INVESTIGATING THE ROLE OF RIDA FAMILY PROTEINS IN *SACCHAROMYCES CEREVISIAE*

by

GREGORY H. WHITAKER

(Under the Direction of Diana Downs)

ABSTRACT

RidA proteins are conserved enamine/imine deaminases found in all domains of life. In both prokaryotic and eukaryotic model organisms, deletion of *ridA* causes a number of phenotypes, including growth defects. Accumulation of 2-aminoacrylate (2AA) occurs in the absence of RidA and results in the respective phenotypes. The reactive 2AA enamine attacks pyridoxal 5'-phosphate (PLP, a B_6 vitamer) in the active site of PLP-dependent enzymes, and covalently inactivates the enzyme. The metabolic impact resulting from damage to PLP-dependent enzymes varies depending on the specific metabolic needs of the organism.

The role of RidA in cellular metabolism has been best characterized in prokaryotes such as *Salmonella enterica,* in which its 2AA deaminase activity was first identified. Even among closely related prokaryotes, consequences of *ridA* lesions vary, from inconsequential to crippling. Disruptions caused by 2AA accumulation are dependent on the needs of the organism and metabolic flux through relevant pathways.

The work described herein was undertaken to improve understanding of the RidA paradigm in the eukaryotic model organism *Saccharomyces cerevisiae*, and by extension eukaryotes in general. The *S. cerevisiae* genome encodes two RidA homologs (Mmf1, Hmf1), but only one generates known phenotypes when deleted. The mitochondrially localized Mmf1 protein was previously shown to prevent 2AA accumulation and deleterious effects. Work presented here built upon prior knowledge to identify Hem1, a PLP-dependent enzyme, as a definitive target of 2AA in the *S. cerevisiae* mitochondria. Damage to Hem1 accounts for a portion of the disruptive effects of 2AA accumulation, including decreasing intracellular heme levels. Beyond the study on Hem1, a role for the cytosolic RidA homolog, Hmf1, was investigated.

In total this work not only furthers our understanding of cellular metabolism in yeast, but also lays the groundwork for defining the role of RidA homologs in complex organisms, about which little is known. Yeast bears many similarities to higher eukaryotes, not least of which is the presence of mitochondria and a mitochondrially localized RidA homolog. Having identified a previously unknown impact of 2AA accumulation on heme biosynthesis, we make the reasonable assumption that this may occur in the mitochondria of other eukaryotic organisms.

INDEX WORDS: Mmf1, *Saccharomyces cerevisiae*

INVESTIGATING THE ROLE OF RIDA FAMILY PROTEINS IN *SACCHAROMYCES CEREVISIAE*

by

GREGORY H. WHITAKER

BS Biology, Texas A&M University, 2009

MS Biology, Texas A&M University, 2015

A Dissertation Submitted to the Graduate Faculty of The University of Georgia in Partial Fulfillment of the Requirements for the Degree

DOCTOR OF PHILOSOPHY

ATHENS, GEORGIA

2023

INVESTIGATING THE ROLE OF RIDA FAMILY PROTEINS IN *SACCHAROMYCES CEREVISIAE*

by

GREGORY H. WHITAKER

Major Professor:	Diana Downs
Committee:	Jorge Escalante-Semerena
	David Garfinkel
	Robert Maier

Electronic Version Approved:

Ron Walcott
Vice Provost for Graduate Education and Dean of the Graduate School
The University of Georgia
May 2023

ACKNOWLEDGEMENTS

I would first like to thank my advisor, Dr. Diana Downs, for the support and instruction, as well as the patience she exhibited during the mentoring process. I chose Dr. Downs as my advisor because it was clear to me from our first meetings that she had not only an enthusiasm for research, but also the understanding that effective mentoring is an integral part of academic science. After my time in her lab, I know that she will do what whatever she thinks will be most effective in achieving her goal of enabling us to be effective as scientists, whether that furthers a future academic career or something different. Her approach with me, which has been direct and honest, is much appreciated.

I would also like to thank Dr. Steve Lockless, as well as the members of the Lockless lab at Texas A&M while I worked there before applying to this program. Dr. Lockless took a chance and enabled me to get back on my feet in research, without which none of this would be possible. Dr. Lockless shares many of the traits I appreciate in Dr. Downs, and my time in his lab vastly broadened my understanding of the scientific process as it applies to dissecting microbial physiology.

I would also like to thank the members of the Downs lab, past and present whom I consider friends (whether unilaterally or not) and have provided considerable mirth and necessary distraction. Their congenial input on science, personal philosophy, and their reactions to my antics have kept me engaged in ways I can't begin to quantify. Especially during the roughest times, when the experiments are not going to the way I would have preferred and motivation is waning, I could always look forward to our interactions.

I won’t list all of my friends and acquaintances in Athens, here, but I would like to give a special mention to Jeff Dodelin, Andrew Duitsman, and Corey Schultz. I appreciate the initial (and continued) reaching out that Jeff did, especially, and the therapeutic dice rolling that occurred and continues to occur on a weekly basis.

Last but far from least I would like to expressly thank my good friend back in College Station, Chuck Rhea. It would be impossible to overstate how much he has helped me to develop a successful philosophy in life, and continued to offer support whenever I reached out. I will be grateful for the rest of my life to Chuck for helping me out of a dark place in which I found myself many years ago, without which none of this would be possible. He would say that I did it myself, but I know that without his help I wouldn’t be here.

TABLE OF CONTENTS

Page

LIST OF TABLES

Page

LIST OF FIGURES

Page

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

Cellular metabolism broadly encompasses all the reactions that occur within a cell. These reactions are the core of life's essential function: the utilization of energy to concentrate and convert chemicals to a useful form. When changes to the precisely balanced chemistry of the cell are made, often through mutation, all varieties of consequences can occur, from nothing impactful to disease to complete loss of viability. While a gene can be identified, its regulation described, and the biochemical activity of its product thoroughly characterized, no computer algorithm is sophisticated or informed enough to reliably predict its role in the complete context of cellular metabolism. The deletion of a gene involved in nonredundant catabolic pathway, for example, would almost certainly preclude utilization of that catabolite, however, the change in concentrations of intermediates and the loss of others frequently have far reaching consequences that are not obvious without empirical examination. Even under close examination, the physiological impact of a metabolic alterations can be elusive and dependent on the context in which the cells are grown.

A reductive approach to metabolism is necessary to dissect this highly complex set of interactions, in which hundreds or thousands of reactions occur simultaneously. Taking a classical genetics approach, a gene for an enzyme is deleted, and information is gathered on the resulting phenotypes; growth, auxotrophies, chemical sensitivities, etc. When these phenotypes cannot be easily explained by the loss of the enzyme's known biochemical

activity, a metabolic interaction with other enzymatic pathways may be the cause. Screens and selections can be used to identify mutations that suppress the observed phenotypes and aid greatly in making the leap from the obvious consequences of a gene deletion and the less direct ones, which can then be further examined both biochemically and genetically. It is this type of approach that has been taken during both the initial discovery and characterization of Rid proteins and is described in the following sections [1-3].

1.1 DEFINITION OF RIDA PROTEIN SUBFAMILY

The Rid (YjgF/YER057c/UK114) superfamily of proteins have been divided into eight subfamilies based on their active site configuration and structural motifs [4, 5]. The RidA subfamily is found in all domains of life, and catalyzes the deamination of imine/enamine intermediates formed during amino acid metabolism (Fig. 1) [6]. RidA was initially characterized as an imine/enamine deaminase in *Salmonella enterica*, and has since been biochemically characterized in *Escherichia coli, Arabidopsis thaliana, Saccharomyces cerevisiae, Campylobacter jejuni,* and *Homo sapiens* among others, in each case being capable of complementing phenotypes associated with a *ridA* deletions in *S. enterica*, fulfilling a role as a 2-aminoacrylate deaminase [7-12].

Initial isolation and proposed functions

RidA was originally isolated from rat liver and kidney based on its unusually high solubility in acid [13]. The function was not determined at this time, but based on its conserved amino acid sequence it was suggested that it may be a heat-shock protein. Several studies followed that isolated the protein and characterized the expression of RidA homologs from other mammalian tissue, observing that it was induced during heat shock,

which seemed to fit its conserved structure [14, 15]. Additional studies have ascribed various functions both correlative and causative to RidA homologs, while none of these have convincingly demonstrated physiological relevance behind them [15-30]. A more thorough compilation of the various investigations of RidA homologs can be found in the review by Irons et al. 2020 [31].

1.2 RIDA DEAMINATES 2-AMINOACRYLATE *IN VIVO*

Initial discovery of imine/enamine deaminase activity.

A definitive and physiologically relevant activity for the RidA family of enzymes was determined serendipitously while investigating the pyrimidine biosynthesis pathway in *S. enterica*. It was found that a null mutation of the *ridA* gene suppressed a requirement for the pentose phosphate pathway during thiamine biosynthesis in *purF* deletion strains using the alternative pyrimidine biosynthesis pathway [32]. The *purF* gene encodes phosphoribosylpyrophosphate amidotransferase, which synthesizes a critical intermediate in thiamine biosynthesis, phosphoribosylamine. RidA was found to contribute to the deamination of the reactive intermediates 2-aminocrotonate (2AC) and 2-aminoacrylate (2AA), which are produced by serine/threonine dehydratases (IlvA) using the substrates threonine and serine, respectively [33]. A subsequent deamination reaction yields their respective products, 2-ketobutyrate (2KB) and pyruvate, which are intermediates in the synthesis of branched-chain amino acids, such as isoleucine. The rescue of thiamine auxotrophy in *ridA* strains was eventually found to be dependent on accumulation of 2AC produced by IlvA, and in addition, to anthranilate synthase (TrpD). This information provided the foundation for a model in which TrpD produces 5-phophoribosylamine

utilizing 2AC and phosphoribosylpyrophosphate, circumventing the loss of PurF in thiamine biosynthesis [33, 34].

Establishment of a physiological role for RidA as an imine/enamine deaminase.

Enos-Berlage et al. also observed that RidA mutants were sensitive to serine and that this sensitivity could be prevented by exogenous isoleucine [32]. In the presence of serine, serine/threonine dehydratases (EC 4.3.1.19) produce 2AA, which can be deaminated by RidA (Fig. 2). 2AA can be hydrolyzed by water in solution to produce pyruvate. 2-aminocrotonate and 2AA are both unstable in aqueous solution, with 2AA possessing a half-life estimated to be as low 1.5s [35]. For this reason, a dedicated deaminase was thought to be unnecessary to produce 2KB and 2AA in branched-chain amino acid biosynthesis, and in fact, *ridA* mutants are not auxotrophic for these amino acids. IlvA itself has often been annotated as a dehydratase/deaminase. However, in *ridA* mutants of *S. enterica*, alanine racemase (Alr), a PLP-dependent enzyme, was shown to be covalently modified by 2AA, resulting in a 2AA/PLP adduct that is incapable of performing the chemistry necessary to enable to enzyme to function, despite the short predicted half-life of 2AA [36]. This modification is dependent on the function of IlvA, which apparently releases the interfering 2AA. A 2AA/PLP adduct can also be observed when 3-chloroalanine (3CA) is used as a substrate with various PLP-dependent enzymes. [37-40]. 3CA is converted in the active site of PLP-dependent enzymes to 2AA. Interestingly, the enzymes that generate 2AA are themselves PLP-dependent, but clearly do not seem to be subject to the same degree of sensitivity as physiologically relevant targets. However, it has recently been shown that PLP-2AA adducts can be found even in the generators of 2AA [41].

The *in vivo* discovery of a PLP-2AA adduct from the active site of Alr was then corroborated by *in vitro* reconstitution of adduct formation using another PLP-dependent target, the branched-chain aminotransferase (BCAT)(EC 2.6.1.42) IlvE [12]. *S. enterica* with a null allele of *ridA* possess only a fraction of the wild type BCAT activity. This phenotype is dependent on the generation of 2AA by the cell and exacerbated by increasing concentrations of serine [12]. Furthermore, the role of IlvA in the generation of 2AA explains the correction of *ridA* phenotypes by the addition of exogenous isoleucine, as IlvA is allosterically down regulated by isoleucine, its downstream metabolic product. Allosteric inhibition, rather than any nutritional requirement, is necessary to prevent phenotypes associated with 2AA accumulation. Thus, the introduction of the feedback-resistant $IlvA^{L447F}$ variant eliminates rescue by isoleucine entirely [42].

The enamine/imine deaminase activity of RidA and RidA homologs has since been extensively examined *in vitro*. Because 2AA is unstable, any reaction with 2AA as a substrate must be carried out as a coupled reaction with a generator of 2AA. Thus, in its first examination by Lambrecht et al. 2012, purified RidA protein was assayed in the presence of IlvA and either threonine or serine, measuring the production of 2KB or pyruvate, respectively [6]. RidA was found to increase the rate of conversion of both threonine and serine to their ketoacids when IlvA was present, justifying its designation as an enamine/imine deaminase.

RidA is one subfamily of the Rid family, which also include Rid(s) 1-7. These proteins have been assigned to families based on the Conserved Domain Database algorithm, which categorizes proteins based on sequence alignment and 3D structures [4]. Like RidA, Rid(s)

1-3 possess an arginine residue located in the active site of the enzyme, which is the sole residue that has been shown to be necessary for catalysis. RidA variants lacking this arginine have no deaminase activity *in vitro* and fail to complement a *ridA* deletions strain [12]. Rid(s) 4-7 lack this arginine residue and in the cases that have been tested, are unable to deaminate imines [5].

1.3 BIOCHEMICAL CHARACTERIZATION OF RID PROTEINS

Rid proteins differ in substrate specificity.

In addition to their activity with 2KB or 2AA, RidA and other Rid family members can hydrolyze other amino-acid derived imines/enamines. Niehaus et al. subjected various amino acids to treatments with L-amino acid oxidases [5]. Imine/enamine deaminase activity was assayed by measuring semicarbazone formation in the presence of semicarbazide, which reacts with imine substrates. Rid-catalyzed deamination competes with semicarbazone formation and thus greater imine deaminase activity reduces the rate of semicarbazone formation. RidA and Rid2 from *S. enterica* were both capable of deaminating imines/enamines from a variety of amino acids, though RidA was roughly 10 times more efficient. A Rid7 from *S. enterica* had no detectable activity. Subsequent to this, further examples of Rid proteins from subfamilies 1, 2, and 3 were isolated and characterized, alongside RidA, for their ability to deaminate imines derived from a variety of amino acids by Hodge-Hanson et al. [43]. Representative examples of proteins from each subfamily were chosen from *Pseudomonas* species, as well as a Rid1 protein from *Acinetobacter baylii*. Each of the Rid tested is capable of deaminating imines, but at different rates. In agreement with Niehaus et al., RidA was unable to efficiently deaminate

aromatic imines, however the examples from the other Rid subfamilies proved more effective. Examples from the Rid subfamilies 4-7 were not tested as they are not expected to have imine deaminase activity due to the lack of the critical active site arginine residue.

1.4 PHYSIOLOGICAL ROLES OF OTHER RID FAMILY MEMBERS

RutC

The broad substrate tolerance and differing specificities among Rid family proteins raises the possibility that RidA or Rid(s) 1-3 could be involved in physiologically relevant catalysis with substrates other than 2AA. *E. coli* RutC, a member of the Rid1 subfamily, is a part of the *rut*ABCDEFG operon, which is necessary for uridine catabolism [27]. RutB hydrolyzes the ureidoacrylate intermediate in this pathway, yielding carbamate and 3-aminoacrylate, both of which have been assumed to be spontaneously hydrolyzed *in vivo*. Buckner et al. demonstrate that RutC is capable of hydrolyzing the 3AA intermediate *in vitro*. RutC is also capable of hydrolyzing 2AA and can partially restore phenotypes of *S. enterica* ridA, while demonstrating differing specificities for a variety of imines generated *in vitro*. No effects on fitness have been discovered in *rutC* mutant strains, however, considering its ureidoacrylate deaminase activity and the presence in the *rut*ABCDEFG operon it is reasonable to expect that conditions exist in which it is beneficial to express RutC.

DadY

Pseudomonas aeruginosa encodes nine Rid family proteins, two of which belong to the RidA subfamily [44]. A member of the Rid2 subfamily, *dadY,* is located in the *dad* locus, which enables effective utilization of alanine as a carbon and nitrogen source [45]. Fulton

and Downs investigated the role of *dadY* in this operon, and found that DadY is an enamine/imine deaminase with a divergent and noncomplementary role to RidA [46]. DadY expressed in *S. enterica ridA* fails to complement the phenotypes associated with 2AA. It is, however, capable of deaminating many imines generated by L-amino acid oxidase *in vitro*. Deletions of *dadY* alone do not have an obvious effect on growth rates when alanine is provided as a carbon or nitrogen source, however competition assays revealed a difference in fitness between *dadY* deletions and wild type *P. aeruginosa* under these conditions [46]. These data represent the first definitively demonstrated physiological role for a Rid family member outside of 2AA deamination. It is also probable that DadY exerts an effect on fitness by modifying flux through the alanine utilization pathway, increasing the availability of reaction products rather than preventing damage. As the RidA homolog is still intact in these strains, the possibility of direct damage from iminoalanine (iminoproprionate) is minimized. To supplement this observation, it has been observed that exogenous alanine causes a growth defect in *ridA P. aeruginosa*, a phenotype on which the presence of DadY has no impact (Fulton and Downs, unpublished data). The addition of isoleucine corrects the sensitivity to alanine, leading to the hypothesis that alanine is converted to serine and subsequently to 2AA by serine/threonine dehydratase, which isoleucine suppresses allosterically.

1.5 SUMMARY OF RIDA AND 2AA STRESS PARADIGM

2AA is an enamine derived from serine and other substrates via the activity of enzymes such as the serine/threonine dehydratases IlvA in *S. enterica*, and Cha1 or Ilv1 in *S. cerevisiae.* In *S. enterica,* other dehydratases such as cysteine desulfhydrase (CdsH) and

diaminoproprionate ammonia-lyase (DpaL) can also contribute to 2AA accumulation, though these enzymes are not present in *S. cerevisiae,* and cysteine or diaminoproprionate do not induce 2AA stress in this yeast. To date, all known phenotypes associated with the absence of RidA enzymes can either be traced to accumulation of 2AA and subsequent damage to PLP-dependent enzymes, or have not been rigorously examined but are possibly the result of damage to PLP-dependent enzymes. 2AA directly attacks PLP bound to the active site of susceptible enzymes, forming a PLP-2AA adduct which negates the activity of the target enzyme [6, 47]. Taking this evidence together, 2AA can be categorized as a reactive metabolite which is an obligatory intermediate in certain reactions, and which causes metabolic disruptions through damage to a small subset (PLP-dependent) enzymes.

Regarding the chemistry of 2AA damage to cellular components, it is not known whether 2AA or its tautomer, the imine 2-iminoproprionate is the relevant compound that reacts with PLP, however they can be expected to tautomerize in the cellular environment, and this particular problem is not addressed in this work [6]. The PLP-2AA adduct can be detected via high-performance liquid chromatography and mass spectrometry after release from the enzyme via treatment with NaOH [36]. Formation of the adduct interferes with the damaged enzymes' function, as the chemistry of the critical Schiff base nitrogen is altered. It is not currently known whether modifications are always permanent, and no mechanisms of repair are currently understood.

1.6 METABOLIC IMPACTS OF RIDA DELETIONS AND MECHANISMS OF RESCUE

Salmonella enterica and *Escherichia coli*

As stated above, deletion of *ridA* results in 2AA accumulation, which causes metabolic disruptions via damage to PLP-dependent enzymes. The exact consequences of damage depend on the metabolic flux and requirements of the organism in question.

In *S. enterica, ridA* mutants are unable to grow with pyruvate as a sole carbon source or in the presence of exogenous serine [48]. These phenotypes are abrogated entirely by the addition of isoleucine. Isoleucine prevents 2AA accumulation in a *ridA* mutant by binding IlvA at its regulatory site. An allele of *ilvA*, *ilvA219*, which encodes a variant deficient in regulatory function ($IlvA^{L447F}$), exerts a dominant effect in *S. enterica*, preventing isoleucine from rescuing *ridA* strains [42, 48].

A number of other mechanisms have been described in *S. enterica* by which 2AA accumulation can be prevented. Exogenous threonine prevents 2AA accumulation indirectly, by increasing the concentration of its downstream product, isoleucine, which in turn allosterically inhibits IlvA [49]. Multicopy suppressor studies indicated that overexpression of cystathione β-lyase (MetC) prevents 2AA accumulation as well. Rescue of a *ridA* strain by MetC overexpression is not due to its canonical activity, but instead is likely due to the production of an unknown product that reacts with 2AA directly [50]. These mechanisms are each similar in that they prevent 2AA accumulation and subsequent damage, but in order to characterize the role of RidA enzymes in the cell i.*e.,* how metabolism is impaired in its absence, it is critical to identify mutants or nutritional

metabolic supplementation that alleviate the phenotypic effects without reducing 2AA accumulation.

The growth defect of *ridA* mutants in *S. enterica* can be rescued by the addition of glycine. This rescue occurs downstream of 2AA production, which is not affected; evidenced by the continued impairment of branched-chain aminotransferase (BCAT, IlvE in *S. enterica*) observed during glycine supplementation [51]. Instead, exogenous glycine bypasses a requirement for glycine created in *ridA* strains as a result of damage to the PLP-dependent serine hydroxymethyltransferase (SHMT) GlyA. In *E. coli* glycine supplementation fails to rescue growth of a 2AA-accumulating strain [52]. This difference is in spite of the observation that *S. enterica* shares a core genome with *E. coli*, in which ~80% of genes are 90% identical in amino acid sequence [53, 54]. Instead, aspartate supplementation improves growth, due to damage to aspartate aminotransferase, another PLP-dependent enzyme [52]. The difference in the effect of 2AA accumulation between these closely related organisms underscores the unreliable nature of any assumptions made based on shared metabolic components. Instead, the final impact on metabolism must be treated as an emergent property resulting from the sum of genes, their regulation, and their biochemical properties, which necessitates empirical and organism-specific experiments.

The exact mechanism by which glycine supplementation rescues growth in *S. enterica ridA* is complex. Glycine is a source of one-carbon units generated by the glycine cleavage system (GCV). Under certain conditions, when 2AA stress is hypothesized to be moderate (0.25 mM cysteine or 0.1 mM diaminoproprionate as substrates), glycine supplementation can restore partial growth of a *ridA* strain through GCV [51]. Supplementation with metabolites that are synthesized from one-carbon building blocks (pantothenate, thiamine,

methionine) also aided growth in a GCV dependent manner, further supporting the hypothesis that 2AA-accumulating strains are deficient in one carbon units as a result of damage to GlyA.

Under heavier 2AA stress (with serine as substrate, supplied at 5mM, which leads to greater 2AA accumulation), the small regulatory RNA *gcvB* is necessary, rather than GCV. It is not known which of the 50+ regulatory targets of *gcvB* contribute to the rescue of *ridA* [55]. What is clear is that a difference in degree of stress elicits a fundamentally different metabolic response. Under moderate 2AA stress, supplementation with metabolites involved in one-carbon metabolism is sufficient to mimic the rescue by glycine, whereas under high levels of 2AA stress an unknown regulatory target responds to glycine levels via *gcvB* and is primarily responsible for the rescue of the growth defect by glycine supplementation.

In addition to the consequences of 2AA accumulation outlined above, 2AA accumulation in *S. enterica ridA* results in reduced motility and a reduction in the expression of genes involved in the production of flagella [56]. This phenotype is not altered by the addition of glycine, indicating that a separate mechanism by which 2AA affects *S. enterica* physiology exists.

Campylobacter jejuni

C. jejuni possess both a RidA and a Rid2 homolog, the function of which was investigated by Irons et al [11]. Both the RidA and Rid2 proteins were able to complement *S. enterica ridA*, though the Rid2 only partially. Both are capable of deaminating 2AA *in vitro*. Unlike *S. enterica*, *C. jejuni ridA* exhibits a flagellar defect on nutrient-rich media and in the presence of isoleucine. The serine-threonine dehydratase, which in *S. enterica* acts as a

major generator of 2AA, lacks a regulatory domain, however deletion of this gene does not prevent deleterious phenotypes from occurring in *C. jejuni ridA*. These deleterious phenotypes include a motility defect, aberrant flagellar structure, and an autoagglutination defect. It is not currently known what specific PLP-dependent targets are damaged as a result of 2AA accumulation in *C. jejuni*. It is also entirely possible that 2AA is not involved at all, and that the role of CjRidA is to deaminate a different enamine/imine, either preventing damage due to a reactive species or altering metabolic flux.

Pseudomonas aeruginosa

In *P. aeruginosa*, suppressor analysis identified a point mutation of the cysteine desulfurase enzyme (IscS) which suppressed growth defects of a *ridA* deletion ($IscS^{Q183P}$) [57]. IscS catalyzes a β-elimination of cysteine, which produces alanine and enzyme-bound persulfide, which is used to produce FeS clusters. IscS is a PLP-dependent enzyme, but prior to this study had not been implicated in phenotypes associated with 2AA accumulation. $IscS^{Q183P}$ was found to exert a dominant effect when expressed as a merodiploid. The PLP cofactor of wild type IscS and $IscS^{Q183P}$ were analyzed by HPLC after purification from *S. enterica ridA*. $IscS^{Q183P}$ was found to be partially resistant to 2AA damage, evidenced by the reduction in the relative amount of PLP-2AA adduct. In both *P. aeruginosa* and *S. enterica*, thiamine supplementation (thiamine is a critical product of FeS-containing enzymes) improved the growth of *ridA* deletion strains. Therefore, the IscS of *S. enterica* is likely to be a physiologically relevant target of 2AA.

Arabidopsis thaliana

Niehaus et al. found that a deletion of the RidA homolog of *A. thaliana* resulted in a growth defect in root development, while having no effect on leaves [58]. Consistent with the RidA paradigm established in *S. enterica,* the BCAT activity of *A. thaliana* extracts was lowered in a *ridA* mutant when compared to wild type. The observed growth defect was exacerbated by increasing concentrations of serine. At low concentrations of serine, isoleucine abrogated the growth defect entirely, while titrating serine to higher concentrations caused the defect to reappear. Based on this, the authors suggest that an *A. thaliana ridA* mutant is deficient in isoleucine synthesis, however, isoleucine concentrations were measured in cell extracts and no significant differences were observed. Given more recent developments in the identification of the most physiologically relevant targets in *S. enterica* and others, it likely that the target is SHMT or another PLP-dependent enzyme, and that isoleucine is acting solely via its allosteric effect on serine/threonine dehydratase in *A. thaliana* [9, 57],[59].

A. thaliana also bears significant similarities with *S. cerevisiae,* in that the known 2AA producers and a RidA homolog are localized to cellular compartments. For yeast this is the mitochondrion, whereas in *A. thaliana* these enzymes are found in chloroplasts. While these compartments fulfill different functions, both are involved in branched chain amino acid metabolism, as well as heme biosynthesis and FeS cluster assembly, which are critical metabolic pathways in which PLP-dependent enzymes play an integral role.

1.7 THE RIDA PARADIGM IN YEAST

Prior investigation of RidA homologs *mmf1* and *hmf1*.

The following chapter describes efforts to understand the specific consequences of 2AA accumulation and the resulting metabolic disruption in the eukaryotic model organism *Saccharomyces cerevisiae*, hereafter referred to as yeast. This work follows the initial characterization of the yeast RidA homolog *mmf1* by Ernst and Downs [9], (Ernst thesis appendix). Prior to this study, it was known that the yeast genome encodes two RidA homologs, *mmf1* and *hmf1*. The product of *mmf1* contains a mitochondrial localization presequence at its N-terminus, and localizes to the mitochondrion [60]. *mmf1* gene deletions result in growth deficiencies and loss of mtDNA, though the mechanism remained unclear at the time. Additionally, the product of *hmf1* was shown to rescue an *mmf1* deletion when it was expressed with a mitochondrial localization sequence [60]. Kim et al. investigated *mmf1* and *hmf1* deletions and found that isoleucine was required for optimal growth on rich undefined medium (YPD) in an *mmf1* strain, and that cell extracts of the same strains were deficient in isoleucine transaminase activity [61]. From this observation they proposed that *mmf1* could be involved in regulation of the specificity of the Bat1/2 transaminases which convert 2-ketomethylvalerate (2KMV) to isoleucine, and that isoleucine metabolism had an indirect effect on mitochondrial DNA stability.

Mmf1 prevents 2AA accumulation *in vivo*.

The work by Ernst and Downs was the first report investigating the function of *Mmf1* after the imine/enamine deaminase role of the RidA subfamily was described in other organisms [9]. Utilizing a model in which Mmf1 plays a crucial role in preventing 2AA accumulation and subsequent damage to PLP-dependent enzymes, many of the phenotypes of an *mmf1*

deletion identified in previous work can be explained. Growth deficiencies are likely the result of damage to PLP-dependent enzymes, as seen in other organisms with RidA homologs. Most notably, the fact that isoleucine (but not the other branched chain amino acids leucine or valine) corrects the phenotypes in an *mmf1* deletion is directly explained by the mechanism of 2AA generation in yeast. *S. cerevisiae* possesses two serine/threonine dehydratases capable of 2AA generation, Ilv1 and Cha1, as mentioned earlier. On defined media without serine or threonine Cha1 is not transcribed, while Ilv1 is present but is allosterically inhibited by isoleucine, as it possesses a regulatory domain much like IlvA in *S. enterica* [62, 63]. Both of these dehydratases are mitochondrially localized [64].

The study by Ernst and Downs [8], informed by previous work on the RidA paradigm in other organisms, went on to definitively link the dependence of the *mmf1* phenotypes on 2AA generation. The authors first established that Ilv1, like IlvA, generates 2AA *in vitro* when supplied with serine as a substrate, by examining pyruvate formation with or without RidA. An Ilv1 mutant lacking a regulatory domain, rendering it insensitive to feedback inhibition by isoleucine, was constructed. Growth of *mmf1* yeast expressing this variant of Ilv1 is not restored by addition of isoleucine when they are grown on defined medium, in which Ilv1 is the relevant producer of 2AA. Therefore, the effects of isoleucine in an *mmf1* deletion are entirely dependent on its regulatory effect on Ilv1 and not related to a nutritional requirement as previously suggested [61]. The addition of serine to a defined medium with isoleucine causes a growth defect which is not present in a *cha1 mmf1* strain. Therefore, Ilv1 and Cha1 are both capable of causing 2AA stress in an *mmf1* strain under specific conditions: Ilv1 when no exogenous isoleucine is present, and Cha1 when exogenous serine is present, regardless of isoleucine addition.

Loss of *MMF1* has pleiotropic effects.

Ernst and Downs investigated additional phenotypes caused by 2AA accumulation in yeast [9]. Using microscopy to visualize mtDNA, they showed that the mtDNA instability phenotype was dependent on the presence of 2AA generators. In addition to this, a variety of nutritional supplements were provided to an *mmf1* strain in an effort to identify specific metabolic pathways impacted by 2AA accumulation [65]. Glycine, which is known to circumvent metabolic disruption caused by 2AA damage to the PLP-dependent glycine hydroxymethyltransferase in *S. enterica,* had no effect. The most impactful supplements tested were aminolevulinic acid (ALA), a precursor to heme biosynthesis, and bathophenanthroline sulfate (BPS), an extracellular iron chelator. In addition to improving growth of an *mmf1* strain, BPS prevents mtDNA loss, suggesting a link between iron, 2AA, and mtDNA stability [9].

1.8 THESIS OUTLINE

This work greatly expands the metabolic impact of RidA homologs in the budding yeast *Saccharomyces cerevisiae*. Yeast is a widely used model organism noted for its sophisticated tool set, extensive understanding of basic processes and simple growth requirments among eukaryotes. Unlike the model prokaryotes for which RidA has been best characterized, yeast possess mitochondria and a number of other organelles, such as endosomes, peroxisomes, and vacuoles. Yeast undergo haploid and diploid life stages, and possess many conserved eukaryotic proteins, which are used as a basis for possible generalizations involving more complex organisms. While we understand certain properties of RidA family enzymes in eukaryotes, no specific PLP-dependent targets of

2AA damage have been rigorously defined [9, 58]. Yeast represent the most reasonable first step in defining the role of RidA homologs in eukaryotic organisms.

Chapter 2 describes the characterization of the PLP-dependent enzyme in yeast responsible for the some of the phenotypes observed by Ernst and Downs, most notably the growth deficiencies resulting from heme depletion [9, 59].

Chapter 3 Summarizes the conclusions reached from the study in Chapter 2, and outlines future experiments to characterize unknown targets of 2AA in mitochondria.

1.9 REFERENCES

1. de Lorenzo, V., A. Sekowska, and A. Danchin, *Chemical reactivity drives spatiotemporal organisation of bacterial metabolism.* FEMS Microbiol Rev, 2015. **39**(1): p. 96-119.

2. Albert, R., H. Jeong, and A.L. Barabasi, *Error and attack tolerance of complex networks.* Nature, 2000. **406**(6794): p. 378-82.

3. Keller, M.A., G. Piedrafita, and M. Ralser, *The widespread role of non-enzymatic reactions in cellular metabolism.* Curr Opin Biotechnol, 2015. **34**: p. 153-61.

4. Marchler-Bauer, A., et al., *CDD: conserved domains and protein three-dimensional structure.* Nucleic Acids Res, 2013. **41**(Database issue): p. D348-52.

5. Niehaus, T.D., et al., *Genomic and experimental evidence for multiple metabolic functions in the RidA/YjgF/YER057c/UK114 (Rid) protein family.* BMC Genomics, 2015. **16**(1): p. 382.

6. Lambrecht, J.A., J.M. Flynn, and D.M. Downs, *Conserved YjgF protein family deaminates reactive enamine/imine intermediates of pyridoxal 5'-phosphate (PLP)-dependent enzyme reactions.* J Biol Chem, 2012. **287**(5): p. 3454-61.

7. Digiovanni, S., et al., *Two novel fish paralogs provide insights into the Rid family of imine deaminases active in pre-empting enamine/imine metabolic damage.* Sci Rep, 2020. **10**(1): p. 10135.

8. ElRamlawy, K.G., et al., *Der f 34, a Novel Major House Dust Mite Allergen Belonging to a Highly Conserved Rid/YjgF/YER057c/UK114 Family of Imine Deaminases.* J Biol Chem, 2016. **291**(41): p. 21607-21615.

9. Ernst, D.C. and D.M. Downs, *Mmf1p Couples Amino Acid Metabolism to Mitochondrial DNA Maintenance in Saccharomyces cerevisiae.* mBio, 2018. **9**(1).

10. Irons, J., K.M. Hodge-Hanson, and D.M. Downs, *PA5339, a RidA Homolog, Is Required for Full Growth in Pseudomonas aeruginosa.* J Bacteriol, 2018. **200**(22).

11. Irons, J., et al., *Cj1388 Is a RidA Homolog and Is Required for Flagella Biosynthesis and/or Function in Campylobacter jejuni.* Front Microbiol, 2019. **10**: p. 2058.

12. Lambrecht, J.A., G.E. Schmitz, and D.M. Downs, *RidA proteins prevent metabolic damage inflicted by PLP-dependent dehydratases in all domains of life.* mBio, 2013. **4**(1): p. e00033-13.

13. Levy-Favatier, F., et al., *Characterization, purification and cDNA cloning of a rat perchloric-acid-soluble 23-kDa protein present only in liver and kidney.* Eur J Biochem, 1993. **212**(3): p. 665-73.

14. Samuel, S.J., S.P. Tzung, and S.A. Cohen, *Hrp12, a novel heat-responsive, tissue-specific, phosphorylated protein isolated from mouse liver.* Hepatology, 1997. **25**(5): p. 1213-22.

15. Schmiedeknecht, G., et al., *Isolation and characterization of a 14.5-kDa trichloroacetic-acid-soluble translational inhibitor protein from human monocytes that is upregulated upon cellular differentiation.* Eur J Biochem, 1996. **242**(2): p. 339-51.

16. Oka, T., et al., *Isolation and characterization of a novel perchloric acid-soluble protein inhibiting cell-free protein synthesis.* J Biol Chem, 1995. **270**(50): p. 30060-7.

17. Morishita, R., et al., *Ribonuclease activity of rat liver perchloric acid-soluble protein, a potent inhibitor of protein synthesis.* J Biol Chem, 1999. **274**(29): p. 20688-92.

18. Dhawan, L., et al., *Y-box binding protein 1 and RNase UK114 mediate monocyte chemoattractant protein 1 mRNA stability in vascular smooth muscle cells.* Mol Cell Biol, 2012. **32**(18): p. 3768-75.

19. He, Z. and J.C. Spain, *A novel 2-aminomuconate deaminase in the nitrobenzene degradation pathway of Pseudomonas pseudoalcaligenes JS45.* J Bacteriol, 1998. **180**(9): p. 2502-6.

20. Davis, J.K., et al., *Genetic and biochemical comparison of 2-aminophenol 1,6-dioxygenase of Pseudomonas pseudoalcaligenes JS45 to meta-cleavage dioxygenases: divergent evolution of 2-aminophenol meta-cleavage pathway.* Arch Microbiol, 1999. **172**(5): p. 330-9.

21. Takenaka, S., et al., *Complete nucleotide sequence and functional analysis of the genes for 2-aminophenol metabolism from Pseudomonas sp. AP-3.* Arch Microbiol, 2000. **174**(4): p. 265-72.

22. Muraki, T., et al., *Prokaryotic homologs of the eukaryotic 3-hydroxyanthranilate 3,4-dioxygenase and 2-amino-3-carboxymuconate-6-semialdehyde decarboxylase in the 2-nitrobenzoate degradation pathway of Pseudomonas fluorescens strain KU-7.* Appl Environ Microbiol, 2003. **69**(3): p. 1564-72.

23. Park, H.S. and H.S. Kim, *Genetic and structural organization of the aminophenol catabolic operon and its implication for evolutionary process.* J Bacteriol, 2001. **183**(17): p. 5074-81.

24. Orii, C., et al., *A novel coupled enzyme assay reveals an enzyme responsible for the deamination of a chemically unstable intermediate in the metabolic pathway of 4-amino-3-hydroxybenzoic acid in Bordetella sp. strain 10d.* Eur J Biochem, 2004. **271**(15): p. 3248-54.

25. Melloni, E., et al., *Molecular and functional properties of a calpain activator protein specific for mu-isoforms.* J Biol Chem, 1998. **273**(21): p. 12827-31.

26. Loh, K.D., et al., *A previously undescribed pathway for pyrimidine catabolism.* Proc Natl Acad Sci U S A, 2006. **103**(13): p. 5114-9.

27. Kim, K.S., et al., *The Rut pathway for pyrimidine degradation: novel chemistry and toxicity problems.* J Bacteriol, 2010. **192**(16): p. 4089-102.

28. Rappu, P., et al., *A role for a highly conserved protein of unknown function in regulation of Bacillus subtilis purA by the purine repressor.* J Bacteriol, 1999. **181**(12): p. 3810-5.

29. Sasagawa, T., et al., *Analysis of the fatty acid components in a perchloric acid-soluble protein.* Biochim Biophys Acta, 1999. **1437**(3): p. 317-24.

30. Farkas, A., et al., *DUK114, the Drosophila orthologue of bovine brain calpain activator protein, is a molecular chaperone.* Biochem J, 2004. **383**(Pt 1): p. 165-70.

31. Irons, J.L., K. Hodge-Hanson, and D.M. Downs, *RidA Proteins Protect against Metabolic Damage by Reactive Intermediates.* Microbiol Mol Biol Rev, 2020. **84**(3).

32. Enos-Berlage, J.L., M.J. Langendorf, and D.M. Downs, *Complex metabolic phenotypes caused by a mutation in yjgF, encoding a member of the highly conserved YER057c/YjgF family of proteins.* J Bacteriol, 1998. **180**(24): p. 6519-28.

33. Christopherson, M.R., et al., *Suppressor analyses identify threonine as a modulator of ridA mutant phenotypes in Salmonella enterica.* PLoS One, 2012. **7**(8): p. e43082.

34. Lambrecht, J.A. and D.M. Downs, *Anthranilate phosphoribosyl transferase (TrpD) generates phosphoribosylamine for thiamine synthesis from enamines and phosphoribosyl pyrophosphate.* ACS Chem Biol, 2013. **8**(1): p. 242-8.

35. Hillebrand, G.G., J.L. Dye, and C.H. Suelter, *Formation of an intermediate and its rate of conversion to pyruvate during the tryptophanase-catalyzed degradation of S-o-nitrophenyl-L-cysteine.* Biochemistry, 1979. **18**(9): p. 1751-5.

36. Flynn, J.M. and D.M. Downs, *In the absence of RidA, endogenous 2-aminoacrylate inactivates alanine racemases by modifying the pyridoxal 5'-phosphate cofactor.* J Bacteriol, 2013. **195**(16): p. 3603-9.

37. Roise, D., et al., *Inactivation of the Pseudomonas striata broad specificity amino acid racemase by D and L isomers of beta-substituted alanines: kinetics, stoichiometry, active site peptide, and mechanistic studies.* Biochemistry, 1984. **23**(22): p. 5195-201.

38. Arfin, S.M. and D.A. Koziell, *Inhibition of growth of Salmonella typhimurium and of threonine deaminase and transaminase B by beta-chloroalanine.* J Bacteriol, 1971. **105**(2): p. 519-22.

39. Relyea, N.M., S.S. Tate, and A. Meister, *Affinity labeling of the active center of L-aspartate-beta-decarboxylase with beta-chloro-L-alanine.* J Biol Chem, 1974. **249**(5): p. 1519-24.

40. Kishore, G.M., *Mechanism-based inactivation of bacterial kynureninase by beta-substituted amino acids.* J Biol Chem, 1984. **259**(17): p. 10669-74.

41. Shen, W., A.J. Borchert, and D.M. Downs, *2-Aminoacrylate stress damages diverse PLP-dependent enzymes in vivo.* J Biol Chem, 2022. **298**(6): p. 101970.

42. Schmitz, G. and D.M. Downs, *Reduced transaminase B (IlvE) activity caused by the lack of yjgF is dependent on the status of threonine deaminase (IlvA) in Salmonella enterica serovar Typhimurium.* J Bacteriol, 2004. **186**(3): p. 803-10.

43. Hodge-Hanson, K.M. and D.M. Downs, *Members of the Rid protein family have broad imine deaminase activity and can accelerate the Pseudomonas aeruginosa D-arginine dehydrogenase (DauA) reaction in vitro.* PLoS One, 2017. **12**(9): p. e0185544.

44. Overbeek, R., et al., *The subsystems approach to genome annotation and its use in the project to annotate 1000 genomes.* Nucleic Acids Res, 2005. **33**(17): p. 5691-702.

45. He, W., C. Li, and C.D. Lu, *Regulation and characterization of the dadRAX locus for D-amino acid catabolism in Pseudomonas aeruginosa PAO1.* J Bacteriol, 2011. **193**(9): p. 2107-15.

46. Fulton, R.L. and D.M. Downs, *DadY (PA5303) is required for fitness of Pseudomonas aeruginosa when growth is dependent on alanine catabolism.* Microb Cell, 2022. **9**(12): p. 190-201.

47. Borchert, A.J., D.C. Ernst, and D.M. Downs, *Reactive Enamines and Imines In Vivo: Lessons from the RidA Paradigm.* Trends Biochem Sci, 2019. **44**(10): p. 849-860.

48. Christopherson, M.R., G.E. Schmitz, and D.M. Downs, *YjgF is required for isoleucine biosynthesis when Salmonella enterica is grown on pyruvate medium.* J Bacteriol, 2008. **190**(8): p. 3057-62.

49. Borchert, A.J. and D.M. Downs, *Analyses of variants of the Ser/Thr dehydratase IlvA provide insight into 2-aminoacrylate metabolism in Salmonella enterica.* J Biol Chem, 2018. **293**(50): p. 19240-19249.

50. Ernst, D.C., M.R. Christopherson, and D.M. Downs, *Increased Activity of Cystathionine beta-Lyase Suppresses 2-Aminoacrylate Stress in Salmonella enterica.* J Bacteriol, 2018. **200**(9).

51. Ernst, D.C. and D.M. Downs, *2-Aminoacrylate Stress Induces a Context-Dependent Glycine Requirement in ridA Strains of Salmonella enterica.* J Bacteriol, 2016. **198**(3): p. 536-43.

52. Borchert, A.J. and D.M. Downs, *The Response to 2-Aminoacrylate Differs in Escherichia coli and Salmonella enterica, despite Shared Metabolic Components.* J Bacteriol, 2017. **199**(14).

53. Anjum, M.F., et al., *Identification of core and variable components of the Salmonella enterica subspecies I genome by microarray.* Infect Immun, 2005. **73**(12): p. 7894-905.

54. Parkhill, J., et al., *Complete genome sequence of a multiple drug resistant Salmonella enterica serovar Typhi CT18.* Nature, 2001. **413**(6858): p. 848-52.

55. Sharma, C.M., et al., *Pervasive post-transcriptional control of genes involved in amino acid metabolism by the Hfq-dependent GcvB small RNA.* Mol Microbiol, 2011. **81**(5): p. 1144-65.

56. Borchert, A.J. and D.M. Downs, *Endogenously generated 2-aminoacrylate inhibits motility in Salmonella enterica.* Sci Rep, 2017. **7**(1): p. 12971.

57. Fulton, R.L., J. Irons, and D.M. Downs, *The Cysteine Desulfurase IscS Is a Significant Target of 2-Aminoacrylate Damage in Pseudomonas aeruginosa.* mBio, 2022. **13**(3): p. e0107122.

58. Niehaus, T.D., et al., *Arabidopsis and maize RidA proteins preempt reactive enamine/imine damage to branched-chain amino acid biosynthesis in plastids.* Plant Cell, 2014. **26**(7): p. 3010-22.

59. Whitaker, G.H., D.C. Ernst, and D.M. Downs, *Absence of MMF1 disrupts heme biosynthesis by targeting Hem1pin Saccharomyces cerevisiae.* Yeast, 2021. **38**(12): p. 615-624.

60. Oxelmark, E., et al., *Mmf1p, a novel yeast mitochondrial protein conserved throughout evolution and involved in maintenance of the mitochondrial genome.* Mol Cell Biol, 2000. **20**(20): p. 7784-97.

61. Kim, J.M., H. Yoshikawa, and K. Shirahige, *A member of the YER057c/yjgf/Uk114 family links isoleucine biosynthesis and intact mitochondria maintenance in Saccharomyces cerevisiae.* Genes Cells, 2001. **6**(6): p. 507-17.

62. Bornaes, C., et al., *A regulatory element in the CHA1 promoter which confers inducibility by serine and threonine on Saccharomyces cerevisiae genes.* Mol Cell Biol, 1993. **13**(12): p. 7604-11.

63. Ahmed, S.I., et al., *Purification and properties of threonine deaminase from Saccharomyces cerevisiae.* Biochimie, 1976. **58**(1-2): p. 225-32.

64. Ryan, E.D. and G.B. Kohlhaw, *Subcellular localization of isoleucine-valine biosynthetic enzymes in yeast.* J Bacteriol, 1974. **120**(2): p. 631-7.

65. Ernst, D.C., *Exploring Sources and Metabolic Consequences of 2-Aminoacrylate Stress in Bacteria and Yeast*, in *Microbiology*. 2017, University of Georgia. p. 256.

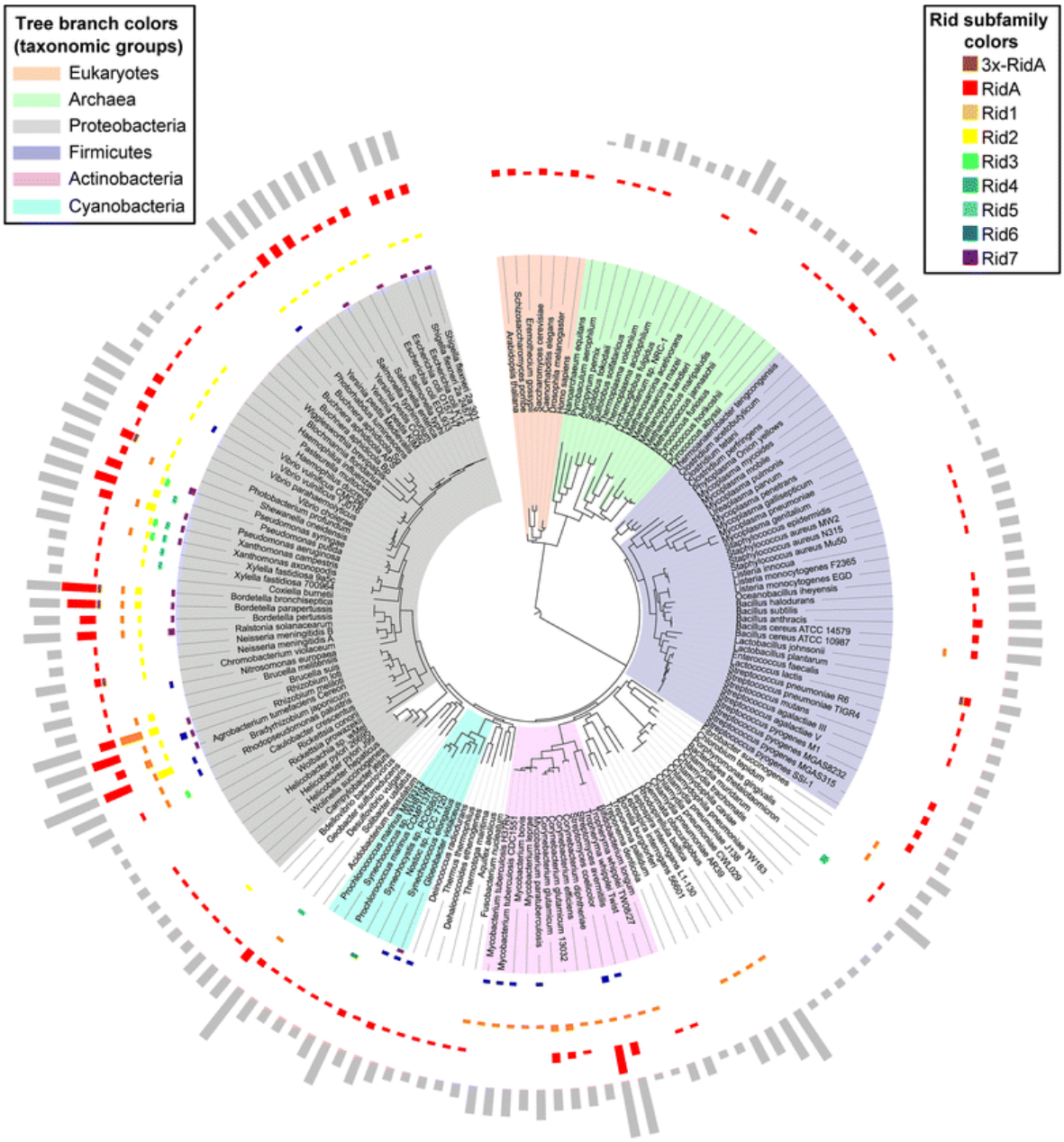

Figure 1

Phylogenetic distribution of the Rid family. Occurrence of RidA and other Rid family homologs in a variety of organisms in which all domains of life are represented. Instances of Rid family members were mapped onto a phylogenetic tree previously constructed by

Cicarelli et al. [1]. Bar size and color indicate the relative number and subtype of Rid family members for each organism. Figure courtesy of Neihaus et al. [2]

1. Ciccarelli, F.D., et al., *Toward automatic reconstruction of a highly resolved tree of life.* Science, 2006. **311**(5765): p. 1283-7.

2. Niehaus, T.D., et al., *Genomic and experimental evidence for multiple metabolic functions in the RidA/YjgF/YER057c/UK114 (Rid) protein family*. BMC Genomics, 2015. **16**(1): p. 382.

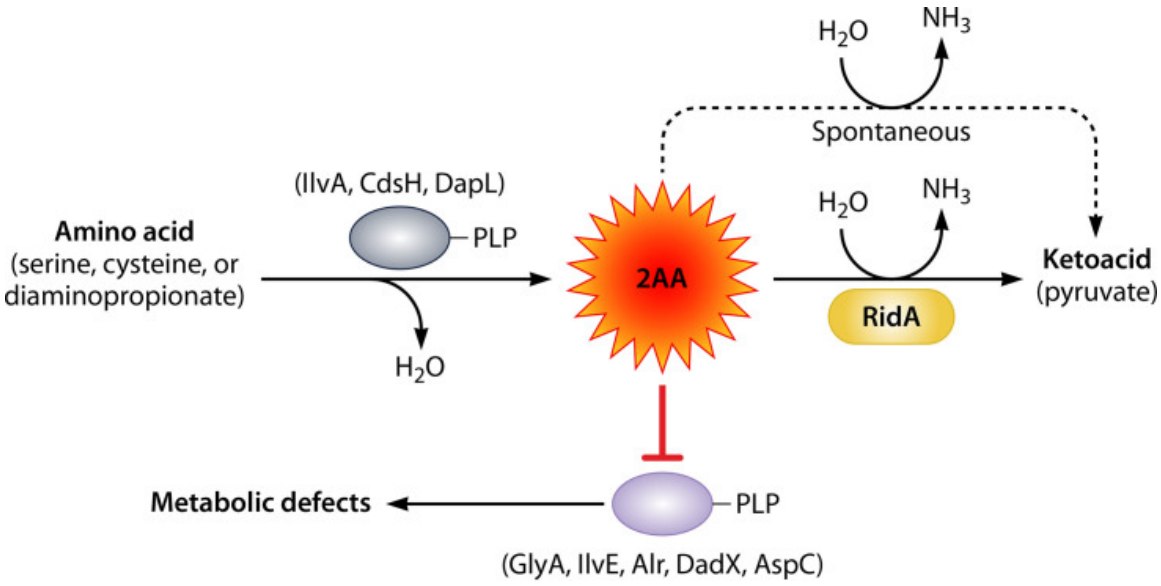

Figure 2

The RidA paradigm. 2-aminoacrylate (2AA) is generated from a variety of amino acid substrates by the action of dehydratase enzymes (grey). 2AA hydrolysis can occur spontaneously to form pyruvate. In the absence of the enamine/imine deaminase RidA (gold), 2AA accumulates and covalently modifies the pyridoxal 5'-phosphate (PLP) cofactor in the active site of a variety of PLP-dependent enzymes, which vary in their susceptibility to damage and their impact on the physiological state of the organism
Figure courtesy of Irons et al. [1]

1. Irons, J.L., K. Hodge-Hanson, and D.M. Downs, *RidA Proteins Protect against Metabolic Damage by Reactive Intermediates.* Microbiol Mol Biol Rev, 2020. **84**(3).

CHAPTER 2

ABSENCE OF *MMF1* DISRUPTS HEME BIOSYNTHESIS BY TARGETING HEM1P IN *SACCHAROMYCES CEREVISIAE* [1]

[1]Whitaker GH, Ernst DC, Downs DM. 2021. *Yeast.* 38(12):615-624.
Reprinted here with permission of the publisher

2.1 ABSTRACT

The RidA subfamily of the Rid (YjgF/YER057c/UK114) superfamily of proteins is broadly distributed and found in all domains of life. RidA proteins are enamine/imine deaminases. In the organisms that have been investigated, lack of RidA results in accumulation of the reactive enamine species 2-aminoacrylate (2AA) and/or its derivative imine 2-iminopropanoate (2IP). The accumulated enamine/imine species can damage specific pyridoxal phosphate (PLP)-dependent target enzymes. The metabolic imbalance resulting from the damaged enzymes is organism specific and based on metabolic network configuration. *Saccharomyces cerevisiae* encodes two RidA homologs, one localized to the cytosol and one to the mitochondria. The mitochondrial RidA homolog, Mmf1p, prevents enamine/imine stress and is important for normal growth and maintenance of mitochondrial DNA. Here we show that Mmf1p is necessary for optimal heme biosynthesis. Biochemical and/or genetic data herein support a model in which accumulation of 2AA and or 2IP, in the absence of Mmf1p, inactivates Hem1p, a mitochondrially located PLP-dependent enzyme required for heme biosynthesis.

2.2 INTRODUCTION

The Rid protein family (YjgF/YER057c/UK114) has been split into nine subfamilies in the NCBI conserved domain database (cd00448) (Marchler-Bauer et al., 2013; Niehaus et al., 2015). Members of the RidA subfamily have enamine/imine deaminase activity and are found in all domains of life (Digiovanni et al., 2020; ElRamlawy et al., 2016; D. C. Ernst & Downs, 2018; J. Irons, Hodge-Hanson, & Downs, 2018; J. Irons, Sacher, Szymanski, & Downs, 2019; Lambrecht, Schmitz, & Downs, 2013; Martínez-

Chavarría et al., 2020; Niehaus et al., 2015). 2-aminoacrylate (2AA) is a reactive enamine that is generated by pyridoxal 5'-phosphate (PLP)-dependent enzymes as an obligatory intermediate in some reactions, specifically the breakdown of serine. Like other enamines, 2AA rapidly tautomerizes to its imine, 2-iminopropanoate (2IP). The imine is subsequently hydrolyzed to form a stable keto acid, which in the case of 2IP is pyruvate. 2AA can react with and irreversibly damage enzymes by forming an adduct with the PLP-enzyme complex (Borchert, Ernst, & Downs, 2019; Esaki & Walsh, 1986; Walsh, 1982). In strains lacking RidA, the accumulation of 2AA/2IP generates endogenous metabolic stress that has diverse phenotypic manifestations characteristic of the organism involved (J. L. Irons, Hodge-Hanson, & Downs, 2020). The inability to easily distinguish between enamine/imine molecules, or control their tautomerization, makes it difficult to definitively assign a role for one over the other *in vivo*. However, *in vitro* mechanistic studies support the assumption that the 2AA enamine is the agent of direct damage in *ridA* mutants (Borchert et al., 2019; Lambrecht et al., 2013).

Saccharomyces cerevisiae encodes two RidA homologs, one localized to the cytoplasm (Hmf1p) and one to the mitochondrion (Mmf1p). There are no phenotypes reported for the lack of Hmf1p, but loss of Mmf1p results in growth defects and the loss of mitochondrial DNA (D. C. Ernst & Downs, 2018; Kim, Yoshikawa, & Shirahige, 2001; Oxelmark et al., 2000). Analysis of *mmf1*-Δ*::KanMX* (*mmf1*Δ) strains showed that the generation and accumulation of 2AA/2IP was responsible for the phenotypes of the mutant (D. C. Ernst & Downs, 2018), closely mirroring the paradigm identified and rigorously characterized in *Salmonella enterica* (Borchert et al., 2019; D. C. Ernst & Downs, 2018; J. L. Irons et al., 2020; Lambrecht et al., 2013). In the case of *S. enterica*, serine/threonine

dehydratase (E.C. 4.3.1.19, IlvA) is the primary generator of 2AA/2IP from endogenous L-serine. While 2AA/2IP can be hydrolyzed nonenzymatically to form pyruvate, low availability of free water is presumed to limit this reaction *in vivo*, and RidA is thus required to expedite the hydrolysis (Lambrecht et al., 2013). Phenotypes of *ridA* mutant strains of *S. enterica* are due to damage of specific PLP-dependent enzymes caused by 2AA (Downs & Ernst, 2015; Lambrecht et al., 2013).

The growth defects and loss of mtDNA in *S. cerevisiae* strains lacking *MMF1* are dependent on the activity of one or both mitochondrial serine/threonine dehydratases (Ilv1p/Cha1p)(D. C. Ernst & Downs, 2018) (Figure 1). The differential roles of these two enzymes allowed modulation of 2AA/2IP formation by changing growth conditions. *CHA1* encodes the catabolic serine dehydratase and is transcribed only in the presence of exogenous L-serine or L-threonine (Bornaes, Ignjatovic, Schjerling, Kielland-Brandt, & Holmberg, 1993). In contrast, the product of *ILV1* is active when no exogenous L-serine is provided and is allosterically inhibited by L-isoleucine (Ahmed, Bollon, Rogers, & Magee, 1976; D. C. Ernst & Downs, 2018). When *mmf1*Δ yeast cells are grown under conditions in which 2AA/2IP accumulates, they grow poorly and lose mitochondrial DNA (mtDNA) at high frequency making these cells unable to respire (cytoplasmic petite) (D. C. Ernst & Downs, 2018). While the phenotypes displayed by the *mmf1*Δ mutants are definitively caused by accumulation or 2AA and/or 2IP, the enzyme(s) targeted by 2AA to cause the metabolic perturbations are not currently known.

This study was initiated to extend our understanding of the 2AA/2IP mediated phenotypes of a *mmf1*Δ mutant and identify targeted enzyme(s) beyond those previously reported. Results herein identified a heme deficiency in *mmf1*Δ yeast strain that contributed

to the growth defect of the mutant. Aminolevulinic acid synthase (ALAS, Hem1p, E.C. 2.3.1.37) catalyzes the condensation of succinyl-CoA and L-glycine to form 5-aminolevulinic acid (ALA) (Hunter et al., 2012). Hem1p is a PLP-dependent enzyme located in the mitochondrion that is responsible for the first step in heme biosynthesis. Here we show that 2AA/2IP caused damage to Hem1p, which contributed to the growth defects and heme deficiency of *mmf1*Δ yeast.

2.3 MATERIALS AND METHODS

Strains, media, and chemicals. *Saccharomyces cerevisiae* strain YJF153 (MATa *ho*::*dsdAMX4*) was derived from an oak tree isolate (YPS163) and provided by Justin Fay (Washington University) (X. C. Li & Fay, 2017). *S. cerevisiae* strain S288c (*MAT*α) (Mortimer & Johnston, 1986) was a gift from David Garfinkel (University of Georgia). Derivative strains are listed in Table S1.

Rich medium (YP) contained 20 g/l peptone (Fisher Scientific) and 10 g/l yeast extract. Minimal medium (S) contained 1.71 g/l yeast nitrogen base without amino acids or nitrogen (Sunrise Science; #1500-100) and ammonium sulfate (5 g/l). Either dextrose (D; 20 g/l) or glycerol (G; 30 g/l) was provided as the sole carbon source. Solid medium included 20 g/l agar (Difco). Antibiotics used for deletion marker selection were added at the following final concentrations: 400 μg/ml Geneticin (G418; Gold Biotechnology), 100 μg/ml nourseothricin sulfate (cloNAT; Gold Biotechnology). Supplements added to SD medium were: L-isoleucine (1 mM), ALA (0.24 mM) and L-glycine (1 mM).

Escherichia coli strain BL21-AI, which contains T7 polymerase under control of the *araBAD* promoter, was used for recombinant protein overproduction. Standard *E. coli* growth medium (LB broth) consisted of 10 g/l tryptone, 5 g/l yeast extract, and 10 g/l NaCl. Superbroth containing tryptone (32 g/l), yeast extract (20 g/l), sodium chloride (5 g/l), and sodium hydroxide (0.2 g/l) was used when high cell densities were desired for protein overproduction. Ampicillin (150 μg/ml) was added to the growth medium as needed. Reagents and chemicals were purchased from Sigma-Aldrich unless otherwise specified.

Genetic techniques and growth methods. Gene disruptions in *S. cerevisiae* were made following the standard gene replacement method described by Hegemann and Heick

(Hegemann & Heick, 2011). Disruption cassettes were amplified using the appropriate primers and plasmid templates listed in Table S2. Purified DNA (1-5 µg) was transformed into *S. cerevisiae* by incubating cells suspended in a mixture of 33% polyethylene glycol 3350 (PEG 3350), lithium acetate (100 mM), and salmon sperm DNA (0.28 mg/ml) at 30°C for 30 min followed by 30 min of heat shock at 42°C. The transformed cells were recovered in rich medium containing dextrose (YPD) or glycerol (YPG) for 1 h at 30°C and were subsequently plated on solid YPD or YPG containing the relevant selective agent. Colonies that arose after 2 to 3 days of incubation were transferred to selective medium, and individual colonies were screened via PCR to identify the appropriate recombinants.

For growth analyses, yeast strains were revived from glycerol stocks stored at −80°C and streaked for isolation on YPG. Selection and propagation of *mmf1*Δ mutants on YPG prevented the loss of the mitochondrial genome that results after growth on dextrose (D. C. Ernst & Downs, 2018). Single colonies were inoculated into 5 ml cultures of SD + L-isoleucine (1 mM) and incubated at 30°C with shaking (200 rpm) overnight. Two µl of overnight cultures were placed into wells of a 96-well microplate. Ninety-eight µl of medium was added to each well and OD_{650} measurements are used to monitor growth using a microplate reader (Biotek). Growth curves were plotted as averages and standard deviations of results from three independent cultures using GraphPad Prism 7.0.

Molecular techniques. Plasmids were constructed using standard molecular techniques. DNA was amplified using Q5 DNA polymerase (New England Biolabs) with primers purchased from Eton Bioscience Inc. (Research Triangle Park, NC). Plasmids were isolated using PureYield plasmid miniprep system (Promega), and PCR products were purified using QIAquick PCR purification kit (Qiagen). Restriction endonucleases used for

molecular cloning were purchased from New England Biolabs. T4 ligase (Thermo Scientific) was used to ligate inserts to vectors. The plasmids and primers used are listed in Table S2. Plasmids pDM1390 and pDM1672 were provided by David Garfinkel (University of Georgia). pDM1479 was constructed by the following method: primers Hem1_NcoI_F and Hem1_XbaI_R were used to amplify *HEM1* from YJF153, omitting the mitochondrial localization sequence (9 amino acids) and introducing *Nco*1 and *Xba*1 restriction sites. The insert was then ligated into pET20b following restriction digest of the insert and vector and transformed into DH5α.

Protein purification. Hem1- His_6 lacking the N-terminal mitochondrial localization sequence was purified from an *E. coli* strain containing pDM1479. IlvA-His_6 was purified from *E. coli* containing pDM1578 (pET20b-IlvA). Both proteins were purified with a similar protocol. An overnight culture of BL21 grown in 50 ml of superbroth (SB) containing ampicillin was inoculated into 6 liters of SB with ampicillin distributed in four 2.8 liter baffled Fernbach flasks. Cultures were grown at 37ºC to an OD_{650} of ~ 0.7. Arabinose (0.2%) was added to induce expression of the inserted gene, and cultures were grown at 30ºC overnight (16 hours). Cells were harvested by centrifugation (15 min at 8k x g) and resuspended in binding buffer containing potassium phosphate pH 8 (100 mM), sodium chloride (100 mM), imidazole (20 mM), PLP (10 μM), TCEP (tris(2-carboxyethyl)phosphine; 1 mM), and glycerol (10% w/v). Lysozyme (1 mg/ml), phenylmethylsulfonyl fluoride (1 mM), and DNase (25 μg/ml) were added, and the cell suspension was placed on ice for 1 h. Cells were mechanically lysed using a One-shot cell disruptor (once at 124 MPa). The resulting lysate was clarified by centrifugation (45 min at 48k x g) and filtered through a membrane (0.45 μm pore size). Filtered lysate was loaded

onto HisTrap HP Ni-Sepharose columns (5 ml) and washed with five column volumes of binding buffer. Protein was eluted by increasing the concentration of imidazole in the elution buffer from 20 to 300 mM over 10 column volumes. Purified protein was concentrated by centrifugation with a 10,000-molecular-weight-cutoff filter unit (Millipore), and the buffer was replaced with potassium phosphate pH 8 (100 mM), containing PLP (10 μM), NaCl (100 mM), and glycerol (10% w/v) using a PD-10 desalting column (GE Healthcare). Protein concentration was determined by BCA assay (Pierce) and a recovery from a typical purification was ~100mg. Protein aliquots were frozen in liquid nitrogen and stored at 80°C. Densitometry estimated purity at >97% for Hem1p and IlvA. Mmf1p of similar purity (D. C. Ernst & Downs, 2018) was available in the laboratory.

Purification of Hem1p expressed in *S. enterica ridA* mutants. Hem1-His_6 was purified from each of two *S. enterica* strains carrying pDM1479, an isogenic pair with (DM13509) or without (DM17050) a functional RidA. Both strains contain T7 polymerase in the chromosome under control of the *araBAD* promoter. Cultures of each strain were grown overnight in LB (50 ml) containing ampicillin. For each strain, 15 ml of the overnight culture was used to inoculate each of three baffled Fernbach flasks (2.8 L) containing 1.5 liters minimal glucose medium with ampicillin (15 μg/ml), L-serine (5 mM) and L-glycine (1 mM). The resulting cultures were grown, with shaking, to an OD_{650} of 0.3 before arabinose (0.08%) was added to induce expression. Cells were harvested by centrifugation (15 min at 7k x g) after 18 hours of growth at 30°C. The cell pellet was resuspended in 2 ml binding buffer / g cell wet weight and subjected to the lysis method described above. Binding buffer contains potassium phosphate pH 8 (50 mM), NaCl (100 mM), imidazole (40 mM), glycerol (10% w/v). Filtered lysates were loaded onto Histrap HP Ni-Sepharose

columns (1 ml), washed with 10 column volumes binding buffer, and eluted with 80 mM imidazole in binding buffer. Densitometry estimated purity at 70% for the protein samples from both WT and *ridA* mutant strains (Figure S1).

Characterization of cofactor content. Hem1-bound cofactors were release from the protein as described previously (Flynn & Downs, 2013). KOH (30 mM final concentration) was added to purified Hem1p (50 nmol protein) and incubated at room temperature for 10 min. Protein was precipitated by addition of 10% trifluoroacetic acid to generate a visible precipitate. The precipitate was removed by centrifugation (3 min at 16k x g). The supernatant was filtered used a 0.45 µm centrifugal tube filter (Costar 8170) and the cofactors separated by high-performance liquid chromatography. Separation was performed on a Shimadzu HPLC equipped with a Luna C18 column (250 by 4.60 mm) (Phenomenex) using a 2-step isocratic method with a flow rate of 0.8 ml/min as follows: 0 to 5 min with 100% buffer A (0.06% [vol/vol] trifluoroacetic acid) and 5 to 18 min with methanol- buffer A (3:97). Between each run, the column was washed for 10 min with methanol-buffer A (60:40). The eluant was monitored at 305 nm using a photodiode array detector (Shimadzu SPD-M20A). Authentic pyridoxal 5'-phosphate (>98% pure; Sigma-Aldrich) and pyruvate/PLP served as standards. Pyruvate/PLP was synthesized as described previously (Schnackerz, Ehrlich, Giesemann, & Reed, 1979) purified by HPLC, and concentrated.

Heme measurements. Intracellular heme levels were measured using a protocol adapted from Hans *et. al.* (Hans, Heinzle, & Wittmann, 2001). Overnight cultures of *S. cerevisiae* (2.5 ml) were used to inoculate 50 ml of SD media in a 500 ml flask and incubated with shaking (200 rpm) until an OD_{650} of 0.4 was reached. Cells were harvested by

centrifugation (3k x g for 5 min), washed with 50 ml of distilled H_2O, resuspended in 1 ml of deionized H_2O, and placed in a microcentrifuge tube. Samples were pelleted (8k x g for 5 min), resuspended in 500 µl of oxalic acid (20 mM), and stored at 4°C for 16 hours. After incubation, 500 µl of warm oxalic acid (2 M) was added and 500 µl of each sample was transferred to a new amber microfuge tube. The original tubes were transferred to a heat block at 95ºC for 30 min, while the new tubes were left at room temperature. Samples were then centrifuged at 16k x g for 2 min. 200 µl of each sample was transferred to a black 96-well microwell plate and top-read fluorescence measurements were obtained using a Spectramax Gemini EM, with 400 nm excitation light and recording emission intensity at 620 nm. Values of each sample before boiling were subtracted from their boiled counterparts. The resulting values were plotted on a standard curve generated with hemin and are presented in nmols heme/OD_{650}. It was formally possible that normalizing heme concentration to OD_{650} could alter the ratios between various strains. Comparison of CFUs/OD between strains failed to suggest that the differences in heme levels reported in Table 1 and 2 were not valid (data not shown). Further, assays of representative strains that normalized heme levels to protein detected trends between strains similar to those reported in Table 1 and 2 (data not shown).

Aminolevulinic acid synthase (ALAS) activity assays. ALAS activity of Hem1p was quantified using a protocol adapted from Whittaker *et. al.* (Whittaker, Penmatsa, & Whittaker, 2015). Reactions consisted of potassium phosphate pH 6.8 (50 mM), L-glycine (100 mM), PLP (10 µM) and the relevant protein (~200-1000 nM) in a total volume of 170 µl. All components were added and allowed to incubate on a heat block at 30ºC for 10 min. When assayed in the presence 3-chloroalanine (3CA), the incubation was at 37ºC and 3CA

was at 1 mM concentration. The reaction was started with the addition of succinyl-CoA (1 mM final concentration). After incubation for either 30 min (with 3CA) or 1 hour (other assays), the reaction was stopped by addition of 10% TCA. Precipitated protein was removed by centrifugation at 17k x g, after which samples were added to 1M sodium acetate (pH 4.6) containing 7.7% v/v acetylacetone. Aminolevulinate in the samples was derivatized by heat treatment (95ºC) for 10 min to form a pyrrole. Ehrlich reagent was added and the resulting pyrrole derivative was detected by absorbance at 553 nm. Aminolevulinate present in each sample was determined by interpolating absorbance values onto a standard curve and reported in μmol.

***In situ* generation of 2AA and assessment of damage to ALAS.** A complete reaction contained IlvA (200 nM), Hem1p (1 μM) in potassium phosphate pH 8 (50 mM) with NaCl (10 mM). Microfuge tubes containing reaction components were incubated at 30ºC for 5 min before adding L-serine (to 100 mM) to start the reaction and bring the total volume to 55 μl. The reaction proceeded for 1 hr at 30ºC followed by cooling on ice for 5 min. Fifty μl of reaction mix was dispensed onto a 0.025 μm MCE membrane filter (MF Millipore VSWP02500) floating on the surface of 25 ml dialysis buffer (KPO_4 pH 8 (50 mM), NaCl (10 mM)) in a petri dish. Dialysis proceeded for approximately one hour in a 4ºC cold room, conditions that were empirically determined to eliminate the inhibitory concentration of L-serine. Forty-five μl was removed and transferred into 108 μl of ALAS reaction buffer (50 mM potassium phosphate (pH 8), 10 mM NaCl, 170 mM L-glycine) and placed on a heat block at 30ºC for 5 min. The ALAS assay was started by the addition of 17 μl 10mM succinyl-CoA, bringing the final reaction mix to a total volume of 170 μl. ALA was quantified after 60 min.

2.4 RESULTS AND DISCUSSION

ρ^+ *mmf1*Δ mutants of *S. cerevisiae* are compromised in synthesis of aminolevulinic acid. ρ^+*mmf1*Δ mutants in both YJF153 and S288c strain backgrounds were used to better understand phenotypic consequences of 2AA/2IP accumulation. Two strain backgrounds were used to provide broader insights into enamine/imine stress by identifying if any effects differed between the strains. Genomic differences in YJF153 and S288c did not significantly impact the growth of a *mmf1*Δ yeast in SD. Parental strains YJF153 and S288c reached a similar final OD_{650} (0.6 and 0.55 respectively), and the *mmf1*Δ mutation caused a growth defect in both strains (Figure 2). In both cases full growth of the *mmf1*Δ mutant was restored when L-isoleucine was added to the medium. This result was previously reported for the YJF153 strains, where L-isoleucine restored growth by allosterically inhibiting Ilv1p, and preventing the accumulation of 2AA/2IP (D. C. Ernst & Downs, 2018). This behavior is similar to that characterized in *S. enterica* and based on the data in Figure 2, is assumed to extend to the S288c strains.

Growth on Yeast extract Peptone medium containing Dextrose (YPD) was also compromised by a *mmf1*Δ (Figure 2). In this case the *mmf1*Δ strains had a period of slower growth before reaching a growth rate that was similar to the parental strains. Due to the presence of L-isoleucine in YPD, there was little to no 2AA/2IP being generated via Ilv1p. However, the presence of L-serine in YPD induces the expression of *CHA1* and would thus allow 2AA/2IP to be generated. Introduction of *cha1*Δ restored growth of the *mmf1*Δ yeast to that of the parental strains on YPD (data not shown), further supporting a role for 2AA/2IP in causing the growth phenotypes seen.

A culture of a ρ^+*mmf1*Δ mutant of YJF153 was grown overnight in YP-Glycerol (YPG) and used to seed a soft agar overlay on minimal-glucose (SD) medium. Informed by knowledge of the RidA paradigm in bacteria, forty-nine nutritional supplements were spotted on the surface and stimulation of growth was noted (Table S3). L-Isoleucine stimulated growth likely due to its allosteric inhibition the major producer of 2AA (Ilv1p) and the iron chelator bathophenanthrolinedisulfonic acid (BPS) stimulated growth reflecting the sensitivity of *mmf1*Δ mutants to iron accumulation (D. C. Ernst & Downs, 2018). While a few additional nutrients showed some growth stimulation, the growth allowed by aminolevulinic acid (ALA), an intermediate in the biosynthesis in heme, was noteworthy. ALA is the product of the mitochondrially located PLP-dependent aminolevulinic acid synthase enzyme, Hem1p. Quantification of growth in liquid SD medium showed that exogenous ALA (240 μM) had a small but reproducible positive effect on the growth of a ρ^+*mmf1*Δ mutant of both YJF153 and of S288c (Figure 2).

Heme biosynthesis is compromised by accumulated 2AA. Partial restoration of growth by an intermediate in heme biosynthesis (ALA) suggested *mmf1*Δ strains might have lowered heme levels. Isogenic *MMF1* and *mmf1*Δ strains in both the S288c and YJF153 background were grown in different media and total heme content was measured (Table 1). In SD medium, deletion of *mmf1* lowered the heme levels significantly in both S288c and YJF153 strain backgrounds. Mutant derivatives of S288c and YJF153 had ~60% and ~35% of the heme found in their respective parental strains. When L-isoleucine was present in the growth medium, heme levels in the *mmf1*Δ mutants were restored to those in the respective wildtype. This result implicated 2AA/2IP in generating the lowered heme levels, since exogenous L-isoleucine allosterically inhibits Ilv1p and prevents the formation of

these molecules (D. C. Ernst & Downs, 2018). When the growth medium contained ALA (240 μM), heme levels in the *mmf1*Δ mutants were restored to ~60-70% of the wild-type levels but notably to the full levels found in the parental strains. The failure of exogenous ALA to fully restore heme levels suggested there was: i) poor transport of ALA, ii) incomplete incorporation of exogenous ALA into the biosynthetic pathway, or iii) an additional bottleneck downstream of Hem1p in heme biosynthesis. A *hem1*Δ mutant of YJF153 was used to distinguish these possibilities. The growth of a *hem1*Δ mutant was restored to near that of wildtype with the addition of 240 μM ALA (Figure 3). Thus, growth occurred despite the fact that internal levels of heme reached only ~50% of the wild-type levels in this condition (Table 1). These data suggested that ALA was inefficiently transported and/or incorporated into the biosynthetic pathway. Importantly, the data also showed the level of heme allowed by this ALA supplementation could support wild-type growth. Thus, the minimal growth stimulation achieved by supplementing *mmf1*Δ mutants with ALA showed there were metabolic defects beyond limited heme biosynthesis that impacted growth of the *mmf1*Δ yeast.

A *mmf1*Δ reduced heme levels in YPD medium in addition to SD (Table 1). The presence of L-isoleucine in YPD medium allosterically inhibits Ilv1p, while L-serine in the medium induces expression of *CHA1.* Thus, Cha1p was the presumed source of 2AA/2IP responsible for the reduced heme levels in YPD (D. C. Ernst & Downs, 2018). In this simple scenario, if *CHA1* were eliminated, it would restore heme levels on YPD by preventing 2AA/2IP formation. However, although deleting *CHA1* increased the levels of heme, they were not restored to those of the parental strains (Table 2). This result appeared to eliminate the simple model above. The formal possibility that Ilv1p was not completely

inhibited by the level of L-isoleucine in YPD was eliminated since adding L-isoleucine to YPD did not increase heme levels in the *mmf1*Δ strain (data not shown). In total, these data suggested that if 2AA/2IP were responsible for the decreased heme levels in a *mmf1*Δ *cha1*Δ yeast in YPD, the source of these metabolites was unknown.

Specific activity of Hem1p decreases in a *mmf1*Δ mutant. Hem1p is a fold type I PLP-dependent enzyme that catalyzes the condensation of succinyl-CoA and L-glycine to generate ALA, which is the first committed step of heme biosynthesis and occurs in the mitochondria (Figure 1). The data above raised the possibility that Hem1p was covalently modified by the 2AA/2IP accumulating in the *mmf1*Δ mutant, consistent with the paradigm established for other PLP-enzymes (Borchert et al., 2019). In this scenario and based on precedent in *S. enterica* (Flynn & Downs, 2013; Schmitz & Downs, 2004), the specific activity of Hem1p was predicted to be lower in an *mmf1*Δ yeast compared to the parental *MMF1* strain. The low level of Hem1p in *S. cerevisiae,* in addition to its mitochondrial location and the inability to easily measure expression, complicated further analysis (Gollub, Liu, Dayan, Adlersberg, & Sprinson, 1977). To circumvent these hurdles, the well-characterized *S. enterica ridA* system was used to determine whether yeast Hem1p was a target of 2AA/2IP *in vivo*. A *S. enterica ridA* mutant (DM17050), and an isogenic wildtype (DM13509) were transformed with pDM1479, which contains *HEM1* expressed by the T7 promoter. Expression of *HEM1* was induced as the wildtype and *ridA* strain grew in minimal glucose medium with added L-serine and L-glycine. L-Serine was added to increase the production of 2AA/2IP, and L-glycine was present to allow growth in the presence of the elevated 2AA/2IP that is present in a *ridA* mutant (Christopherson, Lambrecht, Downs, & Downs, 2012; Dustin C. Ernst & Downs, 2016; Lambrecht et al.,

2013). Hem1-His_6 was purified from each strain and ALA synthase specific activity was determined (Figure 4). Hem1p that was purified from the *S. enterica ridA* mutant had 66% the specific activity of the Hem1p that was purified from cells of wildtype (8.1 ± 0.3 and 12.3 ± 0.8 μmol ALA/mg ALAS, respectively). These data were consistent with damage to Hem1p by 2AA/2IP in a *ridA* mutant.

Cofactors present in the Hem1p purified from each *S. enterica* strain were extracted and separated by HPLC. If a PLP-dependent enzyme is attacked by 2AA, a pyruvate-PLP adduct that can be released from the enzyme after with treatment by base is generated (Flynn & Downs, 2013; Likos, Ueno, Feldhaus, & Metzler, 1982). The data in Figure 5 showed that a pyruvate-PLP adduct (in addition to PLP) was released from Hem1p purified from a *ridA* mutant background. Significantly, the Hem1p purified from wildtype, where 2AA/2IP does not accumulate, released only the PLP cofactor and had a barely detectable peak where the pyruvate-PLP was expected (Figure 5). Together these data supported the hypothesis that Hem1p is attacked *in vivo* by 2AA/2IP, generating a stably modified enzyme that is inactive.

Hem1p is damaged by 2AA *in vitro*. To corroborate the *in vivo* data, Hem1p was purified from *E. coli* strain BL21-AI, which contains wild-type *ridA,* and exposed to 2AA/2IP *in vitro.* Initially, the purified protein was assayed in the presence and absence of 3-chloroalanine (3CA). 3CA reacts with PLP in the active site of some PLP-dependent enzymes, where loss of the chlorine substituent generates 2AA which can damage the active site of target enzymes (Badet, Roise, & Walsh, 1984; Henderson & Johnston, 1976; Relyea, Tate, & Meister, 1974). As such, reaction with 3CA can serve as a predictor of sensitivity to free 2AA or the 2IP tautomer. In the absence of 3CA, Hem1p generated 26.1

± 1.1 μmol ALA/mg Hem1p after 30 min, while in the presence of 3CA (1 mM), ALA synthesis dropped to 7.8 ± 0.1 (Figure 6A).

Analysis of direct damage by 2AA/2IP requires that the enamine/imine is generated *in situ* due to the short half-life (3 sec) of these molecules in aqueous solution (Hillebrand, Dye, & Suelter, 1979). Serine dehydratase (IlvA) and cysteine desulfurase (CdsH) have been used to generate 2AA/2IP *in situ* from L-serine or L-cysteine, respectively (D. C. Ernst, Lambrecht, Schomer, & Downs, 2014; Lambrecht et al., 2013). Control reactions showed that Hem1p was inhibited by both L-serine and L-cysteine, complicating the use of these enzymes and requiring that a two-step assay be implemented. Hem1p was incubated with L-serine and serine dehydratase (IlvA) for 60 min to allow generation of 2AA/2IP and time for it to damage Hem1p. Excess L-serine was then removed by filter dialysis before the components needed to assay ALA formation by Hem1p were added. The data are in Figure 6B. When no 2AA was generated in the initial step (i.e., L-serine or IlvA was not present), 1.4 ± 0.1 and 1.6 +/- 0.1 μmol ALA/mg ALAS was generated, respectively. In contrast, when both L-serine and IlvA were present to generate 2AA, significantly less activity was detected, 0.6 ± 0.2 μmol ALA/mg ALAS. Finally, when Mmf1p was present in the complete assay, ALAS activity was restored to 1.9 ± 0.3 μmol ALA/mg ALAS. Mmf1p has 2AA/2IP deaminase activity (D. C. Ernst & Downs, 2018), and the restoration of ALAS activity supports the conclusion that Hem1p can be attacked by 2AA/2IP and its activity decreased.

Conclusions. Loss of the mitochondrial *ridA* homolog *MMF1* results in numerous phenotypes, which are collectively due to the accumulation of 2AA/2IP in the strain (D. C. Ernst & Downs, 2018). Herein we identify and characterize one target of the accumulated

2AA/2IP as Hem1p, the first enzyme in heme biosynthesis. Our work shows that Hem1p is sensitive to damage by 2AA/2IP *in vivo* and *in vitro.* Further, the data are in support of the conclusion that the reduction of heme levels found in an *mmf1*Δ mutant is at least partially due to damage to Hem1p mediated by 2AA/2IP.

Demonstrating that Hem1p is damaged by 2AA/2IP defines a novel target of enamine/imine damage. The inability of ALA to restore an *mmf1*Δ mutant to full growth on SD showed that Hem1 was not the sole growth determining target of 2AA/2IP in *S. cerevisiae.* Hem1p is the second identified target of 2AA in the yeast mitochondria, with branched chain amino acid transferase (BAT) being the first (D. C. Ernst & Downs, 2018; Kim et al., 2001). Neither the lack of Hem1p or of Bat1p phenocopy the respiratory defects or mtDNA loss associated with a *mmf1*Δ mutant (data not shown). These results suggest multiple small perturbations caused by the accumulation of 2AA/2IP exist in *mmf1*Δ yeast and that they act together to generate phenotypic outcomes. Mitochondrially localized PLP-dependent enzymes are the putative targets for 2AA/2IP in *mmf1*Δ yeast. Several such enzymes are linked directly or indirectly to mitochondrial stability: aspartate amino transferase (EC 2.6.1.1; *AAT1*), mitochondrial serine hydroxymethyltransferase (EC 2.1.2.1; *SHM1*), and cysteine desulfurase (EC 2.8.1.7; *NFS1*) (J. Li, Kogan, Knight, Pain, & Dancis, 1999; Luzzati, 1975; Sliwa, Dairou, Camadro, & Santos, 2012). Homologues of *AAT1* and *SHM1* are inhibited by 2AA/2IP in *S. enterica* and it is likely their eukaryotic counterparts are also targets of enamine/imines attack (Downs & Ernst, 2015). Continued genetic and biochemical studies will define components of the metabolic network in the mitochondria that are impacted by enamine/imine stress and how they combine to generate the multiple phenotypes of an *mmf1*Δ mutant.

ACKNOWLEDGEMENTS.

The authors would like to thank David Garfinkel for plasmids, technical advice and helpful discussions. This work was supported by an award from the competitive grants program at the NIH (GM095837) to DMD. Authors contributed to; (i) the conception or design of the study (DCE, DMD,GHW), (ii) the acquisition, analysis, or interpretation of the data (GHW, DCE, DMD) and (iii) writing of the manuscript (GHW, DMD).

CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest with the contents of this article.

DATA AVAILABILITY

The data that supports the findings of this study are within the manuscript or the supplementary material of this article, and additional information is available upon request from the authors.

2.5 REFERENCES

Ahmed, S. I., Bollon, A. P., Rogers, S. J., & Magee, P. T. (1976). Purification and properties of threonine deaminase from Saccharomyces cerevisiae. *Biochimie, 58*(1-2), 225-232. doi:10.1016/s0300-9084(76)80374-4

Badet, B., Roise, D., & Walsh, C. T. (1984). Inactivation of the *dadB Salmonella typhimurium* alanine racemase by D and L isomers of b-substituted alanines: kinetics, stoichiometry, active site peptide sequencing, and reaction mechanism. *Biochemistry, 23*, 5188-5194.

Borchert, A. J., Ernst, D. C., & Downs, D. M. (2019). Reactive Enamines and Imines In Vivo: Lessons from the RidA Paradigm. *Trends Biochem Sci, 44*(10), 849-860. doi:10.1016/j.tibs.2019.04.011

Bornaes, C., Ignjatovic, M. W., Schjerling, P., Kielland-Brandt, M. C., & Holmberg, S. (1993). A regulatory element in the CHA1 promoter which confers inducibility by serine and threonine on Saccharomyces cerevisiae genes. *Mol Cell Biol, 13*(12), 7604-7611. doi:10.1128/mcb.13.12.7604

Christopherson, M. R., Lambrecht, J. A., Downs, D., & Downs, D. M. (2012). Suppressor analyses identify threonine as a modulator of ridA mutant phenotypes in Salmonella enterica. *PLoS ONE, 7*(8), e43082. doi:10.1371/journal.pone.0043082

Digiovanni, S., Visentin, C., Degani, G., Barbiroli, A., Chiara, M., Regazzoni, L., . . . Popolo, L. (2020). Two novel fish paralogs provide insights into the Rid family of imine deaminases active in pre-empting enamine/imine metabolic damage. *Sci Rep, 10*(1), 10135. doi:10.1038/s41598-020-66663-w

Downs, D. M., & Ernst, D. C. (2015). From microbiology to cancer biology: the Rid protein family prevents cellular damage caused by endogenously generated reactive nitrogen species. *Mol Microbiol, 96*(2), 211-219. doi:10.1111/mmi.12945

ElRamlawy, K. G., Fujimura, T., Baba, K., Kim, J. W., Kawamoto, C., Isobe, T., . . . Kawamoto, S. (2016). Der f 34, a Novel Major House Dust Mite Allergen Belonging to a Highly Conserved Rid/YjgF/YER057c/UK114 Family of Imine Deaminases. *J Biol Chem, 291*(41), 21607-21615. doi:10.1074/jbc.M116.728006

Ernst, D. C., & Downs, D. M. (2016). 2-Aminoacrylate Stress Induces a Context-Dependent Glycine Requirement inridAStrains of Salmonella enterica. *Journal of Bacteriology, 198*(3), 536-543. doi:10.1128/jb.00804-15

Ernst, D. C., & Downs, D. M. (2018). Mmf1p Couples Amino Acid Metabolism to Mitochondrial DNA Maintenance in Saccharomyces cerevisiae. *MBio, 9*(1). doi:10.1128/mBio.00084-18

Ernst, D. C., Lambrecht, J. A., Schomer, R. A., & Downs, D. M. (2014). Endogenous Synthesis of 2-Aminoacrylate Contributes to Cysteine Sensitivity in Salmonella enterica. *Journal of Bacteriology, 196*(18), 3335-3342. doi:10.1128/jb.01960-14

Esaki, N., & Walsh, C. T. (1986). Biosynthetic alanine racemase of Salmonella typhimurium: purification and characterization of the enzyme encoded by the alr gene. *Biochemistry, 25*(11), 3261-3267.

Flynn, J. M., & Downs, D. M. (2013). In the absence of RidA, endogenous 2-aminoacrylate inactivates alanine racemases by modifying the pyridoxal 5'-phosphate cofactor. *J Bacteriol, 195*(16), 3603-3609. doi:10.1128/JB.00463-13

Gollub, E. G., Liu, K. P., Dayan, J., Adlersberg, M., & Sprinson, D. B. (1977). Yeast mutants deficient in heme biosynthesis and a heme mutant additionally blocked in cyclization of 2,3-oxidosqualene. *J Biol Chem, 252*(9), 2846-2854.

Hans, M. A., Heinzle, E., & Wittmann, C. (2001). Quantification of intracellular amino acids in batch cultures of Saccharomyces cerevisiae. *Appl Microbiol Biotechnol, 56*(5-6), 776-779.

Hegemann, J. H., & Heick, S. B. (2011). Delete and repeat: a comprehensive toolkit for sequential gene knockout in the budding yeast Saccharomyces cerevisiae. *Methods Mol Biol, 765*, 189-206. doi:10.1007/978-1-61779-197-0_12

Henderson, L. L., & Johnston, R. B. (1976). Inhibition studies of the enantiomers of beta chloroalanine on purified alanine racemase from B. subtilis. *Biochem Biophys Res Commun, 68*(3), 793-798.

Hillebrand, G. G., Dye, J. L., & Suelter, C. H. (1979). Formation of an intermediate and its rate of conversion to pyruvate during the tryptophanase-catalyzed degradation of S-o-nitrophenyl-L-cysteine. *Biochemistry, 18*(9), 1751-1755.

Hunter, S., Jones, P., Mitchell, A., Apweiler, R., Attwood, T. K., Bateman, A., . . . Yong, S. Y. (2012). InterPro in 2011: new developments in the family and domain prediction database. *Nucleic Acids Res, 40*(Database issue), D306-312. doi:10.1093/nar/gkr948

Irons, J., Hodge-Hanson, K. M., & Downs, D. M. (2018). PA5339, a RidA Homolog, Is Required for Full Growth in Pseudomonas aeruginosa. *J Bacteriol, 200*(22). doi:10.1128/JB.00434-18

Irons, J., Sacher, J. C., Szymanski, C. M., & Downs, D. M. (2019). Cj1388 Is a RidA Homolog and Is Required for Flagella Biosynthesis and/or Function in. *Front Microbiol, 10*, 2058. doi:10.3389/fmicb.2019.02058

Irons, J. L., Hodge-Hanson, K., & Downs, D. M. (2020). RidA Proteins Protect against Metabolic Damage by Reactive Intermediates. *Microbiol Mol Biol Rev, 84*(3). doi:10.1128/MMBR.00024-20

Kim, J. M., Yoshikawa, H., & Shirahige, K. (2001). A member of the YER057c/yjgf/Uk114 family links isoleucine biosynthesis and intact mitochondria maintenance in Saccharomyces cerevisiae. *Genes Cells, 6*(6), 507-517. doi:10.1046/j.1365-2443.2001.00443.x

Lambrecht, J. A., Schmitz, G. E., & Downs, D. M. (2013). RidA proteins prevent metabolic damage inflicted by PLP-dependent dehydratases in all domains of life. *MBio, 4*(1), e00033-00013. doi:10.1128/mBio.00033-13

Li, J., Kogan, M., Knight, S. A., Pain, D., & Dancis, A. (1999). Yeast mitochondrial protein, Nfs1p, coordinately regulates iron-sulfur cluster proteins, cellular iron uptake, and iron distribution. *J Biol Chem, 274*(46), 33025-33034. doi:10.1074/jbc.274.46.33025

Li, X. C., & Fay, J. C. (2017). Cis-Regulatory Divergence in Gene Expression between Two Thermally Divergent Yeast Species. *Genome Biology and Evolution, 9*(5), 1120-1129. doi:10.1093/gbe/evx072

Likos, J. J., Ueno, H., Feldhaus, R. W., & Metzler, D. E. (1982). A novel reaction of the coenzyme of glutamate decarboxylase with L-serine O-sulfate. *Biochemistry, 21*(18), 4377-4386.

Luzzati, M. (1975). Isolation and properties of a thymidylate-less mutant in Saccharomyces cerevisiae. *Eur J Biochem, 56*(2), 533-538. doi:10.1111/j.1432-1033.1975.tb02259.x

Marchler-Bauer, A., Zheng, C., Chitsaz, F., Derbyshire, M. K., Geer, L. Y., Geer, R. C., . . . Bryant, S. H. (2013). CDD: conserved domains and protein three-dimensional structure. *Nucleic Acids Res, 41*(Database issue), D348-352. doi:10.1093/nar/gks1243

Martínez-Chavarría, L. C., Sagawa, J., Irons, J., Hinz, A. K., Lemon, A., Graça, T., . . . Vadyvaloo, V. (2020). Putative Horizontally Acquired Genes, Highly Transcribed during Yersinia pestis Flea Infection, Are Induced by Hyperosmotic Stress and Function in Aromatic Amino Acid Metabolism. *J Bacteriol, 202*(11). doi:10.1128/JB.00733-19

Mortimer, R. K., & Johnston, J. R. (1986). Genealogy of principal strains of the yeast genetic stock center. *Genetics, 113*(1), 35-43.

Niehaus, T. D., Gerdes, S., Hodge-Hanson, K., Zhukov, A., Cooper, A. J., ElBadawi-Sidhu, M., . . . Hanson, A. D. (2015). Genomic and experimental evidence for

multiple metabolic functions in the RidA/YjgF/YER057c/UK114 (Rid) protein family. *BMC Genomics, 16*, 382. doi:10.1186/s12864-015-1584-3

Oxelmark, E., Marchini, A., Malanchi, I., Magherini, F., Jaquet, L., Hajibagheri, M. A., . . . Tommasino, M. (2000). Mmf1p, a novel yeast mitochondrial protein conserved throughout evolution and involved in maintenance of the mitochondrial genome. *Mol Cell Biol, 20*(20), 7784-7797.

Relyea, N. M., Tate, S. S., & Meister, A. (1974). Affinity labeling of the active center of L-aspartate-beta-decarboxylase with beta-chloro-L-alanine. *J Biol Chem, 249*(5), 1519-1524.

Schmitz, G., & Downs, D. M. (2004). Reduced Transaminase B (IlvE) Activity Caused by the Lack of yjgF Is Dependent on the Status of Threonine Deaminase (IlvA) in Salmonella enterica Serovar Typhimurium. *Journal of Bacteriology, 186*(3), 803-810. doi:10.1128/jb.186.3.803-810.2004

Schnackerz, K. D., Ehrlich, J. H., Giesemann, W., & Reed, T. A. (1979). Mechanism of action of D-serine dehydratase. Identification of a transient intermediate. *Biochemistry, 18*(16), 3557-3563. doi:10.1021/bi00583a019

Sliwa, D., Dairou, J., Camadro, J. M., & Santos, R. (2012). Inactivation of mitochondrial aspartate aminotransferase contributes to the respiratory deficit of yeast frataxin-deficient cells. *Biochem J, 441*(3), 945-953. doi:10.1042/BJ20111574

Walsh, C. (1982). Suicide substrates: mechanism-based enzyme inactivators. *Tetrahedron, 38*, 871-909.

Whittaker, M. M., Penmatsa, A., & Whittaker, J. W. (2015). The Mtm1p carrier and pyridoxal 5'-phosphate cofactor trafficking in yeast mitochondria. *Arch Biochem Biophys, 568*, 64-70. doi:10.1016/j.abb.2015.01.021

Table 2.1. An *mmf1*Δ mutant has decreased heme levels.

		Heme levels[a]			
		Medium			
Strain	Genotype	SD	SD + Ile	SD + ALA	YPD
YJF153	YJF153	254 ± 29	238 ± 10	251 ± 26	258 ± 26
Dmy41	YJF153 *mmf1*Δ	90 ± 10	241 ± 13	157 ± 13	80 ± 7
Dmy74	YJF153 *hem1*Δ	ND	ND	124 ± 26	ND
Dmy61	S288c	140 ± 25	186 ± 39	176 ± 12	217 ± 8
Dmy67	S288c *mmf1*Δ	84 ± 8	176 ± 19	125 ± 4	65 ± 9

Mutants lacking *MMF* or *HEM1* in each of two strain backgrounds (YJF153 and S288c) were grown in the indicated medium. When added, L-isoleucine and aminolevulinic acid were at 1 mM and 240 μM, respectively. Data are from a representative experiment with three biological replicates and reported as average plus or minus 1 standard deviation. Abbreviations: SD, synthetic dextrose; YPD, yeast peptone dextrose; Ile, L-isoleucine; ALA, aminolevulinic acid.; ND, not determined.

[a]Heme levels were measured as described in Materials and Methods and are reported in pmol/OD_{650}.

Table 2.2. Cha1p contributes to the decreased heme levels of an *mmf1*Δ mutant on YPD.

Strain	Genotype	Heme levels[a]
YJF153	YJF153	206 ± 26
Dmy41	YJF153 *mmf1*Δ	64 ± 24
Dmy16	YJF153 *cha1*Δ	216 ± 21
Dmy20	YJF153 *mmf1*Δ *cha1*Δ	96 ± 9
Dmy61	S288c	209 ± 4
Dmy67	S288c *mmf1*Δ	69 ± 10
Dmy111	S288c *mmf1*Δ *cha1*Δ	133 ± 16

Mutants lacking *MMF1* and/or *CHA1* in two strain backgrounds (YJF153 and S288c) were grown in YPD medium. Data are from a representative experiment with 4 biological replicates and reported as average plus or minus 1 standard deviation.

[a]Heme levels were measured as described in Materials and Methods and are reported in pmol/OD_{650}.

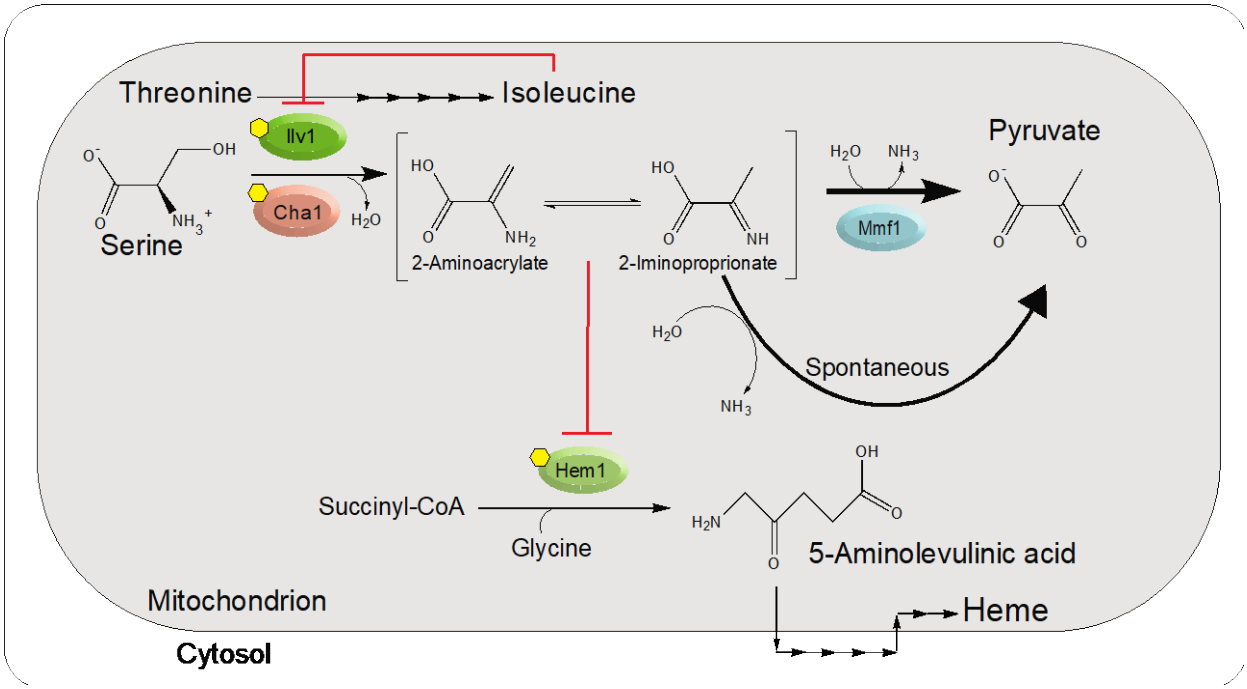

Figure 2.1. Enamine/imine production and the resulting stress in mitochondria. 2-aminoacrylate (2AA) is generated in yeast mitochondria by PLP-dependent serine/threonine dehydratases Ilv1p and Cha1p (D. C. Ernst & Downs, 2018). *CHA1* is transcribed in the presence of exogenous L-serine or L-threonine and has a role in catabolism of those amino acids. Ilv1p is constitutively synthesized, involved in biosynthesis of L-isoleucine and allosterically inhibited by L-isoleucine. Once formed, 2AA tautomerizes to 2-iminoproprionate (2IP) and is ultimately deaminated by solution water, or accelerated by Mmf1p, to pyruvate. In the absence of Mmf1p, 2AA/2IP accumulate *in* vivo to levels sufficient to damage target PLP enzymes. Work herein shows that Hem1p is such a target. Hem1p catalyzes the first committed step in heme biosynthesis, generating 5-aminolevulinic acid in the mitochondria. Subsequent steps in heme synthesis take place in the cytoplasm before the final steps are accomplished in the mitochondrion, as schematically represented. Past work demonstrated that branched chain

amino acid transaminase is also a target for damage by 2AA/2IP (D. C. Ernst & Downs, 2018). The relevant pathways are schematically shown in the figure; red lines indicate points of post translational inhibition, yellow hexagons depict PLP cofactors and the biosynthetic steps to heme are depicted where they occur, either internal, or external to the mitochondrion. Both Ilv1p and Cha1p act on L-threonine and via enamine/imine formation generate the α-ketobutyrate that is an intermediate in the biosynthesis of L-isoleucine.

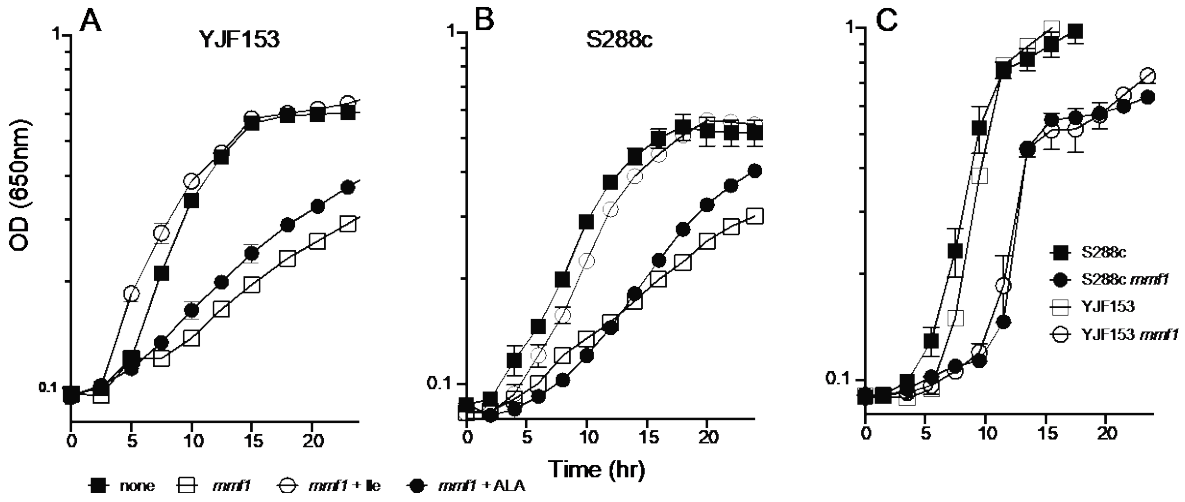

Figure 2.2. Growth of *mmf1*Δ strains is compromised. YJF153, S288c and *mmf1*Δ derivatives of these strains were grown on SD medium (panel A,B) or YPD (panel C). Growth was monitored as the OD_{650} over time. SD medium was supplemented with 5-aminolevulinic acid (ALA, 240 uM), or L-isoleucine (Ile, 1 mM) as indicated. The data represent the average and standard error of three biological replicates.

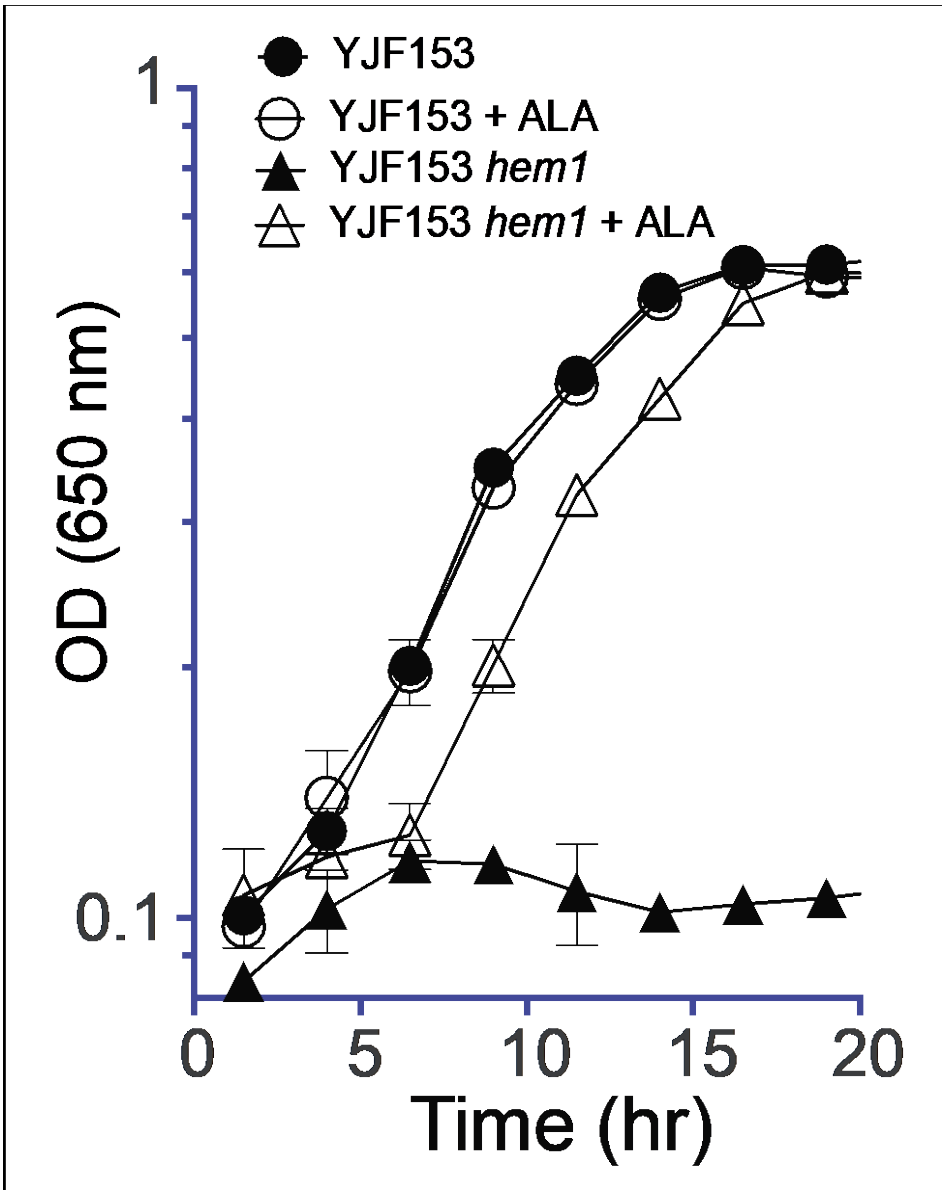

Figure 2.3. 5-aminolevulinic acid restores growth of a *hem1*Δ mutant. Growth of a YJF153 parent and *hem1*Δ mutant strain was monitored in SD media with or without the addition of 5-aminolevulinic acid (ALA) as indicated.

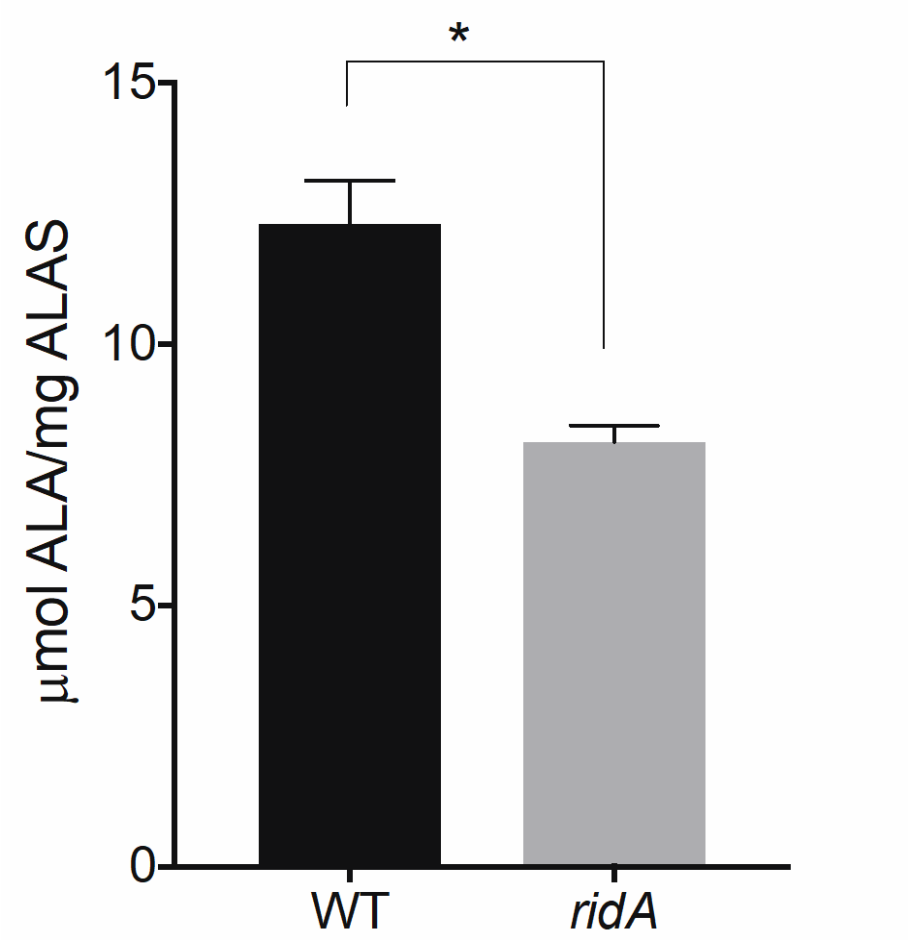

Figure 2.4. Hem1p specific activity is affected by genetic background. Hem1p was purified from either the wildtype (WT) or a *ridA* mutant strain of *Salmonella enterica* as indicated on the X axis. The purified protein was assayed for ALAS activity and the specific activity reported in µmol ALA/mg ALAS. The data shown are averages of triplicate technical replicates and significance (indicated by asterisk) was confirmed when a P value of 0.0042 was determined with an unpaired t test. Data are from a representative experiment that was repeated twice with two independent biological samples.

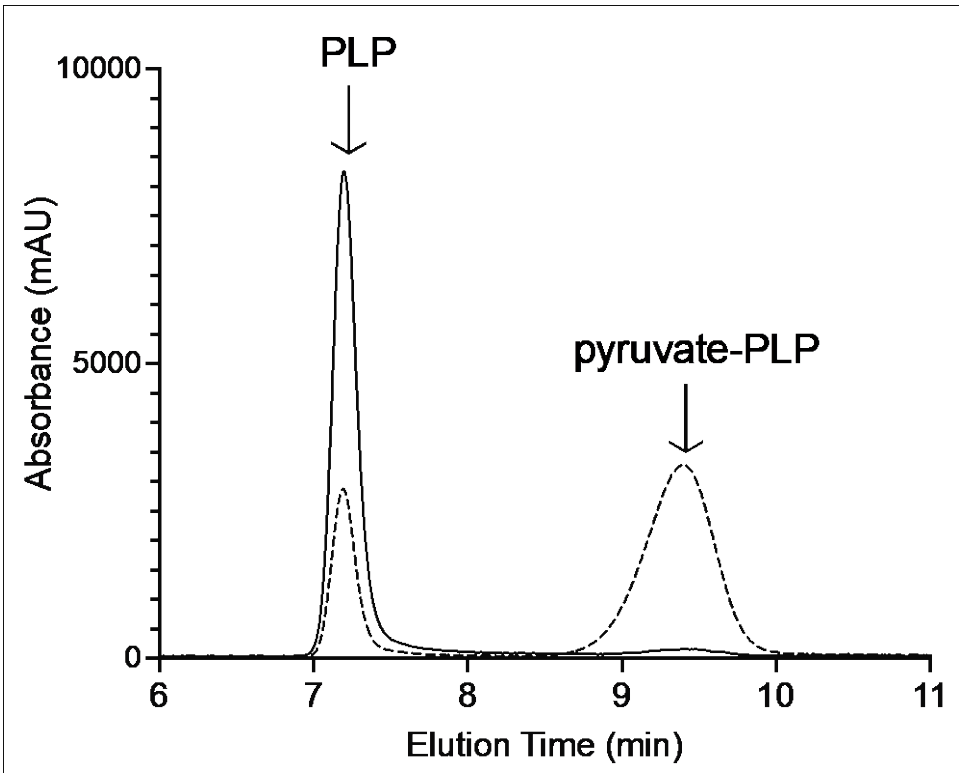

Figure 2.5. Pyruvate-PLP is released from Hem1p after synthesis in a *ridA* mutant. Hem1p was purified from two *S. enterica* strains; wildtype (solid lines) and a *ridA* mutant (dashed lines). Cofactors were released from the protein by treatment with base and separated with HPLC, while monitored by absorbance at 305 nm. The peak eluting with a retention time of ~7.4 min was PLP and the one at ~9.5 min was pyruvate-PLP. Peak assignment was based on retention time, UV-Vis spectra, and co-injection with authentic compounds (Figure S3).

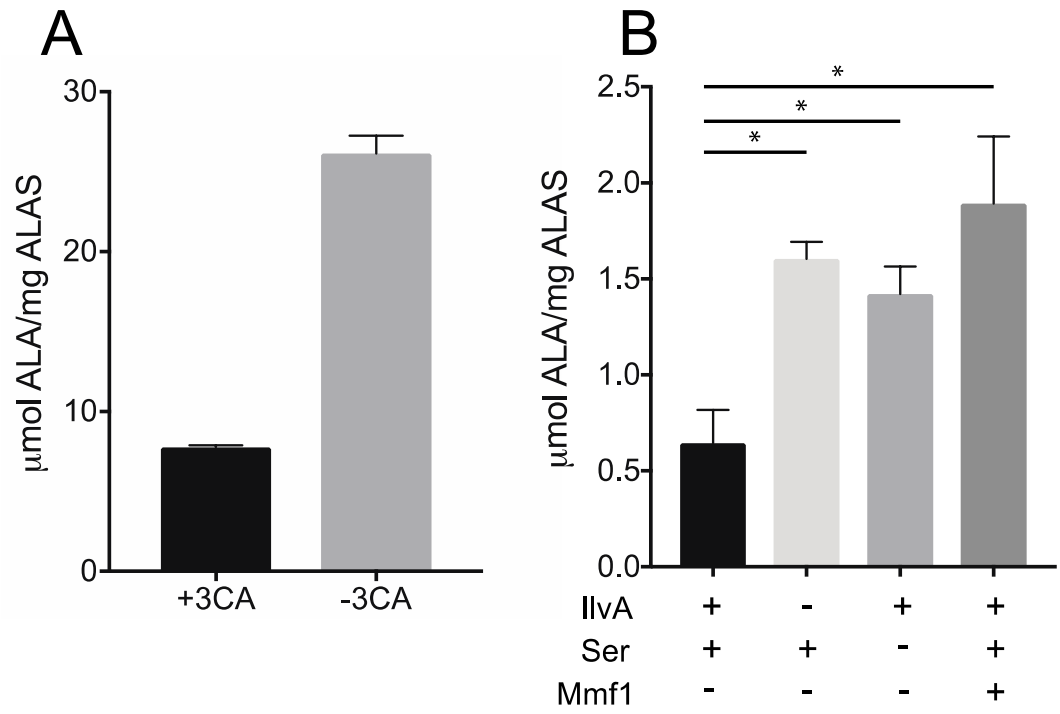

Figure 2.6. Hem1p is sensitive to 2AA *in vitro*. Hem1p was expressed and purified from BL21 *E. coli* and assayed for activity under multiple conditions. (**A)** Hem1p was incubated with 3-chloroalanine (3CA) prior to assaying ALA synthesis. Data are representative averages of three technical replicates. The difference is significant as determined by unpaired t test ($P < .0001$). **(B)** ALAS activity of Hem1p was determined after exposure to the 2AA/2IP generated *in situ* by IlvA as described in Materials and Methods. Components present in the preincubation stage of the protocol are indicated below the Y axis. Data shown are representative of at least 2 independent experiments. Each condition consisted of 4 technical replicates with significance determined by one way ANOVA. * indicates P value < .001.

CHAPTER 3

CONCLUSIONS AND FUTURE DIRECTIONS

As described in Chapter 2, one direct impact of 2AA accumulation in *mmf1* deletion strains of yeast is heme deficiency, caused by inactivation due to the formation of a chemical adduct between 2AA and PLP in the active site of Hem1. This conclusion is justified by the data presented in Chapter 2, combined with previous work on 2AA accumulation in other organisms [1, 2]. Hem1, aminolevulinic acid synthase (EC 2.3.1.37)(ALAS), is a novel target of 2AA. The activity of this enzyme is found in non-plant eukaryotes and α-proteobacteria, whereas other bacteria and plants utilize a PLP-dependent glutamate 1-semialdehyde 2,1-aminomutase (GSAM) (EC 5.4.3.8) to produce aminolevulinic acid from different substrates [3]. *S. enterica ridA* deletion strains, unlike *S. cerevisiae*, do not present with a heme deficiency. While their GSAM (HemL) has not been examined for susceptibility to 2AA damage in vitro, any potential damage had no physiological impact under conditions in which heme synthesis was necessary. Therefore, the identification of Hem1 as susceptible to 2AA represents a target potentially unique to eukaryotes and possibly α-proteobacteria, which have not yet been investigated.

3.1 Identifying other targets of 2AA in *mmf1* strains

As described in Chapter 2, the reduction in ALAS activity does not fully explain the growth defect or additional phenotypes observed in mmf1 mutants. Supplementation

with the product of the ALAS (Hem1) enzyme, ALA, improved growth only slightly in an *mmf1* deletion strain, whereas growth was improved to near wild-type levels in a *hem1* deletion strain. This suggests that damage to other enzymes may be responsible for a significant portion of the growth defect observed in an *mmf1* strain. While it is possible that ALA uptake levels differ in the above strains, heme levels were similarly impacted by ALA supplementation, making uptake of ALA an unlikely source of the difference in growth. 2AA is also known to reduce the activity of Bat1, the mitochondrially localized branched-chain aminotransferase (BCAT, EC 2.6.1.42)[4]. A *bat1* deletion strain, however, shows no growth defect even in the absence of branched-chain amino acids, due to the partially redundant function of its cytosolic homolog Bat2 [5]. Whereas a lowered BCAT activity can be observed as a result of 2AA stress, examination of BCAT activity in a *bat1 mmf1* strain revealed no such loss, demonstrating that the cytosolic Bat2 is unaffected by accumulation of 2AA in the mitochondrion (unpublished data). Therefore, the impaired growth of an *mmf1* strain is not due to the reduction of BCAT activity, as damage to Bat1 alone would not be sufficient to retard growth.

Based on the above observations, we can conclude the damage to both of the known targets of 2AA, Hem1 and Bat1, is not sufficient to account for the entirety of the growth defect observed in an *mmf1* deletion strain. In addition to the growth defect, Ernst reported that *mmf1* deletion strains rapidly lose mtDNA and therefore the ability to respire when grown on fermentative carbon sources [4]. A search of the literature, as well as similar experiments conducted with a *hem1* deletion strain, revealed no such phenotype in either *hem1* or *bat1* deletion strains (unpublished data). The loss of mtDNA in *mmf1* deletions strains can be prevented by the addition of the extracellular iron chelator

bathophenanthroline sulfonate (BPS), which also slightly corrects the growth defect [4](Ernst thesis appendix). These observations suggest that a currently unknown target of 2AA exists that is involved in iron metabolism.

3.2 Possible links to iron metabolism

Although the connection between iron and 2AA metabolism requires further investigation, several studies have linked iron metabolism to mtDNA stability in yeast [6-9]. It is well established that iron is reactive and capable of producing hydroxide radicals that chemically modify and damage DNA [10]. Most likely as a consequence of this, iron uptake and homeostasis are tightly regulated, especially in mitochondria where initiation iron-sulfur (FeS) cluster assembly occurs [11]. Li et al. established a link between Nfs1, a mitochondrially localized cysteine desulfurase (EC 2.8.1.17) involved in the early stages of FeS assembly, and iron homeostasis in yeast mitochondria [12]. A missense mutation of *NFS1* (which encodes an essential enzyme) was isolated in a screen for altered iron regulation. Yeast encoding Nfs1 I191S accumulate iron in the mitochondrion and are deficient in activity of the FeS-dependent enzymes aconitase and succinate dehydrogenase. These phenotypes are recapitulated when Nfs1 expression is under the control of an inducible promoter and deprived of inducer. Relevant to the mechanism of mtDNA loss in 2AA-accumulating yeast, Nfs1 is a PLP-dependent enzyme, and thus is likely to be damaged in the absence of Mmf1. Taken together, this provides a possible explanation for the mtDNA loss phenotype that is attractive in its simplicity, as a reduction in Nfs1 activity due to 2AA accumulation can explain both the mtDNA loss and the rescue of this phenotype by the addition of BPS. More work is required to characterize Nfs1 as a 2AA

target, including but not limited to measurement of mitochondrial iron levels in *mmf1,* overexpression of Nfs1, and evaluation of the activity of iron-sulfur cluster proteins in order to establish whether 2AA accumulation truly phenocopies the effects of a reduction of Nfs1 activity. In addition, Nfs1 should be evaluated *in vitro* for 2AA susceptibility using previously established methods.

3.3 Other candidate 2AA targets

Any PLP-dependent enzyme localized to the mitochondrion is a possible target for 2AA damage. Deficiencies in other PLP-dependent enzymes have been linked to mtDNA stability. Luzzati et al. 1975 identified the tmp3 allele, which was responsible for the generation of rho^- cytoplasmic petites [13]. The petite phenotype is the name given to small colony variants of yeast that have lost the ability to respire, and instead can only grow fermentatively, often as a result of lesions in mtDNA, which encodes components of the electron transport chain. The rho^- designation, which is sometimes referred to as rho-neutral, indicates that when the haploid strain is crossed with a respiring wild type strain to form a diploid zygote, daughter cells resulting from the diploid are respiratory-proficient [14]. This non-Mendelian pattern of inheritance is consistent with and usually indicative of mtDNA loss. Like the YPD-grown *mmf1* mutants, these petites of strains with the *tmp3* allele reliably produce daughter cells that are also unable to respire. *TMP3*, also known as *SHM1*, encodes Shm1, the mitochondrially localized serine hydroxymethyltransferase (EC 2.1.2.1), bacterial homologs of which are known to be susceptible to 2AA damage [15]. The *tmp3* allele, characterized by Zelikson and Luzzati, results in loss of mtDNA [16]. The

PLP-dependent enzyme Aat1, a mitochondrially localized aspartate aminotransferase, has also been linked to respiratory deficient phenotypes, but not to mtDNA loss [17].

A link between SHMT and mtDNA loss is an attractive possibility, as the SHMT known as GlyA in *S. enterica* has been shown to be damaged by PLP adduct formation, resulting in phenotypic consequences. In *S. enterica* these include a growth defect as well as a decrease in CoA levels that can be corrected by the addition of glycine or pantothenate [18]. In *S. enterica ridA* mutants, this is likely due to a limitation in 5,10-methylene tetrahydrofolate (5,10-MTHF), which is produced by SHMT. Glycine likely restores growth by increasing 5,10-MTHF levels via the glycine cleavage system (GCV). Pantothenate restores growth only partially, as supplementation restores CoA levels, but does not correct the low 5,10-MTHF levels, which impact other elements of metabolism [18]

In yeast, pantothenate and glycine have both been added as supplements to *mmf1* cells, but no restoration of growth occurred [19]. The yeast GCV is localized in the mitochondrion, though this is not an obstacle to its utilization of exogenous glycine [20]. This suggests that damage to SHMT in yeast by 2AA is not a physiologically relevant component of the growth phenotype observed in an *mmf1* deletion.

In brief, damage to Hem1 does not account for all the phenotypes of an *mmf1* deletion strain, most notably the iron-dependent mitochondrial DNA loss. While

3.4 References

1. Lambrecht, J.A., G.E. Schmitz, and D.M. Downs, *RidA proteins prevent metabolic damage inflicted by PLP-dependent dehydratases in all domains of life.* mBio, 2013. **4**(1): p. e00033-13.

2. Irons, J.L., K. Hodge-Hanson, and D.M. Downs, *RidA Proteins Protect against Metabolic Damage by Reactive Intermediates.* Microbiol Mol Biol Rev, 2020. **84**(3).

3. Elliott, T., et al., *Cloning and sequence of the Salmonella typhimurium hemL gene and identification of the missing enzyme in hemL mutants as glutamate-1-semialdehyde aminotransferase.* J Bacteriol, 1990. **172**(12): p. 7071-84.

4. Ernst, D.C. and D.M. Downs, *Mmf1p Couples Amino Acid Metabolism to Mitochondrial DNA Maintenance in Saccharomyces cerevisiae.* mBio, 2018. **9**(1).

5. Kispal, G., et al., *Mitochondrial and cytosolic branched-chain amino acid transaminases from yeast, homologs of the myc oncogene-regulated Eca39 protein.* J Biol Chem, 1996. **271**(40): p. 24458-64.

6. Knight, S.A., et al., *Mt-Hsp70 homolog, Ssc2p, required for maturation of yeast frataxin and mitochondrial iron homeostasis.* J Biol Chem, 1998. **273**(29): p. 18389-93.

7. Kispal, G., et al., *The ABC transporter Atm1p is required for mitochondrial iron homeostasis.* FEBS Lett, 1997. **418**(3): p. 346-50.

8. Babcock, M., et al., *Regulation of mitochondrial iron accumulation by Yfh1p, a putative homolog of frataxin.* Science, 1997. **276**(5319): p. 1709-12.

9. Foury, F. and O. Cazzalini, *Deletion of the yeast homologue of the human gene associated with Friedreich's ataxia elicits iron accumulation in mitochondria.* FEBS Lett, 1997. **411**(2-3): p. 373-7.

10. Valko, M., H. Morris, and M.T. Cronin, *Metals, toxicity and oxidative stress.* Curr Med Chem, 2005. **12**(10): p. 1161-208.

11. Muhlenhoff, U., et al., *Compartmentalization of iron between mitochondria and the cytosol and its regulation.* Eur J Cell Biol, 2015. **94**(7-9): p. 292-308.

12. Li, J., et al., *Yeast mitochondrial protein, Nfs1p, coordinately regulates iron-sulfur cluster proteins, cellular iron uptake, and iron distribution.* J Biol Chem, 1999. **274**(46): p. 33025-34.

13. Luzzati, M., *Isolation and properties of a thymidylate-less mutant in Saccharomyces cerevisiae.* Eur J Biochem, 1975. **56**(2): p. 533-8.

14. Chen, X.J. and G.D. Clark-Walker, *The petite mutation in yeasts: 50 years on.* Int Rev Cytol, 2000. **194**: p. 197-238.

15. Ernst, D.C. and D.M. Downs, *2-Aminoacrylate Stress Induces a Context-Dependent Glycine Requirement in ridA Strains of Salmonella enterica.* J Bacteriol, 2016. **198**(3): p. 536-43.

16. Zelikson, R. and M. Luzzati, *Influence of the nuclear gene tmp3 on the loss of mitochondrial genes in Saccharomyces cerevisiae.* Mol Cell Biol, 1982. **2**(4): p. 457-66.

17. Sliwa, D., et al., *Inactivation of mitochondrial aspartate aminotransferase contributes to the respiratory deficit of yeast frataxin-deficient cells.* Biochem J, 2012. **441**(3): p. 945-53.

18. Flynn, J.M., M.R. Christopherson, and D.M. Downs, *Decreased coenzyme A levels in ridA mutant strains of Salmonella enterica result from inactivated serine hydroxymethyltransferase.* Mol Microbiol, 2013. **89**(4): p. 751-9.

19. Ernst, D.C., *Exploring Sources and Metabolic Consequences of 2-Aminoacrylate Stress in Bacteria and Yeast*, in *Microbiology*. 2017, University of Georgia. p. 256.

20. Nagarajan, L. and R.K. Storms, *Molecular characterization of GCV3, the Saccharomyces cerevisiae gene coding for the glycine cleavage system hydrogen carrier protein.* J Biol Chem, 1997. **272**(7): p. 4444-50.

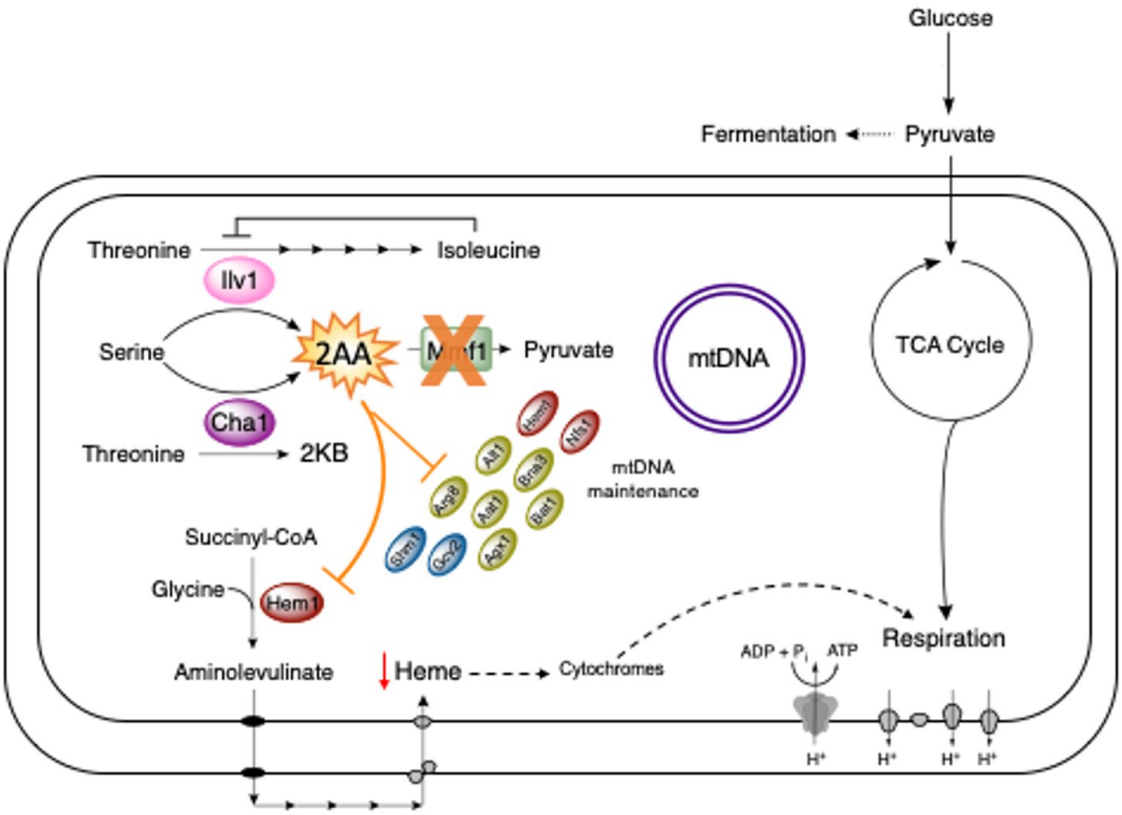

Figure 3.1

The RidA paradigm in yeast. The reactive enamine 2-aminoacrylate (2AA) is produced by the serine/threonine dehydratases Ilv1 and Cha1 within mitochondria (pictured). In the absence of the RidA homolog Mmf1, 2AA damages PLP-dependent enzymes. Hem1 and Bat1 are known targets, all others depicted (yellow, blue, red oval) are PLP-dependent enzymes localized to the mitochondrion. The effects of damage to Hem1 and Bat1 have been examined, but they do not account for all the phenotypes of a *mmf1* deletion strain.