# DISCOVERY AND CHARACTERISATION OF YEAST FERRITIN (*yFer1*) IN *SACCHAROMYCES CEREVISIAE*

# DÉCOUVERTE ET ANALYSE DE LA FERRITINE (*yFer1*) DANS LA LEVURE *SACCHAROMYCES CEREVISIAE*

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by

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{ الحمد لله }

**ABSTRACT**

Ferritin is an important iron chelating protein that is reported to store iron in order to prevent the toxic effects of excess iron inside the cell. Ferritin is found almost ubiquitously in all kingdoms of life except in Fungi where its homologues in different fungal species are very limited. We previously conducted a genetic screen looking for sequences with the ability to suppress the pro-apoptotic effects of Bax in yeast. One of the DNA sequences we found that prevented Bax-mediated cell death in *Saccharomyces cerevisiae* yeast cells was that of the human ferritin heavy chain subunit (H-ferritin). As ferritins appear to be absent in yeast, it was surprising to find that human ferritin was able to function in yeast to prevent the effects of Bax. We therefore set out to determine whether yeast in fact does have a ferritin homologue by conducting a search in the *Saccharomyces* Genome Database (SGD) for sequences homologous to human H-ferritin. We identified *YER067W*, a 161 residue protein with 20% sequence identity with the 183 residue human H-ferritin as a likely ferritin yeast homologue. Like human H-ferritin, we found *YER067W* to be a Bax suppressor and general pro-survival protein. When overexpressed in *S. cerevisiae* separately, both proteins were found to behave similarly under various conditions, and were found to behave like iron scavengers as opposed to iron storage centres in yeast. Overall, these results suggest that *YER067W* is likely to be the long sought-after yeast ferritin (*yFer1*).

**Keywords:** ferritin, programmed cell death, anti-apoptosis, iron, *Saccharomyces cerevisiae*, Bax, fungi

**RÉSUMÉ**

La ferritine est une protéine importante qui chélate et entrepose le fer afin d'éviter les effets toxiques de l'excès de fer dans la cellule. La ferritine se trouve presque ubiquitaire dans tous les règnes de la vie, sauf dans les champignons où ses homologues dans des différentes espèces fongiques sont rares. Nous avons déjà effectué un criblage à la recherche de séquences avec la possibilité de bloquer les effets pro-apoptotiques de Bax. Une des séquences d'ADN que nous avons trouvé qui a empêché la mort cellulaire induite par Bax dans des cellules de levure *Saccharomyces cerevisiae* a été celle qui code pour la chaîne lourde de la ferritine humaine (H-ferritine). Parce que la levure n'est pas connue pour posséder des ferritines, il était surprenant de trouver que la ferritine humaine était en mesure de fonctionner dans la levure pour prévenir les effets de Bax. Nous avons donc cherché à déterminer si la levure, en fait, a un homologue de ferritine en effectuant une recherche dans la base de données du génome de *Saccharomyces* (SGD) pour des séquences qui sont homologues à l'H-ferritine humaine. Nous avons identifié *YER067W*, une protéine de 161 résidus avec 20% d'identité de séquence avec l'H-ferritine humaine de 183 résidus comme un homologue de levure susceptible d'être la ferritine. Comme pour la H-ferritine humaine, nous avons trouvé que *YER067W* est un suppresseur de Bax et une protéine pro-survie générale. Lorsque surexprimée dans *S. cerevisiae* séparément, les deux protéines se comportent de façon similaire dans des conditions diverses et nous avons observé que les deux agissent comme des chélateurs et non comme des entreposeurs du fer. Dans l'ensemble, ces résultats suggèrent que *YER067W* est susceptible d'être la ferritine longuement convoitée de la levure (*yFer1*).

**Mots clés :** ferritine, morte cellulaire programmée, anti-apoptose, fer, *Saccharomyces cerevisiae*, Bax, champignons

**CO-AUTHORSHIP STATEMENT**

The manuscript integrated into Chapter 3 of this thesis was co-first-authored with Rawan Eid, Ph.D. candidate at Queen's University (co-supervised by Dr. Michael T. Greenwood and Dr. Paul G. Young).

As co-first author, I was responsible for the creation of some yeast strains used in the study as well as labour involved in producing figures 1A, 1B, 1C, 2A, 2B, 3B, 4, and S1.

Rawan Eid was responsible for the creation of some yeast strains used in the study as well as labour involved in creating figure 3A.

Sara Sheibani, Natalie Jones, Alex Cerulli, and Jason Lapointe are students in collaborating laboratories at McGill University, led by Dr. Hojatollah Vali and Dr. Craig A. Mandato, and they were responsible for the labour involved in the creation of figure 2C.

Dr. Michael T. Greenwood developed the concept of the study and all the authors of the study contributed to its design and/or assisted in the manuscript's preparation and/or revision.

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**LIST OF ABBREVIATIONS**

Atg – Autophagy related

*ATG* – Autophagy related gene

ATP – Adenosine triphosphate

BH – Bcl-2 homology

cIAP1 – Cellular inhibitor of apoptosis 1

DPS – DNA-binding protein from starved cells

h – Human

H-ferritin – Ferritin heavy chain

HIV – Human immunodeficiency virus

IAPs – Inhibitors of apoptosis

IRE – Iron responsive element

IRP – Iron regulatory protein

L-ferritin – Ferritin light chain

ORF – Open reading frame

PCD – Programmed cell death

RIP – Receptor-interacting protein

ROS – Reactive oxygen species

SGD – *Saccharomyces* Genome Database

TNF – Tumour necrosis factor

TNF-α – Tumour necrosis factor α

TNFR – Tumour necrosis factor receptor

TOR1 – Target of rapamycin 1

UTR – Untranslated region

*yFer1* – Yeast ferritin 1

# 1. INTRODUCTION

## 1.1 Programmed Cell Death (PCD)

In order for an organism to function optimally, programmed cell death (PCD) must occur in an effective and controlled manner [1]. Perhaps one of the most obvious reasons where it is not only beneficial but necessary for a cell to die is when it becomes cancerous. The prevention of cells from becoming oncogenic is beneficial to the organism as a whole since the presence of cancerous cells in a body could cause serious problems that can lead to the death of the entire organism. Therefore, old, damaged, or excess unneeded cells must be removed [1].

Inappropriate PCD, even in just one cell type, can have detrimental effects on the entire organism. For example, the destruction of the pancreatic beta cells in type 1 diabetes [5]. Autoimmune initiation of apoptosis causes the destruction of insulin producing beta cells, resulting in decreases in insulin production levels to a point where insulin production becomes insufficient and the body can no longer tolerate increases in blood glucose levels [5, 6]. It is true that most diseases occur due to improper triggering of apoptosis leading to increased cell death, like the example given of type 1 diabetes; however, the opposite is true as well. The ability of a cell to evade apoptosis can lead to consequences that may cause the development of diseases like cancer. Resistance to apoptosis is an important player in the oncogenesis of cells. Not surprisingly, Bcl-2, a major anti-apoptotic protein (which plays a very important role in cellular anti-apoptotic signalling), was first discovered as an oncogene that was upregulated in cancer cells; subsequently, the oncogenic potential of Bcl-2 was found to be related to its pro-survival properties [1]. Because these cells are under constant stress [1], they use many strategies to not only help them survive, but thrive. One of these strategies is to upregulate genes encoding anti-apoptotic or pro-survival proteins [1] in order to cope with the stresses of a poor environment and a host that is trying to fight them. This increase in the expression of genes encoding pro-survival proteins leads to increased stress resistance and better survival of the cancer cells under conditions that would normally have killed a non-cancerous cell [1]. This leads to a stress resistant phenotype and it becomes more difficult to kill these cells either through host immunity or through cancer drugs and treatments [1].

Different forms of cell death are employed depending, in part, on the severity or type of stress causing the problem. There are three main types of PCD that the cell may use depending on the situation: apoptosis, autophagy, and necrosis [1]. Although these three types of PCD are fundamentally different, they engage in crosstalk in order to initiate the most appropriate response to the present stress [2]. Furthermore, the different types of PCD can also complement each other; when a stress is present that triggers a specific kind of PCD whose pathways may be blocked, another form of PCD can take its place and cause the death of the cell via an alternate pathway [1]. Additionally, the form of cell death to be initiated can depend on many things ranging from the nature of the inducing stimulus to the kind of cell receiving the stress [3, 4]. To illustrate this point, take for example the infection of the motor neurons in the spinal cord by the sindbis virus. This virus will cause the death of the spinal motor neurons via necrosis; however, cortical neurons in the brain infected with this same virus exhibit cell death by apoptosis instead [4]. The same stress, the sindbis virus, causes different forms of cell death depending on which cell type has been infected with it.

Cells constantly monitor their environment in order to decide whether staying alive is more beneficial or whether dying would be better for the organism (or colony in the case of microorganisms) as a whole [1]. Therefore, there is always a balance between pro- and anti-apoptotic signalling from both inside and outside the cell keeping it alive or telling it to die. Before the cell decides that it is time to die, however, it will try to enhance its chances of survival by counteracting the stress in order to avoid unnecessarily killing itself [1]. One way the cell can do this is by increasing the expression of genes that code for pro-survival, or anti-apoptotic, proteins [1]. Our knowledge of the pathways leading from stress to PCD is incomplete and the main focus of our studies, therefore, is to elucidate these pathways by studying genes involved in the negative regulation of the process of PCD.

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# 2. LITERATURE REVIEW

## 2.1 Apoptosis (Type I PCD)

Apoptosis or type I PCD, is a highly regulated process of cell death that occurs only when the cell has made a well-coordinated decision on whether or not death is the best way to proceed [7, 8]. The cell's decision to commit to apoptosis depends on many factors governed by internal cues like pro- and anti-apoptotic proteins and external cues like environmental stressors [7]. There are two main forms of apoptosis that lead to type I PCD known as the intrinsic and extrinsic forms, which are initiated by stress or death inducing cytokines, respectively [9]. Figure 2.1 illustrates the pathways taken by both forms of apoptosis from the detection of stress and the complex web of molecular interactions to the initiation of cell death.

Intrinsic or mitochondrial-based apoptosis in mammals is mainly thought to be controlled by a class of proteins called the Bcl-2 family of proteins (figure 2.1, A) [8, 9]. Bcl-2 family members are recognised by the presence of a Bcl-2 homology (BH) domain, and the approximately 30 family members include both anti-apoptotic proteins like Bcl-2 and pro-apoptotic proteins such as Bax [8]. Pro-apoptotic stimuli activate pro-apoptotic Bcl-2 proteins and inactivate anti-apoptotic members leading to changes in the outer mitochondrial membrane causing the release of numerous pro-apoptotic proteins like cytochrome c, which helps with the formation of a structure called the apoptosome (figure 2.1, B) [8, 9]. The apoptosome recruits signalling molecules called initiator caspases to activate effector caspases and initiate caspase-dependant apoptosis (figure 2.1, C) [8, 9]. Caspases are proteases that exist in the cell in the inactive form of procaspases [10]. During apoptosis, they are cleaved and turned into their activate states; the active caspases are responsible for breaking up the cell via degradation of key proteins inside the cell effectively leading to its disintegration into small cellular blebs and subsequent controlled engulfment by macrophages [10].

The other main form of apoptosis is known as the extrinsic form (figure 2.1, D) [9]. The extrinsic pathway of apoptosis involves external cues for cell death that lead to increased production of cytokines such as tumour necrosis factor α (TNF-α), which activates cell surface death receptors from the tumour necrosis factor (TNF)

receptor family [9]. When these death receptors are activated, initiator caspases turn on downstream complexes leading to the activation of effector caspases [9]. These effector caspases, in turn, cleave molecules called death substrates, initiating cell death and causing the hallmarks of apoptosis [9]. Some of the characteristics of apoptosis include: the inversion of the cell membrane so that the internal cell membrane is exposed to the external environment, the fragmentation of DNA and condensation of chromosomes, and the breakup and packaging of cellular fragments into membrane-bound apoptotic bodies, which later become, as mentioned above, phagocytosed by macrophages [7, 9].

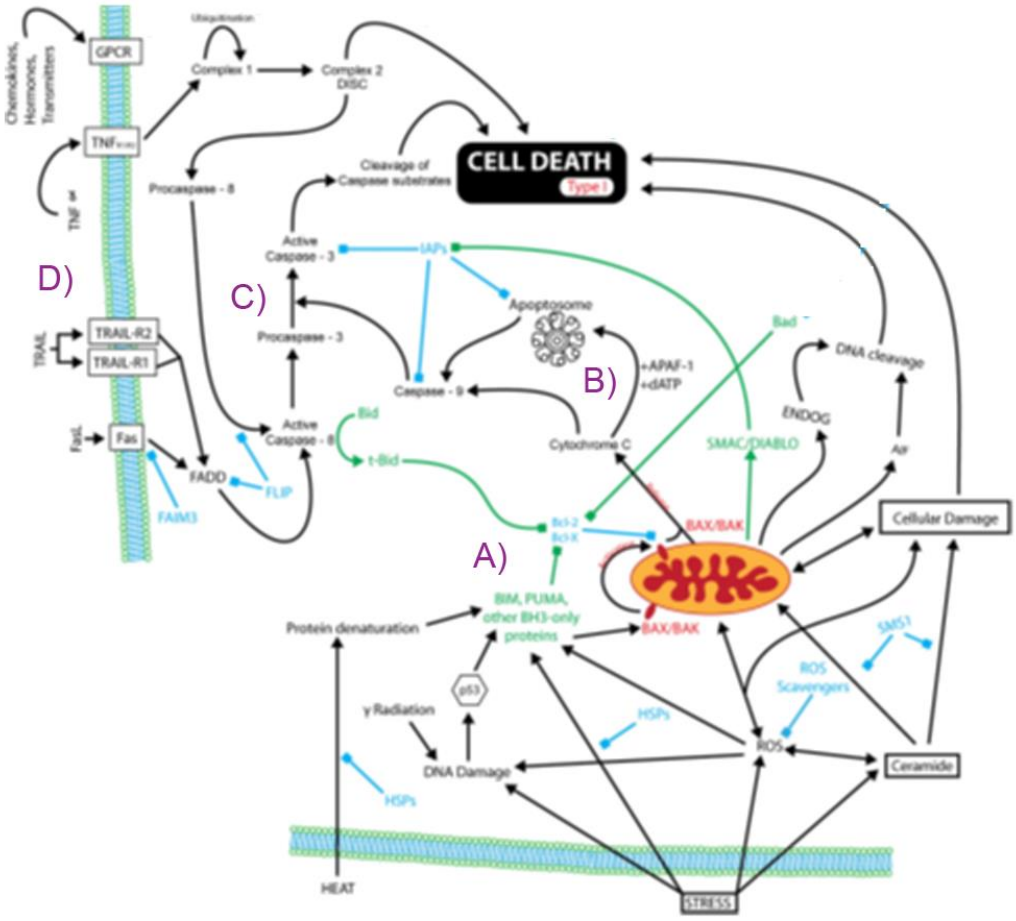


**Figure 2.1** Signalling pathways leading to the initiation of type I programmed cell death (PCD), or apoptosis. There are two main pathways leading to the initiation of type I PCD known as the intrinsic and extrinsic pathways. Both lead to the activation of pro-apoptotic proteins like Bax and the inhibition of anti-apoptotic Bcl-2 proteins like Bcl-2 in response to stress. Bax causes the release of cytochrome c from the mitochondria, which goes on to signal for the formation of the apoptosome leading to the caspase dependent initiation of apoptosis. Anti-apoptotic proteins are shown in blue and pro-apoptotic proteins are shown in green. *Reprinted by permission from Elsevier: Biochimica et Biophysica Acta [reference 9], copyright 2011 (see A1 in Appendix for reuse permission).*

## 2.2 Autophagy (Type II PCD)

Autophagy or type II PCD is an unusual kind of cell death because its main function is to try and save a stressed cell from deterioration [9]. It is not fully understood how the process of autophagy can cause the death of a cell; however, the observation of structures called autophagosomes after cell death is taken as evidence that the cell likely died due to autophagy-induced cell death [9]. The function of these autophagosomes is to act as a site for the accumulation of cellular components to be recycled, as well as being the delivery vehicle that delivers these materials to their degradation sites [9]. Moreover, this form of cellular recycling can be activated by different stresses in order to keep up the cell's normal functioning in difficult circumstances [9]. For example, this can be done in response to situations like organelle damage, misfolded proteins, a period of starvation, or simply organelle ageing [9]. Thus, autophagy can protect cells by getting rid of stress-inducing, damaged cellular debris or by providing recycled amino acids as anabolic building blocks in times of starvation [9]. However, autophagy also serves as an alternate cell death pathway when apoptosis is blocked [9, 10]. Cell death due to autophagy has been hypothesised to occur in situations where apoptosis has been inhibited and a cell is subjected to an apoptosis-inducing stress [9, 10].

It is not clear cut how autophagy results in cell death because it plays mainly a protective role [9]; however, a possible theory is that prolonged, intensive autophagy could lead to a cell's exhaustion and demise due to essentially "eating itself up" [9, 10]. Macrophagy, the specific type of autophagy that is induced by the inactivation of the target of rapamycin 1 (TOR1) and responsible for large-scale recycling, is a caspase independent PCD and is mediated by the actions of the lysosome or vacuole [9, 14]. TOR1 is a kinase that senses the levels of energy available in the cell and influences protein synthesis and cell growth depending on nutrient availability [14]. It is believed that active TOR1 inhibits the onset of autophagy when nutrients are available in sufficient quantities and it is the inactivation of TOR1 that leads to the activation of autophagy [13]. This theory is supported by studies showing that rapamycin, a drug that inhibits TOR1, can induce autophagy even in nutrient rich environments [13]. After the inhibition of TOR1, several autophagy related (Atg) proteins form complexes that are involved in the formation, elongation, and completion stages of autophagosome creation [14]. As shown in figure 2.2, after the complete formation of the autophagosome, specific Atg proteins will signal for the fusion of the autophagosome with a lysosome (or vacuole in the case of yeast), and it then becomes

an autophagolysosome, a structure for the degradation of whatever cellular components it contains [9, 11, 14]. For this reason, the Greek word autophagy means "self-eating"; it is so named because the process of autophagy includes the digestion, or catabolism, of cellular organelles back into their basic building blocks which can then be reused by the cell [9].

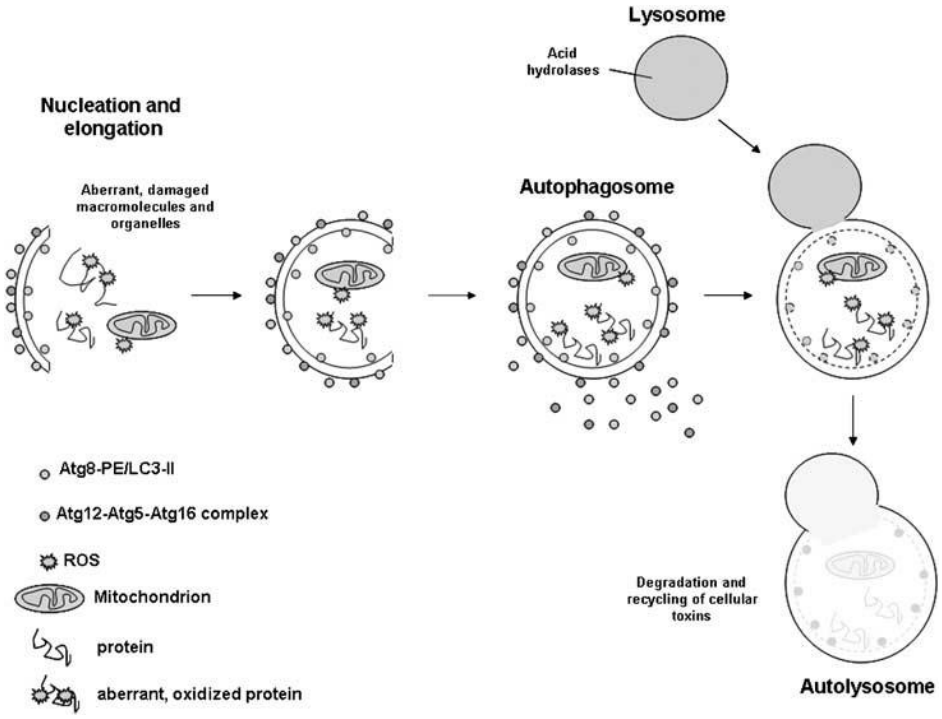


**Figure 2.2** Degradation of cellular components via autophagolysosome formation during macroautophagy. Damaged cellular components are engulfed in a membrane-bound vesicle called the autophagosome. After the completion of autophagosome membrane formation, Atg proteins signal for the fusion of a lysosome filled with acidic hydrolases with the autophagosome, creating an autolysosome where cellular components will be degraded by the hydrolases and subsequently recycled. *Reprinted by permission from Macmillan Publishers Ltd: Cell Death and Differentiation [reference 14], copyright 2009 (see A2 in Appendix for reuse permission).*

### 2.3 Necrosis & Necroptosis (Type III PCD)

The last of the three types of cell death is necrosis. Generally, the word necrosis is used to describe passive or catastrophic forms of cell death [9, 17], usually resulting from inescapable attacks like extreme heat (burning), severe chemical insults, or irreparable physical damage. There is now mounting evidence, however, of another more controlled form of necrosis, usually mediated by so-called death receptors and resulting in the same characteristics as necrotic cell death but via a controlled or regulated pathway that can be inhibited genetically, and is given the name 'necroptosis' [9, 17, 24]. Furthermore, there are proteins, including some that exert some form of control over apoptosis, that have been found to play a role in the initiation or control of necrosis, leading to the suggestion that there is cross-talk between the two forms of cell death [15-17]. In some cases, the same stimulus that can lead to the initiation of apoptosis can also trigger necroptosis and the decision on which pathway to take depends on factors like cell type [15, 16]. The characteristics of necrotic cell death include swelling of the cell and its organelles and rupturing of the plasma membrane leading to the spilling out of cellular contents (usually retaining an intact nucleus without DNA fragmentation), and often resulting in an inflammatory response [15, 17].

Different forms of necroptosis are controlled in several different ways. Although there are other ways necroptosis can be initiated, the most studied is the version initiated by death receptors known as the tumour necrosis factor receptors (TNFR) and their interactions with receptor-interacting protein (RIP) kinases 1 and 3 [15, 17]. The binding of tumour necrosis factor (TNF), a signalling molecule, to the death domain of TNFR, changes the receptor's conformation which then goes on to recruit proteins, forming protein complexes that include RIP1 and cellular inhibitor of apoptosis 1 (cIAP1) [15, 17]. Once TNFR is internalised to the interior of the cell, the members of the protein complex change and now include RIP1, RIP3, and caspase 8 [17]. Necroptosis is generally activated in the presence of caspase inhibitors (which work to inhibit the caspases used in the apoptotic response), as necroptosis is a caspase independent form of PCD [17]. These caspase inhibitors may be artificial pharmacological agents or they may be endogenous caspase inhibitors including the likes of inhibitors of apoptosis (IAPs) [17]. Caspase inhibitors are important in the induction of necroptosis because a lot of the time the receptors that kick-start the necrotic response are also responsible for activating apoptosis; therefore, the presence of caspase inhibitors helps to direct the cell in the direction of a necrotic response

[17]. To elicit TNF-induced necroptosis, caspase 8 must be inhibited due to its ability to inactivate RIP1 and RIP3 and initiate TNF-induced apoptosis [17]. Once caspase 8 is inhibited, RIP1 and RIP3 become phosphorylated and necroptosis proceeds [17]. Thus, the cell can switch between apoptosis and necroptosis using IAPs as regulatory modulators [17].

### 2.4 *Saccharomyces cerevisiae* Yeast as a Model Organism

Due to the fact that mammalian anti-apoptotic pathways are complex and due to all the difficulties encountered while studying biological processes in mammalian cells, a model organism is a useful alternative to examining these mechanisms without the added complications of using mammalian cells. In addition, mammalian cells have many proteins with redundant functions that may step in place of a malfunctioning protein [5] hindering studies performed with gene knockouts whereby another protein may fill the function of the gene product that was deleted or mutated. Another hurdle that stands in the way of pathway studies in mammalian cells is the complex webs of protein-protein interactions that can complicate the process due to the creation of too much background noise [5].

*Saccharomyces cerevisiae* has homologues for many human genes and performs many of the same cellular processes as higher eukaryotes such as DNA repair, mitochondrial respiration, and autophagy [5, 13]. The awarding of Nobel prizes for work on the yeast secretory process and cell cycle regulation demonstrates the usefulness of yeast as a model eukaryotic system that serves to increase our understanding of basic cellular processes that are shared among all eukaryotic cells, including humans. In addition to being a single celled eukaryote, a single yeast cell is also an entire organism *per se*, a characteristic that significantly reduces the complications of intercellular interactions while keeping the whole organism response intact. It is no surprise, then, that yeast as well as humanised yeast (yeast cells expressing human genes) are widely used model organisms [5] as there is a multitude of reasons that make it a very valuable tool for studying basic as well as mammalian cellular pathways.

Aside from being a very low maintenance laboratory organism, one reason why *S. cerevisiae* is such a useful model is due to its fully sequenced genome and the fact that yeast and humans have many homologous proteins that perform similar

functions [4]. Knowing the entire sequence of the yeast's DNA allows for meaningful comparisons and studies to be carried out [4, 12]. Some proteins are so well conserved between humans and yeast, that replacing the yeast protein with the human version retains normal functioning of the eukaryotic yeast cell [4]. In addition to being fully sequenced, the yeast genome is haploid, making it much simpler to knockout genes of interest [1]. Furthermore, yeast has an endogenous plasmid that allows for easy overexpression of any given sequence by the introduction and maintenance of recombinant plasmids. Because yeast only has one copy of each gene, whatever mutation is introduced into a gene or whatever gene is overexpressed, there will be no interference from another copy of that same gene, essentially making the phenotype a direct result of the genotype. As well as being a simpler organism to manipulate genetically, yeast manipulation also comes with a cheaper cost; and because yeast cells grow and divide quickly (doubling every couple of hours or so) they make ideal models for studying human genes, proteins, and signalling pathways because results can generally be witnessed within a maximum timeframe of a few days [4].

Humanised *S. cerevisiae* is a particularly useful system to study PCD regulation as many mammalian pro- and anti-apoptotic genes behave the same in yeast as they do in mammals [5, 8]. Because *S. cerevisiae* undergoes its own form of the three types of PCD, which can be triggered by both internal and external cues and are fundamentally similar to PCD occurring in mammals, it has been widely used as a model for mammalian PCD processes [5, 6, 11]. Specifically, *S. cerevisiae* shows the same hallmarks of apoptotic cell death as mammalian cells such as DNA fragmentation and the externalisation of the internal cell membrane as well as having a multitude of conserved cell death pathways [5-8]. For example, in the process of undergoing apoptosis, yeast cells, like mammalian cells, exhibit intracellular events like increased mitochondrial membrane permeability and the release of the apoptogenic factor cytochrome c, as well as an increase in the presence of apoptotic second messengers like reactive oxygen species (ROS) [5, 8]. In addition to undergoing cell death, yeast can also negatively regulate PCD by anti-apoptotic proteins similar to those found in mammals, paving the way for the study of anti-apoptotic genes in yeast [5, 8]. Moreover, genes involved in the control of autophagy, called autophagy related genes (*ATG*s), were first identified in yeast and are now known to be conserved in higher organisms as well, manifesting once again the importance of yeast in the study of the various forms of PCD [9-13].

## 2.5 Ferritin

Iron is an important element that is needed in human cells as it required by some proteins to carry out their normal functions. For example, iron is a cofactor for some proteins in the electron transport chain, a process involved in cellular energy production. Furthermore, in the case of humans, iron is the site of oxygen binding on the hemoglobin protein, an essential protein for human life. Therefore, due to the importance of iron for living organisms, it is important for cells to have iron available at all times, through the use of iron storage proteins, for example. The ferritin protein is thought to function as an iron storage, chelating, and scavenging protein [2, 3, 20, 23]. Ferritin is composed of 24 subunits and resembles a hollow cage inside which excess iron molecules are stored [18]. The final protein is made up of light chain (L-ferritin) or heavy chain (H-ferritin) subunits or a combination of both [23]. L-ferritin and H-ferritin are related but have some functional differences and are encoded by different genes [18]. H-ferritin has enzymatic activity due to the presence of ferroxidase centres, while L-ferritin contains no ferroxidases and therefore has no enzymatic activity [18]. Ferroxidases are involved in the oxidation of ferrous iron ($Fe^{2+}$) into ferric iron ($Fe^{3+}$) and are active even when the H-ferritin subunit is in monomeric form [18]. In terms of L-ferritin function, studies show that salt bridges in the helical folds of the L-ferritin subunit are responsible for increasing the stability of the ferritin molecule in certain conditions such as acidic environments [18, 19]. Furthermore, there is evidence that the L-ferritin subunit increases the efficiency of the incorporation of iron molecules into the multimeric ferritin protein due to the presence of iron nucleation sites on the light chain subunit [18, 23].

In mammalian cells, ferritin levels are controlled post-transcriptionally by iron regulatory proteins (IRPs) [20]. In the presence of low iron levels in the cytosol, IRPs bind to the iron responsive element (IRE) on the 5' untranslated region (UTR) of the ferritin mRNA [20]. This stops the translation of the mRNA and decreases the amount of ferritin protein [20]. In addition, IRPs signal for the augmentation of the transferrin receptor, which is responsible for iron uptake into the cell, by binding to its 3' UTR [20]. These two actions together act to increase the amount of free iron in the cytosol because it increases iron influx into the cell, and at the same time decreasing ferritin production so that iron remains free in the cytosol and not caged up in ferritin molecules [20]. On the other hand, when there is an excess of iron inside the cell, IRPs no longer bind to the IREs on the ferritin mRNA and this allows for the mRNAs to be translated into ferritin protein; furthermore, IRPs fall off the transferrin

receptor mRNAs as well, decreasing their translation and, consequently, the influx of iron into the cell via the transferrin receptor [20].

Ferritin sequesters free iron inside the cell, and this is thought to be one of the protective mechanisms used against the generation of destructive ROS due to oxidative stress [18]. Iron reacts with hydrogen peroxide ($H_2O_2$), in a reaction known as the Fenton reaction (shown below), to create free radicals that can cause cellular damage inside the cell [18].

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + HO\bullet + OH^-$$
$$Fe^{3+} + H_2O_2 \rightarrow Fe^{2+} + HOO\bullet + H^+$$

The literature seems to be in agreement that ferritin is protective against cell death resulting from heavy metal and oxidative stress, like those caused by iron and $H_2O_2$ [18]. For example, one study done with the nematode *Caenorhabditis elegans* saw increased protection when H-ferritin was overexpressed during oxidative stress and a decrease in *C. elegans* life span when its H-ferritin homologue was knocked out with excess iron in its environment [20]. Another study found that HeLa cell growth rate was decreased due to the overexpression of H-ferritin at 14- to 16-fold higher amounts than control cells, due to its sequestration of iron from labile pools in the cell but showed that, in the presence of oxidative stress, this greatly increased the survival of cells against death due to $H_2O_2$ [23].

### 2.6 Overview of Research Objectives

My project was initiated based on our lab's previous observation that cloned human heavy chain ferritin (H-ferritin) could prevent the negative effects of the overexpression of the pro-apoptotic Bax sequence, in a screen of human cardiac sequences in the yeast *Saccharomyces cerevisiae* (as described in reference 25). Given that we found that numerous such Bax suppressors are pro-survival proteins, my project consisted of using yeast overexpressing H-ferritin as a model to study the largely uncharacterised mechanisms of the pro-survival effects of this protein. In addition, we found it interesting that ferritin was able to function in suppressing the effects of Bax in *S. cerevisiae*, a species of yeast which, like many other fungi, is not known to have a ferritin or a ferritin-like protein. We therefore decided to investigate whether *S. cerevisiae* does, in fact, have its own endogenous version of ferritin. Our methods,

results, and a discussion of our findings are presented in the manuscript that follows in the subsequent chapter.

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## 3. MANUSCRIPT

# The *Saccharomyces cerevisiae* ferritin 1 (*yFer1*) is a novel fungal specific ferritin

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**ABSTRACT**

Ferritin is a sub-family of iron binding proteins that form multi-subunit nanotype cages that take up iron to prevent iron toxicity and to serve as iron storage. Although ferritin has been extensively studied *in vitro*, very little is known about the *in vivo* functions of ferritin except that it can prevent cell death in response to ROS producing stresses. Here we describe the identification of human (h) H-ferritin as a suppressor of the pro-apoptotic murine Bax sequence in yeast. In addition, we demonstrate that ferritin is a general pro-survival sequence since it prevents the effects of other stresses including copper and iron when heterologously expressed in yeast. Although ferritins are phylogenetically widely distributed and are present in most species of Bacteria, Archaea, and Eukarya, ferritin is conspicuously absent in most fungal species, including *Saccharomyces cerevisiae*. Together, these results indicate that either ferritin is self-functioning or that yeast has a yet-to-be uncovered ferritin. An *in silico* analysis of the yeast proteome lead to the identification of the 161 residue *YER067W* encoded protein as a strong candidate for the elusive yeast ferritin (*yFer1*). In addition to sharing 20% sequence identity with the 183 residue h H-ferritin, *yFer1* also has similar functional properties as ferritin when overexpressed in yeast. Phylogenetic analysis reveals that the yeast ferritin (*yFer1*) is conserved but its distribution is restricted to a subset of fungal species in the Saccharomycetaceae (budding yeast) family. Overall, our results suggest that atypical ferritins remain to be identified in many fungal lineages.

### 3.1 Introduction

Iron is an essential co-factor for numerous proteins that are involved in a large variety of different biological functions that range from oxygen and electron transport to DNA synthesis and maintenance [1]. The ferritin-like super-family of proteins is one of the largest groups of iron binding proteins [2, 3]. Members of these 12 sub-families contain a structurally and evolutionary related iron binding domain that is present in a number of different functional proteins. The ferritin sub-family consists of three distinct members including the ferritins, the bacterioferritins, and the DPS (DNA-binding protein from starved cells) [2]. These proteins are capable of binding iron and in the case of ferritins and bacterioferritins, they are also capable of acting as storage molecules. These proteins carry out this function by forming multi-subunit nano-cage like structures that can store as much as 4,000 iron atoms in the 24 subunit structure formed by ferritin [4]. In addition, the ferritin proteins, including the DPSs, can protect cells from the toxic effects of iron as well as other free radical producing stresses such as hydrogen peroxide ($H_2O_2$) [2, 4-6].

The unique chemistry of metals such as iron, which has the ability to switch oxidative states ($Fe^{+3} + ė \leftrightarrow Fe^{+2} – ė$), makes these atoms indispensable for biological systems for such functions as a carrier of electrons [1, 7]. But there are numerous problems associated with the use of iron including the fact that although iron is very abundant, most forms are insoluble and are thus not easily accessible biologically [7]. Of further importance is the toxicity associated with iron *in vivo*. In a series of different reactions, including the Fenton's reactions, both $Fe^{+3}$ and $Fe^{+2}$ can react with other cellular molecules including $H_2O_2$ to produce destructive free radicals including ·OH and ·OHH [1, 4, 7]. Cells have developed a large repertoire of mechanisms to deal with free radicals generated by iron or other cellular stresses [8]. In addition, specific regulatory mechanisms have evolved to allow cells to grow and survive in environments with either excess or limiting levels of iron [9]. One is to coordinately increase and decrease the expression of genes that code for proteins such as iron transporters that alter iron uptake [10]. One other mechanism of importance involves using ferritin cages to safely store excess iron and serve as a reservoir in times of iron limitation [4, 11]. Although the ferritin sub-family may be one of the most well studied iron binding proteins many unanswered questions remain [2, 3]. These include the discrepancy that exists between the iron binding capacity of ferritin *in vitro* and *in vivo*, the mechanisms responsible for the protective effect of ferritin against ROS and why are the ubiquitously distributed ferritins not present in the majority of fungal species

[2, 12-14]. The yeast *S. cerevisiae* is an excellent model to study iron metabolism given that there are a great number of similarities between yeast and humans [9]. One notable difference is the absence of the ferritin iron storage proteins in many fungi including yeast [14]. Hence iron storage is thought to involve the vacuole in yeast while its mammalian counterpart, the lysosome, appears to play a less prominent role than ferritin in iron storage [14, 15].

Here we report the identification of human H-ferritin as a pro-survival protein that was identified in a screen for sequences that prevent Bax mediated cell death in yeast. In our search of the yeast proteome we identified the protein encoded by the yeast *YER067W* gene as a sequence producing significant alignment to human H-ferritin. We renamed this gene yeast ferritin 1 (*yFer1*) because our analysis of *YER067W* revealed that it not only resembles ferritin structurally, but it also behaves like ferritin in a number of assays. In effect, not only does it prevent the cytotoxic effects of Bax, it prevents cell death in response to exogenously applied copper stress and it reverses the iron supersensitivity of a vacuolar defective yeast mutant. In agreement with a growing consensus we find that *in vivo*, ferritin does not serve to store iron, instead it serves as an intracellular scavenger of cellular iron and protector from ROS generating stresses.

## 3.2 Results and Discussion

### *3.2.1 Identification of yeast ferritin*

In our previous screen of a human cardiac cDNA expression library for suppressors of the pro-apoptotic Bax, we isolated an 874 nt cDNA with a 3' poly A tail. The cDNA has a predicted open reading frame (ORF) between nt 138-687 encoding a 183 aa residue protein that represents human ferritin, heavy polypeptide (hFerr) (GenBank BC104643) [16]. The ferritin cDNA was re-transformed into naïve yeast cells with the Bax expressing cDNA. The transformants were grown in liquid glucose media, serially diluted and aliquots were spotted onto nutrient agar media with glucose or galactose. The Bax and ferritin sequences are expressed under the control of the yeast *GAL1* promoter and are thus induced in media that contains galactose. With glucose, all strains grew equally well but cells harbouring Bax alone showed, as expected, a significant inhibition of growth when plated on media containing galactose

(figure 3.1*A*). In contrast, cells that co-express ferritin or human 14-3-3β/α, a previously characterized Bax suppressor [17], show significant growth on galactose. It is worth mentioning that 14-3-3, a chaperone protein that binds several different proteins and is thought to, therefore, have the ability to partake in different cellular processes [17], and ferritin both serve to enhance cell survival under Bax stress but are likely not acting through the same pathway. This confirms the results of the screen showing that hFerr is a Bax suppressor and given that ferritin is a known pro-survival protein, hFerr thus appears to be a functional protein in yeast [4].

The widespread distribution of ferritins in all species except yeast suggests that ferritins were lost or systematically eliminated from fungal genomes [3, 14]. Alternatively, it is possible that yeast has a ferritin but that its sequence has diverged to such an extent that it is not identifiable by routine Blast searches. This is supported by studies reporting a ferritin-like iron binding protein is present in the extracts of *S. cerevisiae* [18]. Although many yeast proteins retain high sequence identity with their human counterparts, many others show more limited identity in the 20% range [19, 20]. In our search of the yeast proteome in the *Saccharomyces* Genome Database (SGD; http://www.yeastgenome.org/), we identified the 161 residue protein encoded by *YER067W* as a sequence that shares 20% identity (40% with the inclusion of chemically similar residues) with hFerr (E value = 0.9) (figure 3.1*C*). Using the Chou and Fasman criteria (http://web.expasy.org/protscale/), the secondary structure of *YER067Wp*, like hFerr, was found to contain five regions of predominant α-helix (figure 3.1*C*) [21]. The H-ferritin protein also contains ferroxidase activity and this can be identified by the presence of seven specific residues within the protein (figure 3.1*C*). Four of these residues can be found in the same region of *yFer1p* when aligned with human H-ferritin with conserved residues present for the other three residues (figure 1*B*). Furthermore, a comparison of the crystal structures of both hFerr and *YER067Wp* was performed using the FATCAT structural alignment tool [46]. The crystal structures of both proteins were retrieved from the Protein Data Bank and compared using FATCAT. The comparison found both structures to be significantly similar with a $p$ value < 0.003.

Although ferroxidase is present in H-ferritin, many other ferritins including human L-ferritin do not have ferroxidase activity [4]. Finally, using spot assays we show that *YER067W*, like hFerr, also serves to promote the growth of cells expressing Bax (figure 3.1*B*). Thus, our results indicate that ferritin is not lost, it has diverged and retains limited conservation in its sequences. Given that its function is unknown we re-named *YER067W* yeast Ferritin1 (*yFer1*) [22].

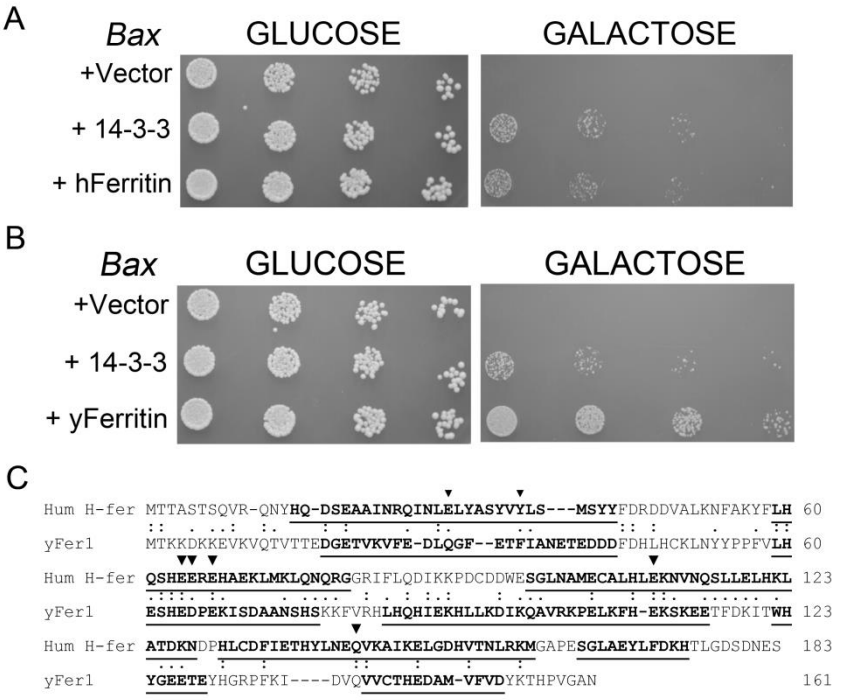


**Figure 3.1.** Identification of the yeast *YER067W* gene as yeast ferritin (*yFer1*). *(A)* Spot assay of cells expressing Bax alone and Bax with human ferritin. Fresh cultures of glucose grown yeast cells harbouring Bax or Bax and the Bax suppressor Bh107 human H-ferritin (hFerritin) were serially diluted and aliquots were spotted on nutrient agar plates with glucose or the *GAL1* promoter inducing galactose media. Photographs of the plates after 5 days of growth at 30° C are shown. *(B)* The spot assay was used to assess the growth of cells expressing Bax alone or Bax with yeast ferritin (yFerritin). Yeast ferritin appears to be more effective at protecting cells from the effects of Bax, possibly due to the fact that it is an endogenous yeast gene while human ferritin is heterologously expressed and may not be fully able to take on its proper conformation in a yeast cell and thus becomes less efficient than its yeast counterpart. In *(A)* and *(B)*, 14-3-3 is a positive control that refers to the previously characterised Bax suppressor human 14-3-3β/α. *(C)* The 183 amino acid sequence of human (h) H-ferritin is shown aligned to the deduced 161 residue amino acid sequence of *YER067W* that we have identified as yFerritin1 (Accessions BC104643 and NM_001178958.1). The sequences were aligned using the Blast program and

manually adjusted to maximize identity. A colon (:) indicates an identical residue, a point (.) represents a biochemically conserved residue and gaps indicate no residue (-). Regions of α-helices deduced by an algorithm using the method of Chou and Fasman (http://web.expasy.org/protscale/) are shown in bold and underlined. Inverted triangles (▼) denote residues that are important for the ferroxidase activity of H-ferritin.

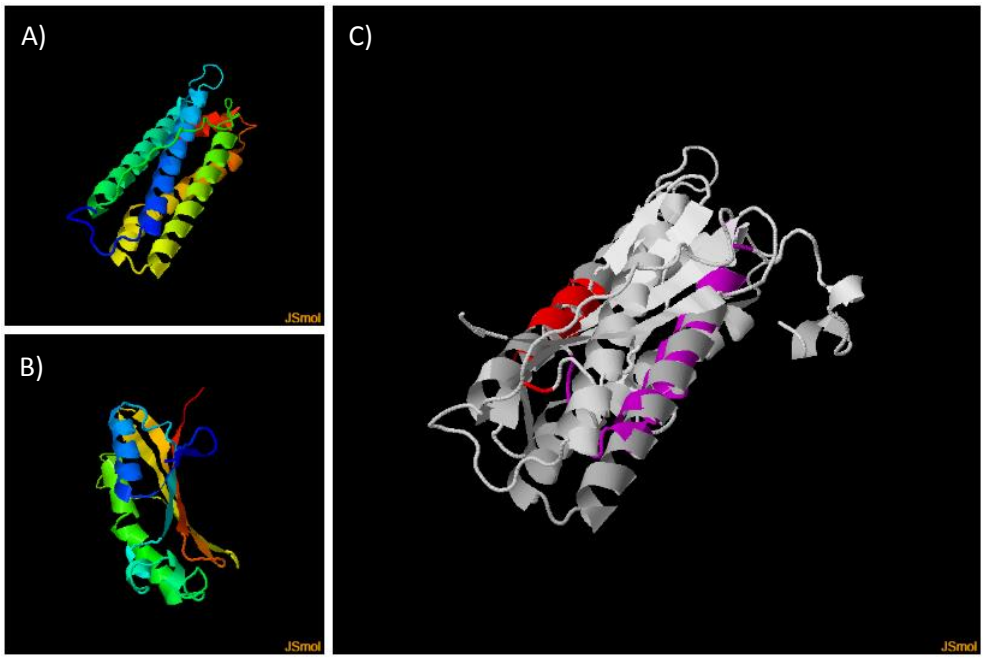


**Figure 3.2.** Comparison of the crystal structures of human heavy chain ferritin and *YER067W*. *(A)* Reported crystal structure of the heavy chain of human ferritin (Protein Data Bank). *(B)* Reported crystal structure of the *YER067W* gene (Protein Data Bank). *(C)* Superposition of human heavy chain ferritin (grey) with *YER067W* (colours). The structural alignments were significantly similar, as calculated by FATCAT [46].

### *3.2.2 Human and yeast ferritins protect against copper and iron stress in yeast*

One of the characteristics of all ferritins, is their ability to decrease stress mediated cell death when overexpressed [4]. To determine whether human and yeast ferritins are anti-apoptotic rather than solely being Bax suppressors, we used the yeast model to test their abilities to prevent cell death in response to copper stress using increasing amounts of the copper salt $CuSO_4$ [20, 23]. Copper is a well-known apoptotic inducer that can be prevented by overexpression of anti-apoptotic sequences [24, 25]. We used $CuSO_4$ as a source of copper but other copper salts may be used in order to confirm that the observed effects are due specifically to the copper and not to any other element or molecule. However, because we are mainly looking for a dose dependent induction of PCD, the specific molecule or chemical responsible for the effect is not the main concern of the experiment. We, therefore, used the spot assay cells containing empty vector or a vector expressing human 14-3-3 β/α, human or yeast ferritin grew well on nutrient agar media containing glucose or galactose (figure 3.3*A*). When challenged with increasing concentrations of $CuSO_4$, the growth of the control cells was noticeably inhibited in a dose dependant manner. In contrast cells harbouring the yeast or the human ferritin expressing constructs were able to grow even at the highest concentration of $CuSO_4$ (figure 3.3*A*).

Because the spot assay only measures growth, we used a vital dye viability assay to assess the ability of ferritins to prevent cell death. The viability of the cells in liquid culture was determined after 18 hours of incubation with or without 1.4 mM $CuSO_4$. Cultures grown without copper all had viabilities above 98% (figure 3.3*B*). The viability of control cells was significantly decreased to 29.2 ± 1.9% by $CuSO_4$. In contrast, cells overexpressing 14-3-3, human or yeast ferritins had respective viabilities of 79.3 ± 6.9%, 70.8 ± 3.3% and 75.3 ± 1.9% when grown with $CuSO_4$ (significant differences with student *t*-test, $p < 0.001$) (figure 3.3*B*). Therefore yeast ferritin is, like other ferritins, a pro-survival protein that can protect cells from stress [4, 6, 26, 27].

Iron is an essential nutrient that must be maintained within a relatively narrow range of concentrations in order to maintain homeostasis [1]. Starvation for iron or its depletion can serve to induce apoptosis [28]. In contrast, excess iron leads to cell damage at least in part by inducing apoptosis [15, 29, 30]. Iron likely mediates its cellular damage by acting as an electron donor or acceptor as it interconverts between the ferric ($Fe^{+3}$) and the ferrous ($Fe^{+2}$) forms and serves to produce ROS [7]. Iron has a limited solubility and a chelating system such as citrate is required to achieve high concentrations in cell growth media [31]. These concentrations of iron are not

sufficiently high for the induction of apoptosis in standard mammalian cell culture systems or in yeast [10, 31, 32]. Thus, iron mediated cell death has largely been studied in combination with other sublethal stresses including chemicals, neurohormones, or serum depleted growth media [28, 33]. Other approaches include the use of a variety of different genetically altered cells, including mammalian and yeast, that are supersensitive to iron [10, 31, 32]. These include mutants defective in the *CCC1* or *VMA3* genes, encoding vacuolar iron/copper transporter and a component of the vacuolar proton pump, respectively [10, 34]. Using a spot assay, we show that *Vma3*Δ is more sensitive to iron ($FeCl_3$) as compared to wild type cells (figure A1). As with $CuSO_4$, it would be necessary to use another iron salt to determine whether the effects are due specifically to iron and not chloride. In effect, yeast *Vma3*Δ cells harbouring empty vector grow well on normal media but they decrease in their ability to grow as the media is supplemented with added iron as low as 1mM and are unable to grow with 3mM iron (figure A1). In contrast, the growth of wild type cells with empty vector alone are unaffected by the added iron. The yeast *Vma3*Δ cells expressing either the human H- or the yeast ferritin are able to grow with 1mM added iron (figure 3.3*B*). Therefore, our results indicate that *yFer1*, like the previously characterised hFerr can reverse the iron mediated growth inhibition of a cell with a defective vacuole [32].

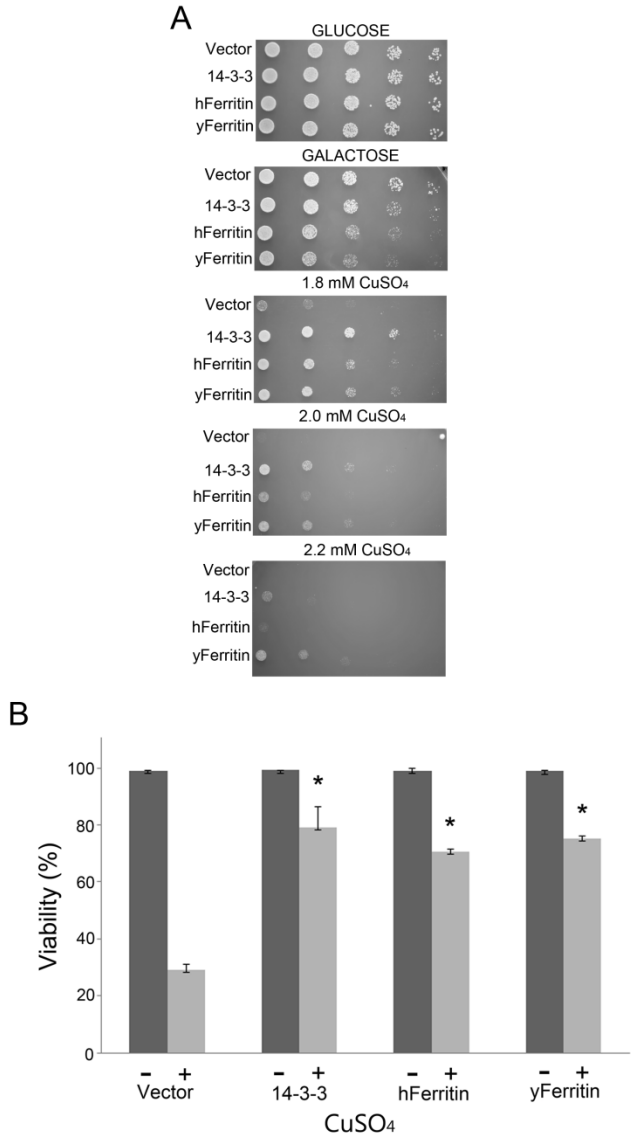


**Figure 3.3.** Human and yeast ferritins protect from copper and iron toxicity. *(A)* The spot assay was used to assess the ability of wild type yeast cells transformed with plasmids expressing human and yeast ferritins for their ability to grow when challenged with copper. Fresh cultures of the strains were serially diluted and aliquots were spotted onto different nutrient agar plates without or with the concentration of copper indicated. *(B)* The same strains were diluted into fresh media and allowed to grow without (-) and with (+) 1.4 mM copper ($CuSO_4$) for 18 hours. Viability was

determined by microscopically examining aliquots of at least 300 cells that were stained with the vital dye trypan blue. Viability is shown as the mean percentage (%) of the cells that survived after treatment in triplicate experiments that were repeated at least 3 times. *, denotes significant differences with copper treated control cells (Vector). (student *t* test, $p < 0.001$).

### *3.2.3 Ferritin is not an iron storage center but an iron scavenger*

Ferritin forms cage like structures that consist of 24 dimers of H-ferritin, L-ferritin or a combination of both subunits [11]. These serve as a reservoir for iron with as many as 4000 atoms stored per structure. To examine if there is an increased iron storage capacity in cells overexpressing ferritins, we grew yeast cells overexpressing ferritins in media with increased iron. After 18 hours, the cells that were still in exponential growth (OD600 between 0.3 and 0.8) were harvested and their iron content determined using a colorimetric assay [35]. When grown with a 10-fold increase in iron, the iron content of both control cells and ferritin overexpressing cells was increased 2-fold (figure A2). The magnitude of the increase for control cells is consistent with published reports indicating that cells likely avoid iron toxicity by limiting the uptake of excess iron [36]. Of interest is the observation that the iron content of *E. coli* cells is increased 2-fold by ferritin in stationary phase compared to logarithmically growing cells [37]. Therefore, we decided to examine the iron content of cells growing with excess iron when they reached stationary phase. The iron content of stationary phase cells were virtually indistinguishable from the results obtained with growing cells as shown in figure 3*A*. In effect, cells expressing ferritins also showed a similar 2-fold increase in the levels of iron when they were grown for 18 hours with a 10-fold excess iron. This indicates that there was no significantly augmented iron storage occurring due to the presence of ferritin. Previous reports had indicated that there was increased iron content of yeast cells heterologously expressing hFerr [38]. This might reflect methodological differences such as washing cells with a divalent ion chelator (EDTA) to decrease non-specific iron binding. Our results are more in agreement with several other studies that have had difficulty demonstrating the iron storage function of ferritin using *in vivo* assays [12, 27, 39, 40]. Hence, although ferritin is widely reported as the cell's iron storage center, the strongest actual evidence for this function was obtained in pioneering work that was carried out *in vitro* using purified protein [41]. In contrast, *in vivo* evidence for this function is severely lacking. For example, a great number of studies showing that overexpressed ferritin leads to resistance to oxidative stress in response to apoptosis inducing agents [6, 26, 27, 40]. There are however, very few studies that explicitly demonstrate an

increase in the levels of intracellular iron in ferritin overexpressing cells with normal or excess levels of iron. Some of the best evidence is provided by work in *E. coli* using mutants that are defective in the *FtnA* encoding ferritin [37, 42]. *E. coli* strains overproducing a 600-fold increase in ferritin only showed a modest 2.5-fold increase in iron content. The iron content increased 5- to 8-fold in the ferritin overproducers when they were grown with excess iron. The purified ferritin complex from these cells contained only 5 to 20 atoms of iron. This low *in vivo* iron content was in contrast to the upwards of 2000 atoms of iron that accumulates in iron challenged ferritin *in vitro*. Similar results were obtained in other studies that looked for an iron storage function of ferritin with transgenic plants and fruit flies either lacking or overexpressing ferritin [12, 39, 40]. Of all the studies examining ferritin in cultured mammalian cells, some of the most explicit data concerning iron uptake was shown using primary corneal epithelial cells [27]. When overexpressed in these cells, ferritin was shown to assemble into higher order structures that could bind iron *in vitro* and protect cells from UV stress. In spite of this, cells with increased ferritin showed a modest 70% increase in iron content. Thus, our results are in agreement with a growing consensus that ferritin is not an actual iron storage protein *in vivo* [12].

Overexpressed ferritin is reported to decrease cell growth [26, 43]. Increasing iron concentration in the growth media partially reverses this effect [26]. A ferritin mediated decrease in the labile iron pools (LIP) suggests that ferritin may not be an iron storage protein but instead it may serve to scavenge free cellular iron. In our spot assays, we noticed a decrease in the growth on nutrient agar plates of yeast cells expressing ferritins (figure3.3*A*). A direct measurement of growth rates showed that cells expressing hFerr or *yFer1* have significantly decreased growth rates. The doubling time of control cells is 202 min ± 11, while cells overexpressing human and yeast ferritins had increased growth rates of 277 min ± 10 and 288 min ± 3 respectively ($p < 0.005$ for control vs. ferritin cells, using a student *t*-test). Microscopic examination of trypan blue stained cells revealed that the viability of all three transformants remained above 97% even after 24 hours of growth in galactose media. Thus, ferritin expression in yeast results in a decrease in the growth of cells. The observed ferritin mediated growth could be partially reversed by the addition of a 10-fold excess iron in the growth media. The growth rate of cells expressing *yFer1* was enhanced to 254 min ± 8 by the addition of iron. We also made use of the iron chelator 2,2'-dipyridyl (DIP) to determine the effect of decreasing the level of iron on the growth of yeast expressing ferritin. Freshly saturated cultures of yeast transformants were serially diluted and spotted on nutrient agar plates containing glucose, galactose or galactose with increasing concentrations of DIP. At 13μg/ml, DIP served to decrease the

observable growth of all yeast transformants but the effect was more pronounced in cells expressing *yFer1* (figure 3.4). Taken together our results indicate that *yFer1* encodes a ferritin like protein that shares structural and functional features and has characteristics that are similar to human ferritin and are reminiscent of the stress protective but non-iron storage DPS-type ferritin family members [2].

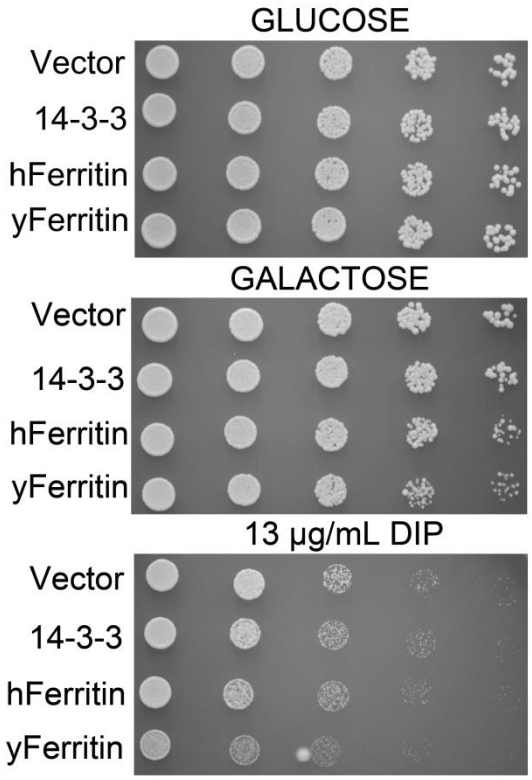


**Figure 3.4.** Human and yeast ferritins reduce cell growth by depleting intracellular iron. The spot assay was used to determine the effect of the iron chelator 2,2'-dipyridyl (DIP) on yeast cells expressing human and yeast ferritin. Serially diluted cultures were spotted onto nutrient agar plates without and with the indicated concentration of DIP.

### *3.2.4 Yeast ferritin (yFer1) is a highly conserved but narrowly distributed fungal specific protein*

Ferritins and/or one of the other related iron binding proteins bacterioferritin and DPS are present in virtually all organisms examined including all three kingdoms of life (Bacteria, Archaea and Eukarya) [2, 3] (figure 4). The similarity of ferritins is such that the *E. coli FtnA* encoded ferritin shows greater than 20% sequence identity with human H-ferritin [21]. In addition to being an ancient protein sub-family, the ferritins are widely distributed and are present in all eukaryotic phyla (figure 4). The situation is somewhat different with the fungi. The fungi represent a highly diverse group that are estimated to number at least 100,000 species [44]. Their classification is not yet complete but the breakdown of the fungi into four different groups is a simple and useful system [45] (http://jgi.doe.gov/our-science/science-programs/fungal-genomics/). Of these, there are three phyla and one group called the Fungi Incertae/Imperfectae. The imperfect fungi include organisms that may require reclassification of some sort. Of the three phyla, Zygomycota is the earliest evolutionary branch (figure 4). The Dikarya phylum contains the Ascomycota and Basidomycota. These are closely related and are actually more often classified as sub-phyla belonging to the Dikarya (figure 4). Analysis of the fungal protein sequences in GenBank with Blast using the sequences of human H-ferritin or *E coli* ferritin identified only 20 different fungal ferritins. The similarities range from a high of 51% with a 184 residue protein from *Rhizopus delemar* (E value = $1.3e^{-39}$; Accession EIE89543.1) to 24% identity over 185 residues with a 217 residue uncharacterised protein from *Penicillium chrysogenum* (E value = $9e^{-4}$; Accession XP_002566549.1). Most of the identified fungal ferritins (12/20) belonged to the division of Fungi Incertae/Imperfectae or to the Zygomycota. The remaining fungal ferritins were restricted to a few genera that belong to only two classes (Eurotiomycetes and Sordamyceta) in the Pezizomycotina sub-phylum of the Ascomycota (figure 4). A simplified phylogenetic depiction of the distribution of the ferritins reveals the widely reported observation of their paucity of in fungi [14] (figure 4). Thus most fungi, including the sub-phylum that contains the yeast *Saccharomyces cerevisiae* the Saccharomycotina, do not have typical ferritins. This is in contrast to what is observed with other conserved sequences. For example, a search using human 14-3-3β/α (NM_139323) revealed that there are over 250 14-3-3-like fungal sequences. This apparent lack of ferritin has been taken as evidence that iron storage is somewhat different in yeast [9, 14].

If *yFer1*p is indeed a novel fungal ferritin, then it should be unique to members of the fungal lineage. A search of the fungal Databases at GenBank and SGD, revealed that there are no non-fungal sequences producing significant alignment to *yFer1*p. In contrast, there are over 45 different fungus that contain a protein sequence producing significant alignment to *YER067W*p (E values < $6e^{-09}$). These *yFer1*p-like sequences are present in a variety of different species with different percentage identities ranging a high of 72% over 98% of the 157 residue of a hypothetical protein from *Candida glabrata* (Accession XP_447838.1) to a low of 31% identity over 82% of 187 residue of the *Rgi1*-like protein in *Dekkera bruxellensis* (Accession EIF48477.1) (figure S1). Finally, all the *yFer1*p-like sequences identified are present in a number of species from a wide range of different fungal genera that all belong to the Saccharomycetaceae family of the order Saccharomyceta of the Ascomycota phylum (figure 4). In addition none of these sequences identify any protein with significant similarity when searched against the human proteome. It should be noted that all of the *yFer1*p-like sequences, including the second *S. cerevisiae yFer1*p-like sequence *YIL057C*, have no known function. Taken together our results suggest *yFer1*p represents the missing and elusive ferritin protein from the yeast *S. cerevisiae*. The fact that *yFer1* is a highly conserved protein that is restricted to a very narrow fungal lineage suggests that other yeast species also contain a ferritin protein that retains ferritin function while having a great deal of sequence divergence with the typical ferritin sequence.

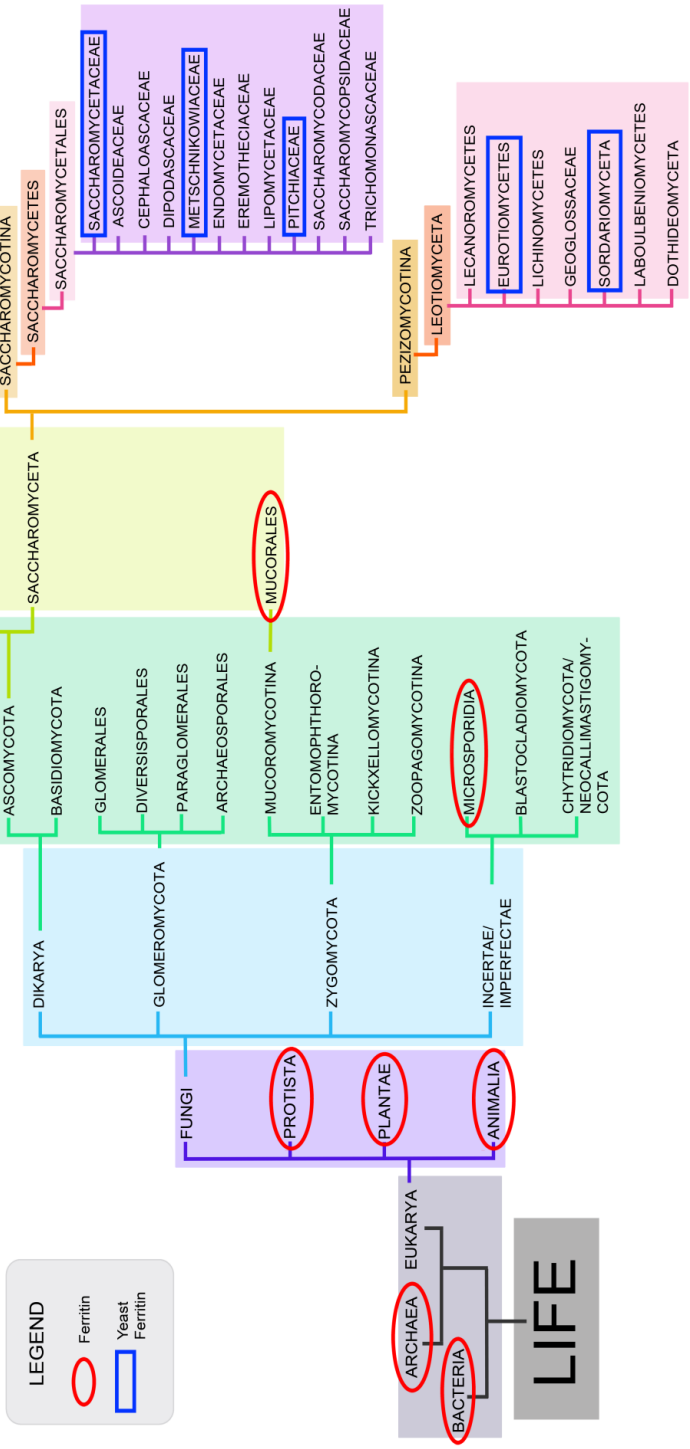
LEGEND
Ferritin
Yeast Ferritin
LIFE
ARCHAEA
BACTERIA
EUKARYA
FUNGI
PROTISTA
PLANTAE
ANIMALIA
DIKARYA
GLOMEROMYCOTA
ZYGOMYCOTA
INCERTAE/ IMPERFECTAE
ASCOMYCOTA
BASIDIOMYCOTA
GLOMERALES
DIVERSISPORALES
PARAGLOMERALES
ARCHAEOSPORALES
MUCOROMYCOTINA
ENTOMOPHTHORO-MYCOTINA
KICKXELLOMYCOTINA
ZOOPAGOMYCOTINA
MICROSPORIDIA
BLASTOCLADIOMYCOTA
CHYTRIDIOMYCOTA/ NEOCALLIMASTIGOMY-COTA
TAPHRINOMYCOTINA
SACCHAROMYCETA
MUCORALES
SACCHAROMYCOTINA
SACCHAROMYCETES
SACCHAROMYCETALES
SACCHAROMYCETACEAE
ASCOIDEACEAE
CEPHALOASCACEAE
DIPODASCACEAE
METSCHNIKOWIACEAE
ENDOMYCETACEAE
EREMOTHECIACEAE
LIPOMYCETACEAE
PITCHIACEAE
SACCHAROMYCODACEAE
SACCHAROMYCOPSIDACEAE
TRICHOMONASCACEAE
PEZIZOMYCOTINA
LEOTIOMYCETA
LECANOROMYCETES
EUROTIOMYCETES
LICHINOMYCETES
GEOGLOSSACEAE
SORDARIOMYCETA
LABOULBENIOMYCETES
DOTHIDEOMYCETA

**Figure 3.5.** Phylogenetic distribution of ferritins and atypical yeast ferritins in the Domains of life with emphasis on the fungal phylum. A diagram showing the three domains of life including Bacteria, Archaea and Eukarya (http://tolweb.org/tree/phylogeny.html). The four phyla of the Eukarya domain, including Animalia, Plantae, Protista and Fungi are shown. The Fungi are further sub-divided into sub-kingdoms. Here we show three including the Zygomycota, Glomeromycota and Dikarya as well as the Fungi Incertae/Imperfectae. The Fungi Incertae/Imperfectae represents a mixture of early lineage fungi and a number of fungi that do not yet have a definitive classification. The Basidomycota and Ascomycota are sub-kingdoms of the Dikarya kingdom that contain closely related species. The Ascomycota is further subdivided into the Saccharomyceta and Taphrinomycotina. The Taphrinomycotina gives rise to at least five Orders of Fungi including the Schizosaccharomycetales or the fission yeast (not shown). The Saccharomyceta are further subdivided into the Saccharomycotina and the Pezizomycotina. The Pezizomycotina contain at least six different classes of fungus including the Eurotiomycetes and the Sordariomyceta. Finally, the Saccharomycotina contain only one fungal Order the Saccharomycetes, which in turn contains the Saccharomycetaceae, the budding yeasts as well as several others including the Metschnikowicaea and Pichiaceae families. A red circle indicates that a classical ferritin is present most members within that branch, while a blue rectangle is used to denote the presence of the yeast ferritin *yFer1* described in this study. A more complete list of the fungal species containing an *yFer1*-like sequence is shown in figure S1.

## 3.3 Materials and Methods

### *3.3.1 Yeast strains and plasmids*

The wild type *S. cerevisiae* strain BY4742 (***MATα*** *his3⊿1 leu2⊿0 lys15⊿0 ura3⊿0*) and the isogenic *VMA3⊿* were used. Plasmids containing the cDNAs for human H-ferritin and 14-3-3β/α were isolated from our previous Bax screen [8, 16]. Plasmid for expressing *yFer1* (*YER067W*) on a *URA3* selectable marker vector was obtained from Thermo Scientific. Murine Bax was expressed on a plasmid with a *HIS3* selectable marker previously described [16]. All clones are expressed under the control of the galactose inducible *GAL1* promoter.

### *3.3.2 Yeast growth, viability and iron assays.*

Yeast cells transformed with the various plasmids were selected for and maintained by excluding the appropriate nutrients from the media. In some instances, yeast was grown in rich media containing 2% Bacto peptone, 1% yeast extract, and 2% glucose (YEPD). However, yeast cells were routinely grown in synthetic minimal media consisting of yeast nitrogen base (YNB), 2% glucose, and the required amino acids or bases. In order to induce gene expression of sequences under the *GAL1* promoter control, glucose was replaced with 2% galactose and 1% raffinose. Spot assays, using serially diluted cells from freshly saturated cultures, were used to assess the ability of different transformants to grow in varying conditions [24, 25]. Viability was determined by microscopic examination of cells stained with the vital dye trypan blue that had grown for 18 hours in YNB galactose media with 1.4mM of copper sulphate ($CuSO_4$) [24, 25]. At least three different samples of 300 cells were scored for each culture and the experiment was repeated at least three different times obtaining similar results. The data from the viability experiments are presented as the mean ± standard deviation of triplicate experiments repeated a minimum of three independent times. Statistical significance of the data was determined using a student t test. For iron assays, cells were inoculated at low or moderate cell densities in fresh galactose media (5µl or 50µl in 10ml). The cultures were grown for 18 hours with or without the addition of 10x more iron than standard YNB media. Cells were harvested and washed 3 times with cold 1mM EDTA, flash frozen and stored at -70° C. The cells were broken with 3% nitric acid (98° C, 18 hours) and iron content was determined using a colorimetric assay using the iron chelator ferrozine [35]. All described experiments were carried out a minimum of three independent times.

```
Saccharomyces_cerevisiae        ----------------------MTKKDKKE-VKVQTVTTEDGE------ 20
Candida_glabrata                ----------------------MAKKDKK--PKVNTIVTKDGE------ 19
Torulaspora_delbrueckii         ----------------------MGKKDKG--PKMTTVTTKEGE------ 19
Kazachstania_naganishii         ----------------------MPKKDKK--PTFTNVTTKSGE------ 19
Saccharomyces_kudriavzevii      ---------------------------------MSTVTTKSGE------ 10
Saccharomyces_arboricola        --------------------MVKKDKKAKG-PKMSTVTTKSGE------ 22
Kazachstania_africana           ----------------------MAKKDNK--PKFSTVLTKSGE------ 19
Vanderwaltozyma_polyspora       ----------------------MTKKDKK--PKVATITTKSGE------ 19
Naumovozyma_dairenensis         ----------------------MPKNKNKG-PKMTTVTTKSGE------ 20
Kluyveromyces_lactis            ----------------------MTKKDKG--PKFANVTTKSGE------ 19
Kluyveromyces_marxianus         ----------------------MTKKDKG--PKFTTVTTKSGE------ 19
Zygosaccharomyces_bailii        ----------------------MTKKNKG--PKVSTVTTKQGE------ 19
Lachancea_thermotolerans        ----------------------MTKKDKK--PKISTIVTKEGD------ 19
Naumovozyma_castellii           ----------------------MPKNKNKG-PKMTTVVTKTGE------ 20
Tetrapisispora_phaffii          ----------------------MTKKDKG--PKIQTITTKSGE------ 19
Zygosaccharomyces_rouxii        ----------------------MAKKNKG--PKVTTVTNKQGE------ 19
Eremothecium_cymbalariae        ----------------------MTKKDKK-KQKINTIETKEGE------ 20
Ashbya_gossypii                 ----------------------MTKKDKKQKVKVKTVETKEGE------ 21
Wickerhamomyces_ciferrii        ----------------------MAKKDKQ-----QKKKLSYG------- 15
Candida_parapsilosis           -MGAQKKKNKSDAVPLDLDNIKPLEHLQAVPKARSSSITSVESVEEPGVV 49
Candida_orthopsilosis          -MGGQKKKNKSDAVPLDLENIKPLEHLQAVPKARSSSITSVESVEEPGVL 49
Komagataella_pastoris          -----MGRRKSQAAAERNLEPIKISTDSIKKRPR---------------- 29
Lodderomyces_elongisporus      -MSG-KKKSKSDTVPLDLDNIKPLDHLQAVPKTRKMSMTSIESADEPGGF 48
Candida_tropicalis             -MAG-KKKSKSDSVPLDLGNIKPLEKLQPVPKTRSSSITSIESADEPGTM 48
Candida_dubliniensis           -MAG-KKKSKSDSLPLDLDNIKPLDHLQPVPKTRSSSITSIESVDEPGTM 48
Scheffersomyces_stipitis       -MAR-AKKSK-DSQPLDLNNCQPLEHLQPVPKTRSASIISVESTDSEGGV 47
Candida_albicans               -MAG-KKKSKSEALPLDLDNIKPMDHLQPVPKTRSSSITSIESADEPGTM 48
Clavispora_lusitaniae          -MTK-GKKK--QTVELDLNKVEKLEHLMPVPKSRQSSITSVESED--GSL 44
Spathaspora_passalidarum       -MAGQKKKSKSQSLPLDLDNIQPLEHLQPVPKTRAMSITSIESADEPSGM 49
Ogataea_parapolymorpha         -----MTKKY-SSYSQT--EPTHLEHLSPVTSRR---------------- 26
Meyerozyma_guilliermondii      MTAK-KSKSGRMTTALDLTTCEKLEHLQPIPKSRSSSITSIESED--GMM 47
Candida_tenuis                 -MGK-KERK--NSVTVDLENCDKMQHLKPIPKSRSSSITSIESN---GSF 43
Candida_maltosa                -MAG-KKKSKSEYEPLDLDNIKPLDKLQAVPKSRSSSITSIESAEAPGTM 48
Debaryomyces_hansenii          -MTK-KNKN--QTLALDLDNCEKLTQLKEIPKSRSSSITSIESE---GSI 43
Millerozyma_farinosa           -MTN-KANKKSKAVELDLDNCERLEHLKEVPKSRSSSITSIESD---GSL 45
Kuraishia_capsulata            -----MARKNKAMIAHT--EPTKVTNLTPKPVR---------------- 26
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Saccharomyces cerevisiae        -------TVKVFEDLQGFETFIANETEDDD-FDHLHCKLNYYPPFVLHES 62
Candida_glabrata                -------SLKVFEDLNDFEMFIKNETEDEE-FDHIHCKLTYYPPFVLHES 61
Torulaspora_delbrueckii         -------TLKVFEDLKDFETFIKQETEDEE-FDNLHCQLKYYPPFVLHEA 61
Kazachstania_naganishii         -------TLKVFDDLDTFETFIKNETEDDD-FSDLHCQVNYLPPFVLHQA 61
Saccharomyces_kudriavzevii      -------SLKVFEDLHDFETYLKGETEDQE-FDHVHCQLKYYPPFVLHDA 52
Saccharomyces_arboricola        -------SLKVFEDLHDFETYLKGETEDQE-FDHVHCQLKYYPPFVLNDA 64
Kazachstania_africana           -------SLKVFEDLDTFETYLKNEIEDDE-FDNVHIQVNYYPPFVMHKN 61
Vanderwaltozyma_polyspora       -------TLKVFEDLAQFEDFLKHETEDDDDFDNVHCQLKYYPPFVLHEA 62
Naumovozyma_dairenensis         -------QLKVFEDLNDFETYIKNETEDNE-FDNVHCHLKYYPPFVLHDA 62
Kluyveromyces_lactis            -------VVKVFEDLNDFETFIKNETEDEE-FDHVHCHLKYYPPFVLHES 61
Kluyveromyces_marxianus         -------VVKVFEDLESFETFIKNETEDEE-FDHVHCHLKYYPPFVLHES 61
Zygosaccharomyces_bailii        -------KVQVFENLGDFETFIKQETEDDE-FDSLHCVLSYYPPFVLHEA 61
Lachancea_thermotolerans        -------TVKVFEDLDTFETFIKNETEDEE-FDHVNCHLKYYPPFVLHES 61
Naumovozyma_castellii           -------SLKLFEDLNDFEMFIRNEVEDDE-FDNIHCQLKYYPPFVLQDA 62
Tetrapisispora_phaffii          -------KIKAFEDLNQFETFLKQETEDDE-FDNLHCQIKYYPPFIMHDC 61
Zygosaccharomyces_rouxii        -------TLQVFETLEDFETFIKQETEDDE-FDHIHCVLNYYPPFLLHHS 61
Eremothecium_cymbalariae        -------KIRVFDDLESFELYIKGETEDEE-FDHVHCQVRYYPPFVLHES 62
Ashbya_gossypii                 -------KIRVFEDLDSFETYLRGETEDEE-FDHVHCQVRYYPPFVLHES 63
Wickerhamomyces_ciferrii        -------KLQEFEDLDTFEQFLKDEREDNE-HNNAHAHINYIPPFVLAAS 57
Candida_parapsilosis            KQVLVPPTIREFDELEQFEAFVRDETWDNE-FDYFHGRLHYYPPFVMKEC 98
Candida_orthopsilosis           KQVLVPPTIREFDELEQFEAFVRDETWDNE-FDYFHGRLHYYPPFVMKEC 98
Komagataella_pastoris           -RDSNEPPFKKFDDLEMFETYLKGESWDND-FDFLHARLDYYPPFIRNEI 77
Lodderomyces_elongisporus       KEVLLPPTIKEFDELEQFEAFVRDETWDND-FDYFHGRLHYYPPFVLKEC 97
Candida_tropicalis              KQVLLPPTIREFDELEQFEAFVRDETWDNE-FDYFHGRLHYYPPFVMKAC 97
Candida_dubliniensis            KQVLLPPTIREFDELDQFESFVRDETWDND-FDYFHGKLHYYPPFVMKSC 97
Scheffersomyces_stipitis        VEVLAPPQVREFDDLTAFEAFVRDETWDND-FDYFHGRLNYYPPFIMKEC 96
Candida_albicans                KQVLLPPTIKEFDELEQFESFVRDETWDND-FDYFHGRLHYYPPFVMKSC 97
Clavispora_lusitaniae           KEVLKPPPRKDFEDLASFESYIRDETWDNE-FDYCHAHLTYYPPFVLKEV 93
Spathaspora_passalidarum        KQVLVPPPIREFDELEQFEAFVRDETWDNE-FDYFHGRLNYYPPFVMKEC 98
Ogataea_parapolymorpha          -NSLEEPPLRKFDDIVAFEQYLKDETWDNE-FDYFHLKVNYLPPFVLHSI 74
Meyerozyma_guilliermondii       STVLKPPPRRDFDDLMAFEGFIRDETWDND-FDYCHAHLTYYPPFIMKEV 96
Candida_tenuis                  ESVLRPPPMKEFDDLRAFEAYIRDETWEND-FDYLHAHLKYYPPFILKEC 92
Candida_maltosa                 MQVLAPPMIREFDELEQFEAFIRDETIDNE-FDYYHCKVRYYPQVILKAS 97
Debaryomyces_hansenii           QSVLKPPPMREFEDVVAFESYIRDETWDND-FDYCHAHLSYYPPFITKEV 92
Millerozyma_farinosa            QKSIKPPPMREFDDLKSFEAYLRDETWDNE-FDYCHAHVMYYPPFIMKTV 94
Kuraishia_capsulata             --PVAGPSTKMFDDLDSFEQFLKDESWDDE-YDFLHAKCAYIPPFVLSEI 73
                                        : *: :  ** ::  *  ::: ..  :    * * .:
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Saccharomyces cerevisiae        HE-DPEKISDAANSHSKKFVRHLHQHIEKHLLKDIKQAVRKPELKFHEKS 111
Candida_glabrata                HD-DPEKIKDTNNSHNKKFVRHLHQHVEKHLLKDIKEALHKPELKFHDKS 110
Torulaspora_delbrueckii         HD-DPEKISTTANSHSRKFVRHLHQHVEKHLLKDIKEAINLRDLKFRNKS 110
Kazachstania_naganishii         HD-DPDKVKDTENSHNKKFVRHLHQHVEKHLLKDLKQALNLPDMKFHEKS 110
Saccharomyces_kudriavzevii      HD-DPEKIKETANSHSKKFVRHLHQHVEKHLLKDIKTAINKPELKFHDKK 101
Saccharomyces_arboricola        HD-DPEKIKETANSHSKKFVRHLHQHVEKHLLKDIKTAINKPELKFHDKK 113
Kazachstania_africana           HD-DPEKIKDTENSHSKKFVRHLHQHVEKHLLKDIKNALHQPDLKFRDKN 110
Vanderwaltozyma_polyspora       HD-DPEKIKDTANSHSKKYVRHLHQHVEKHLLKEIKEALQLPDLKFKDKS 111
Naumovozyma_dairenensis         RD-DPEKIKDTANSHSKKFVRHLHQHVEKHLLKDIKMALDQPDLKFKNKS 111
Kluyveromyces_lactis            HE-DPEKIKDSANSHSKKFVRHLHQHIEKHLLKDIKERIKLPDLKFKDKA 110
Kluyveromyces_marxianus         HD-DPEKIKDSANSHSKKFVRHLHQHIEKHLLKDIKERVHLPDLKFKDKG 110
Zygosaccharomyces_bailii        HD-DPEKISEQANSHSRKFVRHLHQHVEKHLLRDLAVALESPDLKFGDKS 110
Lachancea_thermotolerans        HE-DPEKIKETVNSHNRKFVRHLHQHVEKHLLKDIRERLQLPQLKFNDKS 110
Naumovozyma_castellii           KDHDPEKIKDTENCHSKKFVRHLHQHVEKHLLKDIKEALQQPTLKFHDKS 112
Tetrapisispora_phaffii          HE-DPEKIPNTANSNSKKFVRHLHQHVEKHLLKDIREALDIPELQFKDHS 110
Zygosaccharomyces_rouxii        HN-DPEKISDQNNCHSRKFVRHLHQHVEKHLLKDLAVALQSPDLKFSNKS 110
Eremothecium_cymbalariae        HD-DPDRIKETLNSHNKKFVRHLHQHVEKHLLKDIKESVGLPELKFKDKS 111
Ashbya_gossypii                 HQ-DPEKIKESANSHNKKFVRHLHQHVEKHLLKDIKDSLGVPELKFKDKS 112
Wickerhamomyces_ciferrii        HN-DPDKVKDSQNRKNKKFVRHLHQHVEKHLIKDIKASSG-MDLNFGKPE 105
Candida_parapsilosis            HD-DVEKIKPTTNKNSKKFKRNLQHHIQKHLIKDLQKCCG-YELNFDKGE 146
Candida_orthopsilosis           HD-DVEKIKPTTNKNSKKFKRNLQHHIQKHLIKDLQKCCG-YELNFDKGE 146
Komagataella_pastoris           HD-DPEKIKPTMNNKSKKFVRNLHHHVDKHLLKQINDMVG-IEYKFKRE- 124
Lodderomyces_elongisporus       HD-EIEKIKPTSNKNSSKFKRNLQHHIKKHLIKDLEKCCG-YELNFDKAE 145
Candida_tropicalis              HD-DVEKIKPTVNKNSKKFRRDLQHHIQKHLIKDLEKCCG-YELNFGKGE 145
Candida_dubliniensis            QN-NLEKIKPTMNKNSKKFRRDLHHHIQKHLIKDLEKCCG-YELNFGKGE 145
Scheffersomyces_stipitis        HD-NLDKIKPTANKNSRKFKRNLQHHVARHLIKDLEKCCG-YELHMDKFD 144
Candida_albicans                QN-NLEKIKPTMNKNSKKFRRDLQHHIQKHLIKDLEKCCG-YELNFGKGE 145
Clavispora_lusitaniae           HD-DIEKIKPTMNKNSSKFRRNLQHHIQRHLILEMEKCCG-FEMDFGKAK 141
Spathaspora_passalidarum        QN-NPEKIKTTTNSNSKKFRRNLQHHVKNHLVKDLERCCG-YELNFDKAE 146
Ogataea_parapolymorpha          HD-NPEKIKPTMNSRSRKFVRHLGHHIKRHLLSEINYYTG-VNYNFEKAV 122
Meyerozyma_guilliermondii       HN-NLDKIKPTMNKNSRKFKRNLQHHIKKHLMHDMETCSG-FKMDFNKVG 144
Candida_tenuis                  HE-NLDKVKPTANKNSRKFKRNLQHHVQRHLMKEMAICGG-YNMDFKRVD 140
Candida_maltosa                 HN-EVEKIKPTVNKNSKKFRREIQHHIK-HLTKDLEKCCG-YELNFGKPE 144
Debaryomyces_hansenii           HG-NMDKIKPTMNKKSRKFKRNLQHHIQKHLMPEMEKCSG-FTMDFGKAG 140
Millerozyma_farinosa            HN-DFEKIKPSMNKKSRKFRRNLDHHVKKHLMAEMERCSG-FKMDFDKVG 142
Kuraishia_capsulata             HN-DMEKIKPTMNSRSKKFVRNLHHHLERHLLSEINRMSG-VDYKFEKS- 120
                                :  : :::    * .. *: *.: :*:  **  ::         .:
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Saccharomyces cerevisiae        KEETFD-KITWHYGEETEY---------HGRPFKIDVQVVCTHEDAMVFV 151
Candida_glabrata                KDETFE-HIVWHYGEETEY---------HDRKFRIQVTVTCNHDDAMVDV 150
Torulaspora_delbrueckii         KDESFD-RIVWHYGDDTQY---------HERKFHIEVGVTCTHDDAMVDV 150
Kazachstania_naganishii         KDEQFD-KITWHYAEETEY---------HDKKFKVIVEVTCHHDDALVNV 150
Saccharomyces_kudriavzevii      KQESFD-KIVWNYGEETEL---------NAKKFKVCVEVVCKHDGAMVDV 141
Saccharomyces_arboricola        KQESFD-KIVWNYGEETEL---------NAKKFKVSVEVICKHDGAMVDV 153
Kazachstania_africana           KDESFE-KITWHYGDETEV---------NSRKFKLAVDVTCVHNDAMVSV 150
Vanderwaltozyma_polyspora       KDEDFE-KIVWNYGDTGEF---------HDRKFRISISVTCKHDDAMVDL 151
Naumovozyma_dairenensis         KQETFE-KIVWNYDDETEL---------NDKRFKVTVEVTCNHDGAMVDV 151
Kluyveromyces_lactis            KEESFE-HIVWKYNDVTQY---------HGKDFEIHITVECHNDSAIVDV 150
Kluyveromyces_marxianus         KEETFE-HIVWKYNDVAQY---------HGKDFEIHVTVECHNDSAIVDV 150
Zygosaccharomyces_bailii        KDQSFE-RVVWHYADDANY---------HDKPFHVQVDVTARHEDALVNV 150
Lachancea_thermotolerans        KEETPD-HIVWRYNQRANF---------HERDFDIHVTVECHHDSALVDV 150
Naumovozyma_castellii           KDATFE-KITWHYGEQTEL---------HNKKFKVQIDVSCNNDGAMVDV 152
Tetrapisispora_phaffii          KEKESD-HITWHYHDVTTY---------HDKKFQVNVNVTCNHEDALVNL 150
Zygosaccharomyces_rouxii        KDQTFE-KIVWLYNDDASY---------HDKPFHVAVEVTARHDDALVNV 150
Eremothecium_cymbalariae        KEETFT-HVVWKYHAPTKY---------HNKDFMIHVTVECHNDSAIVDV 151
Ashbya_gossypii                 KEENFN-HVVWRYHAPTAV---------RDREFLVHVTVECHNDTAMVDV 152
Wickerhamomyces_ciferrii        TIEDFD-TITWKYTDSSDHGLG-----LDGEKFKVEVVVKCNSNGAYVDV 149
Candida_parapsilosis            MTESFD-KLSWTFKDETDHGFSKEEEDLYDRHWKLQLDVTCTNDSAMVDV 195
Candida_orthopsilosis           MKETFD-KLSWTFKDDTDHGFSKEEEDLYDRHWKLELDVSCSNDSAMVDV 195
Komagataella_pastoris           EEKLPDGRLIWRYKDESDHGF--EGLDR---KWTVEVDVECSPNDPTVVV 169
Lodderomyces_elongisporus       EIESFD-KLTWKFKDETDHGFSKEEEDKYDRHWKIELDITSHNDSALVDI 194
Candida_tropicalis              VVETDS-KVTWKFKDETDHGFSKEEEDMYDRHWRLELDVTCTNESAMVDV 194
Candida_dubliniensis            IVETDN-KVTWKFKDETDHGFSKEEEDLYDRHWRLELDVSCTNESAMVDV 194
Scheffersomyces_stipitis        TIETPN-RITWKFKDESDHGFSKEEEDEYNRHWRLELSVSCNNENPMVEV 193
Candida_albicans                VVETDN-KVTWKFKDETDHGFSKEEEDMYDRHWRLELDVSCTNESAMVDV 194
Clavispora_lusitaniae           MEETPK-NIIWRYEDSGDHGFPKEEEDKFNRHWKLELEVSCNNENPLVQV 190
Spathaspora_passalidarum        VVEAPG-KVTWKFRDHSDHGFSKEEEDKYNRHWTLELDVSCNSESALVEV 195
Ogataea_parapolymorpha          VEHRPDGSILFHLHDYSDHGYG-EETDKYNRHWKVDLDVMCNPENPYVEV 171
Meyerozyma_guilliermondii       MVETPT-KVKWRFEDMGDHGFSKEEEDMYGRHWKLELEVTCNNENPLVEV 193
Candida_tenuis                  VRETPK-TMVWTYEDSTDHGFNPEEEDAFNRHWKLQLEVCCNNENAMVDV 189
Candida_maltosa                 IVETDK-IYAWKFKDETDHGFSKEEEDLYDRHWKLELEVTCTNESAMIDV 193
Debaryomyces_hansenii           VEETPT-MLKWKFEDTSDHGFSKEEENQFDRHWKLQLEVTCNNENPLVEV 189
Millerozyma_farinosa            LEDNPN-MLKWIYEDTSNHGFDDDEAQRFHRQWKVHLEVSCNNENPMVEV 191
Kuraishia_capsulata             HETKPDGKVVWHYRDYSDHGFG-EKEGE--RKWKVEVDVECNPDSPYVSL 167
                                          :                   : : : : .  : . : :
```

```
Saccharomyces cerevisiae        DYKTHPVGAN---------------------------------------- 161
Candida_glabrata                DYKTIPL------------------------------------------ 157
Torulaspora_delbrueckii         DYKTIPIT----------------------------------------- 158
Kazachstania_naganishii         DYRTVPL------------------------------------------ 157
Saccharomyces_kudriavzevii      DYKTEPVQP---------LI---------------------------- 152
Saccharomyces_arboricola        DYKTEPVQP---------LI---------------------------- 164
Kazachstania_africana           DYKTMPVE----------------------------------------- 158
Vanderwaltozyma_polyspora       DYKTVPLTEESVI------------------------------------ 164
Naumovozyma_dairenensis         DYKTAPVTNNSTTESNEQMI----------------------------- 171
Kluyveromyces_lactis            DYLTKPLTAAVEA------------------------------------ 163
Kluyveromyces_marxianus         DYLTTPVVAAA-------------------------------------- 161
Zygosaccharomyces_bailii        DYRTTPKKIAS-------------------------------------- 161
Lachancea_thermotolerans        DYLTVPVPAVAAA------------------------------------ 163
Naumovozyma_castellii           DYKTVPIKEESEE------------------------------------ 165
Tetrapisispora_phaffii          EYQTLPI------------------------------------------ 157
Zygosaccharomyces_rouxii        DYRTTPRTIAVTETRAPISISPO...----------...DVDYATTPIV 340
Eremothecium_cymbalariae        DYLTEPMVPAQEPVGA--------------------------------- 167
Ashbya_gossypii                 DYLTEPMQPAVQSEQVA-------------------------------- 169
Wickerhamomyces_ciferrii        NYNTLPITEDLQVPV---------------------------------- 164
Candida_parapsilosis            DFKAIPILH---------------------------------------- 204
Candida_orthopsilosis           DFKAIPILHQEEK------------------------------------ 208
Komagataella_pastoris           DMRSIPID----------------------------------------- 177
Lodderomyces_elongisporus       DMKSIPIGH---------------------------------------- 203
Candida_tropicalis              EYKSIPL------------------------------------------ 201
Candida_dubliniensis            EYKSIPM------------------------------------------ 201
Scheffersomyces_stipitis        DYKAIPI------------------------------------------ 200
Candida_albicans                EYKSIPM------------------------------------------ 201
Clavispora_lusitaniae           DYKAIPV------------------------------------------ 197
Spathaspora_passalidarum        DYKAIPL------------------------------------------ 202
Ogataea_parapolymorpha          DMKSVPV------------------------------------------ 178
Meyerozyma_guilliermondii       DYKATPIM----------------------------------------- 201
Candida_tenuis                  DYRAIPL------------------------------------------ 196
Candida_maltosa                 DFKSIPL------------------------------------------ 200
Debaryomyces_hansenii           DYMAIPI------------------------------------------ 196
Millerozyma_farinosa            DYQAIPILD---------------------------------------- 200
Kuraishia_capsulata             EMKSIPL------------------------------------------ 174
                                :  : *
```

**Figure S1.** Alignment of the yeast ferritin1 sequence present in different fungi. The amino acid sequence of *yFer1* was used to search fungal protein databases at GenBank and the *Saccharomyces* Genome Database. Sequences producing high significant identity with *yFer1* were aligned using ClustalW (http://www.ebi.ac.uk/Tools/msa/). The names of the organisms are shown on the left while amino acid numbers are shown on the right. A star (*) indicates a conserved residue, a point (.) represents a conserved residue and a colon (:) indicates a highly conserved residue.

### 3.4 Acknowledgements

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# 4. CONCLUSION

## 4.1 Discussion & Future Direction

Iron is an essential element for many important processes that take place in the human body. Iron is a cofactor in many proteins, like hemoglobin, where it is the site of oxygen binding on red blood cells as well as being present in cytochromes, proteins that are involved in electron transfer in the electron transport chain which generates the adenosine triphosphate (ATP) that is used by the cell for energy. Iron is not always readily available in a biologically accessible form and can often be hard to come across. Furthermore, accumulation of too much iron in the body is toxic to cells, and therefore, iron uptake has to be tightly regulated. Thus, too little iron can cause problems in the cell, and too much of it leads to a different set of problems. Appropriately, the cell has a number of regulatory processes set in place to combat these issues. One way that living systems maintain iron homeostasis is by the use of iron storage or chelating proteins. Perhaps one of the most important weapons in the cell's iron homeostasis arsenal is the ferritin protein, a protein made up of 24 subunits that form a cage wherein iron molecules can be stored [2]. Ferritin is thought to not only store iron for later use, but is also thought of as a chelator of iron, hence preventing iron's toxicity within the cell [3].

Any perturbation in ferritin's ability to store iron can lead to problems and disease onset. For example, mutations in the genes of the ferritin subunits can result in diseases, one of which is a neurodegenerative disease by the name of neuroferritinopathy [7]. The disease is characterised by involuntary movements in adult patients due to toxicity caused by free radical accumulation in the basal ganglia region of the brain [7, 9]. The reason for the disease is the insertion of a single adenine base in the gene of the human ferritin light chain (L-ferritin) resulting in the change of the structure of the L-ferritin subunit's carboxy-terminus [9]. This alteration in the protein structure causes abnormalities in the formation of the iron channels in the ferritin multimer protein [7]. The inability of ferritin to properly allow iron storage in these cells is thought to result in the accumulation of iron in the neurons of the basal ganglia, causing free radical formation and consequent damage [7]. Furthermore, these increases in free intracellular iron signal for increased ferritin production in order to cope with the excess iron, which further exacerbates the problem by causing too much ferritin protein accumulation in these cells as well [7].

Studying ferritin is of importance because there are many other diseases involved with ferritin and iron overload or deficiency. For example, many cancers are found to have upregulated ferritins without a corresponding increase in iron levels, which is an unusual phenomenon in non-disease states [4, 5]. Growing cells need to uptake iron in order to be able to supply cellular enzymes, like ribonucleotide reductase, with iron as a cofactor or to be incorporated as part of a prosthetic group, like the heme prosthetic group that is present in cytochromes, so that these proteins are able to perform their functions in DNA synthesis and energy metabolism, respectively. Since cancer cells grow very rapidly, they increase their expression of cell surface transferrin receptors, hence bringing more iron into the cell [6]. The increase in free iron inside the cell consequently causes an upregulation of human heavy chain ferritin (H-ferritin) expression, and due to its protective nature against ROS, ferritin is thought to provide cancer cells with increased resistance to chemotherapeutic agents that work by inducing oxidative stress [6]. There is evidence to suggest that some of the ferritins overexpressed in cancer cells have a different subunit ratio composition than their non-cancerous counterparts reflecting a ferritin that is more acidic due to an increase in H-ferritin subunit incorporation [2, 4]. This increase in ferritin levels, and possibly the tell-tale combinations of subunits giving rise to more acidic ferritins, may be used as cancer markers for tumour targeting and identification [4, 5]. Therefore, a thorough knowledge of what normal ferritin levels, and which ferritin compositions belong to which cell type, may facilitate the detection of abnormalities involving ferritin and which disease state these changes in ferritin may belong to. A greater understanding of ferritin's normal functioning and composition in different cell types may aid in understanding diseases involving ferritin dysregulation, and as a result, allows for the discovery of new therapies for these diseases of which many are cancers.

Diseases involving iron overload or excess iron accumulation in cells may not always be caused by problems with the ferritin gene or gene product, but ferritin may be a viable solution. As an example, classical hereditary (type 1) hemochromatosis is a very prevalent genetic disease, most likely due to its late-onset [7]. It is characterised by iron overload resulting in excess oxidative stress to tissues and organs, causing damage or even organ failure [7]. Classical hemochromatosis is caused by a mutation in the *HFE* gene encoding the hemochromatosis protein, which is thought to be responsible for the sequestration of transferrin receptors in response to increased iron levels in the body [7, 8]. Cell surface transferrin receptors are responsible for binding the iron carrier protein transferrin, thus bringing iron into the cell [8]. Therefore, individuals with a mutated *HFE* gene cannot properly control iron intake into cells,

leading to iron overload [7]. Being an iron chelator, overexpression of ferritin sequences might be a useful therapeutic strategy to research for the use in the problem of iron overload in this and other overload diseases.

Finding yeast ferritin (*yFer1*) can greatly aid in these kinds of studies. At first, it is pertinent to establish that *yFer1* is, in fact, the yeast homologue of human H-ferritin. One way to test this is to delete the predicted ferroxidase centres in the proposed yeast ferritin. Ferroxidase centres in H-ferritin function in catalysing $Fe^{2+}$ to $Fe^{3+}$ [2]. Thus, deleting the sequences in the ferritin protein encoding the ferroxidase centres should abolish its enzymatic activity. As a result of this, we could measure the levels of $Fe^{2+}$ and $Fe^{3+}$ before and after the deletion of the ferroxidase centres to see whether the activity of the enzyme has been abolished. If *yFer1* is found to be a homologue of human H-ferritin, we may use it to study the functions of H-ferritin in yeast. Using *yFer1*, we can study an endogenous ferritin *in vivo*. This means that the protein remains in its native environment, allowing us to observe its actions without having to take into account possible changes in its behaviour due to a change in its environment. Furthermore, one yeast cell is also a whole organism *per se* and this allows for the study of ferritin in a whole organism setting; in other words, we can observe how it would affect the organism as a whole and not just its effects on one cell type from the organism. This being said, the behaviour of yeast ferritin, or any other organism's ferritin, will most likely not perfectly translate into that of human ferritin, but it may give us valuable insight. Our study shows that both human and yeast ferritins generally behave the same in yeast; for example, they are both Bax suppressors and both protect against copper stress, and so, it follows that the behaviour of yeast ferritin may be used, to an extent, to predict human ferritin behaviour. This is further supported by the observation that many yeast and human genes pertaining to transition metal metabolism are conserved between the two species and the homologues usually share similar biochemistry and genetics with one another [7].

Moreover, finding ferritin in *Saccharomyces cerevisiae* opens the door to the possibility that ferritin, or ferritin like proteins, may be found in other members of fungal lineages where they once were thought to be absent. In addition, this may have implications for the study of iron anemia that occurs during pathogenic fungal infections. Pathogenic microorganisms, including many types of yeast, take advantage during many human disease states involving iron overload and become increasingly pathogenic in the presence of surplus iron [11]. Most of the iron inside the human body is bound to cytochromes, hemoglobin, and myoglobin while the majority of the

remaining unbound iron is caged up in iron storage molecules like ferritin, essentially decreasing the amount of free iron available for pathogenic microorganisms [11]. However, if excess free iron becomes present because of certain clinical conditions like hemochromatosis, it may encourage microorganism proliferation and pathogenic infection which could lead to a form of anemia known as anemia of inflammation [11]. Therefore, a clear understanding of the mechanisms that human cells have in place to combat excess free iron and an understanding of how these microorganisms, like pathogenic yeast, scavenge, and metabolise free iron from host cells is a useful tool for the development of new therapies to combat this issue. Indeed, therapies involving the disruption of iron acquisition by the pathogen could be of great use, especially if they are specific to the pathogen's own iron metabolism pathways, therefore, possibly decreasing the negative side effects experienced by the patient.

In addition to all of this, ferritin is a pro-survival protein and likely an anti-apoptotic protein because of its role in the prevention of apoptosis occurring due to oxidative stress [2, 10, 3]. Our finding that ferritin protects against Bax and copper stress further supports its pro-survival role. Therefore, ferritin can be used to study the pro-survival pathways it is involved in, with the goal that this may increase our knowledge of the pathways leading from stress induction to a pro-survival response. One way to go about this is to test the pro-survival abilities of ferritin against a diversity of stresses to determine how broad its actions are or if there are patterns in its protective abilities (ie. does it only protect against ROS producing stresses?) Furthermore, deleting the sequences of the ferritin protein that thought to be responsible for its pro-survival abilities, like its ferroxidase centres for example, in order to see if in fact, this is how ferritin protects against stress. It is in this way that the roles of many anti-apoptotic proteins, including the highly researched Bcl-2 protein, were uncovered [1]. The Bcl-2 protein was identified as a cell death inhibiting oncogene through gene expression profiles of apoptosis resistant cancer cells, as its overexpression was seen to inhibit cell death as opposed to promoting growth and proliferation like classical category I oncogenes [1, 12, 13]. This led to the discovery of a new category of oncogenes: ones that inhibit cell death, like Bcl-2 [13]. Further studies showed that Bcl-2 overexpression promoted prolonged survival of various cell types in response to different apoptotic signals, branding it as a general pro-survival and anti-apoptotic protein [12]. Therefore, studies such as these can increase our understanding of the way the cell signals for an anti-apoptotic response, as well as giving us insight into the specific functioning of different anti-apoptotic proteins and possibly lending a hand in coming up with new therapies to protect cells from stress [1].

**4.2 References**


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# APPENDIX

## APPENDIX

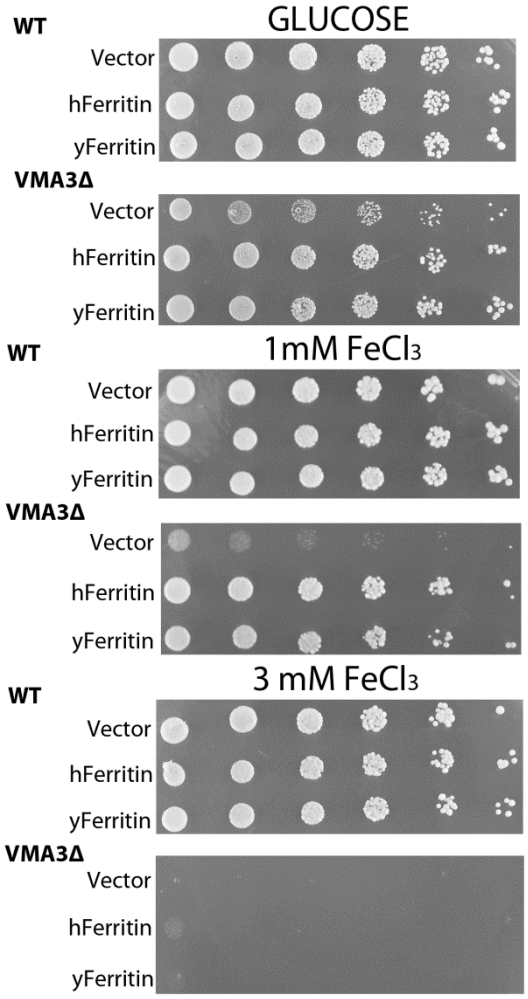


**Figure A1.** The spot assay was used to assess the ability of wild type (WT) and *VMA3*⊿ mutant (*VMA3*⊿) yeast cells transformed with plasmids expressing human and yeast ferritins for their ability to grow when challenged with iron. Fresh cultures of the strains were serially diluted and aliquots were spotted onto different nutrient agar plates without or with 1 or 3 mM iron ($FeCl_3$).

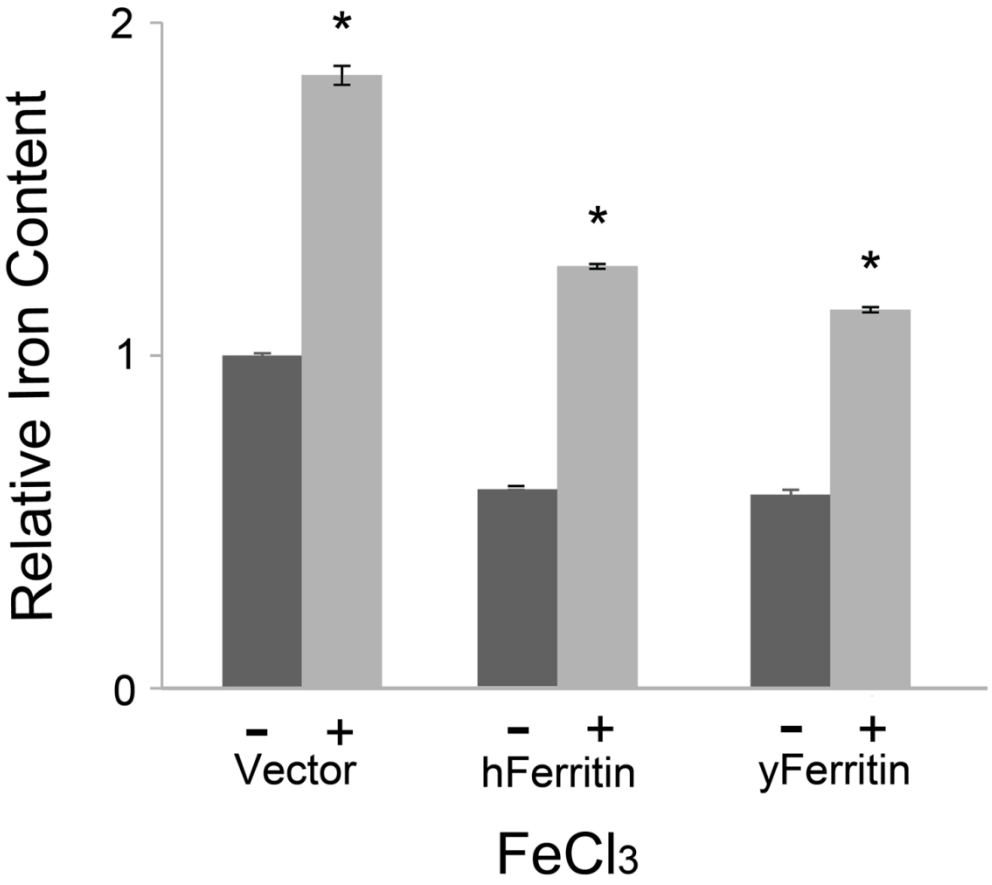


**Figure A2.** Yeast cells harbouring empty vector or human (Ferritin) or yeast ferritin were grown for 18 hours in absence or presence of a 10-fold excess of iron than is found in yeast media. The cells were harvested, lysed with nitric acid and total iron was determined using a ferrozine based colorimetric assay. The data are presented relative to the iron content found in control yeast cells growing in normal YNB galactose media (1 = 5.2 ± 0.4 ng iron/$10^8$ cells). The data are shown as the mean of triplicate experiments that were repeated at least 3 times. *, indicates that the values obtained with cells grown in the presence of 10-fold excess iron are significantly different than the values obtained with the same strain grown in media with normal iron content (student t test, $p < 0.05$).

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# CURRICULUM VITAE

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## EDUCATION

2012 - Present ROYAL MILITARY COLLEGE OF CANADA (RMC)
KINGSTON, ON
***Master of Science Candidate – Department of Chemistry and Chemical Engineering***

- Molecular and cell biology research conducted in the area of anti-apoptosis, specifically looking at the pathways and genes responsible for inhibition of stress-mediated apoptosis using the model organism *Saccharomyces cerevisiae*
- Thesis title: Discovery and characterisation of yeast ferritin (*yFer1*) in *Saccharomyces cerevisiae*
- Courses taken: Endocrinology (RMC), Cell Structure and Basic Tissues (Queen's), Environmental Issues (RMC), and Selected Topics in Molecular Genetics (Queen's)

2007 - 2011 QUEEN'S UNIVERSITY
KINGSTON, ON
***Bachelor of Science (Honours) – Biology Major***

- Genetic and Molecular Biology stream
- Courses taken cover the following areas of Biological sciences: Cell and Molecular Biology, Genetics and Genomics, Biochemistry, Plant and Animal Physiology, Microbiology, Pharmacology, and Human Anatomy

## LABORATORY EXPERIENCE

2012 - Present DR. MICHAEL T. GREENWOOD LAB, RMC
KINGSTON, ON
***Master of Science Candidate***

- Conducting *S. cerevisiae* yeast viability assays in both liquid media and on agar plates

- Isolating plasmid DNA from *Escherichia coli* and transforming plasmid DNA into both *E. coli* and *S. cerevisiae*
- Performing PCR reactions, restriction enzyme digestion, and using gel electrophoresis for DNA fragment identification
- Preparing *E. coli* competent cells

2010 DR. PAUL G. YOUNG LAB, QUEEN'S UNIVERSITY
KINGSTON, ON
***Summer Lab Volunteer***

- Carried out phenotypic screens for the incorporation of desired genes into *Schizosaccharomyces pombe* yeast
- Measured size of cell cycle mutant *S. pombe* yeast using microscopic imaging
- Dissected *S. pombe* yeast tetrads using dissection microscope

## AWARDS & SCHOLARSHIPS

- Queen's University Excellence Scholarship (2007)
- Queen Elizabeth II Aiming for the Top Scholarship (2007)

## STUDENT TRAINING & SUPERVISION

2013 - 2014 ROYAL MILITARY COLLEGE OF CANADA (RMC)
KINGSTON, ON
***Undergraduate Thesis Students***

- Thinh Nguyen (Queen's University)
- OCdt Adelaide Fearnley (RMC)
- NCdt Jessica Henry (RMC)

2013 ROYAL MILITARY COLLEGE OF CANADA (RMC)
KINGSTON, ON
***Undergraduate Summer Lab Volunteer***

- Sophia Attwells (University of Toronto)

## OTHER SKILLS

- Proficient in creating, editing, and manipulating figures in photo editing programs like Adobe Photoshop, Adobe Illustrator, and Paint.NET
- Experience using Adobe Flash and ActionScript programming

## PUBLICATIONS, PRESENTATIONS & POSTERS

### *PUBLICATIONS*

Sheibani S, Jones NK, Eid R, **Gharib N**, Lapointe JF, Titorenko V, Vali H, Mandato CA, Young PG, & Greenwood MT. Inhibition of stress induced lactate mediated cell death by human Lactate Dehydrogenase B (LDHB) and 14-3-3 β/α in yeast. *Manuscript submitted for publication*.

Eid R, Sheibani S, **Gharib N**, Lapointe JF, Horowitz A, Vali H, Mondato CA, & Greenwood MT. Human ribosomal protein L9 is a Bax suppressor that promotes cell survival in yeast. *FEMS Yeast Research* 14(3): 495-507. **2014**

Horowitz A, Lapointe JF, Eid R, Sheibani S, **Gharib N**, Jones NK, Vali H, Mondato CA, & Greenwood MT. The human septin7 and the yeast *CDC10* septin prevent Bax and copper mediated cell death in yeast. *Biochimica et Biophysica Acta* 1833: 3186-3194. **2013**

### *PRESENTATIONS*

Greenwood MT, **Gharib N**, Jones NK, Eid R, Lapointe JF, Titorenko V, Vali H, Mandato CA, & Sheibani S. Identification of human lactate dehydrogenase B (LDHB) as a Bax suppressor provides further evidence that lactate is a stress induced pro-apoptotic second messenger in yeast. Paper presented at: 10th International Meeting on Yeast Apoptosis (IMYA); May 14-18, **2014**; Chalmers University of Technology, Gothenburg, Sweden.

### *POSTERS*

Henry JL, **Gharib N**, Eid R, & Greenwood MT. The interrelationship between hormesis and anti-apoptosis. Poster presented at: Gananoque Environmental Sciences and Engineering Conference; February 1-2, **2014**; Gananoque, Ontario.