subject of research for some time. The only bona fide mitochondrial heme exporter thus far described is the “b” isoform of the feline leukemia virus subgroup C receptor 1 (FLVCR1b), a splice variant of the *FLVCR1* gene whose protein product is termed FLVCR1a.[44] Genetic studies in mammalian cells and mice showed FLVCR1b to be critical for mitochondrial heme export during erythropoiesis.[44] While the

question of the exact localization of FLVCR1b (IMM and/or OMM) and how it obtains heme for export remain open,[1,2] recent work in HEK293T cells has confirmed FLVCR1b to be critical for heme delivery to the cytosolic heme chaperone GAPDH and its downstream clients.[45] The progesterone receptor membrane component 1 and 2 (PGRMC1; PGRMC2) proteins have both been suggested as candidates for trafficking heme out of the mitochondria but it is unclear if they function in the capacity of membrane transport proteins or as chaperones.[2] They are reviewed more extensively in Chapter 2.

Once outside the mitochondria, heme is known to access the cytosol where studies using FRET and activity-based fluorescence reporters have placed the concentration of cytosolic labile heme in the range of about 20-430 nM.[46–49] The full landscape of cytosolic buffers and chaperones is not known at this time, but the identities of certain players have been revealed while others have been suggested, including GAPDH, HO-2, GST, FABP, HEBP1 and PRDX1.

The glycolytic enzyme GAPDH has been identified to function both as a buffer and chaperone for heme in the cytosol.[2] Its buffering capacity is in the range for labile heme estimates,[49] and it has been shown to be required for heme delivery to cytosolic clients such nitric oxide synthase (NOS) and guanylyl cyclase.[12,50] Additionally, GAPDH regulates heme availability to nuclear transcription factors[51] and is important for as an accepting heme chaperone from FLVCR1b.[45]

HO-2 has recently emerged as an unlikely player in the cytosolic labile heme speciation arena. Hanna and colleagues found that HO-2 binds and buffers heme,

leaving much of its canonical heme degradative function to HO-1. Under conditions which do not overwhelm HO-1, HO-2 regulates heme bioavailability by acting as a heme buffering factor.[52] Other proteins like GST, FABP, HEBP1 and PRDX1 have been shown to bind heme with affinities that would enable them to participate in regulating cytosolic labile heme, but strong evidence of their exact roles is currently lacking.[2,4]

In addition to the cytosol, labile heme pools have been observed in other organelles such as the nucleus, mitochondria, ER and Golgi.[48,49,53–57] The mechanisms by which heme accesses these intracellular membraned compartments is still unclear but speculations on the importance of membrane contact sites (MCSs) in this regard have been made, as they are critical for the exchange of other metabolites such as lipids.[2] The central role of the ER in this MCS network has been emphasized[2,56] and is discussed in more detail in Chapter 2. Cells are also known to uptake heme-laden vesicles via endocytosis as is the case with some yeast like *C. albicans* and *S. pombe*, as well as with hemopexin and haptoglobin complexes in vertebrates.[43,58,59] It is therefore possible that these systems already in place for distributing exogenous heme can be utilized for the trafficking of endogenous heme.[2] Like intracellular vesicle trafficking, mitochondria-derived vesicles (MDVs) are an interesting possibility as a heme trafficking conduit because they can bundle specific cargo and appear to be targetable.[2] Yet another new and intriguing player in intracellular heme mobilization are guanine quadruplexes (G4s) which are secondary structural elements common to both DNA and RNA. They have been found to bind and

sequester heme, and their perturbation was shown to affect expression of heme and iron genes, as well as cytosolic labile heme.[60,61] As many of these candidates are still emerging, much work still needs to be done to clearly delineate their involvement in intracellular heme homeostasis.

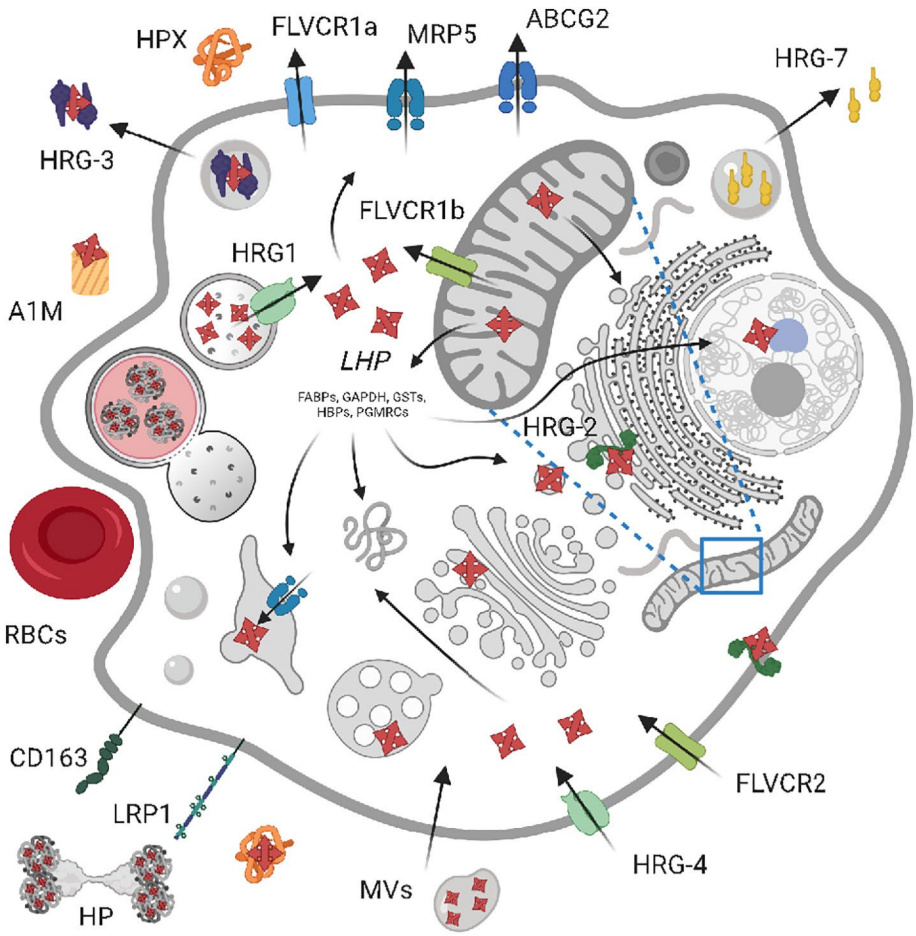

Figure 3. Comprehensive model of heme trafficking and transport in metazoa. Details are as discussed in the text. Figure reproduced with permission from Chambers et al, Copyright © 2021 Biochimica et Biophysica Acta (BBA) - Molecular Cell Research.[2]

1.3.2 Intercellular Heme Trafficking

The discovery that many eukaryotic cells can import and export heme presents the likelihood that these cells can share their heme to help regulate system-wide heme homeostasis. Briefly reviewed here are findings from *C. elegans* while other transport factors are discussed in more detail in Chapter 3.

The bloodless worm, *C. elegans*, is a heme auxotroph that must obtain its heme extracellularly to survive. One of the heme chaperones in this organism, HRG-3, binds and delivers maternal heme to developing oocytes and is critical for their survival as its absence leads to embryonic lethality or early developmental arrest. While there are no vertebrate analogs for HRG-3, it does present the possibility of similar systems existing in other organisms.[62] Another *C. elegans* protein, HRG-2, is believed to be an oxidoreductase that facilitates heme import and utilization.[63] A third protein, HRG-7, indeed acts an intercellular signal that is released from the intestine and communicates intestinal heme status to distal tissues in the worm.[64]

1.4 Heme Measurement Methods

Historically, the study of heme has employed traditional biochemical and analytical methods such as various chromatographic and spectroscopic techniques.[11,65] The spectral characteristics of heme make it amenable to analysis by UV/visible spectroscopy, and because heme is also sensitive to its iron oxidation state and the chemistry of coordinating ligands, it is possible to distinguish between bound and unbound forms.[66] Scientific improvements to UV/visible spectrophotometry have increased measurement sensitivity, allowing the detection of heme even

within untreated biological samples. The OLIS CLARiTY employs a specialized quartz cuvette that effectively increases the path length within an integrating cavity absorption meter (ICAM), thereby increasing sensitivity by several orders of magnitude.[67]

In many cases, because heme is complexed with other molecules in biological matrices, it needs to be extracted to be accurately measured, after which HPLC with UV detection against hemin standards can be used for quantification.[68] Since heme itself is non-fluorescent, it requires derivatization to become amenable to measurement by fluorescence techniques. A longstanding method for heme detection is that of the pyridine hemochromagen assay which is based upon the distinct spectral features of the bis-pyridine ferrous heme complex formed following the reaction of pyridine with heme, first in the ferric state before reduction to the ferrous state.[69] Another time-tested technique for heme determination is the porphyrin assay that exploits the inherent fluorescence of the protoporphyrin IX ring after demetallation of its iron quota.[70]

While these aforementioned methods have been instrumental in laying the groundwork for expanding our knowledge on heme homeostasis, they suffer from several disadvantages such as the need for lengthy sample preparation, destruction of “interfering” matrix components, loss of organellar and subpopulation heme concentration information, as well as the inability to differentiate between total and labile (bioavailable) heme. To surmount these shortcomings, the unique chemical characteristics of heme have been harnessed to develop tools and techniques capable of quantifying heme *in situ*.

1.4.1 Assessing Labile Heme Homeostasis

To date, only a handful of tools for *in situ* determination of labile heme have been developed, and they fall generally into the classes of small molecule probes and genetically encoded sensors. The small molecule probes are much fewer and more recent, and as such have enjoyed far less utility than the more extensive genetically encoded sensors.

Newton and coworkers developed a synthetic peptide-based labile heme sensor based on residues including and flanking Cys-Pro (CP) motifs of Bach1. One of the successful constructs (CP3) had 7-azatryptophan incorporated as the fluorescence reporter and this was applied in the detection of changes to labile heme following UV treatment of FEK4 skin cell lysates.[71] Taking inspiration from the mechanism of activation of artemisinin within the malaria parasite, Xu et al. developed HNG, a small-molecule fluorescent probe based on a 4-amino-1,8-naphthalimide fluorophore. HNG was highly selective for labile heme without interference from hemin, protein-bound (inert) heme, and zinc protoporphyrin.[72] Another molecule, H-FluNox, is a selective activity-based fluorescent probe that senses labile heme using a heme-dependent biomimetic *N*-oxide deoxygenation reaction to trigger the production of the fluorescent molecule DFP-rhodol. H-FluNox is >100-fold more selective for labile heme over Fe(II), enabling the discrimination of labile heme from the labile Fe(II) pool in living cells.[73]

Genetically encoded fluorescent sensors have been used extensively for the detection and quantification of metals,[74] as well as the assessment of key

contributors to metabolic status such as pH and redox status.[75] They are of benefit for their tunability and versatility, in addition to their *in situ* biological production.[75] In the realm of labile heme monitoring, several molecules have been developed.

Yuan and colleagues first used peroxidase reporters to quantify labile heme in HEK293 cells and *C. elegans*. Horse radish peroxidase (HRP) and ascorbate peroxidase (APX) reporters were expressed and targeted to various intracellular locales such as the ER, Golgi and the plasma membrane (HRP), as well as the cytosol, peroxisome and mitochondrial matrix (APX) to determine organellar labile heme pools.[48] Since the activity of peroxidases is dependent on heme as a cofactor, hemylation of the apoenzyme in the presence of H_2O_2 and a compound like *o*-dianisidine or 3,3'-diaminobenzidine (DAB) gives a colorimetric readout which is proportional to labile heme concentration. The activities of both reporters were found to be heme-responsive.[48] These sensors have also been applied to the monitoring of labile heme changes in brown fat adipocytes.[76]

Another genetically encoded heme sensor, termed CISDY, is a chimeric "turn-on" construct that utilizes the NEAT heme-binding domains of two heme transfer chaperones, IsdX1 and IsdC from *B. anthracis*, tethered by a linker and flanked by ECFP and EYFP at the N- and C-termini, respectively. Heme binding to IsdX1 and IsdC causes their heterodimerization which increases FRET efficiency between ECFP and EYFP in a dose-dependent manner.[47] CISDY was successfully used to characterize the distribution of labile heme in different intracellular compartments like the cytosol, nucleus, mitochondria, and ER, as well as in various human cell lines.[47]

In a bid to better understand heme homeostasis in the malaria parasite, Abshire *et. al.* developed CHY which consists of a *P. falciparum* heme binding protein (histidine-rich protein II (HRP2)) connecting ECFP and EYFP. CHY binds 15 heme molecules per sensor monomer, and in the apo state it exhibits FRET which is quenched when heme is bound.[77] Fluorescence lifetime imaging (FLIM) was used as a sensor readout to circumvent issues with intensity-based fluorescence which is typically complicated due to impacts to signal from inner filter effects and photobleaching. CHY informed on labile heme dynamics in *P. falciparum* during various stages of its life cycle.[77]

Also using FLIM as their readout, Leung and coworkers designed a sensor that had monomeric APX fused to monomeric GFP (mAPxmEGFP), with the former serving as the heme binding module and the latter as the heme reporter. Binding of heme to mAPX causes FRET from mEGFP to heme and the decay parameters can be used to accurately determine concentration as photon emission times are independent of fluorescence intensity.[78] This sensor was used to determine that the free (unbound) heme concentration is less than one molecule per compartment, leading to the suggestion that heme is tightly buffered in these systems and that heme transfer follows a ligand-exchange mechanism with the exchangeable/labile/regulatory heme pool.[78]

To better understand heme transfer after uptake by the bacterial pathogen *Corynebacterium diphtheriae* and other actinobacteria, researchers drew inspiration from the integral fusion design of the CG6 construct[79] (discussed below) and developed CiG, which incorporates the C-terminal Conserved Region (CR2)

domain of the HtaA protein into the β2-β3 turn of EGFP.[80] Utility of the sensor was then demonstrated by monitoring heme transfer from the sensor to the CR domains located within the HtaA or HtaB proteins in the *C. diphtheriae* heme-uptake system as measured by a ~ 60% increase in sensor fluorescence and native mass spectrometry.[80]

1.4.1.1 First Generation Heme Sensor (HS1)

Arguably the most widely used genetically encoded fluorescent labile heme sensor is that developed by Hanna and colleagues.[49] The first-generation heme sensor, HS1 is a tri-modular chimeric protein consisting of a heme binding domain in the form of the His/Met coordinating cytochrome b_{562} (cyt b_{562}) fused to enhanced green fluorescent protein (EGFP) (as the combinatorial domain insertion-designed CG6)[79], and monomeric Katushka 2 (mKATE2)[81](Fig. 1). While EGFP fluorescence is quenched by the binding of heme to cyt b_{562}, mKATE2 fluorescence is relatively unperturbed. As such, HS1 is an excitation-emission ratiometric (EGFP: mKATE2 fluorescence) FRET probe that provides a readout of labile heme concentration independent of sensor expression.[49]

In HS1, bound heme is coordinated to the sensor by the interactions of Met7 and His102 of the cyt b_{562} moiety to the iron center of heme. Experiments to determine the sensor dissociation constants for ferric and ferrous heme showed that HS1 was completely saturated (low and sub-nM range, respectively) with heme in cells making it ineffective for use as an intracellular heme probe. To address this, a weaker binding mutant, HS1-M7A which has the Met coordinating ligand mutated

to Ala, was developed and found to be 20-50% saturated (μM and nM range for Fe(III) and Fe(II), respectively), and hence more suitable for dynamic labile heme monitoring.[49]

HS1 has been targeted to different intracellular compartments such as the cytosol, mitochondrial matrix and nucleus, where it was used to characterize the labile heme pools in these locales. HS1 has also been employed for the identification of heme chaperones (e.g., GAPDH),[49,51] heme buffers (e.g., GAPDH, rRNA G4s and HO-2)[49,51,52,61] and signaling molecules capable of mobilizing labile heme (e.g., NO).[49] Additionally, HS1 has improved our understanding of heme utilization under lead stress[82] and labile heme mobilization to the nucleus through ER contact sites.[56] It has also shown versatility, having been utilized across different species including Baker's yeast, mammalian cells, plants and pathogenic organisms.[49,51,56,83–87]

1.5 Scope of Thesis

The essentiality of heme is very well known, together with its potential cytotoxicity. Indeed, the properties which make heme such a versatile molecule also confer it with deleterious tendencies. Organisms must carefully tread this delicate balance as dysregulation of heme homeostasis leads to various pathological states. There is a pressing need, therefore, to better understand heme homeostasis across different organisms as this will enable the development of healthcare solutions for heme-related conditions.

The traditional view of heme as a static cofactor led to the development of techniques to study it in vitro, which have been invaluable in laying the foundations for our early understanding of heme processes. New technologies, however, have engendered a growing appreciation for heme as a dynamic molecule capable of mobilization within and between cells, as a nutrient or signaling molecule. Consequently, the development of tools and methods that allow the study of heme in this new context is needed. Genetically encoded fluorescent heme sensors have gained widespread utility in this regard and their continued improvement is necessary for the expansion of knowledge within the field.

In this thesis, traditional and advanced techniques were used to expand the sensor toolkit for heme trafficking studies and apply these sensors towards novel discoveries. Herein, to shed more light on the involvement of the endomembrane pathway in intracellular heme trafficking, efforts towards the development of labile heme sensors targeted to the ER in different organisms will be discussed. Next, the proof-of-concept expansion of the color palette of HS1 will be presented. Lastly, HS1 will be used to explore intercellular heme exchange in *S. cerevisiae*.

CHAPTER 2. DEVELOPMENT OF ER-TARGETED LABILE HEME SENSORS

2.1 Introduction

A fundamental open question in heme biology is that of how biosynthesized heme exits the mitochondrial matrix,[88,89] setting the initiation point of endogenous heme trafficking. Such trafficking must exist because, on one hand, hemoproteins synthesized by the ER which co- or post-translationally acquire their heme cargo, are found in virtually all locales within the cell. On another hand, labile heme pools in several organelles have been identified and characterized in different cell types,[90,91] and especially in controlled conditions where endogenous heme is the only source of heme within the system.[91]

The exceedingly low amount of labile heme (< 1 nM) in the mitochondrial matrix[91] vis-a-vis the exponentially larger amounts of heme that mitochondria have been shown to accumulate (~ 30 μM)[92] suggests that the mechanisms that govern the trafficking of heme within and out of the mitochondria must be very efficient. Considering the critical role of the mitochondria in the cell, its high exposure to oxygen for energy production, and the propensity for heme to cause deleterious cell damage via Fenton and Haber-Weiss chemistry, such tightly controlled heme trafficking would be necessary.

Indeed, once synthesized on the matrix side of the inner mitochondrial membrane, heme must be transported across both the inner and outer mitochondrial

membranes to be able to access all its extra-mitochondrial clients and fulfil its signaling roles. Herein, a short overview of the known, suggested and plausible roles of the endomembrane system/secretory pathway in intracellular heme trafficking will be presented, highlighting the proposed trafficking factors and mechanisms, including unpublished work from our group.

Early work had shown that in liver samples from rats treated with ^{14}C-ALA, radioactivity (confirmed as heme) was observed predominantly in mitochondria and mitochondria associated with ER, with the cytosolic fraction having significantly less label. This suggested the ER as a preferred destination for the trafficking of nascently synthesized heme, majorly bypassing the cytosol.[93]

Two attractive mitochondrial heme export candidates that are related to the endomembrane system/secretory pathway are progesterone receptor membrane component 1 and 2 (PGRMC1; PGRMC2). Both proteins are members of the membrane-associated progesterone receptor (MAPR) family, together with neudesin and neuferricin, all of which are characterized by the presence of a cytochrome b_5-like heme/steroid-binding domain.[94,95] Both PGRMCs are generally accepted as membrane-anchored proteins[94] that have been found to interact with each other,[96,97] ER and mitochondrial membranes,[98,99] and are present in mitochondrial-associated membranes.[100]

PGRMC1 has been shown to interact with ferrochelatase (FECH), the terminal enzyme in heme biosynthesis, and can localize to the outer mitochondrial membrane, suggesting that it may span both membranes and can traffic

endogenous heme out of the mitochondria.[99,101] The details of how this might occur are unclear, however, and require further investigation. Notably, the interaction of PGRMC1 with FECH was observed to be in the open conformation of the latter, and the activity of FECH was found to be diminished in the presence of PGRMC1.[99] This raises the interesting possibility that PGRMC1 may serve a regulatory role for heme synthesis by controlling release of heme from FECH. It was also demonstrated that PGRMC1 can transfer heme to apo-cytochrome b_5 *in vitro*, ascribing it as a likely heme chaperone.[99] Additionally, PGRMC1 has been found at contact sites between the mitochondria and endoplasmic reticulum (ER),[98] suggesting a possible pathway for heme into the endomembrane system. In yeast, there is only one member of the of the MAPR family, termed as Damage Response Protein 1 (Dap1) and it is homologous to PGRMC1.[102,103] Like its mammalian homolog, Dap1p binds heme and this heme-binding capacity has been shown to be involved in ergosterol biosynthesis via interaction with the cytochrome p450 enzyme Erg11p, either in the capacity of a heme chaperone or via some other mechanism independent of heme transfer.[103–105] Unpublished work by Willoughby in the Reddi lab concluded that Dap1p facilitates heme trafficking between the mitochondria and nucleus but does not appear to affect steady state labile heme in yeast.[106] The work also showed that the ability of Dap1p to function in this manner is conserved in higher organisms as complementation of a *dap1Δ* strain with PGRMC1 was able to rescue both *dap1Δ* nuclear heme trafficking and fluconazole growth phenotypes.[106] The data from this work supports the postulation of Dap1p/PGRMC1 as a heme chaperone and though it shows a connection to the

nucleus and not the ER, it is instructive in that it supports a heme trafficking route that bypasses the cytosol, as suggested by Martinez-Guzman et al.[107] It is worth noting that Gem1 which is a subunit of the ERMES complex (an ER-mitochondria contact site) was also fingered as a regulator of mitochondrial-nuclear heme trafficking along with GTPases that control mitochondrial dynamics (Mgm1 and Dnm1),[107] indicating that organelle membrane contact sites might be leveraged for intracellular heme trafficking and predicting the plausible involvement of the ER in this process.

Data on PGRMC2 is less available but as earlier mentioned, it has been shown to be associated with both the ER and outer mitochondrial membranes.[98] It was also identified as an interacting partner with FECH[99] but this relationship requires further investigation. Galmozzi and colleagues produced data that functionally designated PGRMC2 as a heme chaperone in brown fat adipocytes, where it is required for the movement of heme to the nucleus for transcriptional activation of genes involved with thermogenesis.[96] They also suggest a model whereby PGRMC1 transfers heme to PGRMC2 based on purportedly different localizations of the proteins (mitochondria vs ER, respectively)[96] but this hypothesis is overly simplistic, failing to take into account the varied localizations reported for both proteins.[108,109] It was also recently reported that PGRMC2 was not involved in the delivery of mitochondrial heme either directly to the known cytosolic heme chaperone GAPDH, or any of its downstream clients.[110] The combination of this finding with that of Galmozzi et al., together with the reported localizations of PGRMC2 (ER, mitochondria and nucleus) might suggest that PGRMC2 is only

involved in heme trafficking along the mitochondria-ER-nucleus axis and does not participate in cytosolic heme delivery.

Another player that may be involved in the trafficking of heme out of the mitochondria is the mitochondrial contact site and cristae organizing system (MICOS) which is a large subunit complex (six proteins in yeast and nine in humans) responsible for maintaining cristae inner membrane (IM) architecture, as well as contact sites between the inner and outer mitochondrial membranes.[111,112] In mammals, MICOS is part of a larger group of ER-mitochondrial membrane sites referred to as mitochondria-associated membranes (MAMs).[113] In this manner, it has been reported to enable the bidirectional movement of hydrophobic molecules such as phosphatidic acid and coenzyme Q biosynthetic intermediates.[114,115] Phospholipids synthesized by the ER are critical for mitochondrial function,[116] and it has been shown that the lipid transport proteins ORP5 and ORP8 physically link with MICOS and that this connection is important for their ability to mediate the transport of phosphatidylserine from the ER to mitochondria.[117] Mic19, a core subunit of MICOS, regulates the formation of ER-mitochondria contact sites via interactions with EMC2, a subunit of the ER membrane complex, and SLC25A46 (Ugo1 in yeast), a coordinator of mitochondrial fusion.[118] Additionally, it has been observed that the MICOS subcomplex composed of Mic27/Mic26/Mic10/Mic12 requires ERMES to assemble, and that Mic60 which is a critical member of MICOS,[119] self-localizes close to the ER and redistribute to mitochondria-vacuole contact sites.[120] In relation to heme biochemistry, Mic60 was identified as a physical interacting partner of FECH/Hem15p.[99,121] Mic60 appears to control the

delivery of tetrapyrrole precursors to Hem15, as a deletion of Mic60 leads to significantly elevated porphyrin levels with concomitant effects on respiratory fitness and growth.[121] It was also found that Mic60 is required for the optimal activity of Hem15[121] but does not affect heme trafficking to the cytosol, mitochondrial matrix or nucleus.[106,121] Considering the role of MICOS in ER-mitochondria contacts, it would be intriguing to examine the role of Mic60 and other key MICOS components on ER heme homeostasis.

Originally described in *C. elegans*, multidrug resistance protein 5 (MRP5) is a member of the ABCC subfamily of transporters found to be expressed on the plasma membrane and endosomal compartments where it modulates heme levels in the secretory pathway.[122] These worms, which are naturally auxotrophic for heme, die off in the absence of MRP5 as extraintestinal tissues are unable to obtain the essential nutrient, and the importance of the transporter in regulating systemic heme homeostasis was demonstrated in other eukaryotic systems.[122] In mice, the additional knockout of the MRP5 homolog, MRP9 is required to observe any phenotypic dysfunctionality presenting as mitochondrial dysfunction and aberrant levels of succinyl-CoA, leading to male reproductive defects.[123] Interestingly, both MRP5 and MRP9 localize to MAMs in testis.[123] While much still needs to be done to elucidate the role of MRP5 and MRP9 in heme homeostasis, their interaction with MAMs opens possibilities for their involvement in heme trafficking through the endomembrane system.

While it is still unclear how organelles of the secretory pathway obtain heme, there are several reports wherein the presence of heme within organelles in the pathway

has been presented. For instance, in work describing the phagolysosomal heme transporter HRG1, a Golgi-HRP reporter was shown to respond to modulations in intracellular heme levels.[124] In studies of MRP5, confocal microscopy in yeast revealed an expression pattern of the protein consistent with endosomal compartments and the activity of ferric reductase (which requires a heme cofactor for functionality) as a heme reporter in the secretory pathway was shown to be altered in relation to heme levels.[122] Additionally, MRP5 colocalized with Golgi and endosomal compartments in MEFs, with a Golgi-HRP reporter correspondingly reading out on the presence of heme within that compartment.[122] Yuan and colleagues also targeted peroxidase-based activity reporters to secretory (ER and Golgi) and non-secretory (cytosol, mitochondria, nucleus and plasma membrane) locales in mammalian cells, and their findings suggested that the ER and Golgi were involved in trafficking of both endogenous and exogenous heme.[90] Finally, as part of experiments delineating the heme chaperone functionality of PGRMC2 in brown fat adipocytes, the ER-HRP reporter described previously[90] was deployed and confirmed heme response within that organelle.[96]

In a genome-wide screen of a yeast non-essential gene deletion library, cytosolic variants of the HS1 sensor were used to identify new regulators of heme synthesis and degradation, as well as factors that influence heme trafficking and distribution.[125] Of the 4470 mutants screened, it was found that 323 of them had significantly lower levels of cytosolic labile heme when compared to WT, and the cellular component PANTHER analysis revealed most of the mutants were associated with the endosomal sorting complex required for transport (ESCRT)

pathway, endosomal membranes, and intracellular vesicles.[125] These data suggest that heme may be trapped in the endomembrane system due to the loss of these proteins, thereby implicating the secretory pathway in intracellular heme trafficking and indicating a preference or hierarchy of endogenous heme distribution via this route. Indeed, work from our group has shown nuclear heme trafficking to occur faster than that to the cytoplasm and mitochondrial matrix, with factors that control mitochondrial dynamics and ER-mitochondria contact sites found to be key regulators of the process.[107] The research described herein, therefore, probes the hypothesis that intracellular heme is made bioavailable via the endomembrane system, by [detailing attempts at] developing ER-targeted heme sensors to characterize ER heme pools in yeast strains and human cells.

It should be noted that, separate from any purported roles that the endomembrane system may play in the trafficking of endogenously synthesized heme, the uptake of heme from exogenous sources by eukaryotic species has been better studied. As many of these species are parasitic or saprophytic in nature, endocytic mechanisms have been shown to be involved in exogenous heme acquisition, such as for protozoa[126] and fungi.[127] While intracellular mobilization of this externally-sourced heme cargo is less well understood, based on its role in heme uptake, the plausibility that species would employ the endomembrane system for movement of mitochondrial heme is high, in a bid to maximize metabolic efficiency.

2.2 Results

2.2.1 "Oxidation-stable" ER-targeted Constructs are Non-Fluorescent

The initial approach to targeting a heme sensor to the ER lumen took into consideration the highly oxidizing environment of the ER which is required for the formation of disulfide bridges. The cysteine substitutions implemented according to Suzuki et al.[128] (EGFP: C48S/C70M; mKATE2: C26A/C114M/C172A/C222S) were made on the canonical HS1 gene scaffold and codon-optimized for yeast expression. ER targeting for all yeast constructs was achieved by appending the Kar2p signal sequence to the N-terminus (Kar2-ss) and the HDEL ER retention motif (for yeast) to the C-terminus. The "oxidation-stable" ER-HS1 on p415GPD (415-ER-HS1-ox) was then transformed into WT and *hem1Δ* yeast for characterization. Surprisingly, plate reader fluorescence of exponential-phase WT and *hem1Δ* ER-HS1-ox cells showed no fluorescence above background levels in both the EGFP and mKATE2 channels (Figure 4A). Background fluorescence was obtained from WT cells expressing an empty p415GPD vector. To determine if there was an effect of construct size on fluorescence within the ER lumen, or if the combination of EGFP and mKATE2 was problematic, a construct of just the "oxidation-stable" mKATE2 on p415GPD targeted to the ER (415-ER-mKATE2-ox) was also made and it was observed that this construct was also non-fluorescent above background (Figure 4B). Attempts to make an "oxidation-stable" ER-targeted EGFP were unsuccessful.

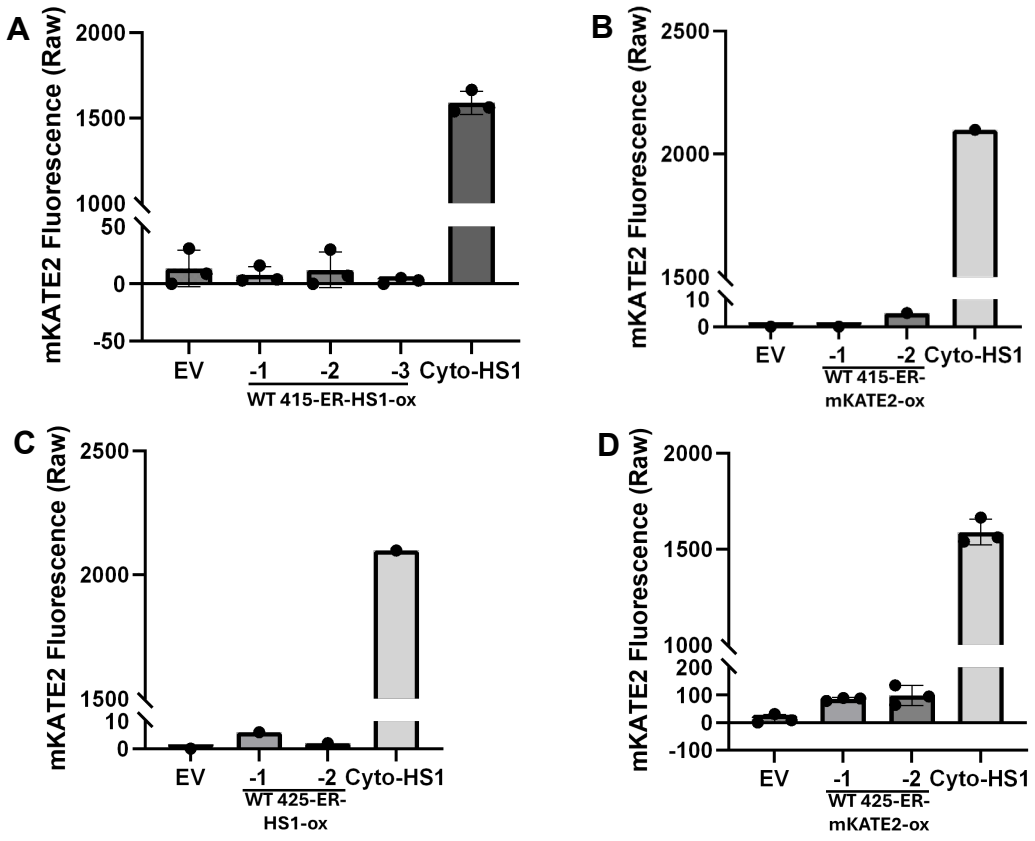

Figure 4."Oxidation-stable" ER-targeted constructs fail to properly express. ER-HS1 (**A**, **C**) and ER-mKATE2 (**B**, **D**) constructs with residue mutations intended to prevent ER oxidation fail to fluoresce within the ER lumen.

Considering the possibility that placing these constructs on a single copy plasmid (p415GPD) might be insufficient to drive their expression within the ER, “oxidation-stable” ER-HS1, ER-CG6 and ER-mKATE2 constructs on multi-copy vectors (p425GPD) were made (425-ER-HS1-ox, 425-ER-CG6-ox and 425-ER-mKATE2-ox, respectively). Like the p415GPD constructs, the 425-ER-HS1-ox, 425-ER-CG6-ox and 425-ER-mKATE2-ox were once again non-fluorescent above background levels (Figure 4C,D). The next task was to decipher if the ER environment itself was impeding the ability of the “oxidation-stable” constructs to fluoresce. To do this, a cytosolic version of CG6 with the “oxidation-stable”

mutations (C48S/C70M) in the EGFP module was made on p425GPD (425-CG6-ox). Here, it was observed that even 425-CG6-ox was not fluorescent above background (Figure 5A,B), leading to the conclusion that the “oxidation stabilizing” mutations introduced to both EGFP and mKATE2 inadvertently affected their fluorescent properties, at least in the context of *S. cerevisiae*.

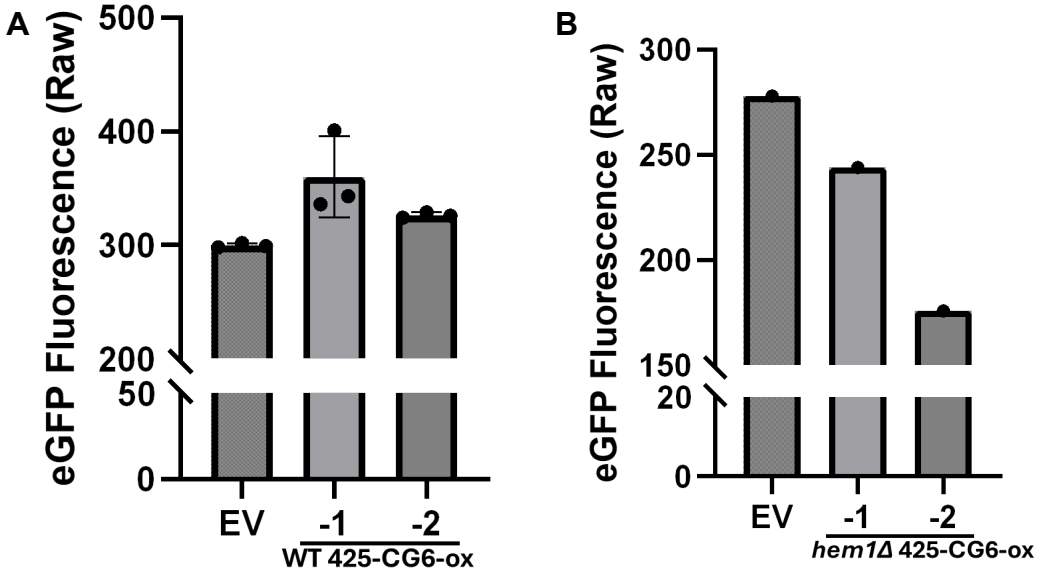

Figure 5. Cytosolic "oxidation-stable" constructs are non-fluorescent. WT **(A)** and *hem1Δ* **(B)** strains expressing cytosolic CG6 with C48S/C70M mutations on a p425GPD plasmid are non-fluorescent above background levels (EV).

2.2.2 Improper Folding of CG6 in ER Lumen (Due to Integral Fusion Design) Abolishes its Fluorescence

In parallel with work on the “oxidation stable” constructs, attempts were being made to target and express the canonical HS1 gene in the ER of Baker’s yeast. This was a plausible step because some EGFP-based constructs and other

fluorescent proteins had been successfully deployed to the ER in *S. cerevisiae*.[129,130] Targeting HS1 to the ER employed the same strategy as the "oxidation stable" constructs – Kar2p signal sequence at the N-terminus and a C-terminal HDEL retention sequence. The resulting insert was then cloned into p425GPD, and the generated plasmid (425G-ER-HS1) transformed into WT and *hem1Δ* yeast. While it was observed that there was some expression of the protein (Figure 8), fluorimetry data showed that this construct was non-fluorescent (Figure 7A). To assess if there was a construct size effect on expression, CG6 (~40 kDa) and EGFP (~27 kDa) were targeted to the ER on p425GPD plasmids (425G-ER-CG6 and 425G-ER-GFP, respectively). Similar to the observation for 425G-ER-HS1 (~65 kDa), 425G-ER-CG6 was also non-fluorescent as read out by plate reader fluorimetry and confocal microscopy (data not shown), despite protein expression (Figure 6). Surprisingly, 425G-ER-GFP showed robust fluorescence and ER localization (Figure 9).

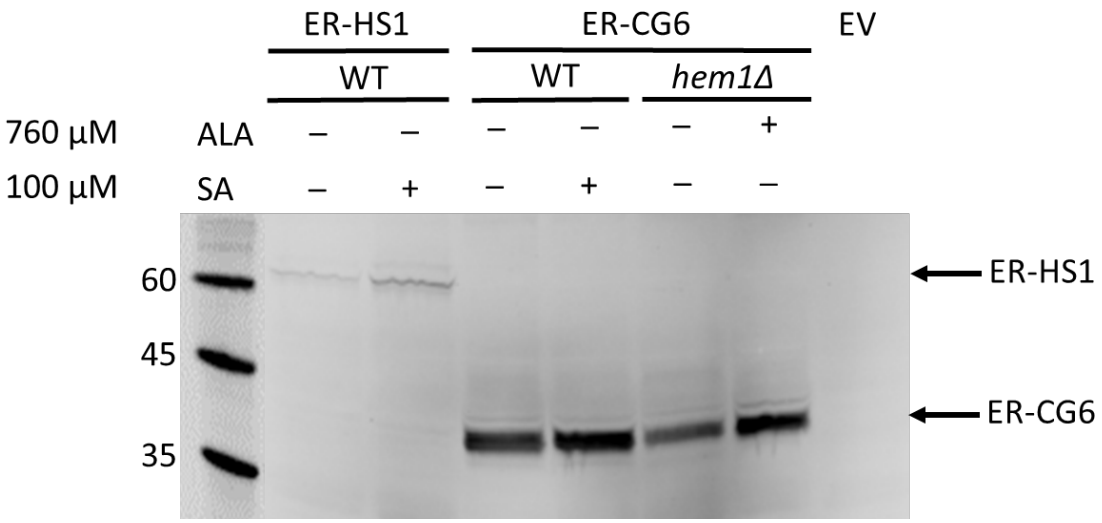

Figure 6. Weak vs robust protein expression of ER-HS1 and ER-CG6 constructs on p415GPD. 415G-ER-HS1 is weakly expressed in WT cells while 415G-ER-CG6 shows relatively stronger expression in both WT and *hem1Δ* cells.

Based off the foregoing results, the next consideration was whether tuned expression of the fluorescent protein constructs within the host might offer improved outcomes. The p425GPD vector is a multi-copy plasmid with a high strength GPD promoter, and their combination leads to very robust protein expression.[131] The p425GPD vector is the strongest of a suite of vectors designed for heterologous protein expression in yeast, wherein different combinations of copy number and promoter strength were used to construct yeast protein expression vectors of graded strengths.[131] The surmise was that high strength protein expression as driven by p425GPD might be overwhelming the steady state protein homeostasis within the compacted environment of the ER, leading to increased protein mishandling, misfolding and/or degradation. This could also have been happening based off a size threshold as observed from the 425G-ER-GFP vs 425G-ER-CG6 and 425G-ER-HS1 data. The vectors with immediately

lower strengths from p425GPD are p425TEF (multi-copy plasmid, intermediate strength promoter) and p415GPD (single-copy plasmid, high strength promoter),[131] and ER-HS1 (425T-ER-HS1; 415G-ER-HS1), ER-CG6 (425T-ER-CG6; 415G-ER-CG6) and ER-GFP (425T-ER-GFP; 415G-ER-GFP) were all transformed into both vectors (p425TEF and p415GPD, respectively).

Fluorimetry and confocal microscopy data for the p425TEF and p415GPD ER-HS1 and ER-CG6 (425T-ER-HS1, 415G-ER-HS1, 425T-ER-CG6 and 415G-ER-CG6) constructs were strikingly similar to that of 425G-ER-HS1 and 425G-ER-CG6, showing no discernable fluorescence above background despite protein expression (Figure 6; Figure 7B,C). The EGFP constructs for both p425TEF and p415GPD (425T-ER-GFP and 415G-ER-GFP), just like their p425GPD counterpart (425G-ER-GFP), were fluorescent and ER-localized (Figure 9).

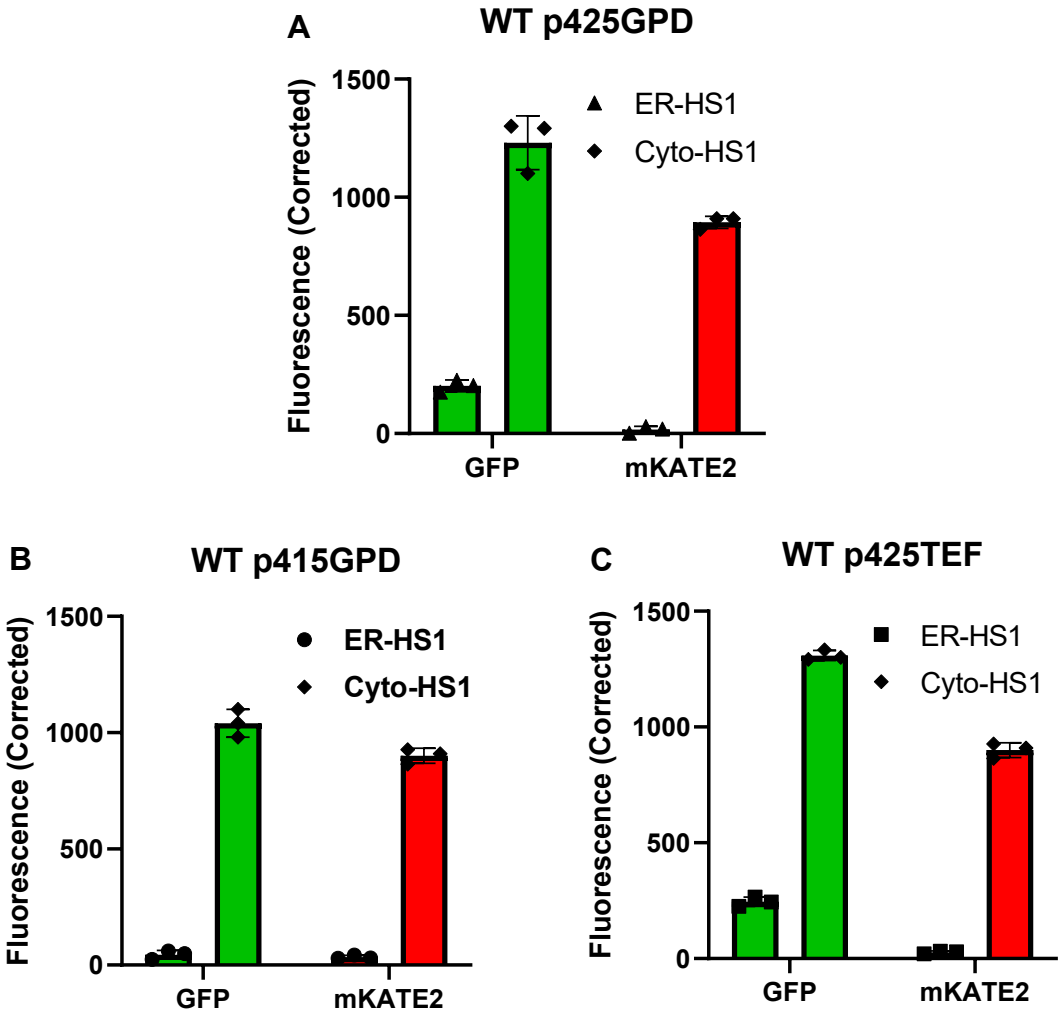

Figure 7. Tuned expression levels fail to produce well folded and fluorescent ER sensor constructs. ER-HS1 expressed on plasmids with different expression strengths (p425GPD **(A)** > p425TEF **(C)** > p415GPD **(B)**) is unable to fold sufficiently well to produce a functional protein, as indicated by lack of mKATE2 fluorescence.

The data from the tuned expression attempts across ER-HS1, ER-CG6 and ER-GFP offered certain insights into the lack of fluorescence of the sensor constructs. It was evident that varying the expression levels of the constructs did not affect their ability to fluoresce despite the theoretical ~50% and ~75% decreases in expression of p425TEF and p415GPD below that of p425GPD, respectively.[131] It

should be noted, however, that the confirmation of the differences in expression

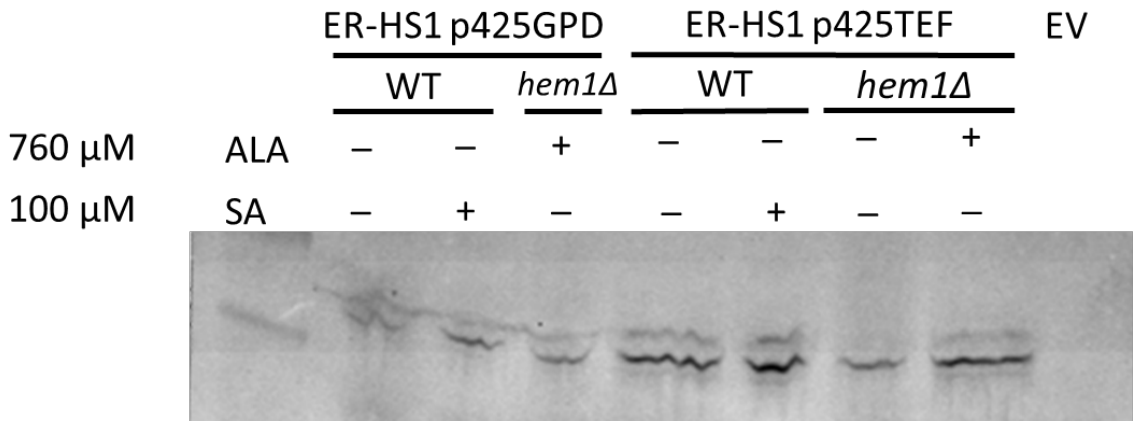

Figure 8. ER-HS1 is expressed on p425GPD and p425TEF vectors. 425G-ER-HS1 and 425T-ER-HS1 show expression in WT and *hem1Δ* backgrounds.

described by Mumberg and colleagues was based off β-galactosidase activity rather than actual protein expression, even though they mention "considerable levels of expression in *E. coli*."[131] While there is generally good correlation between enzyme expression and activity,[132] it is possible that this may not always be the case. The data obtained from Western blot analysis of the ER-HS1, ER-CG6 and ER-GFP constructs across p425GPD, p425TEF and p415GPD showed only increases in expression from p415GPD to p425TEF/p425GPD but not between p425TEF and p425GPD. Additionally, these expression differences were only observed for the ER-CG6 and ER-HS1 constructs. Another important observation was that the non-fluorescent constructs were based off the CG6 heme sensing module which has an integral fusion design wherein the primary sequence of cytochrome b_{562} is inserted into that of EGFP at loop regions. The EGFP module (along with cytochrome b_{562}) is still able to fold properly and independently, and is structurally and functionally the same as an EGFP-only construct. It appears,

however, that this integral fusion design becomes problematic with respect to folding during or after importation into the ER lumen. The reason for this folding difficulty requires further investigation. It could also be suggested that there might be a size threshold for proper folding within the ER based off the data, but this is very unlikely because the Kar2p chaperone protein (from which the signal sequence for these constructs was derived) is ER-resident, has β-sheet elements critical for its function[133] (like EGFP), and at ~74kDa, it is larger than the ER-HS1, ER-CG6 and ER-GFP constructs.

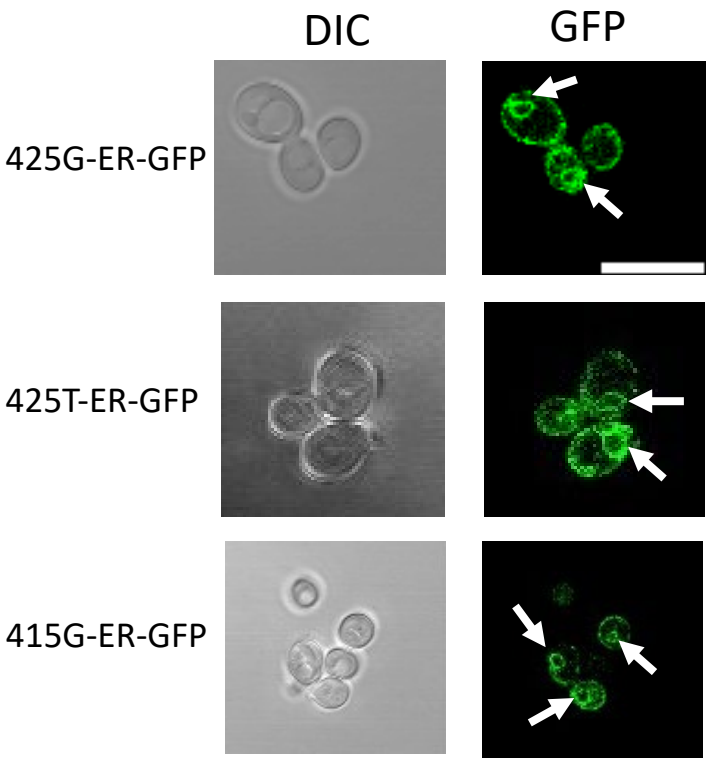

Figure 9. GFP expresses robustly in yeast ER. WT yeast expressing ER-GFP on p425GPD (top), p425TEF (middle) and p415GPD (bottom) plasmids show typical yeast ER expression pattern in the GFP channel, with cortical and perinuclear staining. White arrows point to perinuclear ER-GFP expression. Scale bar represents 10 μm and all images are the same scale.

2.2.3 Linear Fusion Ratiometric ER Sensor Constructs are Differently Problematic

Realizing that an ER-HS1 would be impossible in its current iteration, efforts were shifted to the development of linear fusion constructs. It was already known that heme bound to cytochrome b_{562} fused linearly to EGFP could serve as a FRET acceptor but not as efficiently as the integral fusion construct (~60-70% vs ~99% quenching, respectively).[134] The first designed ER-targeted heme sensor construct

in this group consisted of a cytochrome b_{562} flanked by an N-terminal EGFP and a C-terminal mKATE2 (ER-EGFP- cyt b_{562}-mKATE2). Microscopy data for this construct showed robust fluorescence and ER localization, and while there was a modest heme dose response within a narrow range for the WT strain (Figure 10A), the response of the sensor in a *hem1Δ* strain was unusual where the EGFP heme response was dose-dependent, but the mKATE2 signal in a zero-heme background was relatively high and dropped significantly (~80%) in the presence of heme (76-760 μM 5-ALA) (Figure 10B). This led to a relatively low ratio at zero heme which climbed significantly and responded modestly to heme over 76-760 μM 5-ALA (Figure 10B). Attempts to find a low dose of 5-ALA that would produce the expected sensor response without significantly increasing total heme above the zero-heme level were unsuccessful. It should also be noted that the range of sensor ratio values for the *hem1Δ* strain were relatively lower than those for the WT, a pattern uncharacteristic of the heme-dependent ratiometric relationship between EGFP and mKATE2. While the result from the WT strain for this construct might suggest the presence of mechanisms by the ER to withhold its heme load, much remains to be confirmed. Additionally, the data from the *hem1Δ* strain complicates the use of this construct for ER heme sensing.

Efforts then moved to the development of an ER-targeted heme sensor consisting of an N-terminal mKATE2 linearly fused to the EGFP module with a C-terminal cytochrome b_{562} (ER-mKATE2-EGFP-cyt b_{562}). This molecule was also fluorescent and localized to the ER but was surprisingly heme responsive in *both* the EGFP and mKATE2 channels in WT and *hem1Δ* strains, which affected its ability to be

used as a ratiometric heme sensor. The assumption was that in the final tertiary structure of this construct, the mKATE2 module was sufficiently proximal to that of cytochrome b_{562} to enable the transfer of energy to heme, since mKATE2 and heme have limited wavelength overlap with each other.

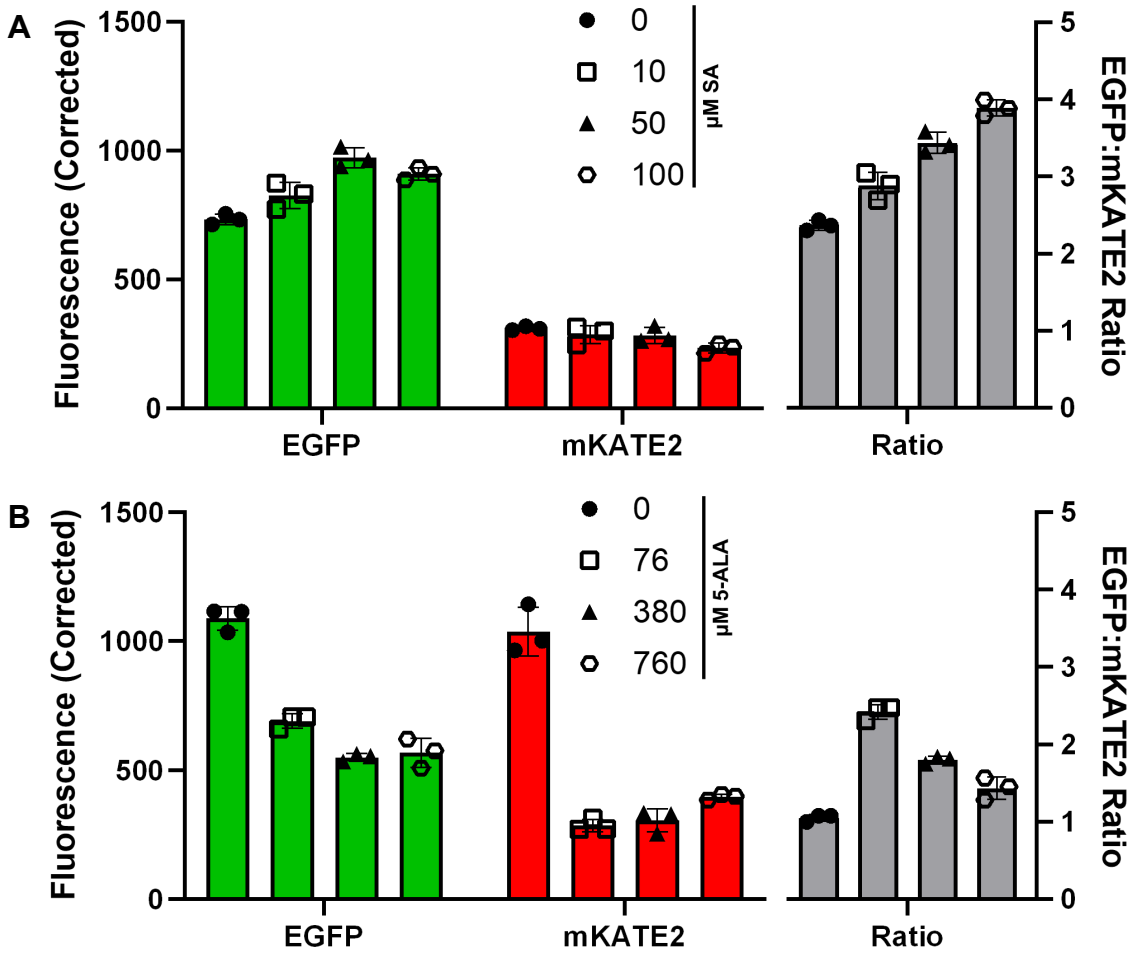

Figure 10. ER-EGFP-cyt b_{562}-mKATE2 shows an irregular heme response. ER-EGFP-cyt b_{562}-mKATE2 has an apparent heme response in **(A)** WT when titrated with SA but not in **(B)** *hem1Δ* titrated with 5-ALA.

Following from the observation on ER-mKATE2-EGFP-cyt b_{562} and the assumption for its explanation, two remedies were attempted. One action was to reduce the proximity of mKATE2 to cyt b_{562} by increasing the linker length between both modules, and the other action was to replace mKATE2 with a more red-shifted fluorescent protein to reduce the wavelength overlap between the red fluorescent protein and heme. For the linker length extension, two approaches were taken – using either a flexible or rigid linker. The flexible linker attempt added a GS (Gly-Ser) dipeptide between mKATE2 and EGFP (ER-mKATE2-GS-EGFP-cyt b_{562}) while the rigid linker addition was PAP (Pro-Ala-Pro) in the same location (ER-mKATE2-PAP-EGFP-cyt b_{562}). It should be noted that a GS linker already existed between mKATE2 and EGFP and this was not altered for either the GS or PAP addition.

The ER-mKATE2-GS-EGFP-cyt b_{562} construct in both WT and *hem1Δ* strains had similar levels of EGFP fluorescence at steady state. While it would typically be expected that the *hem1Δ* cells have higher EGFP fluorescence than the WT due to the absence of heme, the absolute fluorescence values do not reflect sensor expression, hence the necessity to normalize by mKATE2. Interestingly, the *hem1Δ* cells showed relatively higher fluorescence than the WT, leading to the WT cells having sensor ratios above those of *hem1Δ* and negating the use of this construct as a viable ER heme sensor. The ER-mKATE2-PAP-EGFP-cyt b_{562} construct also had similar EGFP fluorescence values in WT and *hem1Δ* strains but suffered from very low mKATE2 fluorescence in both strains as well. This led to

sensor ratios that were overall higher than the ER-mKATE2-GS-EGFP-cyt b_{562} constructs but similar in trend with the *hem1Δ* strains having lower ratios than the WT. Additionally, the very low mKATE2 fluorescence complicated the use of any resulting ratio values as it suggested that either sensor expression as a whole was affected or the introduction of the PAP linker disrupted mKATE2 folding specifically, perhaps by way of steric interference with the rest of the molecule. Ultimately, this complication made the ER-mKATE2-PAP-EGFP-cyt b_{562} construct a poor candidate as an ER heme sensor.

With respect to the replacement of mKATE2, the choice of mCardinal was decided upon because it has slightly red-shifted fluorescence (ex.605 nm/em.657 nm vs ex.588 nm/em.620 nm for mKATE2) and has a chromophore that is not dependent on biliverdin,[135] as biliverdin is a breakdown product of heme and its presence as a component of a heme sensor would complicate its use for the same purpose. The mCardinal construct (ER-mCard-EGFP-cyt b_{562}) was ordered commercially in p415GPD (GenScript) and then transformed into WT and *hem1Δ* cells. The ER-mCard-EGFP-cyt b_{562} construct responded similarly to its ER-mKATE2-GS-EGFP-cyt b_{562} counterpart with a modest heme response in both WT and *hem1Δ* cells, despite relatively low mCardinal fluorescence which might indicate expression difficulties. Nevertheless, regardless of heme levels, sensor ratios for this construct were similar within and between strains, suggesting the ineffectiveness of this construct for ER heme sensing.

2.2.4 Normalization Issues Affect a Heme-responsive EGFP-cytochrome b_{562} Construct

Due to the challenges experienced with trying to obtain a ratiometric linear-fusion heme sensor, a linear construct consisting of just an N-terminal EGFP and a C-terminal cytochrome b_{562} was targeted to the ER (ER-EGFP-cyt b_{562}) and transformed into WT and *hem1Δ* cells. As mentioned earlier, previous work had shown *in vitro* that this linear fusion construct could report on heme with intermediate efficiency[134] and as such, there was some confidence that it would work, although it was unclear how the yeast ER environment might affect the heme sensor. Confocal microscopy data showed that the sensor constructs were fluorescent and ER-localized in both strains. Plate reader fluorimetry indicated that in *hem1Δ* cells there was a dose-dependent decrease in EGFP fluorescence when titrated with 0-760 μM 5-ALA, but no further fluorescence changes at higher doses of 5-ALA. This suggested that endogenous heme was populating the ER but the sensor construct was unable to report on the maximum levels attained. In WT cells, an increase in EGFP fluorescence was observed between 0 and 10 μM SA but no further significant changes at higher doses of 50 and 100 μM SA. This might point to the ER having mechanisms in place to sequester heme despite a cell-wide depletion of the essential nutrient. It should be noted that this ER-EGFP-cyt b_{562} sensor construct has no normalization component hence one could not make any conclusions from the fluorescence data. Without normalization for sensor expression, it would be impossible to discern if changes in EGFP fluorescence are due to its FRET relationship with heme or differences in expression of the sensor.

Normalization for the ER-EGFP-cyt b_{562} sensor construct was approached by comparison with an ER-targeted EGFP (ER-EGFP), an ER-targeted nonheme-binding EGFP-cytochrome b_{562} (ER-EGFP- cyt b_{562}-M7A/H102A) and sensor expression as determined by Western blot against EGFP. The most consistent results were obtained by normalizing using Western blot.

In addition to the WT and *hem1Δ* cells, four other strains of interest were transformed with the ER-EGFP-cyt b_{562} sensor construct: *pep12Δ*, a target membrane receptor for vesicular intermediates traveling between the Golgi apparatus and the vacuole, *snf7Δ*, one of four subunits of the ESCRT-III complex which is involved in the sorting of transmembrane proteins into the multivesicular body (MVB) pathway, *vph1Δ*, subunit a of the vacuolar-ATPase V0 domain, and *vps36Δ*, a component of the ESCRT-II complex which is involved in interactions with ESCRT-I and ubiquitin-dependent sorting of proteins into endosomes. These mutants had been shown to have low levels of cytosolic labile heme but WT levels of total heme,[125] suggesting that heme may be trapped in the endomembrane pathway due to the loss of these proteins. Data obtained showed that *snf7Δ* and *vph1Δ* had normalized sensor values that were significantly lower and higher than WT respectively, while *pep12Δ* and *vps36Δ* fluorescence resembled that of WT (Figure 11). Treatment of all strains with 100 μM SA led to an increase in normalized EGFP fluorescence, but the trend observed at steady state remained (Figure 11). These results could suggest that heme is being held up in the earlier parts of the endomembrane pathway. The ER-EGFP-cyt b_{562} data from these

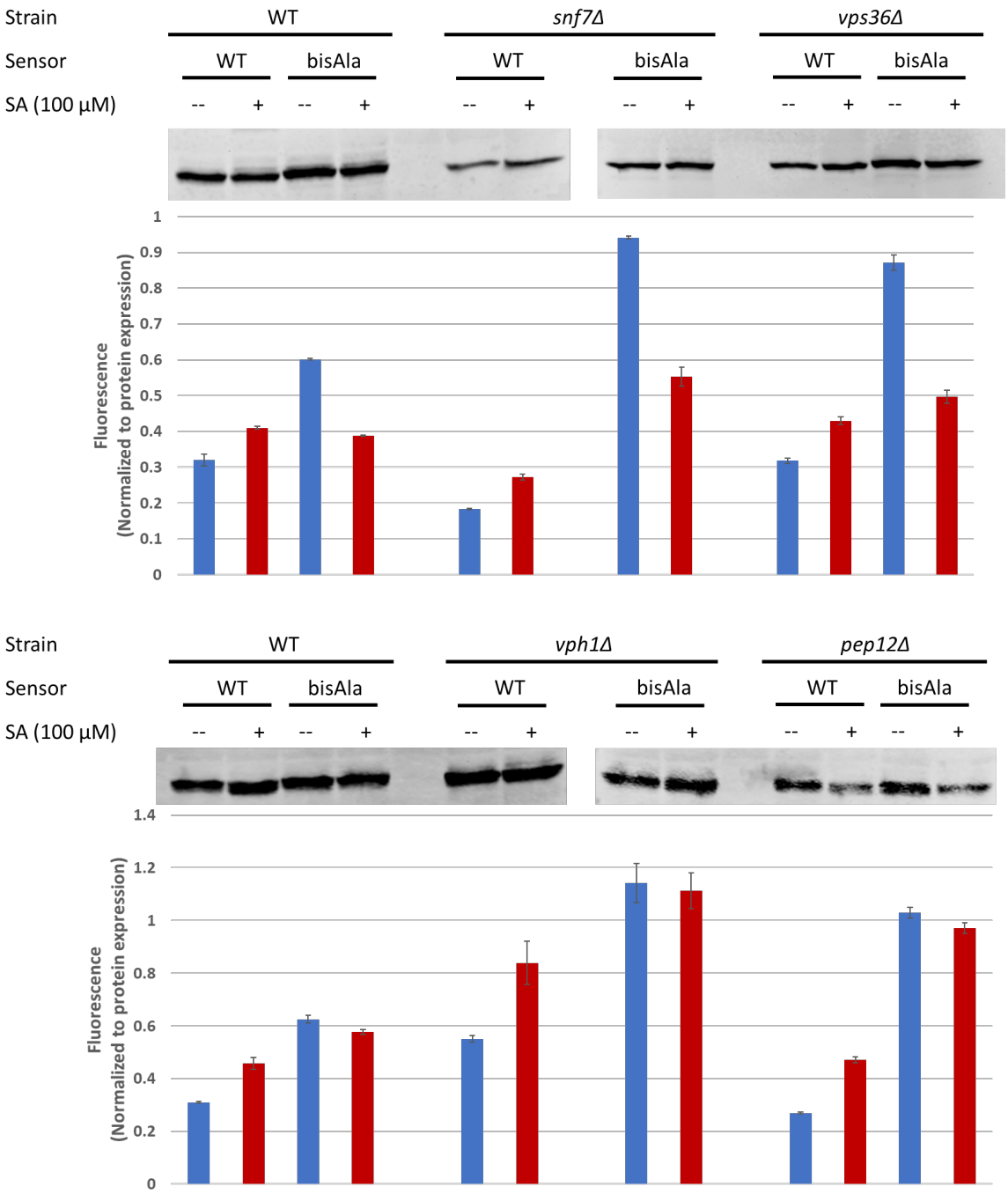

Figure 11. Inconsistent fluorescence normalization by Western blot for ER-GFP-cyt b_{562} constructs in different strains. Normalizing fluorescence of the linear fusion construct ER-GFP-cyt b562 by Western blot protein expression was problematic, particularly with respect to the nonheme-binding bisAla constructs.

knockout strains was, however, complicated by results from ER-EGFP-cyt b_{562}-M7A/H102A constructs wherein there was inconsistent normalized fluorescence

across all the constructs (Figure 11). Considering that this sensor is nonheme-binding, it would have been expected that the normalized EGFP fluorescence for all the strains would be similar if they have similar levels of labile heme. To this end, further investigation would be needed to fine-tune the use of these sensor constructs for clearer interpretation of data obtained from their use.

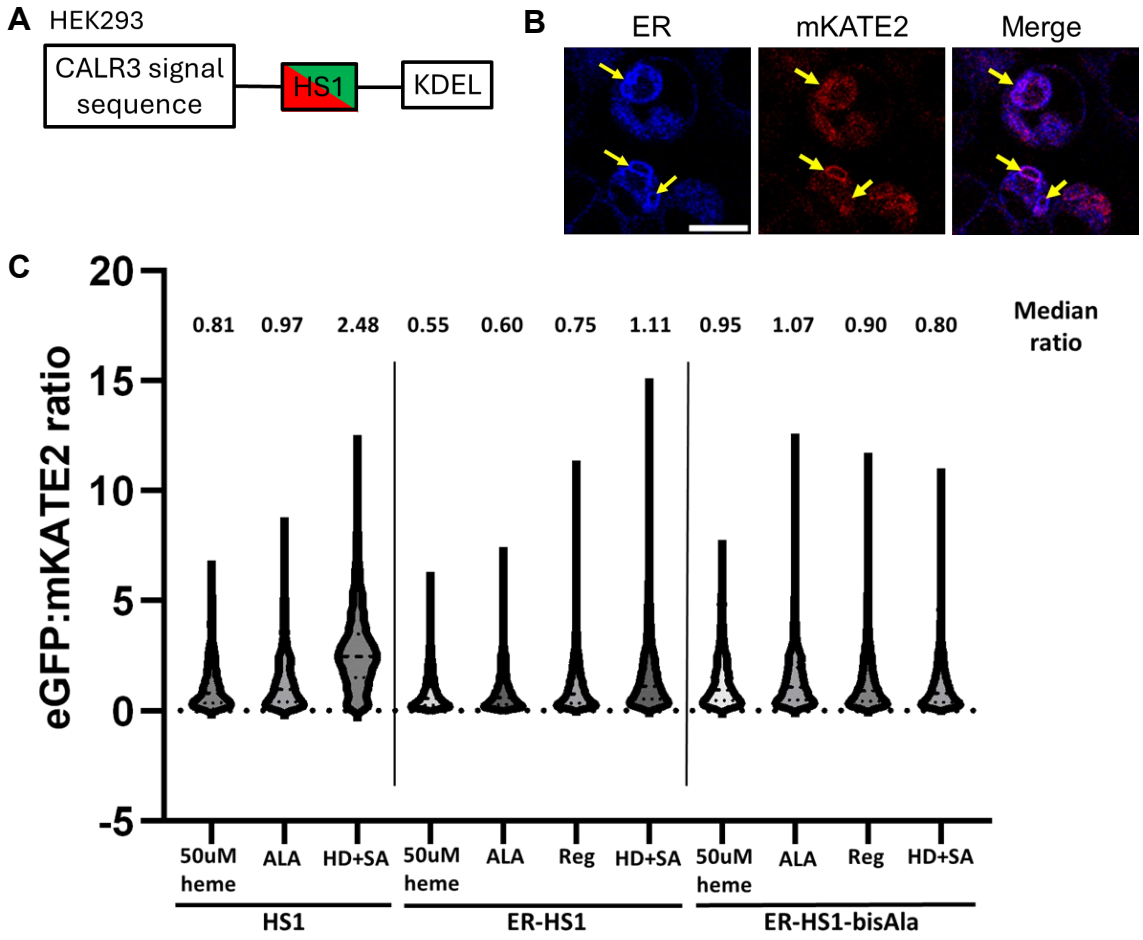

Figure 12. Human ER-HS1 is ER-localized, fluorescent and heme responsive in HEK293 cells. (A) The HS1 sensor is targeted to the ER via N-terminus calreticulin targeting and C-terminus KDEL retention sequences. **(B)** Confocal microscopy images show co-localization of ER stain with mKATE2 expression of the ER-HS1 sensor. Yellow arrows point to perinuclear ER expression. Scale bar represents 10 μm. **(C)** The ER-HS1 sensor is responsive to intracellular heme levels while a nonheme-binding construct is invariant.

2.2.5 ER-targeted HS1 is Functional in Mammalian Cells

In parallel with the attempts to obtain a functional ER heme sensor in yeast, efforts to develop a ratiometric ER heme sensor in human cells were undertaken. The design consisted of the calreticulin signal sequence fused N-terminally to HS1, together with a C-terminal KDEL retention sequence (hER-HS1) (Figure 12A). When transfected into HEK293 cells, ER localization was observed (Figure 12B) and analysis by flow cytometry revealed that it was also responsive to modulation of heme levels when treated with exogenous heme, 5-ALA, or SA in heme-depleted (HD+SA) media (Figure 12C). Additionally, an analogous construct without heme binding capacity (hER-HS1-M7A/H102A) was relatively invariant as regards heme response when given the same heme modulation treatments (Figure 12C), thereby validating the functionality of hER-HS1.

2.3 Discussion

The endomembrane system plays a crucial role in numerous cellular processes, such as processing proteins intended for export, handling proteins imported into the cell, synthesizing lipids, and facilitating various signaling activities. With respect to heme homeostasis, it is implicated in heme uptake[126,127] and there is data to suggest its involvement in mitochondrial heme trafficking. In a bid to probe the role of the endomembrane system in heme trafficking, the need for robust heme sensors localized to organelles of the pathway was identified. The ER plays an important role in the synthesis, folding, and post-translational modification of

proteins in eukaryotic cells, and strong evidence presented earlier suggests that it'll be a likely key organelle in any intracellular heme trafficking function.

Several approaches were taken to obtain a functional ER labile heme sensor in Baker's yeast. Importantly, targeting the constructs for luminal ER expression was primary. A common strategy to achieve the localization of heterologously expressed proteins in model systems is the use of signal sequences of proteins native to the organelle of interest. In this case, the 42-amino acid signal sequence of the ER-resident protein folding chaperone Kar2p (BiP) was chosen (Kar2-ss), with the addition of the C-terminal HDEL ER retention signal.[136] Kar2-ss has been successfully utilized in several yeast studies to target different sized constructs to the ER.[137–141] Despite this, could the choice of ER signal sequence have affected the proper targeting of the ER-HS1-ox, ER-HS1 and related constructs? For the ER-HS1-ox constructs, the only readout of targeting and proper folding in this work was fluorescence, while Western blots were additionally carried out for the ER-HS1 (and related) constructs. If *only* improper targeting was implicated, then the sensors would have accumulated in the ER (due to the retention tag), leading to ER stress, and been marked for degradation.[142,143] Depending on the rate of degradation, it might have been possible to observe fluorescence above background of the accumulated sensors, but this was never the case. In fact, the Western blot data for ER-HS1 and ER-CG6 confirm their expression, and this, together with the observation of proper ER-GFP localization and fluorescence, suggests that the non-fluorescence issue involved the improper folding of ER-HS1 and ER-CG6 in the ER lumen, i.e., the integral positioning of cytochrome b_{562}

within the EGFP primary sequence, hindered the formation of the β-barrel structure required for maturation of the EGFP chromophore.[144] It would be interesting, however, to probe the cells expressing non-functional ER sensors for markers of ER stress response[142,145] to further clarify their fates and confirm this finding.

Again, despite the apparent successful targeting of the ER-GFP and linear ER constructs, another interesting point to clarify would be the actual cleaving of the signal peptide from the constructs, as their presence could be deleterious to the cell.[146] In the design for the ER-targeted yeast constructs, the terminal amino acids of Kar2-ss (VRG), which serve as the recognition sequence for signal peptidase,[147] immediately precede the first amino acid of the mature protein construct (fig). In yeast and higher eukaryotes, it is unclear if there are any recognition features of importance beyond the C-terminus of the signal peptide, though these might be important in bacteria.[148] Indeed, a comparison of the mature protein N-terminal amino acids of selected ER-resident proteins in *S. cerevisiae* shows a lack of pattern (fig), suggesting that they might hold no relevance regarding the site of signal peptidase cleavage. A size comparison of mature ER-HS1 and cytosolic HS1 should provide a definitive answer to the *in vivo* intralumenal processing of ER-HS1, since both are of equivalent size (~65 kDa) vs an ER-HS1 still tagged with Kar2-ss (~70 kDa).

There have been reports whereby the oxidizing environment of the ER acted upon solvent-exposed cysteines of fluorescent proteins, leading to their oligomerization and thereby affecting their functionality.[128,149] Suzuki and colleagues successfully designed cysteine-free fluorescent proteins (SGFP2 and mKATE2) that retained

their brightness within the ER and inspiration was drawn from their work.[128] It should be noted that while the work by Suzuki et al. was conducted in the COS-7 mammalian cell line and on strongly enhanced GFP (SGFP2)[150], the attempts here were for EGFP expression in *S. cerevisiae*. Despite the potential for effects of species differences, it was believed that it was a worthwhile attempt even though targeting of EGFP constructs to the ER had been demonstrated in yeast either as a stand-alone module[130] or as a tag to another protein.[129] To some extent, it is possible that the non-fluorescence observed for the ER-HS1-ox constructs could be attributable to folding issues within the ER already described, but it is unclear if this would extend to the ER-mKATE2-ox constructs as they are single-domain fluorescent proteins without any integral fusion elements. Interestingly, a cytosolic CG6 with the “oxidation stabilizing” mutations applied to C48 and C70 of the EGFP scaffold was also non-fluorescent above background levels, suggesting that those changes were not tolerated for *in vivo* expression in *S. cerevisiae*. *In vitro* studies have shown the spectroscopic properties of C48S and C70S to be like that of native EGFP, with increased susceptibility of both mutants to aggregation in *E. coli* and the C70S mutant being less folding-efficient and denaturation-resistant.[151] This highlights both the importance of C48/C70 to EGFP stability and functionality, as well as the tolerability of any changes in those positions in different species.

It was interesting to observe the relative ease of expression of ER-HS1 in HEK293 cells whereas significant difficulties were experienced for the same construct in *S. cerevisiae*. As earlier mentioned, there did not appear to be a size threshold for heterologous expression using the Kar2-ss, as the linear fusion constructs

containing the EGFP and mKATE2 modules displayed proper ER localization and fluorescence, suggesting once again some inherent deficiency of the yeast system to properly fold the integral fusion constructs. One difference between the yeast and human ER-HS1 constructs is the signal sequence used, wherein the calreticulin signal sequence was used for the human ER-HS1 in HEK293 cells. While *S. cerevisiae* does not possess a calreticulin homolog, it does express calnexin (CNE1) which is the yeast homolog. It has been shown that different signal sequences can affect secretory protein production in yeast,[152] but it is unclear if this is relevant for *in vivo* protein expression within an organelle. In general, mammalian cells perform better for the expression of more complex proteins, outscoring yeast in improved folding and assembly of large polypeptides.[153] This is quite understandable, considering that the human proteome, for instance, is about three times the size that of yeast. One strategy that might be helpful for *S. cerevisiae* in this regard is the co-expression of the heterologous protein of interest with folding chaperones to alleviate increased protein expression and quality control pressure.[154,155] Indeed, this method has been shown to be effective for GFP-tagged aggregation-prone constructs in *E. coli*.[156] Culturing at sub-physiological temperature has also been shown to be effective for improved expression of difficult-to-express proteins in *S. cerevisiae*,[157] and is another tenable suggestion.

2.4 Materials and Methods

2.4.1 E. coli strains, Media, and Growth Conditions

For routine cloning, sub-cloning grade chemically competent *E. coli* cells, strain 10G *E. cloni* (Lucigen), were used according to the manufacturer's specifications. For site-directed mutagenesis, high efficiency grade chemically competent *E. coli* cells, strain 10G DUOS *E. cloni* (Lucigen), were used according to the manufacturer's specifications. All *E. coli* strains were cultured in Lysogeny broth (LB) with appropriate antibiotic selection (50 µg/mL ampicillin) at 37 °C.

2.4.2 Yeast Strains, Transformation and Growth Conditions

S. cerevisiae strains used in this work were derived from BY4741 (*MATa, his3Δ1, leu2Δ0, met15Δ0, ura3Δ0*). The *hem1Δ* (*hem1::HIS3*) strain DH001b-3 has been previously described.[91] Yeast transformations were performed according to the lithium acetate procedure.[158] Strains were maintained at 30 °C on either enriched yeast extract-peptone based medium supplemented with 2 % glucose (YPD) or synthetic complete medium (SC) supplemented with 2% glucose, with appropriate amino acids to maintain selection. Culturing of *hem1Δ* cells required supplementing YPD or SC media with 50 µg/mL 5-aminolevulinic acid (5-ALA), or 15 mg/mL ergosterol and 0.5% Tween-80 (YPDE or SCE, respectively).[159] Growth in liquid culture was for ~14-16 h overnight. Cells cultured on solid media plates were done with the appropriate media supplemented with 2% agar.

2.4.3 Human Embryonic Kidney (HEK293) cells, Media, and Growth Conditions

HEK293 cells were cultured and treated as described elsewhere.[160] Briefly, HEK293 cells (ATCC Cat. # CRL-1573) were plated and transfected in 6-well sterile plates (Corning) for flow cytometry, and in 35 mm glass bottom dishes (MatTek) for microscopy experiments. The cells were plated in basal growth medium (Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum). Groups designated as HD + SA were grown in DMEM containing heme-depleted serum (10%) and 0.5 mM succinyl acetone (SA), an inhibitor of heme biosynthesis. All cells were maintained at 37 °C and 5% CO_2.

2.4.4 Plasmids

The "oxidation-stable" ER-HS1 (ER-HS1-ox) was obtained commercially (SynBio Technologies) as a codon-optimized construct on a pUC57-Amp plasmid and was then subcloned into p415GPD and p425GPD[131] using appropriate restriction sites. ER-mKATE2-ox was made from ER-HS1-ox by amplifying the Kar2-ss and mKATE2 region with a C-terminal HDEL sequence, and subcloned into p415GPD and p425GPD. CG6-ox was made from ER-HS1-ox by amplifying the CG6 region without the C-terminal HDEL sequence, and subcloned into p425GPD. 425-ER-HS1 was made by amplifying the Kar2-ss from yeast genomic DNA (ER targeting sequence), HS1 from pDH013[91] with a C-terminal HDEL sequence (ER retention sequence), and ligating both into p425GPD. 425-ER-CG6 was made by amplifying the Kar2-ss from yeast genomic DNA, CG6 from pDH005[91] with a C-terminal HDEL sequence, and ligating both into p425GPD. 425-ER-GFP was made by amplifying

the Kar2-ss from yeast genomic DNA, EGFP from pID010 (this thesis) with a C-terminal HDEL sequence, and ligating both into p425GPD. The p415GPD and p425TEF[131] ER-HS1, ER-CG6 and ER-GFP constructs were made by amplifying out the inserts from the p425GPD plasmids and ligating them into the respective vectors.

2.4.5 Instrumentation and Data Analysis

All growth measurements ($OD_{600\ nm}$) and UV/visible absorbance readings/spectra were recorded on a Cary 60 spectrophotometer (Agilent Technologies). Fluorescence measurements were collected on a Biotek Synergy Mx multi-modal plate reader (Agilent Technologies).

Flow cytometric measurements were performed using a BD FACS Aria IIIu Cell Sorter or BD LSRFortessa flow cytometer, both equipped with an argon laser (ex 488nm) and yellow-green laser (ex 561nm). EGFP was excited using the argon laser and was measured using a 530/30nm bandpass filter. mKATE2 was excited using the yellow-green laser and was measured using a 610/20nm bandpass filter. Data evaluation was conducted using FlowJo v10.9.3 software. The number of cells measured per experiment was set to 50,000 – 100,000 unless otherwise stated. Only mKATE2 positive cells were selected for analysis.[160]

Confocal laser scanning microscopy was accomplished using a Zeiss ELYRA LSM 780 super-resolution microscope or a Zeiss LSM 710 NLO confocal microscope configured for live cell imaging. Both were equipped with a 63x, 1.4 numerical aperture oil objective. EGFP was excited with the 488 nm line of an argon ion laser,

while mKATE2 was excited using the 594 nm line of a HeNe laser. The 494-571nm and 606-686 nm band pass filters were used to filter emission for EGFP and mKATE2, respectively. Images were collected using Zeiss software and analyzed with ImageJ v1.54f. Ratio images were generated using ImageJ as described.[160]

2.4.6 Characterization of Heme Sensors in Yeast

For all sensor fluorescence measurements, WT and *hem1Δ* yeast cells expressing the heme sensors were cultured in SCE-LEU media for ~14-16 hours (overnight) to mid-exponential phase (an optical density at 600 nm ($OD_{600\ nm}$) of ~ 1-2). Unless otherwise noted, all measurements were accomplished with the indicated sensor proteins expressed on the p415-GPD plasmid, a low copy centromeric (CEN) plasmid with a GPD promoter.[131] Modulation of heme levels was done by supplementing triplicate biological samples with 5-ALA or SA (as appropriate, to designated final concentrations) at the beginning of overnight culture. After culturing, cells were harvested, washed in water, and resuspended in phosphate buffered saline (PBS) solution at 5 $OD_{600\ nm}$/mL, or 1 × 10^8 cells/mL. For fluorimetry measurements on a population of cells, fluorescence was recorded on a Synergy Mx multi-modal plate reader using black Greiner Bio-one flat bottom fluorescence plates. EGFP and mKATE2 fluorescence were recorded using excitation and emission wavelength pairs of 488 nm and 510 nm, and 588 nm and 620 nm, respectively. Background fluorescence of cells not expressing the heme sensors were recorded and subtracted from the EGFP and mKATE2 fluorescence values. Heme sensor fluorescence ratios were monitored by recording the ratio of EGFP

to mKATE2 fluorescence values. For microscopy, cells were prepared as previously described.[160]

2.4.7 Characterization of Heme Sensors in HEK293 cells

HEK293 cells were plated on day 0 at 30-50% confluency in basal growth medium (Dulbecco's modified Eagle medium (DMEM) containing 10% fetal bovine serum). On day 1, growth medium either remained as basal or was changed to HD + SA before transfection. Cells were then transfected as described[160] but using 1.5 µg of plasmid, 2.5 µL of Plus reagent (Invitrogen) and 5 µL of Lipofectamine LTX (Invitrogen) per well. Forty-eight hours after transfection, groups designated as Reg + 5-ALA were given fresh basal growth medium and treated with 760 µM 5-ALA for 24 h. Groups designated as Reg + Heme remained in basal growth medium for 72 h after transfection and were supplemented with 50 µM hemin 1 h before cell harvest. HEK293 cells transfected without plasmid were used as a negative control for fluorescence. For flow cytometry, cells were detached from the plate with PBS, washed and resuspended in the same, then filtered into 5 mL round-bottom tubes with 35 µm cell strainer cap. For microscopy, cells were gently washed with pre-warmed (37 °C) PBS and wash solution replaced with the same for viewing. ER sensor localization was confirmed by co-localization with ER-Tracker™ Blue-White DPX (Invitrogen).

2.4.8 Immunoblotting

Yeast were cultured in 5-10 mL of SCE-LEU media as described above. Cells (10 OD_{600} pellet or 2×10^8 cells) were harvested, washed in deionized water, and lysed

in 100 µL of PBS supplemented with protease inhibitors and PMSF as described previously.[161] Lysis was achieved at 4 °C using one pellet volume of 0.5 mm zirconium oxide beads (Next Advance) and a bead beater (Bullet Blender, Next Advance) on a setting of 8 for two rounds of 3 minutes each. Lysate protein concentrations were determined by the Bradford method and 50 µg of protein lysate samples were prepared for SDS-PAGE, boiled at 100 °C for 5 min, and briefly centrifuged. Samples were resolved on 14% tris-glycine gels (Invitrogen) in tris-glycine SDS buffer at room temperature at a constant 20 mA. After resolving, proteins were transferred onto nitrocellulose membranes overnight (~16 – 18 h) at 4 °C at 20 mA, and all remaining steps were conducted at room temperature. Following the overnight transfer, membranes were first normalized using Revert™ 700 Total Protein Stain (LI-COR Biotech) according to the manufacturer's instructions, then blocked for 1 h with Intercept (TBS) Blocking Buffer (LI-COR Biotech), followed by primary antibody incubation (rabbit anti-GFP polyclonal antibody (Genetex) diluted 1:5000 in blocking buffer), both rocking. After two, 10-min washes were performed using 1X TBST (TBS with 0.1% Tween-20), membranes were incubated for 1 h with secondary antibody (goat anti-rabbit conjugated to a 680 nm emitting fluorophore (Biotium Cat. # 20067), diluted 1:10000 in blocking buffer) and given another two, 10-min washes in 1X TBST. Lastly, membranes were imaged on an Odyssey® CLx (LI-COR Biotech) imager and analyzed by Image Studio™ (LI-COR Biotech) or ImageJ v1.54f.

CHAPTER 3. EXPANDING THE HEME SENSOR COLOR PALETTE

3.1 Introduction

The necessity of heme for aerobic life requires that it is either synthesized *de novo*, or in some species and under certain conditions, acquired from the environment to be used intact or as a source of iron. In metazoa, where cells are not in opportunistic relationships with each other, it is unclear whether there is a need for such heme import and export mechanisms, especially since these cells possess heme oxygenase systems to degrade heme and can employ this as a means of intracellular heme regulation.[162] However, it is known that certain cell and animal systems can uptake and utilize exogenous heme in conditions of heme deficiency.[163–165] Additionally, the discovery of putative plasma membrane heme transport proteins suggests that the maintenance of heme homeostasis may involve more than just synthesis and degradation mechanisms.[113] Heme import mechanisms may involve heme transporters like HRG1 and FLVCR2, receptor-mediated endocytosis of hemoproteins, and alternative pathways involving lipoproteins and microparticles. The primary players implicated in heme export include FLVCR1a, MRP5/ABCC5 and ABCG2.

HRG1 is one of the most studied heme transporters, initially identified in the heme auxotroph *Caenorhabditis elegans*, which relies entirely on environmental heme due to its inability to synthesize heme.[166,167] HRG1 and its paralog HRG4 mediate

the uptake of heme in *C. elegans*, with HRG4 localized on the apical membrane facilitating direct heme absorption from the intestinal lumen,[167] and HRG1 residing in endolysosomal compartments to transport heme into the cytosol.[124] HRG1's function is tightly coupled with the vacuolar proton ATPase pump, interacting in a pH-dependent manner to facilitate heme binding and translocation.[168] In mammals, HRG1 is highly conserved and it is found highly expressed in macrophages of the reticuloendothelial system (RES). During erythrophagocytosis, HRG1 imports heme into the cytosol, allowing for its subsequent degradation by heme oxygenase. This process is crucial for recycling iron from senescent red blood cells.[113] HRG1-deficient mice accumulate hemozoin crystals, highlighting the essential role of HRG1 in preventing toxic heme accumulation.[169] Additionally, HRG1 is expressed in other tissues, including the brain, intestine, kidney, and smooth muscle, though its precise functions in these organs remain under investigation.[170]

FLVCR2 is another protein postulated as a heme importer, associated with Fowler syndrome, a rare proliferative vasculopathy of the brain. The substrate specificity and cellular localization of FLVCR2, however, have recently become the subject of debate. Earlier studies showed that cells expressing FLVCR2 exhibited increased heme uptake and heightened sensitivity to heme toxicity, while FLVCR2 silencing reduced heme import,[171] suggesting a role for plasma membrane heme import. In later *in vitro* work, heme binding was determined to occur via the N-terminal domain of FLVCR2 but the protein was found to be mitochondrially located and involved in switching ATP production to thermogenesis in response to

heme.[172] Interestingly, unlike HRG1 and HRG4, complementation of heme-deficient yeast strains with FLVCR2 did not restore growth,[173] indicating neither a role in heme transport nor plasma membrane localization. Indeed, more recent studies suggest FLVCR2 to be a plasma membrane choline and ethanolamine importer, with critical functions at the blood-brain barrier.[174–176] These conflicting reports mean that more work is needed to delineate the function(s) and localization(s) of FLVCR2, with considerations of the choice of experimental methods and conditions, as well as species and cell-type differences.

Heme import is not solely reliant on dedicated heme transporters. Receptor-mediated endocytosis of hemoproteins provides another critical pathway for heme acquisition, especially during states of increased heme release, such as hemolysis or cellular injury. Key serum proteins, including hemopexin and haptoglobin, bind free heme and hemoglobin, respectively, forming complexes that are recognized and internalized by specific cell surface receptors like CD163 (for hemoglobin-haptoglobin)[177] and LRP1/CD91 (for heme-hemopexin).[178] These receptors are predominantly expressed in macrophages and hepatocytes, playing pivotal roles in heme clearance, iron recycling, and protecting tissues from heme-induced oxidative damage. Additionally, heme-bound microparticles released from erythrocytes, particularly under conditions such as sickle-cell disease, are highly inflammatory and can be taken up by endothelial cells through endocytic pathways involving Rab5.[179,180] This mechanism adds to the diverse array of heme import pathways that ensure cellular heme homeostasis and prevent heme toxicity. Lipoproteins such as low-density lipoproteins (LDL) and high-density lipoproteins

(HDL) can also bind free heme in the bloodstream, facilitating its uptake into cells. Despite the presence of high-affinity heme scavengers like hemopexin, heme can partition into these lipoproteins, which are then internalized via endocytosis. Once inside the acidic endosomal compartments, heme is released and can be utilized or degraded.[181] This route of heme uptake highlights the adaptability of cells in managing extracellular heme and underscores the multifaceted nature of heme import mechanisms.

FLVCR1a, initially identified as a receptor for feline leukemia virus, has been described to function as a plasma membrane heme exporter. It plays a crucial role in managing intracellular heme levels by regulating its efflux from the cytosol to the extracellular environment and maintaining proper heme synthesis.[182,183] While the function of FLVCR1a is particularly vital in erythroid precursors,[184–186] it is also believed to be of importance in other tissues like the liver and nervous system where it is found to be highly expressed.[32,187,188] FLVCR1a is believed to primarily serve as a cellular protection mechanism against heme toxicity, for instance, by exporting heme from macrophages during the process of erythrophagocytosis. Notably, FLVCR1a's heme export efficiency is significantly enhanced (>100-fold) in the presence of hemopexin, an extracellular heme scavenger with which it interacts.[189] Emerging research also suggests that FLVCR1a-mediated heme metabolism regulation is critical for meeting the energy demands of highly proliferative cells, supported by elevated FLVCR1a expression during embryonic development and in cancer cells.[190,191] Additionally, like FLVCR2, recent studies have uncovered a role for FLVCR1a in choline transport, with important roles in

mitochondrial function and murine embryogenesis.[192–194] Further research is therefore needed to provide explanations for the varied findings.

MRP-5/ABCC5, a member of the ABC transporter superfamily, plays a crucial role in heme export across various species. Initially discovered in *C. elegans*, this protein has been found to be conserved in vertebrates, including zebrafish and mammals. In worms, MRP-5 is located on the basolateral membrane of intestinal cells, while in vertebrates, it is present on the plasma membrane, Golgi complex, and recycling endosomes.[54] The importance of MRP-5 is highlighted by the severe consequences of its deficiency: embryonic lethality in worms due to heme export failure and subsequent heme deficiency in extra-intestinal tissues, and severe anemia in zebrafish when MRP-5 is knocked down.[54] Interestingly, despite its near-ubiquitous expression in mammals, MRP-5 knockout mice do not exhibit obvious phenotypes, suggesting potential compensatory mechanisms.[54] One such mechanism may involve MRP-9, a close relative of MRP-5, which has been implicated in maintaining heme homeostasis, particularly in the testes and mitochondrial metabolism.[195]

ABCG2, also known as the breast cancer resistance protein (BCRP), is a versatile member of the ATP-binding cassette transporter family. This protein has been suggested as a key player in heme and porphyrin transport, alongside its role in multidrug resistance. ABCG2 is expressed in various tissues, including the placenta, brain, small intestines, ovary, liver, and hematopoietic stem cells, as well as in numerous cancer cell types.[196] Its ability to export heme to serum albumin suggests a protective function against heme accumulation.[197] Notably, ABCG2-

deficient mice exhibit protoporphyria, characterized by a significant increase in protoporphyrin within erythrocytes, and show extreme photosensitivity when exposed to pheophorbide, a chlorophyll-breakdown product structurally similar to protoporphyrin.[198] Recent structural studies using cryoelectron microscopy have provided insights into the conformational changes and transport mechanisms of ABCG2,[199] enhancing our understanding of its function in porphyrin efflux and cellular protection.

The existence of these known and putative heme import and export factors suggests that heme may be shuttled intercellularly, perhaps as a signal of nutritional or metabolic status. This opens up new and exciting areas of research and allows us to broaden our overall understanding of heme homeostasis within and across species. Such intercellular trafficking of heme might be important in various complex multicellular microenvironments where heme is not readily available, such as at the host-pathogen interface, the gut microbiome, or within complex organs like the brain where multiple cells must cohabitate and function in concert. Regardless of the specific reasons for intercellular heme exchange, it is important to have the right tools to probe these processes simultaneously in different cell populations for real-time, dynamic monitoring of heme levels. However, the availability of such tools is currently limited. For instance, the HS1 labile heme sensor developed by the Reddi group offers only an EGFP heme-sensing fluorescence readout, thereby restricting its utility in this regard. Expanding the heme sensing and imaging toolkit with HS1-type sensors of different colors (fluorescence) was therefore embarked upon to complement the

prototype and offer a solution for multiplexing applications. Indeed, in addition to intercellular heme imaging, such tools could also be effective in elucidating inter-organellar heme trafficking at physiological timescales, and generally offer researchers multiplexing options with other fluorescent tools for nonheme-related studies. In this chapter, the development and application of an enhanced cyan fluorescent protein (ECFP) HS1-type sensor is described, as a proof-of-concept for the expansion of the heme sensor color palette.

3.2 Results

3.2.1 Development of an HS1-type Sensor based off ECFP

Early work from the research group of Roger Tsien had detailed the necessary mutations required to blue-shift the fluorescence profile of EGFP to cyan (G65T/Y66W/N149I/M153T/V163A),[200] and these were effected onto a previously described yeast cytoplasmic HS1 plasmid.[49] Surprisingly, the resulting construct was observed to be non-fluorescent. Initial troubleshooting attempts primarily involved assessing the individual and combined effects of the core chromophore mutations (G65T and Y66W), followed by the addition of the other enabling mutations (N149I/M153T/V163A). When none of these produced a functional construct, efforts were directed towards varying the linker length between mKATE2 and ECFP-CG6 (CG6 with the eCFP mutations made to the EGFP module), as it had been observed that a standalone ECFP-CG6 construct was indeed fluorescent. The template construct possessed a GS (Gly-Ser) linker, and the variations made were shortened (G) and extended (double and triple GS (GSGS

and GSGSGS, respectively)) in nature. While the shortened linker construct was non-fluorescent, both extended linker variants similarly exhibited robust fluorescence above background levels. Further characterization was performed on the GSGS construct (hereafter referred to as ECFP-HS1, Figure 13) as it structurally deviated less from HS1 than the GSGSGS construct.

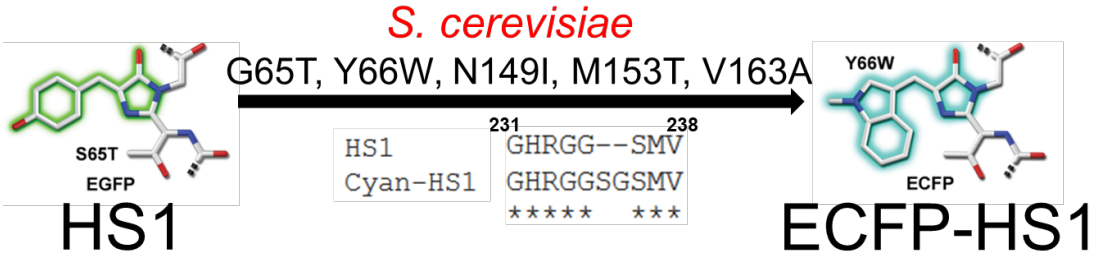

Figure 13. Development of yeast ECFP-HS1. ECFP-HS1 on the yeast vector p415GPD was made by mutagenesis (G65T/Y66W/N149I/M153T/V163A) to the CG6 scaffold of HS1 and a two-amino acid extension (GS) of the mKATE2-CG6 linker.

3.2.2 ECFP-HS1 is Functionally Similar to HS1

The ECFP-HS1 construct was transformed into WT and *hem1Δ* yeast and the sensor fluorescence ratios in these strains were characteristically low and high (respectively) (Figure 14A), comparable to readouts obtained with the canonical HS1 sensor.[49] Heme-dependent responses were also observed when the *hem1Δ* and WT strains were titrated with 5-ALA and SA, respectively (Figure 14B,C). It was also seen that data from confocal microscopy corroborated our findings from plate reader fluorimetry (Figure 15), indicating that ECFP-HS1 could serve as a bona fide heme sensor in *S. cerevisiae*.

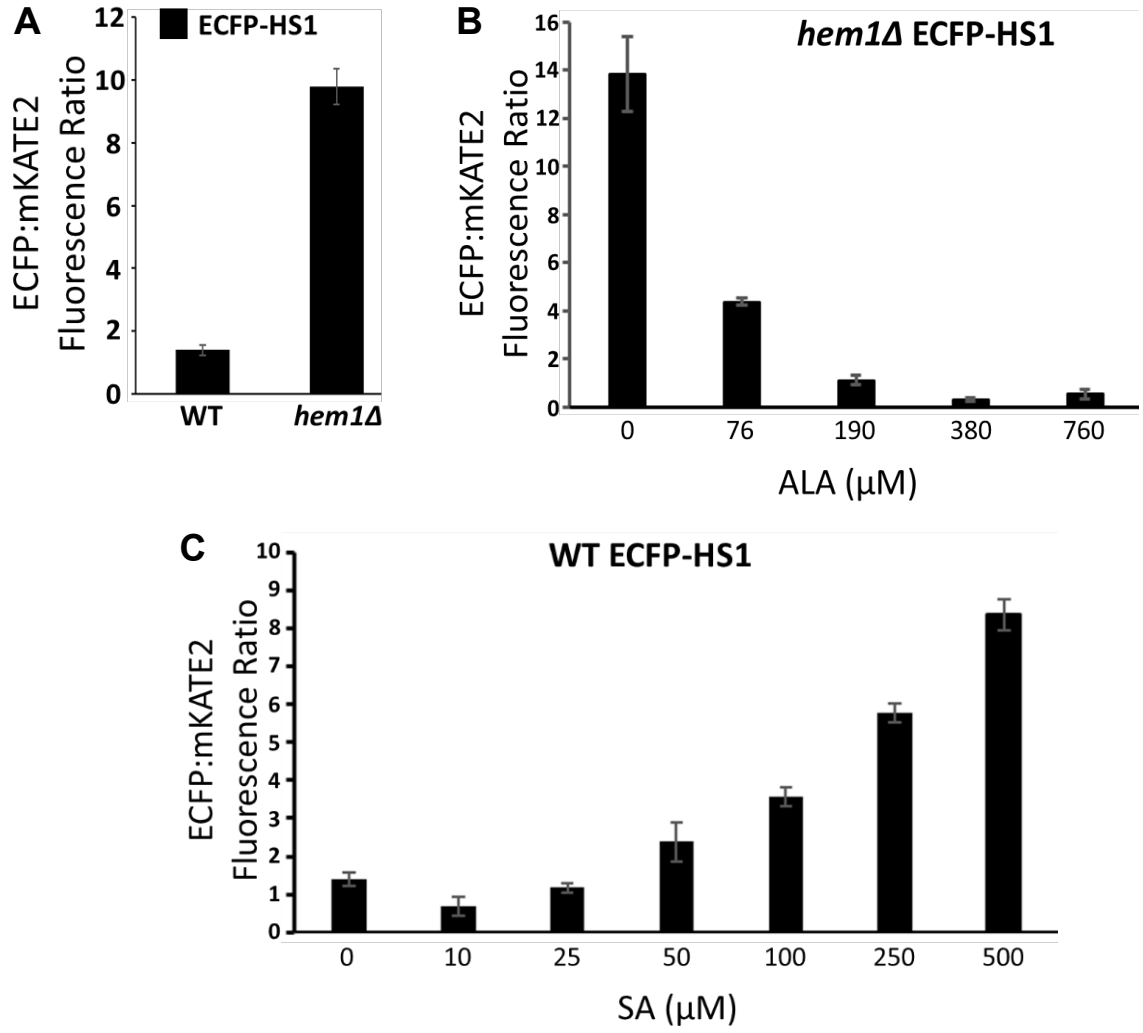

Figure 14. ECFP-HS1 is heme responsive. (A) When expressed in WT cells, ECFP-HS1 gives a low ECFP:mKATE2 sensor ratio, indicative of high heme levels, and the sensor in *hem1Δ* yeast gives a high ratio readout indicative of a heme depleted state. (B) Titration of *hem1Δ*-ECFP-HS1 yeast cells with 5-ALA shows a dose-dependent decrease in sensor ratio that corresponds to increasing cytosolic heme levels. (C) WT ECFP-HS1 treated with increasing doses of SA give incrementally rising sensor ratios that point to decreasing intracellular heme concentrations.

3.2.3 ECFP-HS1 is Spectrally Distinguishable from HS1 in Mixed Cell Populations

To use both the prototype HS1 sensor and ECFP-HS1 simultaneously for dual (multiplexing) imaging applications, it was necessary to adjust the fluorescence acquisition settings. This was because there is sufficient overlap between the ECFP and EGFP excitation and emission spectra. For plate reader fluorimetry, this was effected by slightly red-shifting the EGFP parameters wherein excitation was increased from 488 nm to 500 nm, and emission from 510 nm to 525 nm. In this way, it was possible to spectrally distinguish between WT and *hem1Δ* strains expressing different constructs (either ECFP-HS1 or HS1) (Figure 16A,B), suggesting that the sensors could be used for qualitative assessment of heme

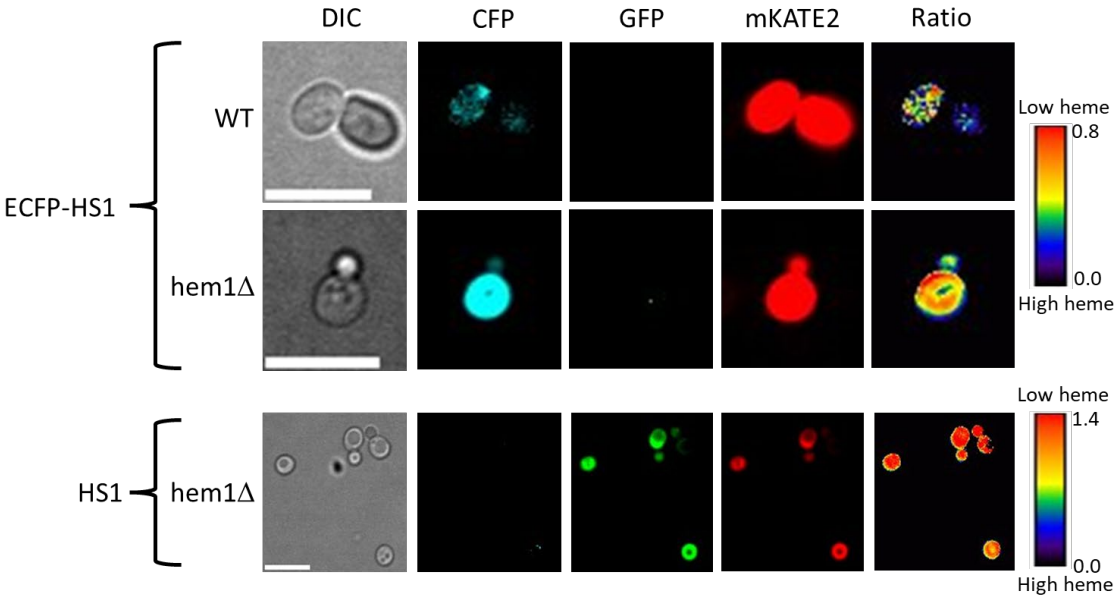

Figure 15. Heme-dependent ECFP-HS1 fluorescence ratios can be measured by confocal microscopy. Determination of sensor ratios can be achieved by confocal microscopy as shown by WT and *hem1Δ* yeast cells expressing the ECFP-HS1 sensor (top and middle panels). HS1-expressing *hem1Δ* cells are shown for comparison (bottom panel). Scale bar represents 10 μm.

levels in different cell types by this method. The application was further extended to a mixed population of cells with differing heme levels and expressing either ECFP-HS1 or HS1. After data collection by confocal microscopy, spectral unmixing algorithms were applied to the images to deconvolute the ECFP and EGFP signals, and thereby achieve semi-quantitation of heme levels for the mixed cell populations (Figure 16C).

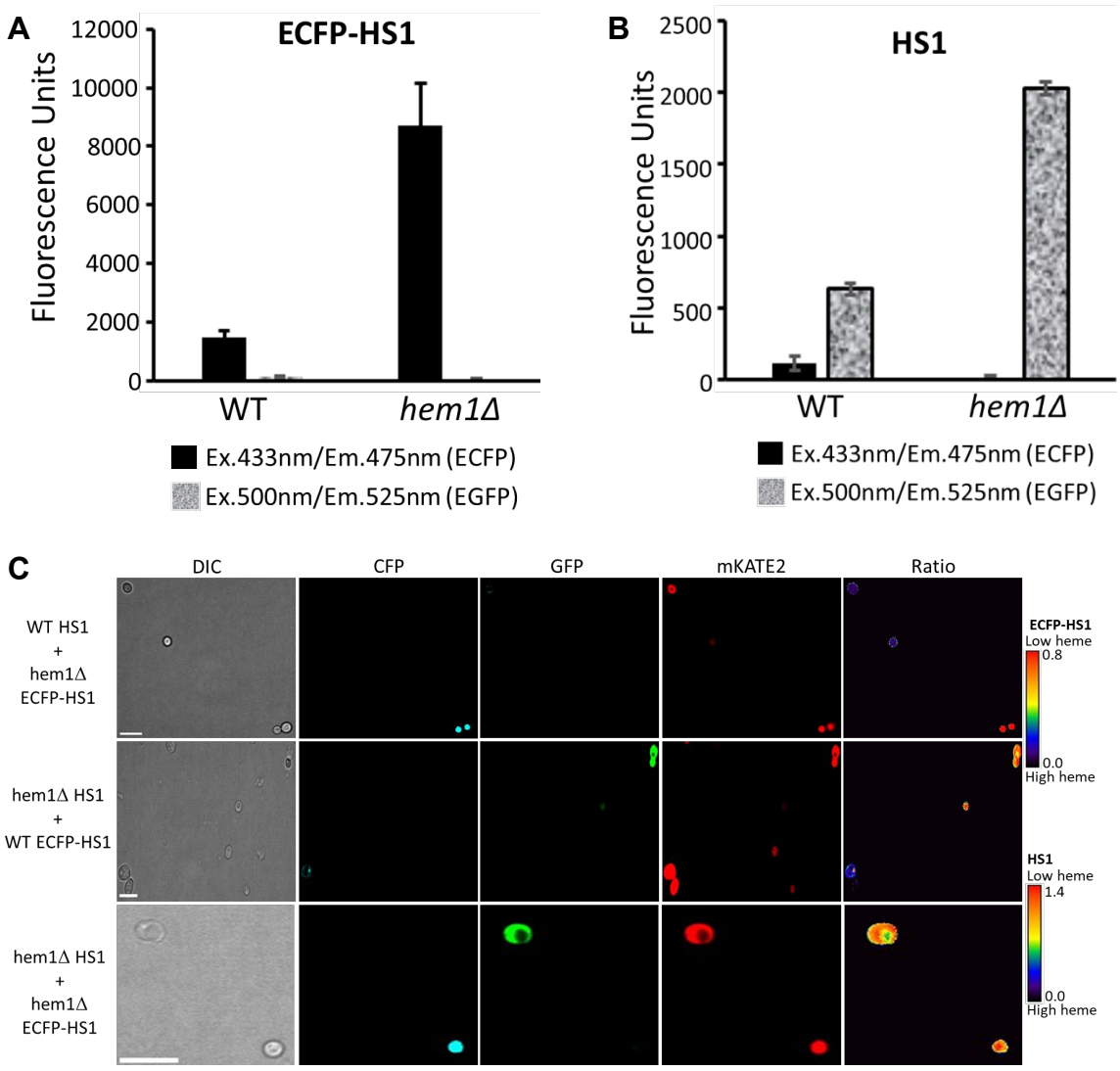

Figure 16. ECFP-HS1 and HS1 can be used simultaneously for multiplexing applications. WT and *hem1Δ* cells expressing the ECFP-HS1 **(A)** and HS1 **(B)** sensors can be spectrally distinguished from each other by fluorimetry with negligible interference from the other fluorophore. **(C)** Mixed populations of cells expressing both ECFP-HS1 and HS1 and with different heme loading can be clearly differentiated by confocal microscopy. Scale bar represents 10 μm.

3.3 Discussion

Fluorescent sensors have become veritable tools in cell biology for studying the dynamics of metal ions and small molecules within living cells as they allow for real-time imaging, providing high spatial and temporal resolution, and the ability to monitor multiple analytes simultaneously through multiplexing. Such multiplexing applications could be between different analytes in the same locale, or the same analyte in different organelles or cells. In this work, the HS1 and ECFP-HS1 sensors were used to assess labile heme levels in different yeast strains as a proof-of-concept towards expanding the color palette of HS1-type sensors for multiplexing applications to broaden our understanding of heme homeostasis.

One important area to pay attention to in the expansion of this work would be the overall design of the sensor variants. In the case of ECFP-HS1, it was expected that only direct mutagenesis of the residues needed to alter the chromophore and stabilize the molecule would be required,[200] but additional manipulations to the domain arrangement were necessary to achieve a functional molecule. While such linker considerations would be made in the general design of multi-domain proteins,[201] it was an unexpected factor for the ECFP-HS1 construct because its creation simply required mutagenesis of residues of an already-functional structural template (HS1). Since there is no crystal structure of the ECFP-HS1 molecule, it is unclear why more physical distance was needed to produce a functional construct vs HS1. It is possible that one or a combination of the non-chromophore mutations (N149I/M153T/V163A) introduced interactions that were unfavorable to the overall structure of the molecule or disrupted those that were

beneficial. This is especially likely considering the relative proximity of the mutations to each other. Going forward therefore, linker length manipulation should be made a core consideration in troubleshooting issues with the development of HS1-type sensors.

For this proof-of-concept sensor development, the cyan version of the EGFP-based HS1 was used. This construct, however, has relatively low brightness compared to EGFP which causes issues when it comes to fluorescence detection. And while EGFP itself remains a gold standard for fluorescent proteins,[202] several improvements *have* been made to EGFP and its direct color variants (EBFP, ECFP and EYFP) in terms of fluorescent properties. For example, we have mTagBFP2[203] (blue), mCerulean[204] and mTurquoise2[205] (cyan), mEmerald,[206] mWasabi[207] and mNeonGreen[208] (green), and mCitrine[209] and YPet[210] (yellow). Further expansion of the heme sensor color palette should directly employ these variants as they are the brightest and most chemically robust molecules, and their use will minimize imaging challenges.

3.4 Materials and Methods

3.4.1 E. coli strains, Media, and Growth Conditions

For routine cloning, sub-cloning grade chemically competent *E. coli* cells, strain 10G *E. cloni* (Lucigen), were used according to the manufacturer's specifications. For site-directed mutagenesis, high efficiency grade chemically competent *E. coli* cells, strain 10G DUOS *E. cloni* (Lucigen), were used according to the

manufacturer's specifications. All *E. coli* strains were cultured in Lysogeny broth (LB) with appropriate antibiotic selection (50 µg/mL ampicillin) at 37 °C.

3.4.2 Yeast Strains, Transformation and Growth Conditions

S. cerevisiae strains used in this work were derived from BY4741 (*MATa, his3Δ1, leu2Δ0, met15Δ0, ura3Δ0*). The *hem1Δ* (*hem1::HIS3*) strain DH001b-3 has been previously described.[91] Yeast transformations were performed according to the lithium acetate procedure.[158] Strains were maintained at 30 °C on either enriched yeast extract-peptone based medium supplemented with 2% glucose (YPD) or synthetic complete medium (SC) supplemented with 2 % glucose, with appropriate amino acids to maintain selection. Culturing of *hem1Δ* cells required supplementing YPD or SC media with 50 µg/mL 5-aminolevulinic acid (5-ALA), or 15 mg/mL ergosterol and 0.5 % Tween-80 (YPDE or SCE, respectively).[159] Growth in liquid culture was for ~14-16 h overnight. Cells cultured on solid media plates were done with the appropriate media supplemented with 2% agar.

3.4.3 Plasmids

The pID028 (ECFP-HS1) plasmid was made by performing several rounds of mutagenesis on pDH013 (HS1) (G65T, Y66W, N149I, M153T, V163A, and a GS insertion in the linker region between mKATE2 and CG6). The pDH013 (HS1)[49] and p415GPD (EV)[211] plasmids were obtained as described previously.

3.4.4 Instrumentation and Data Analysis

All growth measurements ($OD_{600\ nm}$) and UV/visible absorbance readings/spectra were recorded on a Cary 60 spectrophotometer (Agilent Technologies). Fluorescence measurements were collected on a Biotek Synergy Mx multi-modal plate reader (Agilent Technologies).

Confocal laser scanning microscopy was accomplished using a PerkinElmer UltraVIEW VoX spinning disk confocal microscope configured for live cell imaging. It was equipped with 20X, 0.75 NA air and 60X, 1.4 NA oil objectives. ECFP was excited with a 440 nm laser, EGFP with a 488 nm laser, while mKATE2 was excited using a 561 nm laser line. The 415-475 nm, 455-515 nm and 580-650 nm band pass filters were used to filter emission for ECFP, EGFP and mKATE2, respectively. Images were collected using Volocity software and analyzed with ImageJ v1.54f. Ratio images were generated using ImageJ as described.[160]

3.4.5 Characterization of Heme Sensors in Yeast

For all sensor fluorescence measurements, WT and *hem1Δ* yeast cells expressing the heme sensors were cultured in SCE-LEU media for ~14-16 hours (overnight) to mid-exponential phase (an optical density at 600 nm ($OD_{600\ nm}$) of ~ 1-2). Unless otherwise noted, all measurements were accomplished with the indicated sensor proteins expressed on the p415GPD plasmid, a low copy centromeric (CEN) plasmid with a GPD promoter.[131] Modulation of heme levels was done by supplementing triplicate biological samples with 5-ALA or SA (as appropriate, to designated final concentrations) at the beginning of overnight culture. After

culturing, cells were harvested, washed in water, and resuspended in phosphate buffered saline (PBS) solution at 5 $OD_{600\ nm}$/mL, or 1 × 10^8 cells/mL. For fluorimetry measurements on a population of cells, fluorescence was recorded on a Synergy Mx multi-modal plate reader using black Greiner Bio-one flat bottom fluorescence plates. ECFP, EGFP and mKATE2 fluorescence were recorded using excitation and emission wavelength pairs of 433 nm and 475 nm (gain=130), 488 nm and 510 nm (gain=100), and 588 nm and 620 nm (gain=100), respectively. When doing mixed culture experiments, EGFP was recorded using excitation and emission wavelength pairs of 500 nm and 525 nm (gain=100). Background fluorescence of cells not expressing the heme sensors were recorded and subtracted from the ECFP, EGFP and mKATE2 fluorescence values. Heme sensor fluorescence ratios were monitored by recording the ratio of ECFP or EGFP to mKATE2 fluorescence values. For microscopy, cells were prepared as previously described.[70]

CHAPTER 4. INTERCELLULAR HEME TRANSFER IN *S. CEREVISIAE*

4.1 Introduction

Intercellular communication in yeast – studied most extensively in *S. cerevisiae* – plays a vital role in regulating growth, adaptation, and coordinated behavior among cells, allowing yeast populations to respond effectively to environmental conditions. Of these conditions, nutrient availability is critical, and yeast have evolved sophisticated mechanisms in this regard, using signaling molecules and metabolic regulators to control cellular pathways accordingly, thereby ensuring optimal resource use.

In one form of intercellular communication, yeast cells undergo sexual reproduction between two haploid cells of different mating types (a and α). These cells secrete mating pheromones that attach to receptors on the corresponding cell type, triggering a series of signaling events.[212] This sequence utilizes mitogen-activated protein kinase (MAPK) pathways, resulting in cell shape changes, growth arrest, and activating the expression of genes specific to mating. This process guarantees that compatible mating type cells locate one another and merge to create diploid cells.[212] Transmission of such mating signals has also been demonstrated using a light-inducible system between two spatially separated yeast populations.[213]

In another important form of communication termed quorum sensing, yeast cells regulate population density in response to depleting nutrient levels or other unfavorable environmental conditions.[214] This involves the release of signaling molecules such as the aromatic alcohols phenylethanol, tryptophol and tyrosol which trigger appropriate changes to various cellular behaviors, including biofilm formation, growth, and stress response.[215] It has also been shown that small molecule signals like acetaldehyde and cyanide can mediate glycolytic synchronizations amongst yeast subpopulations.[216] In these manners, quorum sensing helps yeast cells manage metabolic resources collectively, reducing their growth and enhancing survival chances.

More recently emerging in the field of yeast intercellular communication are extracellular vesicles (EVs). These membrane-bound particles are typically laden with macromolecules such as proteins, RNA and lipids. These cargos have been shown to elicit downstream bioactivity: proteins may possess enzymatic activity or modulate immune responses, RNA can affect gene expression in recipient cells, while lipids can influence the structural stability of cells via membrane integrity.[217–219] Increasingly, the metabolomes of EVs are being studied for improved understanding of their roles in cell-cell communication.[220]

As with all other organisms, access to nutrients is critical to yeast survival, and genetic control of yeast populations impinges heavily on the availability of usable nitrogen and carbon sources as they must rapidly adapt their growth and metabolism in response to these environmental cues. A central regulator of cell growth is the TOR (**t**arget **o**f **r**apamycin) signaling pathway, which informs primarily

on amino acid status through TORC1 (**TOR c**omplex **1**).[221,222] Under nutrient-replete conditions, TORC1 promotes protein synthesis, ribosome biogenesis, and inhibits autophagy. However, when these nutrients are in short supply, TORC1 activity is downregulated, and the cell shifts towards catabolic processes such as autophagy to conserve resources.[221,223] Available data specifically identify leucine, glutamine and methionine as signals, but it is unclear if the scope of regulators extends beyond these.[221]

Two parallel pathways in yeast are also involved in signaling amino acid availability. The general amino acid control (GAAC) pathway is a starvation response system that is sensed via uncharged tRNAs, leading to the upregulation of genes involved in amino acid transport, metabolism, and autophagy.[224] Conversely, the yeast SPS (Ssy1, Ptr3 and Ssy5) pathway is activated by increase in amino acid levels and controls nutrient uptake. Some crosstalk between TOR and both the GAAC and SPS pathways has been identified,[221] suggesting a complex interplay of processes for the regulation of amino acid homeostasis.

With respect to carbon sources, the protein kinase A (PKA) pathway plays a central regulatory role in yeast. Glucose, which is the preferred carbon source for yeast, activates the PKA pathway, which in turn drives several downstream processes such as cell growth, proliferation, and energy production. Under glucose deprivation, the PKA pathway is inhibited, reducing growth and promoting stress tolerance mechanisms.[225] Another important glucose regulator is the Snf1 (AMPK) pathway which is activated under glucose deprivation and helps yeast cells adapt by promoting the usage of alternative carbon sources such as ethanol or fatty

acids. Snf1 facilitates the transition between fermentative and respiratory metabolism in response to glucose availability by controlling gene expression levels.[221,226] Just like the nitrogen sensing pathways, there is also significant crosstalk between TOR and the PKA and AMPK pathways, [221,227,228] highlighting the necessity for broader regulatory control of overall nutrient availability.

Heme is a cofactor essential for all aerobic life. In this sense, heme can also be viewed as a critical nutrient and of similar relevance as amino acids and glucose. For a molecule of such importance, little is known about whether intracellular heme status can be communicated in *S. cerevisiae*. Specifically, can heme deficiency in one subpopulation be signaled, leading to heme exchange from heme-sufficient cells? It is an intriguing concept, and the answers could reveal novel factors and pathways in heme regulation, thereby not only furthering our understanding of overall cellular heme homeostasis, but also providing new insight into the general yeast regulatory framework for nutrients.

4.2 Results

To probe the ability of heme-deficient yeast to uptake heme from heme-replete cells, a liquid co-culture system in heme-free media (SC-LEU) was used, consisting of WT cells transformed with an EV plasmid (WT EV; donor) and *hem1Δ* yeast expressing the HS1 sensor (*hem1Δ*-HS1; recipient). This system mitigated confounding data analysis factors by having only one strain from which fluorescence data can be obtained. Additionally, the choice of having the HS1 sensor in the *hem1Δ* strain was purposeful as it is easier to monitor labile heme

increases against a zero-level background. Only mKATE2-positive cells were selected for final analysis.

4.2.1 WT Yeast Can Donate Heme to hem1Δ Cells

After growth for 24 h, it was observed that *hem1Δ*-HS1 yeast in the co-culture had median sensor ratios that were similar to WT or *hem1Δ* cells treated with either 1.52 mM 5-ALA or 50 μM hemin (Figure 17A), indicating high labile heme levels in the *hem1Δ* yeast. To confirm that the WT cells were indeed serving as heme donors, WT cells were pre-cultured overnight with 500 μM SA to inhibit new heme synthesis and a subculture of those cells were used for the co-culture system, to which 500 μM SA was also added. Here, a significant decrease in *hem1Δ*-HS1 heme levels was seen as evidenced by increased sensor ratios almost to levels of the untreated *hem1Δ*-HS1 negative control (Figure 17B).

It had previously been observed that the detergent Tween-80 affects heme uptake in *hem1Δ* cells (unpublished data), perhaps by solubilizing heme and preventing its physical interaction with the yeast cell wall. To better understand the *hem1Δ* heme uptake phenomenon in WT co-culture, 0.5% Tween-80 was added, and this was found to decrease heme uptake but not totally abolish it (Figure 17C). Additionally, it was desirable to determine if the uptake first required secretion of heme into the media. To this end, *hem1Δ*-HS1 cells were grown in filtered spent media from a 24-hour culture of WT cells but no effect on heme levels were observed (Figure 17D). These findings suggest that physical cell contact (to some

degree) is required between the yeast subpopulations for the heme transfer to occur.

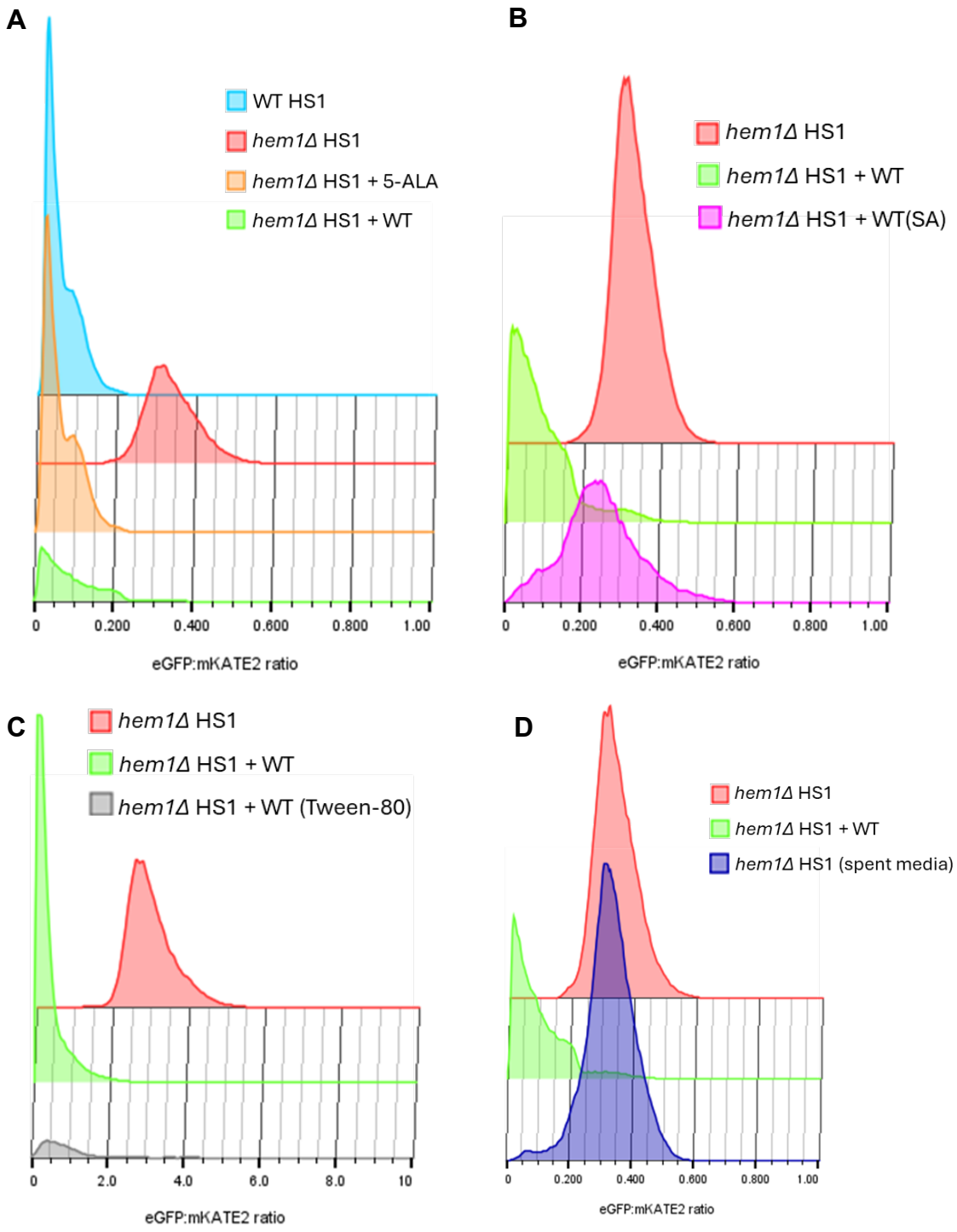

Figure 17. Contact-dependent heme exchange occurs between WT and *hem1Δ* cells in a mixed culture. (A) Sensor (HS1)-expressing *hem1Δ* cells exhibit WT-like sensor ratios in a mixed culture with WT cells. **(B)** Inhibition of heme synthesis in WT cells significantly abrogates heme loading of the *hem1Δ*-HS1 sensors in the co-culture system. **(C)** Supplementation of the mixed culture with Tween-80 slightly affects *hem1Δ*-HS1 heme levels. **(D)** Heme levels in HS1-expressing *hem1Δ* cells are unperturbed when grown in spent and filtered media from 24 h culture of WT cells.

4.2.2 Heme Transfer is Dependent on Heme Deficiency and Strain Traits

It was unclear whether the uptake of heme by the *hem1Δ*-HS1 cells was due to their heme-deficient state or some trait resulting from the genetic deletion of the *hem1* gene. To determine this, exponential phase WT cells expressing the HS1 sensor (WT-HS1) were first pre-cultured with 500 μM SA to inhibit new heme synthesis and a subculture of those cells were used for a co-culture system with WT EV cells, to which 500 μM SA was also added. Here, the heme-starved WT-HS1 served as the heme recipient strain. In comparison to a single culture WT-

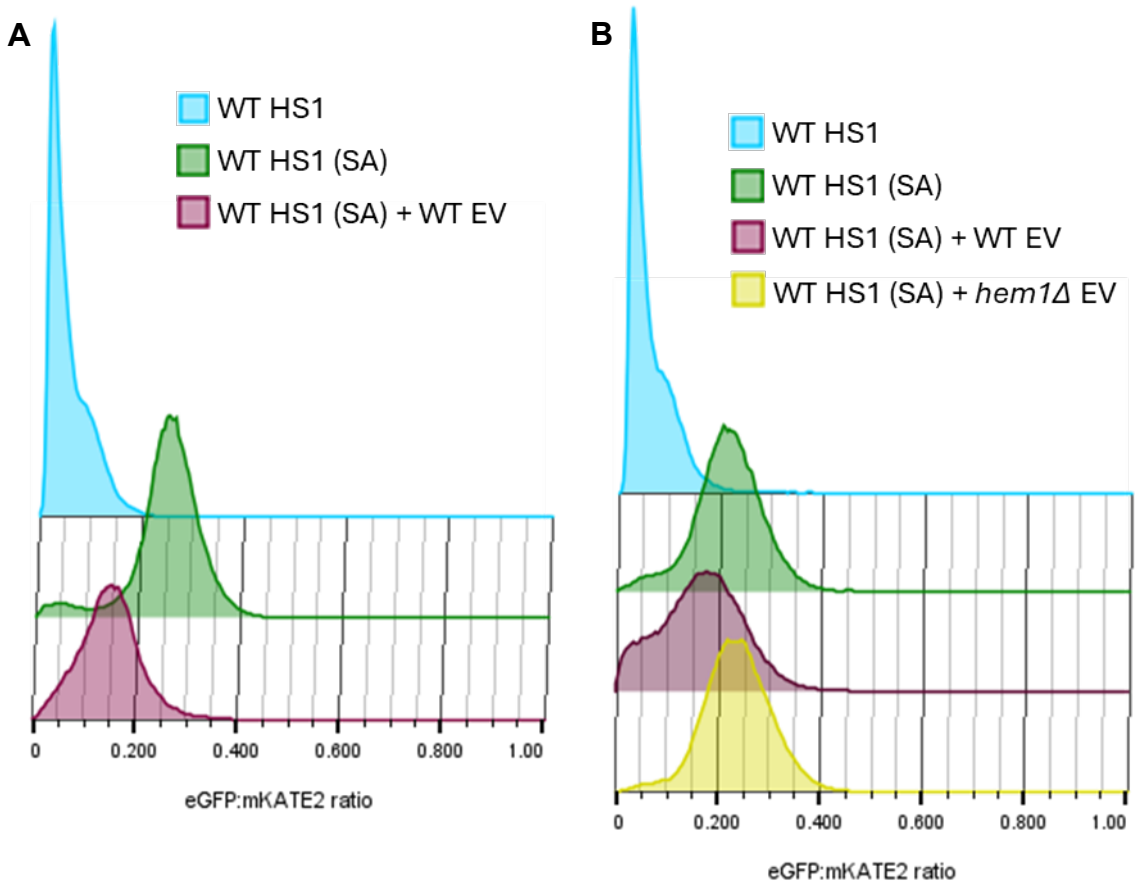

Figure 18. The heme-deficient state is recognized in the intercellular heme exchange phenomenon. (A) WT-HS1 cells starved of heme by SA treatment are able to uptake heme from heme-replete WT cells. (B) Heme-deficient WT-HS1 cells are unable to obtain heme when co-cultured with *hem1Δ* cells.

HS1 control group pre-dosed and continually treated with 500 μM SA, a significant decrease in sensor ratio was observed (Figure 18A), indicating higher cytosolic labile heme levels in the co-cultured WT-HS1 cells.

To confirm that the difference in sensor ratio seen in the co-cultured WT-HS1 cells was indeed sourced from the WT EV strain, a similar co-culture experiment was set up wherein the donor WT EV cells were substituted with *hem1Δ* cells transformed with EV plasmid (*hem1Δ*-EV). In this system, it was necessary to use SC-LEU media supplemented with 0.5% Tween-80 and 0.0015% ergosterol (SCE-LEU) to allow the growth of the *hem1Δ*-EV cells in the absence of heme, to ensure the availability of a sufficient population of donor *hem1Δ* cells. It was discovered that the WT-HS1 cells co-cultured with the *hem1Δ*-EV strain had significantly higher sensor ratios than those grown with WT EV (Figure 18B), suggesting that heme transfer from the WT EV cells was responsible for the increased heme levels in the heme-starved WT-HS1.

4.3 Discussion

Cell-cell communication in eukaryotic microbes, such as fungi and protists, plays a crucial role in coordinating population-level behaviors like mating, nutrient sensing, and pathogenesis. This communication often occurs through chemical signaling, where cells release molecules (e.g., pheromones) that bind to receptors on neighboring cells, triggering intracellular responses. For example, quorum sensing in the pathogenic fungus *Candida albicans* regulates biofilm formation and virulence, allowing the population to coordinate responses based on cell density.[229]

These signaling pathways allow microbial populations to adapt collectively to environmental changes and host interactions, ensuring their survival and proliferation.

The outcome of signaling events is typically some downstream process that is mediated by protein effectors in response to the signal. In many cases, the process being acted upon involves the regulation of nutrient utilization as these are critical for the continued survival of the cell population. While the most studied nutrients in this regard include glucose, amino acids and lipids, the requirement of essential cofactors by these cells, and hence their regulation in response to environmental stimuli, has not received significant attention.

Heme is a well-known metallo-nutrient that is critical for the survival of aerobic organisms. Systemic regulation of heme homeostasis has been best documented for the heme auxotroph, *C. elegans* which must acquire heme from external sources. In *C. elegans*, tissues responsible for the uptake and storage of heme in the intestine are distal from those with high heme demand, such as those involved in respiration and detoxification.[230] Ensuring that adequate heme supply is available to these distal tissues requires some form of communication between the demand location and the supplier port and depot. Intercellular signaling, involving proteins like HRG-7, helps regulate this trafficking, maintaining heme homeostasis and preventing toxicity from free heme. This coordinated transport is crucial for cellular metabolism and overall organismal health.[64] HRG-7 is a protein with homology to aspartic proteases that mediates inter-organ signaling between the intestine and extra-intestinal tissues in *C. elegans*. HRG-7 functions as a secreted

signaling factor during heme starvation in extra-intestinal tissues and is itself regulated through neuronal signaling.[64]

In this work, it was observed that *hem1Δ* yeast cells defective in their ability to synthesize heme can receive heme from heme-sufficient WT cells. This finding integrates the concepts of cell-cell communication and intercellular nutrient exchange. Additionally, it appeared that this cross-feeding relationship was contact dependent. While cell-cell communication is known to occur in yeast for population control processes like mating and quorum sensing, not much is known in relation to direct nutrient exchange. In general, the occurrence of metabolite exchange is quite widespread across unicellular and multicellular organisms, typically involving uptake from an extracellular milieu of shared resources that provides constituent members with a degree of freedom to undergo metabolic shift and division of labor.[231] This leads to diversification within the community wherein certain subpopulations become auxotrophic for essential metabolites.[231,232] This behavior, therefore, lends biological relevance to the co-culture system adopted in this work as well as the findings for heme exchange.

Surprisingly, it appeared that metabolic deficiency uniquely played some role in this cross-feeding phenomenon as chemical inhibition of heme synthesis in healthy WT cells was able to induce the uptake behavior. It is likely, however, that this occurs in conjunction with some other phenotype that results from the genetic inability to make heme, as chemically-induced heme deficiency in the WT failed to fully recapitulate the level of heme repletion seen with the *hem1Δ* yeast. Could the accumulation of 5-ALA from inhibition of Hem2p be causing a specific response

different from the build-up of succinyl-CoA and glycine? Does Hem1p play a regulatory role in heme homeostasis that leads to increased heme uptake when it is absent? The answers to these questions could help us better understand this phenomenon.

It was interesting to discover that cell-cell contact might be important for heme transfer within this cross-feeding system. The formation of cell-cell connections has been observed in a well-mixed population of bacteria, for the transfer of cytoplasmic content based on an auxotrophy-prototrophy relationship.[233] Yeast can also develop such contacts, in both intra- and inter-species communities, and the formation of these contacts has also been shown to elicit gene transcription changes such as those involved in maintaining cell wall integrity, cell wall structural components, and the production of H_2S.[234,235] What signals lead to the formation of these contacts, and does heme, despite being the exchange cargo, also play a role in the contact formation process? Considering the role of flocculins in cell-cell contacts, it is rather noteworthy that preliminary work herein showed a relatively higher frequency of flocculant colonies amongst *hem1Δ* yeast cells during transformation procedures. The relevance of this observation is unclear, however, and further work is needed to improve our understanding.

4.4 Materials and Methods

4.4.1 E. coli strains, Media, and Growth Conditions

For routine cloning, sub-cloning grade chemically competent *E. coli* cells, strain 10G *E. cloni* (Lucigen), were used according to the manufacturer's specifications.

All *E. coli* strains were cultured in Lysogeny broth (LB) with appropriate antibiotic selection (50 µg/mL ampicillin) at 37 °C.

4.4.2 Yeast Strains, Transformation and Growth Conditions

S. cerevisiae strains used in this work were derived from BY4741 (*MATa, his3Δ1, leu2Δ0, met15Δ0, ura3Δ0*). The *hem1Δ* (*hem1::HIS3*) strain DH001b-3 has been previously described.[91] Yeast transformations were performed according to the lithium acetate procedure.[158] Strains were maintained at 30 °C on synthetic complete medium (SC) supplemented with 2 % glucose, with appropriate amino acids to maintain selection. Culturing of *hem1Δ* cells was also done as aforementioned, except when pre-cultured or specifically stated otherwise wherein SC media with 1.52 mM 5-aminolevulinic acid (5-ALA), or 15 mg/mL ergosterol and 0.5 % Tween-80 (SCE) was used.[159] Growth for pre-culture was in liquid culture for ~14-16 h overnight while experiment culture time was 24 h. Cells cultured on solid media plates were done with the appropriate media supplemented with 2% agar.

4.4.3 Plasmids

The pDH013 (HS1)[49] and p415GPD (EV)[211] plasmids were obtained as described previously.

4.4.4 Instrumentation and Data Analysis

All growth measurements ($OD_{600\ nm}$) and UV/visible absorbance readings/spectra were recorded on a Cary 60 spectrophotometer (Agilent Technologies).

Fluorescence measurements were collected on a Biotek Synergy Mx multi-modal plate reader (Agilent Technologies).

Flow cytometry measurements were performed using a BD LSRFortessa flow cytometer or Cytek Aurora spectral cytometer. Both cytometers were equipped with an argon laser (ex 488nm) for EGFP and yellow-green laser (ex 561nm) for mKATE2. On the BD LSRFortessa, EGFP emission was measured using a 530/30nm bandpass filter and mKATE2 was measured using a 610/20nm bandpass filter. For the Cytek Aurora, EGFP emission was recorded from the B1 channel (~515 nm) and mKATE2 emission from the YG3 channel (~610 nm). Data evaluation was conducted using FlowJo v10.10 software. The number of cells measured per experiment was set to 50,000 – 100,000 unless otherwise stated. Only single, mKATE2 positive cells were selected for analysis.[160]

CHAPTER 5. CONCLUSION AND FUTURE WORK

Our understanding of heme homeostasis has evolved significantly over the last two decades. Early assertions held that heme is primarily a protein cofactor, that it is only made when needed and rapidly degraded thereafter, and that each cell makes and breaks its own heme. Scientific advancements, however, have provided data that challenge these long-held beliefs. Indeed, it is now known that heme exists in buffered labile pools within cells in such a manner that its toxicity is mitigated but it is readily available and can be rapidly mobilized for biological activity. Heme chaperones have also been identified that mediate this exchangeability. In addition, there is an appreciation that heme can serve as a biological signal, regulating gene expression and directly affecting protein activity, and that it can as well be shared between cells. Despite all this progress, much remains to be discovered as fundamental questions about overall heme homeostasis are yet unanswered. The work in this thesis extends the frontier in this regard by introducing new tools to probe intra- and inter-cellular heme trafficking, and by exploring the concept of heme as an exchangeable nutrient.

The endomembrane system plays a vital role in various cellular processes, including protein processing, lipid synthesis, and heme uptake. One of the projects herein aimed to investigate the role of the endomembrane system in heme trafficking, focusing on the development of heme sensors localized to the endoplasmic reticulum. Attempts in yeast showed the yeast ER to not be very permissive of the expression of complex luminal protein structures (in this case,

HS1 with its integral fusion design), which might be due to its relatively simple genome. Surprisingly, expression of ER-HS1 in human cells occurred without incident and this construct can now be calibrated *in situ*, following which it can be utilized to characterize the ER heme pool and how it is affected by metabolic dysregulation such as ER stress, oxidative stress and nutrient deprivation. In fact, the success achieved from ER heme sensor development in human cells buttresses the suggestion of folding machinery incapability in yeast, therefore strategies such as the co-expression of folding chaperones or culturing at sub-physiological temperatures could potentially improve protein expression and folding in yeast.

Fluorescent sensors have become important for studying metal ions and small molecules in living cells, offering real-time imaging capabilities and enabling multiplexing for tracking multiple analytes. Some of the work in this thesis used HS1 and ECFP-HS1 sensors to measure labile heme levels in different yeast strains, the latter aiming to expand the color palette of HS1-type sensors for greater utility, with the goal of accelerating our pace towards an improved understanding of heme homeostasis. A key challenge encountered in ECFP-HS1 development was the unexpected domain arrangement adjustments required to produce a functional sensor, likely due to disruptive interactions caused by non-chromophore mutations. Going forward, linker length adjustments should be a core consideration in sensor development. It should also be noted that ECFP has relatively low brightness which complicated fluorescence detection. While ECFP-HS1 was a proof-of-concept for HS1 color palette expansion (with HS1 being

based on EGFP), newer FP variants of the same or different lineage from EGFP have been developed which offer improved brightness and stability. Future sensor designs should incorporate these improved variants to minimize imaging challenges and enhance performance.

Cells living in communities have developed ways of communication that ensure survival at the population level. A critical aspect of this cell communication involves the regulation of key nutrients. In one of the studies in this thesis, *hem1Δ* yeast cells were found to receive heme from heme-sufficient wild-type cells through contact-dependent cross-feeding, integrating the concepts of cell-cell communication and nutrient exchange. Metabolite exchange is common in microbial communities and often involves division of labor, leading to auxotrophy in subpopulations. Interestingly, both metabolic and genetic deficiency appeared to regulate this heme exchange phenomenon for which cell-cell contact was found to be important. Further work needs to be done to determine the physiological relevance of this process and the genetic determinants of its occurrence. In the former instance, assessment of heme acquisition on indices such as growth and survival should be undertaken, while the co-culture system can be adapted for multi-well plate reader format to genetically screen for factors that regulate heme exchange.

Taken together, this thesis research has contributed to knowledge in the field of heme homeostasis via the development of tools that will be used to answer more questions, as well as shedding light on previously uncharacterized processes. Fitting together the pieces of the heme homeostasis puzzle will be pertinent

towards the development of therapeutic and diagnostic solutions for conditions wherein heme is implicated.

APPENDIX A. SUPPLEMENTAL INFORMATION

A.1 Chapter 2

A.1.1 Yeast Strains Used in Chapter 2

Table 1. List of *S. cerevisiae* strains and plasmids used in Chapter 2.

Strain	Cell Type	Plasmid Name	Plasmid Construction
DH003	BY4741 WT	p425GPD	NA
DH013	BY4741 *hem1::HIS3*	pDH013	p415-GPD-HS1
DH018	BY4741 WT	pDH013	p415-GPD-HS1
ID037	BY4741 WT	pID035	p415GPD-ER-HS1-oxstable
ID038	BY4741 *hem1::HIS3*	pID035	p415GPD-ER-HS1-oxstable
ID039	BY4741 WT	pID038	p415GPD-ER-mKATE2-oxstable
ID040	BY4741 *hem1::HIS3*	pID038	p415GPD-ER-mKATE2-oxstable
ID041	BY4741 WT	pID039	p425GPD-ER-HS1-oxstable
ID042	BY4741 WT	pID041	p425GPD-CG6-oxstable
ID043	BY4741 WT	pID042	p425GPD-ER-mKATE2-oxstable
ID044	BY4741 *hem1::HIS3*	pID039	p425GPD-ER-HS1-oxstable
ID045	BY4741 *hem1::HIS3*	pID041	p425GPD-CG6-oxstable
ID046	BY4741 *hem1::HIS3*	pID042	p425GPD-ER-mKATE2-oxstable
ID047	BY4741 WT	pID037	p425GPD-ER-GFP
ID048	BY4741 *hem1::HIS3*	pID037	p425GPD-ER-GFP

ID049	BY4741 WT	pID045	p425GPD ER-CG6-oxstable
ID050	BY4741 *hem1::HIS3*	pID045	p425GPD ER-CG6-oxstable
ID051	BY4741 WT	pID036	p425GPD ER-CG6
ID052	BY4741 *hem1::HIS3*	pID036	p425GPD ER-CG6
ID053	BY4741 WT	pID033	p425GPD ER-HS1
ID056	BY4741 *hem1::HIS3*	pID033	p425GPD ER-HS1
ID073	BY4741 WT	pID052	p415GPD ER-HS1
ID074	BY4741 *hem1::HIS3*	pID052	p415GPD ER-HS1
ID075	BY4741 WT	pID053	p425TEF ER-HS1
ID076	BY4741 *hem1::HIS3*	pID053	p425TEF ER-HS1
ID077	BY4741 WT	pID054	p415GPD ER-GFP
ID078	BY4741 *hem1::HIS3*	pID054	p415GPD ER-GFP
ID079	BY4741 WT	pID055	p415GPD ER-CG6
ID080	BY4741 *hem1::HIS3*	pID055	p415GPD ER-CG6
ID081	BY4741 WT	pID056	p425TEF ER-CG6
ID082	BY4741 *hem1::HIS3*	pID056	p425TEF ER-CG6
ID083	BY4741 WT	pID057	p425TEF ER-GFP
ID084	BY4741 *hem1::HIS3*	pID057	p425TEF ER-GFP
ID095	BY4741 WT	pID062	p415GPD ER-GFP-cyt b562
ID096	BY4741 *hem1::HIS3*	pID062	p415GPD ER-GFP-cyt b562

ID097	BY4741 WT	pID063	p415GPD cyt b562-GFP
ID098	BY4741 *hem1::HIS3*	pID063	p415GPD cyt b562-GFP
ID099	BY4741 WT	pID064	p415GPD ER-GFP-cyt b562
ID100	BY4741 *hem1::HIS3*	pID064	p415GPD ER-GFP-cyt b562
ID101	*vph1Δ*	pID064	p415GPD ER-GFP-cyt b562
ID102	*pep12Δ*	pID064	p415GPD ER-GFP-cyt b562
ID103	*snf7Δ*	pID064	p415GPD ER-GFP-cyt b562
ID104	*vps36 Δ*	pID064	p415GPD ER-GFP-cyt b562
ID105	*vph1Δ*	pID037	p425GPD ER-GFP
ID106	*pep12Δ*	pID037	p425GPD ER-GFP
ID107	*snf7Δ*	pID037	p425GPD ER-GFP
ID108	*vps36 Δ*	pID037	p425GPD ER-GFP
ID115	BY4741 WT	pID069	p415GPD ER-mKATE2-GFP-cyt b562
ID116	BY4741 *hem1::HIS3*	pID069	p415GPD ER-mKATE2-GFP-cyt b562
ID125	BY4741 WT	pID079	p415GPD ER-mKATE2-GS-GFP-cyt b562
ID126	BY4741 *hem1::HIS3*	pID079	p415GPD ER-mKATE2-GS-GFP-cyt b562
ID131	*vph1Δ*	pID054	p415GPD ER-GFP
ID132	*snf7Δ*	pID054	p415GPD ER-GFP
ID133	*pep12Δ*	pID054	p415GPD ER-GFP
ID134	*vps36 Δ*	pID054	p415GPD ER-GFP
ID135	BY4741 WT	pID082	p415GPD ER-GFP-cyt b562-M7A
ID136	BY4741 *hem1::HIS3*	pID082	p415GPD ER-GFP-cyt b562-M7A

ID137	BY4741 WT	pID083	p415GPD ER-GFP-cyt b562-M7A-H102A
ID138	BY4741 *hem1::HIS3*	pID083	p415GPD ER-GFP-cyt b562-M7A-H102A
ID144	*vph1Δ*	pID082	p415GPD ER-GFP-cyt b562-M7A
ID145	*snf7Δ*	pID082	p415GPD ER-GFP-cyt b562-M7A
ID146	*pep12Δ*	pID082	p415GPD ER-GFP-cyt b562-M7A
ID147	*vps36 Δ*	pID082	p415GPD ER-GFP-cyt b562-M7A
ID148	*vph1Δ*	pID083	p415GPD ER-GFP-cyt b562-M7A-H102A
ID149	*snf7Δ*	pID083	p415GPD ER-GFP-cyt b562-M7A-H102A
ID150	*pep12Δ*	pID083	p415GPD ER-GFP-cyt b562-M7A-H102A
ID151	*vps36 Δ*	pID083	p415GPD ER-GFP-cyt b562-M7A-H102A
ID152	BY4741 WT	p415GPD	NA

A.2 Chapter 3

A.2.1 Yeast Strains Used in Chapter 3

Table 2. List of *S. cerevisiae* strains and plasmids used in Chapter 3.

Strain	Cell Type	Plasmid Name	Plasmid Construction
DH003	BY4741 WT	p425GPD	NA
DH013	BY4741 *hem1::HIS3*	pDH013	p415-GPD-HS1
DH018	BY4741 WT	pDH013	p415-GPD-HS1
ID033	BY4741 WT	pID028	p415GPD-ECFP HS1
ID034	BY4741 *hem1::HIS3*	pID028	p415GPD-ECFP HS1

A.3 Chapter 4

A.2.1 Yeast Strains Used in Chapter 4

Table 3. List of *S. cerevisiae* strains and plasmids used in Chapter 4.

Strain	Cell Type	Plasmid Name	Plasmid Construction
ID152	BY4741 WT	p415GPD	NA
DH013	BY4741 *hem1::HIS3*	pDH013	p415-GPD-HS1
DH018	BY4741 WT	pDH013	p415-GPD-HS1

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