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## Characterization of p62, HAH1 and their Interaction with the Wilson Disease Protein

Paul Sifuna Oshule

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CHARACTERIZATION OF p62, HAH1 AND THEIR INTERACTION WITH  
THE WILSON DISEASE PROTEIN

by

Paul Sifuna Oshule

A Thesis  
Submitted to the  
Faculty of The Graduate College  
in partial fulfillment of the  
requirements for the  
Degree of Master of Science  
Department of Chemistry

Western Michigan University  
Kalamazoo, Michigan  
August 2008

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2008

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Paul Sifuna Oshule

## CHARACTERIZATION OF p62, HAH1 AND THEIR INTERACTION WITH THE WILSON DISEASE PROTEIN

Paul Sifuna Oshule, M.S.

Western Michigan University, 2008

Wilson disease is an autosomal recessive disorder resulting in the accumulation of copper in the body primarily in the liver and brain. In order to understand the pathogenesis of Wilson disease, our laboratory is studying several different proteins. Wilson disease protein (ATP7B) is a P-type ATPase which acts to remove excess copper from the cells. The p62 subunit of the dynactin complex is a protein whose function is still unknown but it has been proposed to have a copper-dependent interaction with ATP7B. HAH1 is a human metallochaperone that binds copper as it comes into the cells from CTR1, a permease. HAH1 then shuttles copper ions to one or more of the domains at the N-terminus of ATP7B. Copper binding occurs via the thiol groups on two cysteines of HAH1 and the copper-binding domains of ATP7B.

In order to study protein-protein interactions we have cloned and expressed these proteins. Three different vector constructs of p62 protein were prepared and tested. Using site-directed mutagenesis we also generated mutants of both HAH1 and WLN4 (copper-binding domain 4 of ATP7B) and used them to study copper-dependent interactions. We have used the two mutants pHAH1(C11A,C14A) and pTRX-WLN4(C15A,C18A) to serve as controls in the study of copper exchange between HAH1 and WLN4.

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## LIST OF ABBREVIATIONS

ATOX1 (HAH1).....	Human copper chaperone
ATX1.....	<i>Saccharomyces cerevisiae</i> copper chaperone
CCS.....	copper chaperone for superoxide dismutase
COMMD1.....	Copper metabolism (MURR1) domain containing 1
cDNA.....	Complementary DNA
DEAE.....	Diethylaminoethyl
EXAFS.....	Extended X-Ray Absorption Fine Structure
GST.....	Glutathione-S-transferase
IPTG.....	Isopropyl $\beta$ -D-1-thiogalactopyranoside
LB.....	Luria-Bertani broth
LIC.....	Ligation independent cloning
MBD.....	Metal binding domain
MBP.....	Maltose binding protein
MBS.....	Metal binding site
MNK (ATP7A).....	Menkes disease protein
MURR1.....	Mouse U2af1-rs1 region
PCR.....	Polymerase chain reaction
SDS-PAGE....	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
WLN.....	Wilson disease N-Terminal metal-binding domain
WLN4.....	Wilson disease N-Terminal metal-binding domain 4, aa 356-429

## CHAPTER I

### INTRODUCTION

One in 30,000 people suffer from Wilson's disease. Wilson's disease was described in 1912 by Samuel Kinner Wilson (Wilson, 1912). Clinical manifestations of Wilson's disease include liver disease, neurological symptoms and Kayser-Fleischer rings caused by disrupted copper metabolism. Described as an autosomal recessive genetic disorder of copper transport Wilson's disease leads to copper accumulation in the liver, brain, and kidneys (Cumings, 1948). It is caused by a mutation of the Wilson disease protein (mapped to gene *atp7b* on chromosome 13). The human Wilson protein (WLNP) is a copper-transporting P-type ATPase located in the secretory pathway and plays an important role in copper transport and homeostasis.

The Wilson disease protein plays a key role in copper distribution in the liver, kidney, and the brain. WLNP utilizes the energy of ATP hydrolysis to transport the metal into the secretory pathway for incorporation into such copper-dependent enzymes as ceruloplasmin or be expelled from the cell if copper is in excess (Lutsenko & Petris, 2003). WLNP and other eukaryotic copper-ATPases are unique among the P-type ATPases because they do not bind copper directly from the cytosol; where the amounts of free copper are extremely low. WLNP receives the metal ion from a small cytosolic protein called a metallochaperone through direct protein-protein interactions with human Atox1 (Achila *et al.*, 2006; Larin *et al.*, 1999; Pufahl *et al.*, 1997). The focus of this thesis was to study interactions between the

human Wilson disease protein and its partners, namely: HAH1, a copper metallochaperone (Klomp *et al.*, 1997) and a recently discovered p62 subunit of the dynactin complex (Lim *et al.*, 2006).

### 1.1 Significance of copper metabolism

Many trace elements require a delicate homeostatic balance to ensure the cellular processes work efficiently. However, variations affecting these processes lead to toxicity, due to excessive accumulation of these elements. Copper is required for numerous cellular processes, including mitochondrial respiration, antioxidant defense, neurotransmitter synthesis, connective tissue formation, pigmentation, peptide amidation and iron metabolism as seen in Table 1 (Kaler *et al.*, 1994; Kaler, 1998; Linder, 1991).

**Table 1**  
Functions of copper-dependent enzymes.

Enzyme	Function	Consequences of deficiency
Ceruloplasmin	Iron and copper transport	Decreased circulating copper levels, iron deficiency
Cytochrome C oxidase	Mitochondrial respiration	Hypothermia, muscle weakness
Dopamine $\beta$ -hydroxylase	Catecholamine production	Hypothermia, neurological defects
Lysyl oxidase	Connective tissue	Laxity of skin and joints
Peptidylglycine $\alpha$ -amidating mono-oxygenase	Peptide amidation	Neuroendocrine defects
Superoxide dismutase	Antioxidant	Diminished protection against oxidative stress
Tyrosinase	Pigment formation	Hypopigmentation of hair and skin

Dietary copper is absorbed into the body through the intestinal mucosa where it joins recycled endogenous copper secreted into the gastrointestinal tract from other tissues (Linder, 1991). In general, dietary copper absorption is dependent upon the amount of copper reabsorbed from the fluids of other tissues. Newly absorbed copper is transported to body tissues in two phases. First, albumin, transcuprein, amino acids, and a group of uncharacterized low molecular weight proteins transport the majority of exchangeable copper to the liver (Linder, 1991). After traversing the basolateral membrane of hepatocytes, copper is distributed to endogenous copper-requiring enzymes and to secreted cuproproteins such as ceruloplasmin, which is thereafter released to plasma for delivery of copper to other tissues. Copper's ability to exist in distinct redox states allows it to function as a critical catalyst. The diversity of function and tissue expression of most of the copper proteins reveal an important role for copper in mammalian systems. Any excess copper is excreted to the bile by the way of the canalicular (apical) plasma membrane (Shim & Harris, 2003).

In amounts that exceed cellular needs copper is highly toxic, owing to its potential to facilitate the production of reactive oxygen species by means of Fenton chemistry (Stohs & Bagchi, 1995). Cu(I) and Cu(II) ions can bind with high affinity to adventitious sites in partially folded proteins as well as catalyze auto-oxidation of lipids, proteins, and nucleic acids (Pufahl *et al.*, 1997). Thus, intracellular copper concentrations must be rigorously controlled such that copper is provided to essential enzymes (Table 1), but does not accumulate to toxic levels (Rosenzweig, 2001).

Refined mechanisms have evolved to regulate intake, excretion and the cellular distribution of copper. The importance of these regulatory mechanisms is underlined by several hereditary human disorders of copper homeostasis. These disorders can broadly be divided into two classes: (1) diseases associated with copper

deficiency (Menkes disease, (Menkes *et al.*, 1962)) and occipital horn syndrome (Kaler *et al.*, 1994; Menkes *et al.*, 1962) and (2) diseases associated with copper excess, Wilson disease (Wilson, 1912).

## 1.2 Menkes disease protein

The Menkes protein belongs to a large family of cation-transporting P-type ATPases, a human copper(I)-transporting P-type ATPase also known as ATP7A. Mutations in ATP7A may lead to a fatal X-linked copper deficiency syndrome, the Menkes disease (Menkes *et al.*, 1962). Hence, ATP7A is often referred to as the Menkes protein (MNK). Menkes disease causes a defect with copper transport across membranes, particularly in intestinal mucosal cells where copper efflux is impaired (Voskoboinik *et al.*, 2001).

This protein has two important cellular functions: first, to facilitate the export of copper from non-hepatic tissues and its absorption into the circulation, and secondly to deliver copper to the secretory pathway for incorporation into copper-dependent enzymes. To carry out both functions, MNK must be correctly localized within distinct subcellular compartments, which is achieved through a copper-dependent trafficking of the polypeptide (Petrakis *et al.*, 1996).

MNK contains four major regions/domains (the N-terminal copper binding region, eight transmembrane domains, the ATP-binding domain, and the activator domain) and a short C-terminal tail. The N-terminal copper binding cytosolic tail of MNK is ~650 amino acids long and contains six 70-amino acid independently folded domains, which have a sequence similarity and structure homology (Arnesano *et al.*, 2002; Banci *et al.*, 2004; Banci *et al.*, 2005). Each domain harbors the conserved



sequence motif GMXCXXC, through which it can bind one equivalent of copper(I) (DiDonato *et al.*, 2000; Lutsenko *et al.*, 1997).

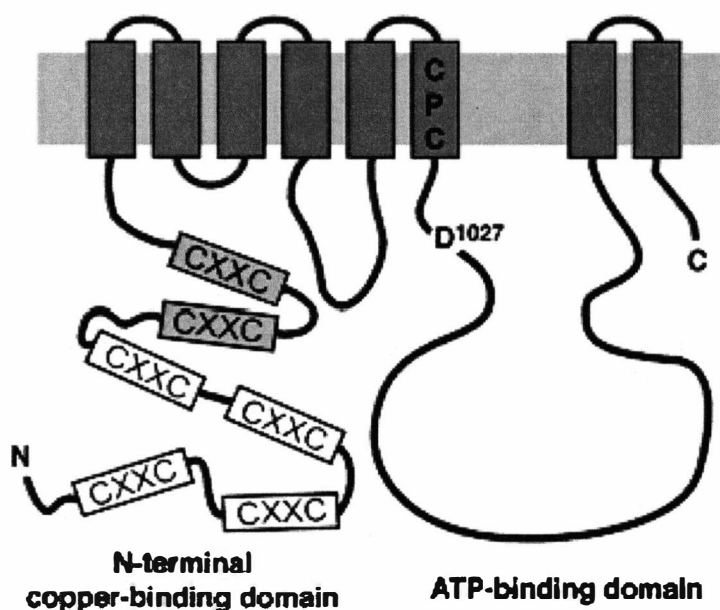
### 1.3 Wilson disease protein

The copper transporting P-type ATPases (designated CPx-type ATPases) are distinguished from other P-type ATPases (Arguello *et al.*, 2007), by the presence of a large N-terminal metal-binding domain with six metal binding sites (MBS) (Figure 1). These domains contain repeats of a GMXCXXCXXXIE motif evolutionarily conserved in heavy metal-binding proteins. The number of these motifs varies between phylogeny (Arnesano *et al.*, 2001). The Wilson disease protein (WLN) and the Menkes protein (MNK) are each comprised of six metal binding domains (WLN) which are about 70 amino acids long and share homology (Bull *et al.*, 1993).

According to yeast two-hybrid assays using Wilson disease N-terminal domains (WLN) and MNK (Larin *et al.*, 1999; van Dongen *et al.*, 2004), domains 1 to 4 of the N-terminal (WLN1–4) interact most strongly with Atox1, whereas no interaction is observed with domains 5 and 6. This observation has been verified by high resolution NMR experiments on the individual domains WLN2 and WLN4 (Achila *et al.*, 2006).

The interaction is dependent on the conserved cysteine residues, as verified in NMR titrations with HAH1 and MNK (Banci *et al.*, 2007). In contrast to the two-hybrid data, an interaction between MNK, domains 5 and 6, and Atox1 was detected by surface plasmon resonance studies (Strausak *et al.*, 2003). Copper transfer studies between HAH1 and WLN1-6 also show transfer to each of the individual domains (Yatsunyk & Rosenzweig, 2007). The latter experiment is, however, not capable of resolving complex formation as seen by a number of  $^1\text{H}$ - $^{15}\text{N}$  HSQC titrations between

the metallochaperones and their target domains (Achila *et al.*, 2006; Arnesano *et al.*, 2001; Banci *et al.*, 2006).



**Figure 1** Transmembrane organization of the six metal-binding sites in the N-terminal domain of WLNP with metal binding motifs indicated by the letters CXXC (Huster & Lutsenko, 2003).

#### 1.4 Mutations in the Wilson disease protein gene

It is known that copper binding requires two cysteine residues for a linear digonal environment; a transient docking of metallochaperone and target is thought to be mediated by a transient three coordinate complex, with the third ligand given by the target or donor (in the case of transfer to target). The absence of one residue will cause a failure of copper acquisition. In their study of interactions of the third metal binding domain of the Menkes disease protein Banci and coworkers using a homologous system, Atx1-Ccc2 found in yeast, concluded the that removal of

cysteines eliminates copper-dependent interaction between WLN and HAH1 (Banci *et al.*, 2006). This mutation of the metal binding motif in WLN4 may also inhibit HAH1 delivery of copper. Pufahl and coworkers have proposed a mechanism by which copper is delivered, through metal mediated protein-protein interaction, to its target protein in a bucket brigade mechanism (Pufahl *et al.*, 1997). Recent studies, showing that all the copper-binding domains have similar apparent affinity constants argue against this (Wernimont *et al.*, 2004; Yatsunyk & Rosenzweig, 2007). A bucket brigade requires flowing of Cu(I) ions down a shallow thermodynamic gradient. It is more likely that Le Chatelier's principle is at work, since Cu(I) is being removed from one compartment into another via the action of ATP; such a mechanism was alluded to later (Huffman & O'Halloran, 2000).

The human copper chaperone HAH1 is capable of delivering copper(I) both to the Menkes and the Wilson disease proteins (Hamza *et al.*, 1999). These two proteins are membrane-bound P-type ATPases which translocate copper in the *trans*-Golgi network or across the plasma membrane, depending on the environmental conditions. Homologues of both HAH1 and ATP7A/ATP7B are found in a number of prokaryotic and eukaryotic organisms; however, it is noteworthy that the genome of some organisms may encode only one of the two partners (Anastassopoulou *et al.*, 2004; Arnesano *et al.*, 2001).

The p62 subunit of the dynactin complex has been cloned and expressed (Lim *et al.*, 2006). p62 subunit of the dynactin complex is one of the other proteins that have been shown to interact with the Wilson disease protein (ATP7B) (Lim *et al.*, 2006). The most striking feature of the p62 polypeptide is a very highly conserved pattern of cysteine residues (Karki *et al.*, 2000). The primary sequence of p62 subunit of the dynactin complex predicts a protein of 53 kDa with a zinc-binding motif near

the N terminus. Such motifs are commonly known to support protein-protein interactions (Karki *et al.*, 2000; Schroer, 2004). It was proposed that p62 uses this region to bind other dynactin subunits, or membrane-associated phospholipids (Karki *et al.*, 2000; Schroer, 2004). p62 has two additional cysteines in the C-terminal which form the highly conserved, functionally important, CxxC motif which may be implicated in the copper-dependent interaction between p62 and ATP7B (Lim *et al.*, 2006).

The copper chaperone HAH1 interacts with the N terminus of both ATP7A and ATP7B in a copper-dependent manner, and this interaction assists copper delivery to the secretory pathway. COMMD1 (MURR1), which is mutated in Bedlington terriers suffering from copper toxicosis (van De Sluis *et al.*, 2002), interacts with the N terminus of ATP7B (Tao *et al.*, 2003). The fact that COMMD1 interacts with ATP7B but not with HAH1 or ATP7A is consistent with its proposed role in copper excretion from the liver (Tao *et al.*, 2003).

Despite their sequence and structural similarity and the presence of six MBS within the N-terminus of the copper ATPases, there are differences in sequence as well as structure that confer specificity of interactions with proteins such as COMMD1 and differences in their trafficking pathways. The identification of a putative targeting signal in ATP7B that is not present or is different in ATP7A suggests that additional specific protein interactions are likely to impact on their activity (Tao *et al.*, 2003).

### 1.5 HAH1 Metallochaperone (Human homolog of Atx1)

Metallochaperones are a newly discovered and important class of enzymes that transport metal ions into specific partners (O'Halloran & Culotta, 2000). After

uptake, distribution of intracellular copper is facilitated by a group of proteins called copper chaperones, which function to deliver free copper to pertinent sites around the cell. Until recently it was thought that once copper is taken inside the cell it would be sequestered by sulfhydryl copper ligands, like those found in metallothioneins and glutathione, which are abundant in most cell types (Camakaris *et al.*, 1999; Freedman *et al.*, 1989). The role of metallothioneins in normal copper homeostasis was cast into doubt by the absence of a copper-related phenotype in the metallothionein knock-out mice (Kelly & Palmiter, 1996). However, metallothionein knock-out mice which also carry the Mottled-Brindled allele (MNK mutant allele in mouse) showed embryolethality suggesting metallothioneins do play a role in protection against copper toxicity (Freedman *et al.*, 1989). Little was known about the intracellular transport of Cu until the recent identification of several copper binding proteins termed Cu chaperones. These proteins were first identified in yeast, and their homologues were subsequently discovered in mammalian cells. In contrast to metallothioneins which sequester Cu into an extremely stable heptacopper thiolate cluster buried within the tertiary fold, chaperones bind Cu in labile complexes which enable ligand exchange when docking to a target protein (Portnoy *et al.*, 1999; Pufahl *et al.*, 1997; Srinivasan *et al.*, 1998). These copper chaperones protect the cell from potential cytotoxic reactions caused by free metal ions. Several mutations in WLN originally found in Wilson's disease patients were shown to disrupt the HAH1-WLN interaction, suggesting that HAH1 is required for Wilson disease protein function (Hung *et al.*, 1997).

These copper chaperones were first isolated in the baker's yeast *Saccharomyces cerevisiae* (Lin & Culotta, 1995; Pufahl *et al.*, 1997), and functional homologs have been noted in humans (Klomp *et al.*, 1997), sheep (Lockhart *et al.*,

2000), mice (Nishihara *et al.*, 1998), *Arabidopsis thaliana* (Himmelblau *et al.*, 1998), and *Caenorhabditis elegans* (Wakabayashi *et al.*, 1998). Both the yeast Atx1 and the human Atx1 homolog (HAH1 or Atox1) specifically traffic copper to the secretory pathway for incorporation into copper enzymes destined for the cell surface or extracellular environment (Hung *et al.*, 1997; Klomp *et al.*, 1997; Pufahl *et al.*, 1997).

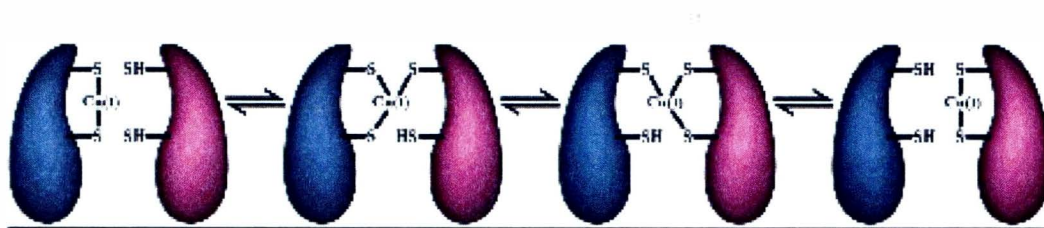
HAH1 is a small soluble, single-domain cytoplasmic protein containing a conserved CxxC motif that binds Cu(I). The bound Cu(I) is then transferred from HAH1 to a WLN metal binding domain through direct and specific HAH1-WLN4 protein-protein interactions (Achila *et al.*, 2006).

### 1.6 Copper transfer between the HAH1 and WLN4

It is likely that the role of each Wilson disease N-terminal domain is dependent on and defined by its environment. Direct evidence for the presence of a copper exchange mechanism between a Cu chaperone and acceptor protein has been provided recently by several groups (Huffman & O'Halloran, 2000; Yatsunyk & Rosenzweig, 2007). For example, Cobine and coworkers (Cobine *et al.*, 2002) demonstrated that the CopY repressor accepts Cu from the CopZ chaperone in bacteria. The described yeast and human Cu chaperones have similar MBSs, therefore it is likely that other surface motifs (or individual amino acid residues) on these proteins may be responsible for a target-specific docking of Cu-loaded chaperones (Bunce *et al.*, 2006; Huffman & O'Halloran, 2001).

As stated before Atx1, HAH1, and the other “Atx1-like” chaperones and target domains bind metal ions with the two cysteine residues in the conserved MT/HCXXC motif which help in the binding of copper. Structural evidence for

coordination by the conserved cysteines initially was suggested by the NMR structure of the fourth domain of the Menkes protein in the presence of Ag(I) (Gitschier *et al.*, 1998); however, it is noted that direct coordination was not demonstrated – for example, by interrogation of the metal center by X-ray absorption or emission.

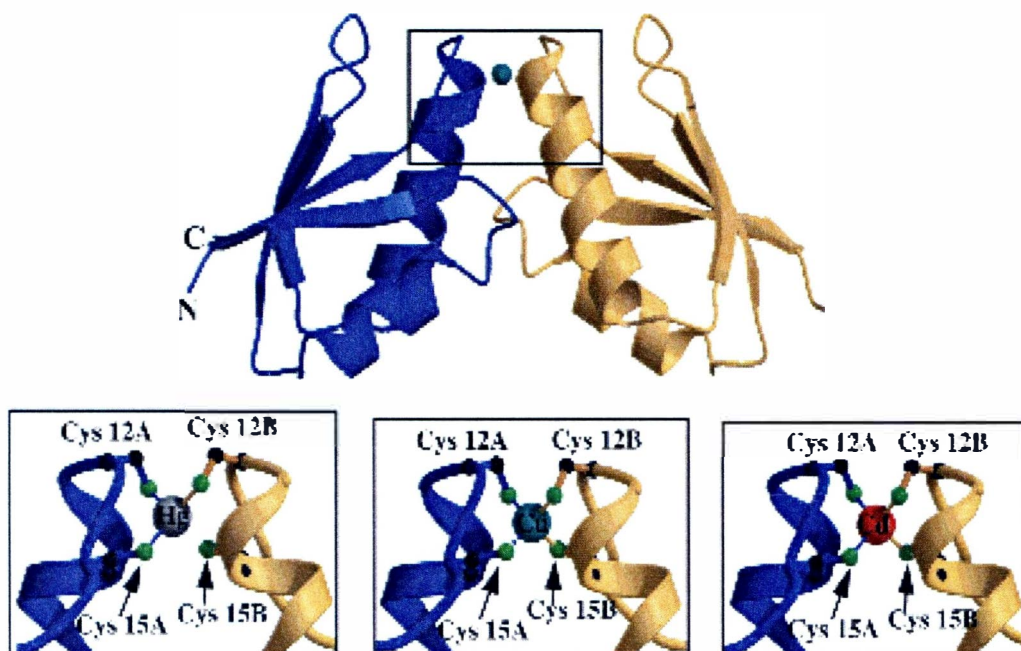


**Figure 2** The proposed mechanism of copper transfer between HAH1 and a domain of the Menkes or Wilson protein. HAH1 is shown on the right, and the target domain is shown on the left (Wernimont *et al.*, 2000).

The first X-ray structure of a metallochaperone was that of Hg(II)-Atx1 (Rosenzweig *et al.*, 1999), and then several metal-bound structures of HAH1 followed (Figure 3) (Wernimont *et al.*, 2000). The HAH1 structures also provide a three-dimensional model for target recognition and docking. Since Atx1 and HAH1 are very similar to the fourth Menkes domain in both overall fold and metal binding site, the observed HAH1 dimer can be considered a model for the chaperone-target protein complex. The two HAH1 molecules contact one another primarily at the site of the shared metal ion as shown in Figure 3 above (Wernimont *et al.*, 2000).

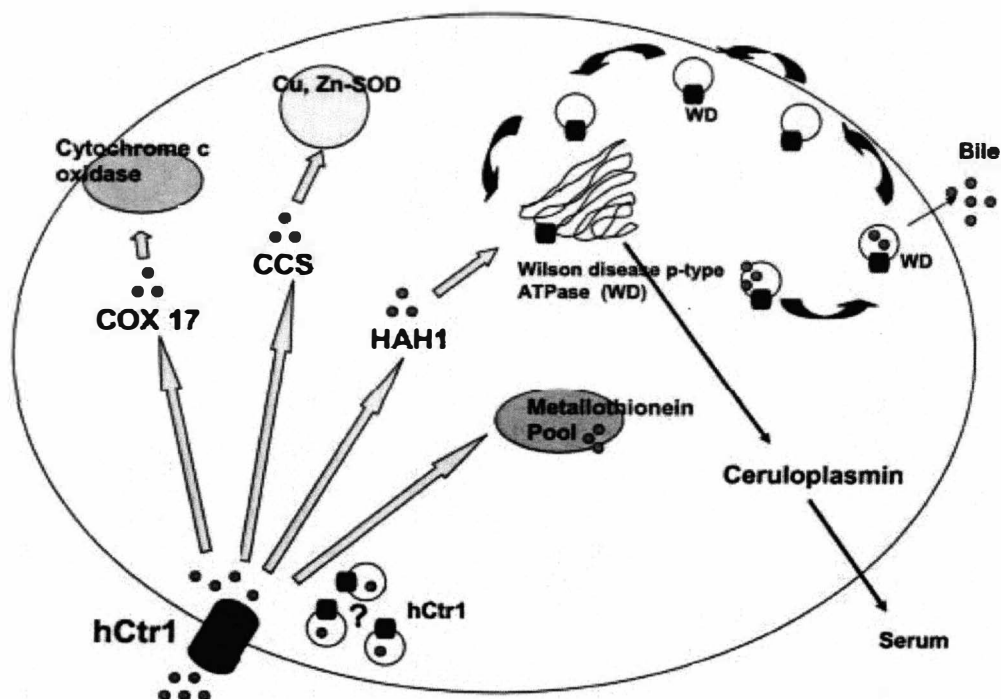
Once copper enters the hepatocyte bound to either albumin or histidine it traverses the cell via the high-affinity human copper transporter, hCtr1 (Figure 4) (Zhou & Gitschier, 1997). Characterization of hCtr1 confirms localization on the plasma membrane and also suggests the presence of hCtr1 on a separate intracellular perinuclear vesicular compartment (Shim & Harris, 2003). Copper, once inside the

hepatocyte, has one of three possible fates: i) joining the copper/metallothionein pool, ii) binding to CCS for delivery to Cu, Zn-SOD, or iii) trafficking to the Wilson disease P-type ATPase, which resides in the trans-Golgi network by HAH1 for subsequent copper incorporation into the cuproprotein ceruloplasmin. However, the copper chaperone that delivers copper to cytochrome c oxidase is still unknown. Recent studies indicate the possibility of mitochondrion matrix copper being the copper source for cytochrome c oxidase (Cobine *et al.*, 2006). Localization studies of the Wilson P-type ATPase reveal redistribution of the ATPase from the trans-Golgi network to a vesicular compartment that moves out toward the biliary epithelium under conditions of high copper concentration providing a mechanism for copper excretion in bile.



**Figure 3** The structure of the Atx1 human homologue, HAH1. The metal ion is coordinated by conserved cysteine residues (Wernimont *et al.*, 2000).





**Figure 4** Model of copper trafficking in a hepatocyte.

## 1.7 Other proteins that interact with the Wilson disease protein

### 1.7.1 p62 subunit of the dynactin complex

This study also involved the isolation, characterization and purification of C-terminal portion of p62 subunit of the dynactin complex. The amino acid sequence of the C-terminus portion of p62 is shown (Figure 5). p62 is a protein with a molecular weight of about 25 kDa in size.

The p62 subunit contains a CxxC motif in the C-terminus that might be involved in Cu(I) binding. There are also a number of potential histidine copper ligands in this protein. It has been shown to interact with a portion of the N-terminus that includes metal-binding domains 4-6 of the Wilson disease protein (Lim *et al.*,

2006). Since the potential copper binding properties of the p62 protein has been postulated by Lim and coworkers, this project aimed at isolating the p62 protein, then use it to find out its metal-binding environment as well as its interaction with the Wilson protein (Lim *et al.*, 2006).

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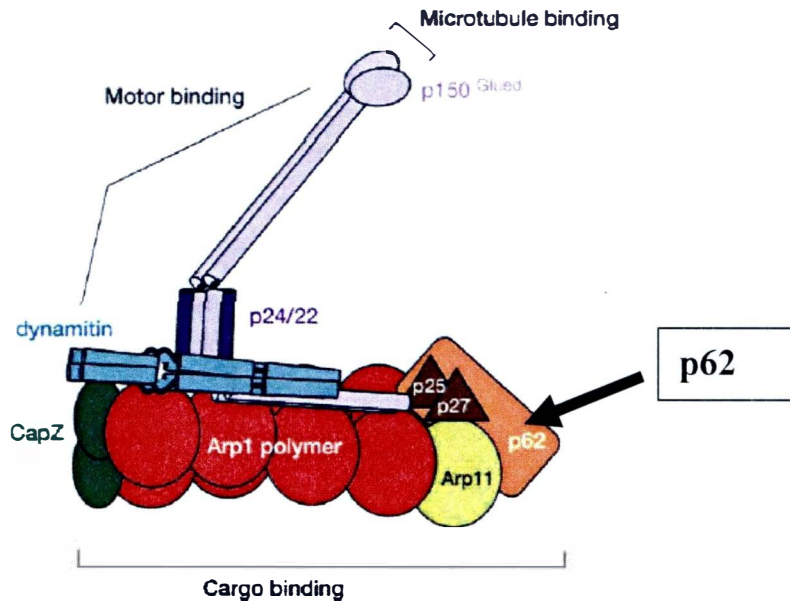
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51 NPTSIKFKIQ LVAVNYIPEV RIMSIPNLRY MKESQVLLTL TNPVENLTHV
101 TLFECEEGDP DDINSTAKVV VPPKELVLAG KDAAA EYDEL AEPQDFQDDP
151 DIIAFRKANK VGIFIKVTPQ REEGEVTVCF KMKHDFKNLA APIRPIEESD
201 QGTEVIWLTQ HVELSLGPLL P

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**Figure 5** Amino acid sequence of the p62 subunit of the dynactin complex. Note a CXXC motif in bold and underlined above.

Dynactin is a multi-subunit protein complex necessary for the function of the cytoplasmic molecular motor, dynein, found in all eukaryotic cells. The coordinated trafficking of organelles along microtubules is critical to the viability of cell and is powered by the mechanochemical ATPases, kinesin and cytoplasmic dynein (Karki & Holzbaur, 1995).

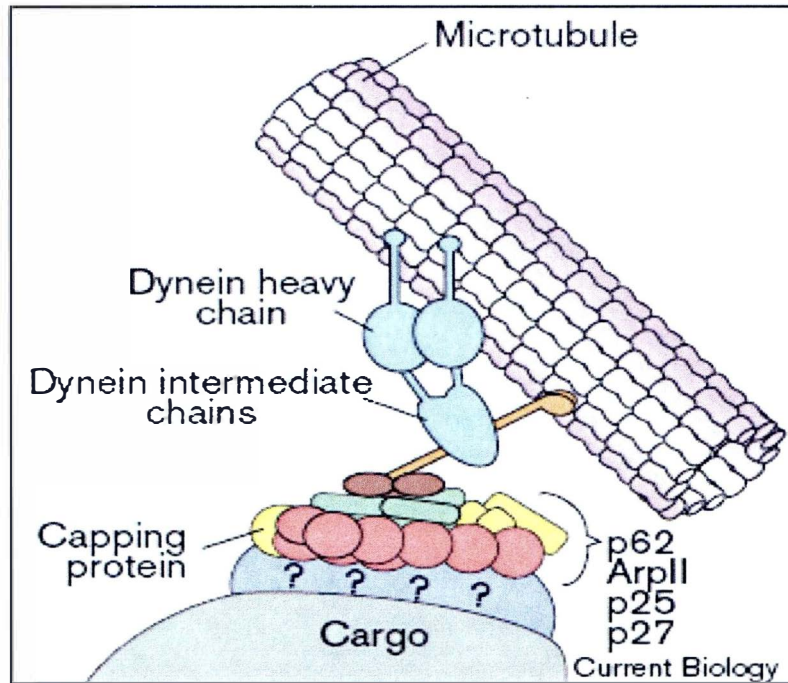
The dynactin is an important component of the cytoplasmic dynein motor machinery (Holleran *et al.*, 1998). It has two distinct structural domains: a projecting sidearm that interacts with dynein and an actin-like minifilament backbone that is thought to bind cargo (Karki *et al.*, 2000). The dynactin contains 11 different subunits which are known to interact with the cargo molecules (Figures 6, 7). The complex binds to the motor and then carries it along microtubules along with the numerous and diverse cargo molecules (Allan, 2000).



**Figure 6** Scheme illustrating the location and approximate structural features of dynactin units (Schroer, 2004).

The most striking feature of the p62 polypeptide is a very highly conserved pattern of cysteine residues. Comparisons of the sequences of p62 from human, mouse, *Drosophila*, *C. elegans*, and *N. crassa* indicate a pattern of 11 highly conserved cysteine residues (Karki *et al.*, 2000). These cysteines are the ones most likely responsible for the copper-dependent interaction with this protein. This particular interaction is key to copper-regulated trafficking pathway that delivers ATP7B to sub-apical vesicles of the hepatocytes for the removal of excess copper. This has been proposed by Lim and coworkers who studied the N-terminus of ATP7B and p62 interaction using a yeast two-hybrid technique. By mutating the cysteines in all the six metal binding domains, the interaction was disrupted. The mutation abolished the copper-regulated redistribution of ATP7B from the trans-Golgi network

to sub-apical vesicular compartments and the copper transport activity (Forbes *et al.*, 1999; Lim *et al.*, 2006; Petris *et al.*, 2002).



**Figure 7** The model of the dynactin complex and its proposed interaction with microtubules and cytoplasmic dynein (Allan, 2000).

#### 1.7.2 MURR1 (COMMD1)

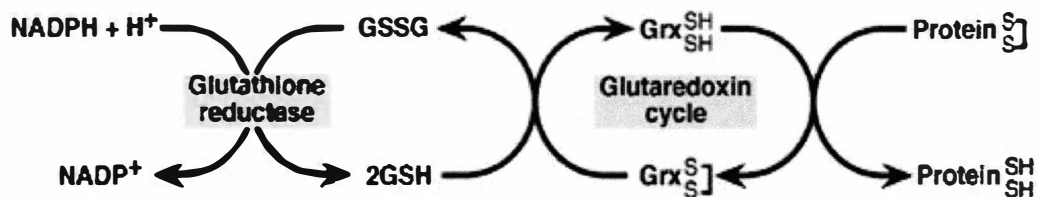
Recently, the discovery of a mutated MURR1 gene in Bedlington terriers (inbred canine strain) with copper toxicosis provides a new lead to disentangling the complexities of copper metabolism in mammals (van De Sluis *et al.*, 2002). Bedlington terriers are purebred dogs known to possess a high incidence of copper toxicosis due to a defect in biliary copper excretion. MURR1 functions in the regulation of the transcription factor NF- $\kappa$ B and control of copper metabolism. Mutations in this gene are responsible for copper toxicosis in the Bedlington terriers.

These species of dogs do not have mutations in ATP7B but display a phenotype which is consistent with inactivation of copper export by ATP7B (van De Sluis *et al.*, 2002). In their coimmunoprecipitation studies of COMMD1, Tao and coworkers have demonstrated that COMMD1 interacts with ATP7B through the N-terminal region of the protein. Since COMMD1 does not coimmunoprecipitate with ATP7A it could be a specific regulator of hepatic ATP7B and provide a better understanding of the copper homeostasis pathway (Burstein *et al.*, 2005; Tao *et al.*, 2003).

### 1.7.3 Glutaredoxin

Glutaredoxin, an example of a sulfhydryl protein similar to thioredoxin, was first discovered in 1976 as a thiol donor system for ribonucleic reductase in a mutant lacking thioredoxin 1 (Trx1) in *E. coli* (Holmgren, 1976). Glutaredoxin (Grx1) is a small cytosolic protein that catalyzes the reduction of disulfide bonds in proteins, oxidizing glutathione (GSH) to glutathione disulfide (GSSG). GSSG is in turn recycled to GSH by the enzyme glutathione reductase at the expense of NADPH. During the reaction cycle it is thought that a cysteine pair in the active site of glutaredoxin is converted to a disulfide (Shelton *et al.*, 2005).

Using yeast-two hybrid technology and co-immunoprecipitation, Lim and coworkers proposed that glutaredoxin interacts with the N-terminus the copper ATPases ATP7A and ATP7B. This interaction is copper –dependent and it requires a CxxC motif and could have an essential role in maintaining the activity of the copper ATPases (Lim *et al.*, 2006). Glutaredoxin may act to reduce intramolecular disulphide bonds formed by the cysteine residues to allow copper coordination by the thiol groups during the copper translocation by these ATPases (Lim *et al.*, 2006).



**Figure 8** A schematic representation of the glutaredoxin pathway (Carmel-Harel & Storz, 2000).

### 1.8 Significance of this study

This study focused on trying to understand the various known proteins that interact with the Wilson disease protein and how these copper-dependent interactions will help elucidate copper homeostasis. The p62 subunit of the dynactin complex has initially been identified as an interacting partner of ATP7B using yeast two-hybrid assay (Lim *et al.*, 2006). WLN4 is known to interact with HAH1 directly for Cu(I) transfer (Achila *et al.*, 2006; Yatsunyk & Rosenzweig, 2007). An insightful study of HAH1- WLN4 interactions will help understand how HAH1 and the full length of WLN interact for Cu(I) transfer.

The results of these experiments will generate new information regarding key copper transporters in mammalian cells and will advance understanding of basic mechanisms regulating intracellular copper distribution. Studies of interacting partners of the Cu-ATPases will give more insights into the molecular mechanism of regulating localization of these copper transporting proteins in the cell which have a significant role in Wilson's disease and Menkes disease.

## 1.9 Objectives of this study

This research was based on the study of copper-dependent interaction partners with the Wilson disease protein: p62 subunit of the dynactin complex and HAH1, a copper-chaperone, which delivers copper specifically to copper-transporting ATPases in mammalian cells. One objective was to clone, over express and characterize the p62 subunit protein of the dynactin complex. Among the studies that we will carry out include copper interaction studies of the p62 protein with the N-terminal domains of the Wilson disease protein.

The second objective was to generate mutants of HAH1 and the fourth domain of the Wilson disease protein (WLN4) by changing cysteines of the metal binding motif CxxC to AxxA. Mutations will alter the properties of the holoHAH1. This will be used in various copper binding studies to investigate the role played by the cysteine metal binding motif in transfer of copper between HAH1 and WLN4, especially in the development of control studies.

## CHAPTER II

### MATERIALS AND METHODS

#### 2.1 Materials

A plasmid containing HAH1 cloned into pET11d was available in our laboratory (pET11d-HAH1) and also the single mutant of Wilson disease protein metal-binding domain four. The template for site-directed mutagenesis of pTRX-WLN4(C15A,C18A) was single mutant (WLN4(C15)) that Ms. Sarah Dickenson and Mr. Joshua Muia produced in the Huffman laboratory. The p62 gene was obtained as a donation from Dr. Julian Mercer and Dr. Sharon LaFontaine of Deakin University, Australia.

All the primers used were purchased from Integrated DNA Technologies, Inc. Restriction endonucleases and other enzymes with their respective buffers were purchased from New England Biolabs Inc. and Life Technologies. The nucleotides were purchased from Sigma Chemicals Co., while bacterial strains and plasmids were obtained from Novagen. The QIAGEN plasmid isolation and DNA isolation kits were purchased from QIAGEN Inc. QuikChange® II XL (Stratagene, #200521) the site-directed mutagenesis kit was obtained from Stratagene. Dimethylsulfoxide, dibasic potassium phosphate and sodium phosphate (monobasic and dibasic) all were purchased from J.T. Baker Chemical Co. Other significant reagents were IPTG (Isopropyl- $\beta$ -D-thiogalactopyranoside, INALCO, #1758-1400), Agarose™ (Invitrogen, #15510-019), tryptone (MO BIO Laboratories, #12111-1), Bacto-yeast extract (Fisher, #212720), NaCl (Fischer, #BP330-1), yeast extract (Fisher,



#7264672, dextrose (Fisher, #BP350-1) and MES (2-(4-morpholino)-ethane sulphonic acid, #BP300-100). Bio-Rad dye (#500-0006) for the Bradford assay was obtained from Bio-Rad Laboratories while Coomassie plus dye (#B-0149) was obtained from Pierce. Ampicillin (#A-9518) and Bacto-Agar (#214010) were from DIFCO Laboratories. YM3 membranes for protein concentration were purchased from Millipore.

## 2.2 Cloning of genes

Before cloning of the p62 gene into the pET24d plasmid vector, both forward and reverse primers for p62 amplification were designed as described below.

### 2.2.1 Primer design

The primers were designed with the DNA Strider program. The sequence for the forward primer was: 5'-CAA GAC CAG TAA CCA TGG CAG AGG TAA CAA CCC TTC AGC AGC -3'. This primer design included a start codon ATG (underlined) which codes for methionine and the NcoI restriction site CCATGG. The sequence of the reverse primer was 5- CCC TCC AGT GGA TCC TTT TAA GGA AGA AGT G -3'. This design incorporated a stop codon TGA and a unique restriction site for BamHI, GGATCC.

### 2.2.2 p62 gene amplification

The gene encoding p62 was amplified using the Polymerase Chain Reaction (PCR) utilizing the above designed primers (forward and reverse). Taking into account the Guanine-Cytosine (GC) content and the melting temperatures of the respective primers, the following written program was used to facilitate the polymerization activity.

**Table 2**  
PCR reaction conditions for the p62 gene amplification.

Name	Temperature	Time
First denaturing	95°C	5 min
Annealing	65°C	1 min
Extension	72°C	40 sec
Second denaturing	95°C	40 sec
Annealing	65°C	1 min
Extension	72°C	1 min

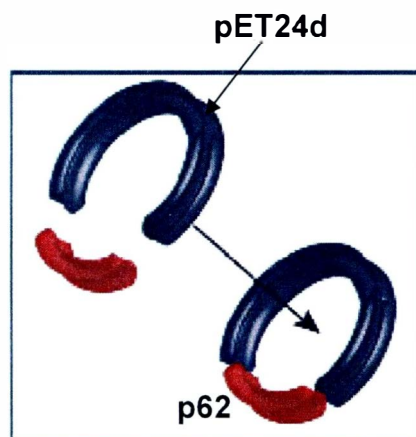
The last two steps were repeated 30 times. This was followed by storage at 4°C. The annealing temperature ( $T_m$ ) was calculated from the composition of the primer according to the Wallace rule (Equation 2.1).

$$T_m (^{\circ}\text{C}) = [(G + C) \times 4] + [(A + T) \times 2] \quad 2.1$$

A PCR product of 700 bp was obtained and visualized in an agarose gel before purification. The product was subjected to purification using the QIAquick Spin PCR purification kit. Purified DNA was analyzed using 1% Agarose gel electrophoresis. The gel was stained in ethidium bromide and visualized under UV light. The correct mass of the DNA was observed.

### 2.2.3 Ligation of p62 gene into pET24d

pET24d (Novagen) expression vector was digested using restriction enzymes NcoI and BamHI. The purified p62 gene from the PCR reaction was then ligated see figure below into the vector pET24d at 25°C for 4 hr and the reaction conditions maintained at 4°C.



**Figure 9** Ligation of p62 into the pET24d vector.

The ligation products were analyzed by 1% agarose gel electrophoresis. The ligation was successful due to the agarose gel electrophoresis analysis showing a band of 700 bp size corresponding to the p62 gene.

The recombinant p62 gene was then expressed in DH5 $\alpha$  *E. coli* cells using the Invitrogen protocol described in the transformation kit and a time course induction was carried out for 4 hr and the protein was induced using 1mM IPTG. The resultant protein was analyzed by 10% SDS-PAGE gel electrophoresis.

### 2.3 Ligation Independent Cloning (LIC) of p62 gene

Due to difficulty in expressing the p62 protein, the cloning method was changed to use a fusion protein following the protocols described by Novagen book. The second strategy of Ligation Independent Cloning was employed with vectors coding for glutathione transferase (GST) and the thioredoxin tag respectively. These LIC vectors are created by treating a linearized backbone with T4 DNA polymerase

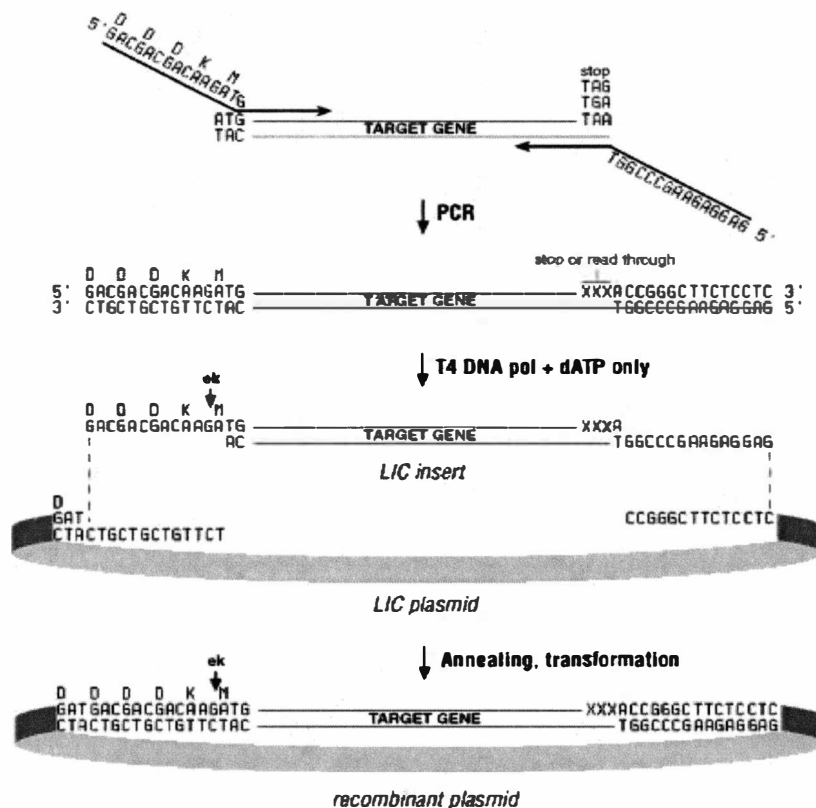
in the presence of dATP. The 3' to 5' exonuclease activity of T4 DNA polymerase removes nucleotides until it encounters a residue that corresponds to the single dATP present in the reaction mixture. At this point, the 5' to 3' polymerase activity of the enzyme counteracts the exonuclease activity to prevent further excision. Plasmid sequences adjacent to the site of linearization are designed to give specific non complementary overhangs which in most cases are normally 13 to 14 base pairs in the LIC vector. Fusion proteins are known to improve protein solubility and also enhance protein purification. They also help in stabilizing the protein so that it gets made efficiently. The illustration of Ligation Independent Cloning is shown in Figure 10.

Both pET-41 Ek/LIC which is fused with N-terminal GST·Tag™ sequence and pET-32 Xa/LIC fused with Trx·Tag™ thioredoxin protein (LaVallie *et al.*, 1993) were used in this study. These two vectors are designed for rapid and directional cloning of PCR amplified DNA for high level expression of polypeptides with a fusion protein. Specifically designed primers were inserted without the need for restriction digestion or ligation and the resultant fusion protein can be cleaved by proteases enterokinase in the case of pET-41 Ek/LIC and Factor Xa in the case of pET32-Xa/LIC (LaVallie *et al.*, 1993).

The sense primer was designed to encode the last four amino acids of the enterokinase (Ek) cleavage site plus the C-terminal flanking amino acid Met. The sequence of the GST fusion forward primer was: 5'- GAC GAC AAG ATG GCA GAG GTA ACA ACC CTT CAG CAG CGT CTG – 3' and the sequence for the GST fusion reverse primer was: 5'- GAG GAG AAG CCC GGT TTA AGG AAG AAG TGG GCC CAA GCT AAG TTC – 3'. The primers for pET32-Xa/LIC were 5' – GGT ATT GAG GGT CGC ATG GCA GAG GTA ACA ACC CTT CAG CAG CG

– 3' (forward) and 5' – AGA GGA GAG TTA GAG CCT TAA GGA AGA AGT  
GGG CCC AAG CTA AG – 3' (reverse).

Two PCR tubes were set up: The first tube contained 58  $\mu$ L H<sub>2</sub>O, 10  $\mu$ L Thermopol buffer<sup>TM</sup>(10x), 10  $\mu$ L 2 mM dNTP mix, 10  $\mu$ L Ek/LIC p62 forward primer (10  $\mu$ M), 10  $\mu$ L Ek/LICp62 reverse primer (10  $\mu$ M), 2  $\mu$ L template (p62), and 0.9  $\mu$ L DNA polymerase (Deep Vent). This mixture was overlaid with 30  $\mu$ L mineral oil. The second tube contained: 58  $\mu$ L H<sub>2</sub>O, 10  $\mu$ L thermopol buffer (10x), 10  $\mu$ L 2mM dNTP mix, 10  $\mu$ L Xa/LIC p62 Fwd primer (10  $\mu$ M), 10  $\mu$ L Xa/LIC p62 reverse primer( 10  $\mu$ M), 2  $\mu$ L templatep62), and 0.9  $\mu$ L DNA polymerase (Deep Vent). The two tubes were set in the PCR instrument and the desired DNA piece was amplified using the following PCR program: Melting temperature of 95°C for 5 min, annealing temperature of 65°C for 1 min, extension temperature of 72°C for 40 sec, melting at 95°C for 40 sec, annealing of at 65°C for 1 min and Extension at 72°C for 1 min. The last 3 cycles were repeated 27 times (Table 2).



**Figure 10** Schematic representation of Ligation Independent Cloning technique. (LaVallie *et al.*, 1993).

After the DNA amplification, an agarose gel electrophoresis was run to ascertain the presence of the DNA piece. The PCR product was then purified using the Qiagen mini elute protocol. The purified product was also quantified by agarose gel electrophoresis.

### 2.3.1 T4 DNA polymerase treatment of target insert

The following components were assembled in a PCR tube: 10  $\mu$ L purified PCR product, 2  $\mu$ L T4 DNA polymerase buffer (10X), 2  $\mu$ L 25 mM dATP, 1  $\mu$ L 100 mM DTT 0.4  $\mu$ L T4 DNA polymerase (LIC-qualified) and 4.6  $\mu$ L nuclease-free water. The total volume of the components was 20  $\mu$ L. The reaction was started by

adding the enzyme and stirring with the pipette tip for 2 min. The mixture was then incubated at 22°C for 30 min. Finally the temperature was raised to 75°C for 20 min to inactivate the enzyme.

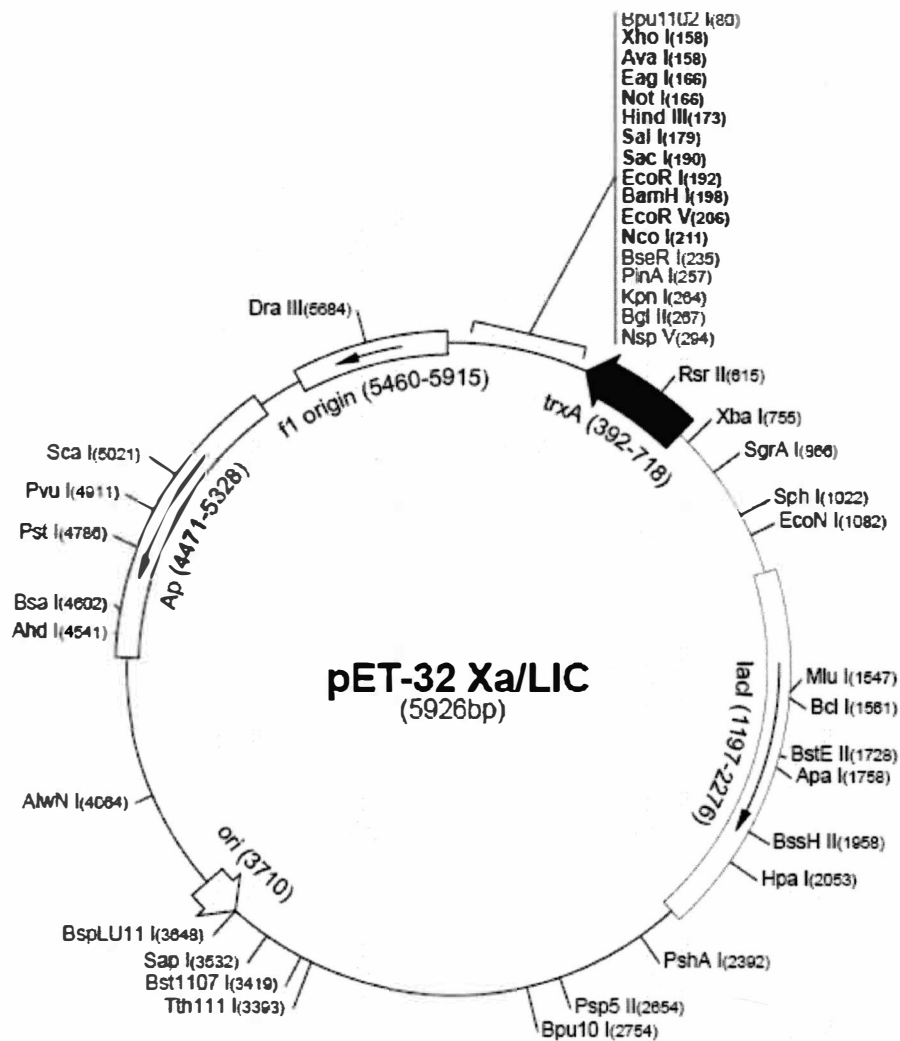
**Table 3**  
PCR reaction conditions for the LIC experiment.

Name	Temperature	Time
First denaturing	94°C	5 min
Annealing	65°C	1 min
Extension	72°C	40 sec
Second denaturing	95°C	40 sec
Annealing	65°C	1 min
Extension	72°C	1 min

### 2.3.2 Annealing the vector and the treated insert (Ek/LICp62)

Annealing of the pET41Ek/LIC vector and the treated insert above (Ek/LICp62) was done by assembling the following components in a sterile PCR tube: 1 µL Ek/LIC vector (pET 41) (Figure 12) and 2 µL T4 DNA polymerase-treated Ek/LICp62 insert. These were incubated at 22°C for 5 min then 1 µL 25 mM EDTA was added to the mixture with stirring followed by incubation at 22°C for 5 min. Annealing continued for 1 hr. We repeated the same process above separately with the pET-32Xa/LIC insert of p62 using the same reaction conditions as described above. The clones were digested by restriction enzymes and analyzed by agarose gel electrophoresis to test the success of the LIC technique. A sample was sent for DNA

sequencing to verify cloning which was successful for both pET41Ek/LIC and pET32Xa/LIC.



**Figure 11** Diagrammatic representation of the pET32Xa/LIC vector showing its various restriction sites (LaVallie *et al.*, 1993).



## 2.4 Construction of double mutant by site-directed mutagenesis

The HAH1-containing plasmid was obtained from our laboratory freezer. Mutagenesis was performed sequentially on the HAH1 plasmid by Peter Sande and Jubril Ayodele respectfully by mutating the cysteine at position 11 to alanine (C11A) followed by cysteine 14 to alanine (C14A). The site-directed mutants of both HAH1 and the Wilson disease protein domain 4 (WLN4) were prepared starting with single mutants HAH1(C11A) and WLN4(C15A) respectfully using the Stratagene kit. Mismatched primers with the corresponding mutations were used with primer extension by the proofreading polymerase *PfuI*. *DpnI* restriction enzyme digests were used to remove the parental plasmid before transformation into *Escherichia coli* Nova Blue GigaSingles™ competent cells. The mutants were confirmed by dideoxynucleotide sequencing. The Stratagene Quick-Change site-directed mutagenesis kit was used to introduce the C11A and C14A mutation. The primers were designed with the DNA Strider program. The sequence of the forward mutagenic primers for pHAH1(C11A,C14A) were as follows 5' – G ACT GCC GGT GGT GCC GCT GAA GCT GTA TCC CGG G -3' and the sequence of the reverse mutagenic primer was 5'- CCC GGG ATA CAG CTT CAG CGG CAC CAC CGG CAG TC -3'. Those for pTRX-WLN4(C15A,C18A) leading strand, 5'- G ACC GCT GCA TCC GCT GTC CAT TCC ATT GAA GG C -3' and complementary strand, 5' – GCC TTC AAT GGA ATG GAC AGC GGA TGC AGC GGT C -3'. The changed amino acid codons are underlined. The reagents were mixed in a 1 mL microcentrifuge tube following the protocol described in the Stratagene kit. The solution was then overlaid with 30 µL of mineral oil and subjected to the PCR for several cycles.

#### 2.4.1 Digestion of DNA by Dpn1 enzyme

The non-mutated parental DNA was digested with Dpn1 restriction enzyme (10 U/ $\mu$ L). One  $\mu$ L (10 U/ $\mu$ L) of the restriction enzyme was added to the PCR product. The reaction mixture was then centrifuged at 10,000 rpm for 1 min in a microcentrifuge. The mixture was then incubated at 37°C for 1 hr.

The plasmid containing the mutation construct was transformed into NovaBlue GigaSingles *E. coli* cells according to the protocol: An aliquot of 200  $\mu$ L NovaBlue GigaSingles<sup>TM</sup> competent cells (Novagen) was thawed on ice and mixed gently to ensure that cells were evenly suspended and then added 1  $\mu$ L (100 ng) of DNA solution containing pHAH1(C11A,C14A) to the cells and stirred to mix. The tubes were placed on ice for 5 min, heated for 30 sec in a 42°C water bath then placed on ice for another 2 min before adding 250  $\mu$ L of room temperature SOC media. The cells were then incubated at 37°C while shaking at 250 rpm for 60 min prior to plating.

The transformation product was plated on Luria Bertini broth (LB) agar plates with 100  $\mu$ g/mL Ampicillin and incubated at 37°C overnight. A few colonies obtained from the transformation were inoculated into a 5 mL starter culture of liquid LB for plasmid recovery. The pure plasmid, pHAH(C11A,C14A) was recovered after isolation and purification using Qiagen mini elute protocol.

Similarly, a site-directed mutagenesis was carried out on a single mutant of the fourth domain of Wilson disease protein WLN4(C15A) to generate the pTRX-WLN4(C15A,C18A) a double mutant.

## 2.5 Protein expression

The plasmid of p62 was transformed in *E. coli* cells Tuner(DE3) and the product plated on LB culture containing 30 µg/mL kanamycin and incubated overnight at 37°C. An antibiotic, which is vector specific was used to make sure only the resultant colonies are those of the desired protein. Several colonies observed on the plates were inoculated and tested for protein expression. When the cells were grown up to an OD of 0.6-1.0 an induction test was done with addition of 1 mM IPTG for a period of 4 hr. The harvested bacterial cultures were centrifuged at 6000xg and the pellets were stored at -20°C. An aliquot withdrawn at each hr interval were analyzed by SDS-PAGE.

After purifying the mutant gene of pTRX-WLN4(C15A,C18A), it was transformed into Rosetta(DE3) cells using the following protocol described previously above. A time-course induction in 1 mM IPTG (a synthetic mimic of the natural inducer, 1,6- allolactose which binds to the *lac repressor*) was used to study the protein expression on the 100 ml LB culture inoculated with mutated pTRX-WLN4(C15A,C18A) plasmid containing 100 µg/mL ampicillin. Aliquots of 500 µL were withdrawn every 1 hr and centrifuged and stored at -20°C until loaded onto a 15% SDS-PAGE gel in a Hoefer SE250 mini vertical gel electrophoresis unit. The pellets were treated with 5 µL of loading buffer 2x and 20 µL of water then heated at 92°C for 15 min before loading onto the gel which was then run for 1 hr at 98 mV then stained with Coomassie blue stain for four hr and the gel was dried.

The pHAH1(C11A,C14A) plasmid was transformed into BL21 (DE3) cells for protein expression using the protocol described above.

### 2.5.1 Large scale protein expression

After the successful induction of pHAH1(C11A,C14A) in a 100 mL aliquot of BL21(DE3) cells, the cell culture was then over expressed in 10 L of LB. A fresh colony of BL21(DE3) cells transformed with pHAH1(C11A,C14A) plasmid was inoculated in 5 mL LB media containing 100 µg/mL of ampicillin and grown in a 37°C incubator to an OD<sub>600</sub> of 0.6 and this was used as a starter culture to inoculate 10 L of LB. This batch culture of BL21(DE3) transformed with pHAH1(C11A,C14A) was grown aerobically in a New Brunswick Scientific Microferm® fermentor to an OD<sub>600</sub> of 0.6 before induction with 1 M IPTG to a final concentration of 1 mM. After 4 hr the cells were harvested by centrifugation at 6,000 rpm for 15 min using an SLA 3,000 rotor. The pellets were then stored at -20°C prior to protein extraction.

## 2.6 Protein purification

### 2.6.1 Freeze-thaw extraction

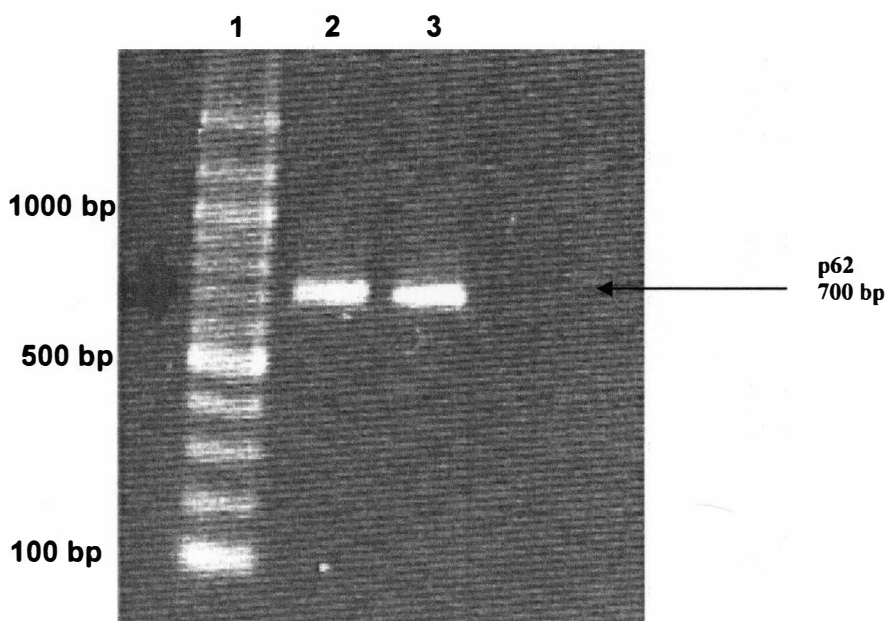
The freeze-thaw extraction buffer was 20 mM MES/NA, 1 mM EDTA, 10 mM dithiothreitol (DTT), pH 6. Protein extraction was performed by resuspending the cell pellet in the freeze-thaw extraction buffer (24 mL of buffer per liter of induction culture). The suspension was gently agitated in ice for 1 hr. The non-soluble portion was pelleted by centrifugation at 6000 g for 15 min. Both the supernatant and the pellet were tested analytically by SDS-PAGE for the mutant pHAH1(C11A,C14A) protein. A positive result was finding the appropriate protein in the supernatant sample, representing solubilized protein.

## CHAPTER III

### RESULTS

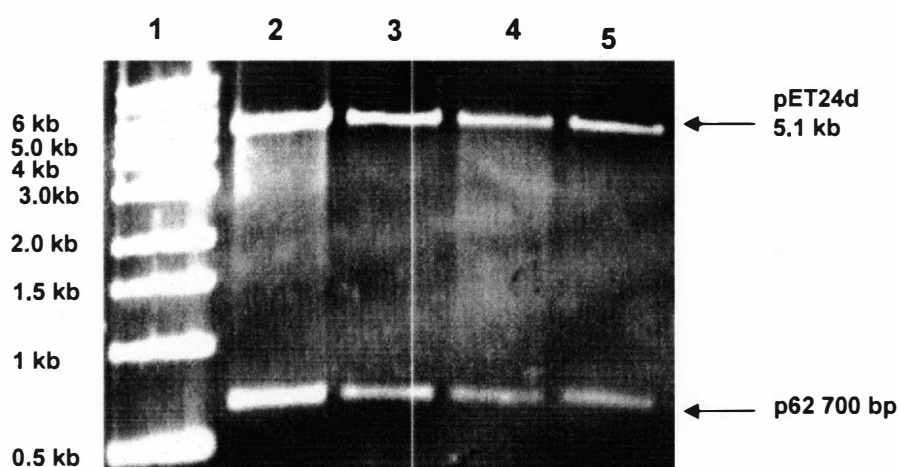
#### 3.1 Cloning of p62 gene

The gene coding for p62 was amplified through 28 PCR cycles in a MiniCycle using Deep Vent DNA polymerase (New England Biolabs). After running the agarose gel electrophoresis with the PCR product it was found to be the appropriate size of 700 base pairs, (Figure 12).



**Figure 12** 1% agarose gel electrophoresis of p62 PCR product. Lane 1, is the 100bp marker, Lanes 2 & 3 represent the p62 gene (~700 bp).

The resultant PCR products were purified, double digested with restriction endonucleases BamHI and NcoI to get it out of the PCR fragment then cloned into similarly digested pET24d *E. coli* expression vector. This ligation was digested with BamHI and NcoI then analyzed by agarose gel electrophoresis and the right size of p62 (700 bp) was visualized (Figure 14). The plasmid was then isolated for further protein expression.



**Figure 13** Ligation product of p62 into pET24d after restriction enzyme digestion with BamHI and NcoI. Lane 1 represents 1 kb DNA ladder. Lanes 2-5 are the ligation products (p62 size 700 bp).

The successful cloning in pET24d was verified by sequencing the gene (by Retrogen) and also by restriction analysis of the recovered plasmid, both analyses gave positive results. The cloned p62 gene was transformed into Rosetta cells and a time-course induction test was performed. The induction did not work since no expression of the protein was visualized.

The difficulty of the above expression prompted another ligation method using fusion techniques where we used Ligation Independent Cloning (LIC). First we created two p62 constructs using the vectors pET41Ek/LIC and pET32Xa/LIC.

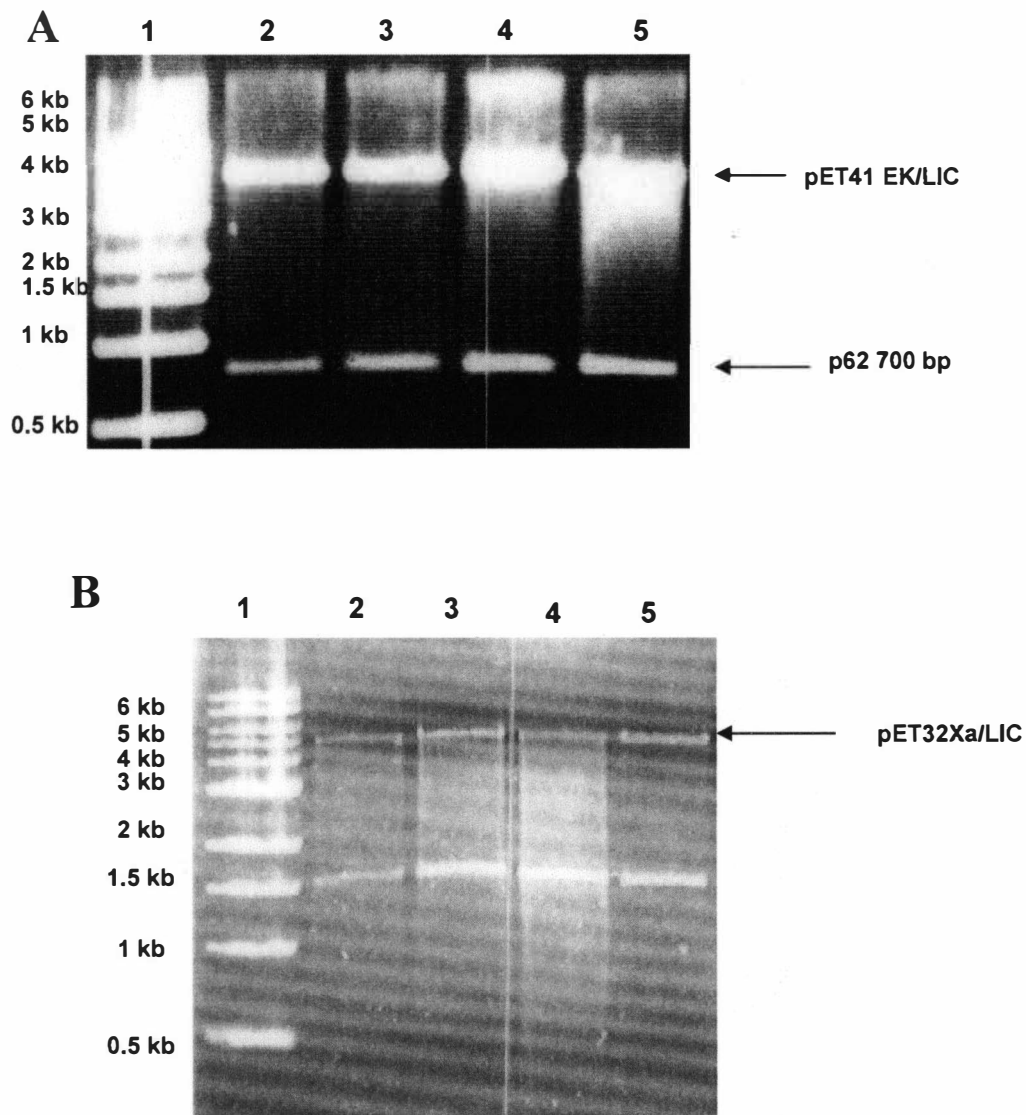
PCR products with the resultant complementary overhangs were created by building 5' extensions into the primers. The PCR product was purified to remove dNTPs (and template plasmid). After annealing of the LIC vector, it was transformed into *E. coli* cells. Positive clones were transformed into Nova Blue GigaSingles *E. coli* strains. Approximately 10 colonies appeared in the plates. Five colonies were selected for screening by inoculation in 5 mL of LB containing 100 µg/mL ampicillin. After reaching an OD of 0.6, the cell culture was centrifuged and the pellet was recovered. The p62 plasmid DNA was then extracted using a Qiagen kit before subjecting to restriction mapping.

The results of the Ligation Independent Cloning (LIC) where the cloned gene was double-digested using restriction enzymes BamHI and NcoI showed that the ligation was successful.

A band representing a fragment of the recombinant plasmid was observed on a 1% agarose electrophoresis gel (Figure 15 A & B). This confirmed that p62 was successfully inserted into the vectors pET41Ek/LIC and pET32Xa/LIC respectively.

### 3.2 Induction of p62 in LB media

After the successful ligation using the LIC technique, the p62 gene was cloned into Rosetta2(DE3) competent *E. coli* cells. An induction test using IPTG was carried out with randomly selected colonies which were inoculated in LB media containing 30 µg/mL of kanamycin. The cultures were incubated at 37°C with shaking. Kanamycin was chosen as the appropriate antibiotic because the plasmid contained



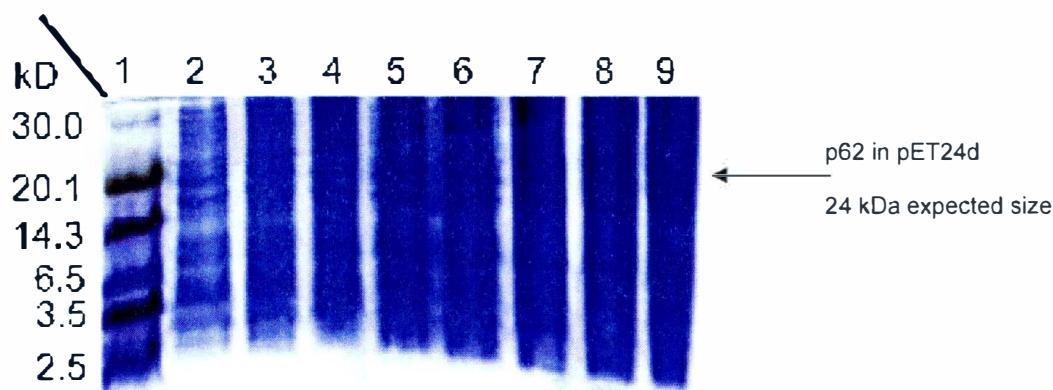
**Figure 14** Confirmation of ligation both in:(A) pET41Ek/LIC and (B) pET32Xa/LIC with a 1% agarose gel electrophoresis showing the 700 base pair p62. Lanes 2-5 show identical results.



$$\frac{OD_{600}(\text{at time "zero"})}{OD_{600}(\text{at time t})} \times 15\mu\text{L} \quad 3.2$$

genes for kanamycin resistance. We monitored the growth of the cells during induction by measuring optical density at 600 nm ( $OD_{600}$ ). To induce the expression of p62 gene we added 1 mM IPTG when the OD reached 0.6. IPTG is known to bind and inactivate the *lac operon* repressor and therefore induces gene transcription by RNA polymerase. Measuring of OD stopped after 4 hours. Samples withdrawn in each time interval were centrifuged and analyzed by SDS-PAGE gel for the p62 induction. Loading on SDS-PAGE was normalized by the OD reading, equation below.

The normalized loading on SDS-PAGE did not show the desired band with time, thus, the expression was unsuccessful (Figure 16). This was evident from the missing band 25 kDa on the gel above which corresponds with the size of p62.

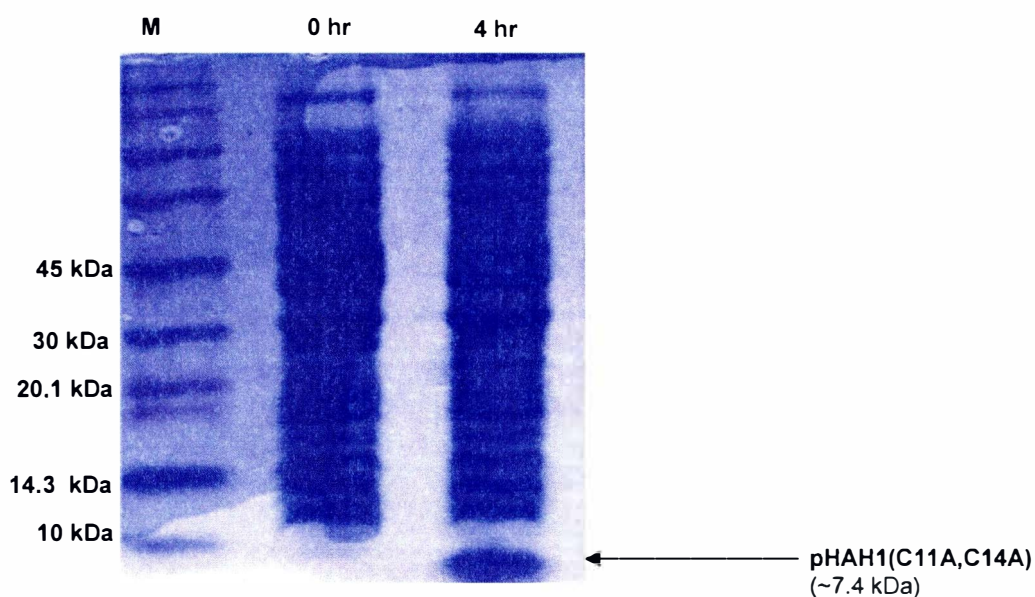


**Figure 15** 10 % SDS-PAGE electrophoresis results of p62 induction in *E. coli*. Lanes 2-9 represent the time-course induction while lane 1 represents the Rainbow marker. There is no band corresponding to the induced protein as expected with the horizontal arrow.

### 3.3 Site-directed mutagenesis of pHAH1(C11A,C14A) and pTRX-WLN4

#### 3.3.1 Protein expression of pHAH1(C11A,C14A) in *E. coli*

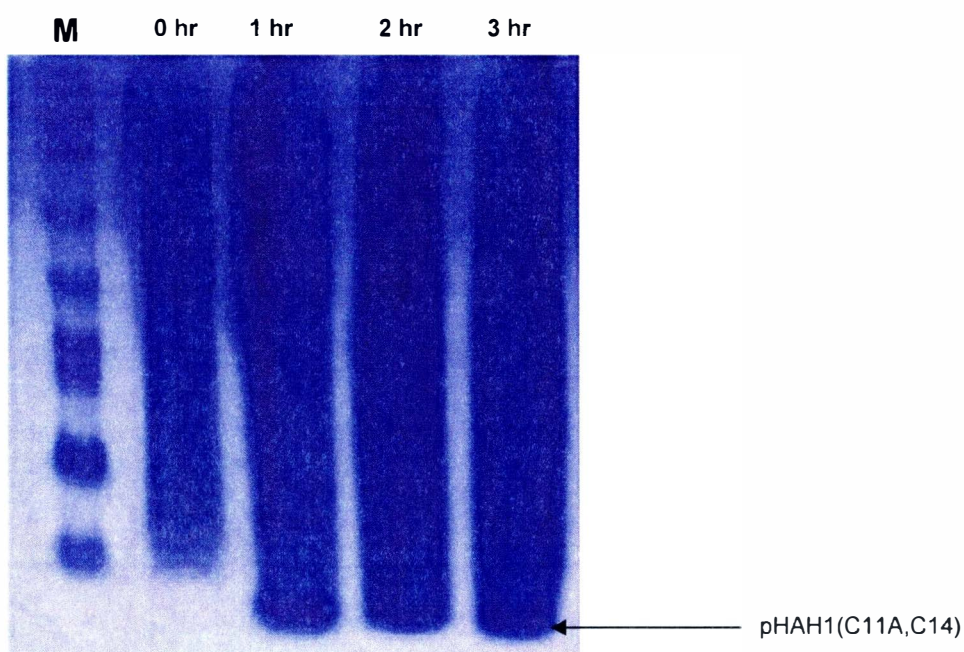
Upon successful site-directed mutagenesis of the HAH1(C11A) single mutant template to replace cysteine to alanine at position 14 recombinant plasmid pHAH1(C11A,C14A) was transformed into NovaBlue GigaSingles cells of *E. coli*. The plasmid was isolated and purified. This plasmid was then transformed into BL21(DE3) cells inoculated in LB and induced with IPTG for protein expression. The expression of this protein was then analyzed by SDS-PAGE (Figure 17). A band was visualized on SDS-PAGE gel to show that the four hour protein induction worked.



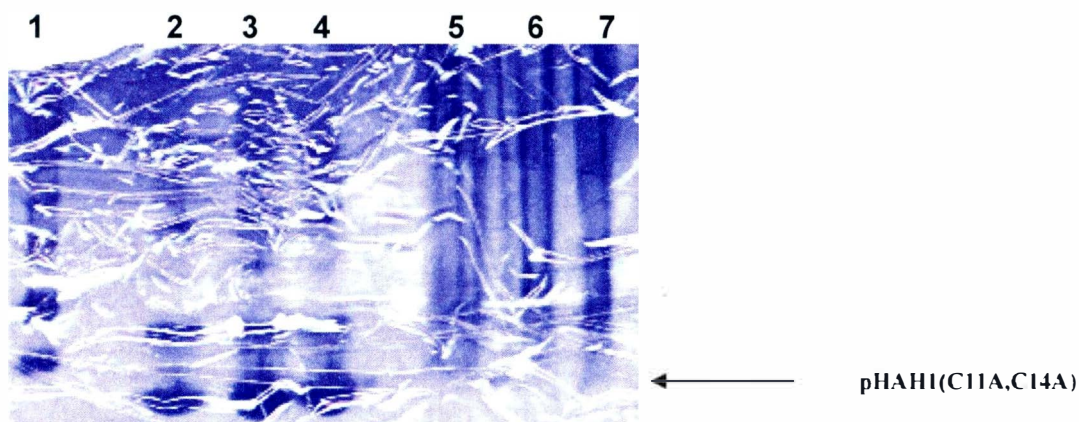
**Figure 16** 10% SDS-PAGE electrophoresis of the small scale induction of the pHAH1(C11A,C14A) gene. M is the marker.

### 3.3.2 pHAH1(C11A,C14A) protein purification

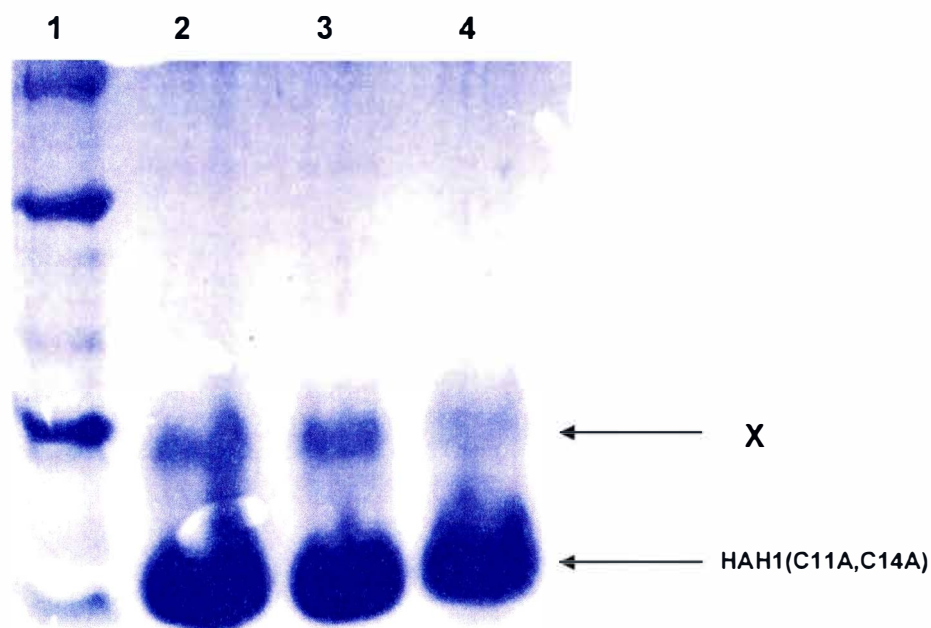
Over expression on a large scale was conducted for pHAH1(C11A,C14A) in BL21(DE3 using a 10 liter LB culture for induction. This was performed using a New Brunswick Microferm® Fermentor. Incubation conditions were set at 37°C with 300 rpm, and 10 psi of aeration. After 2 hr the cultures were induced with 1 mM IPTG when the OD<sub>600</sub> was 0.6. Bacterial pellets were treated to a freeze-thaw extraction to selectively release soluble proteins.



**Figure 17** 10% SDS-PAGE electrophoresis of the 10 L 3 hr induction of the pHAH1(C11A,C14A) showing the bands corresponding with the protein. The first lane is the full range mol wt. protein marker.



**Figure 18** 10% SDS-PAGE electrophoresis showing the freeze-thaw results of pHAH1(C11A,C14A) protein purification. Lanes 2-4 are the supernatant while lanes 5-7 pellet. Lane 1 represents the standard marker (Rainbow).



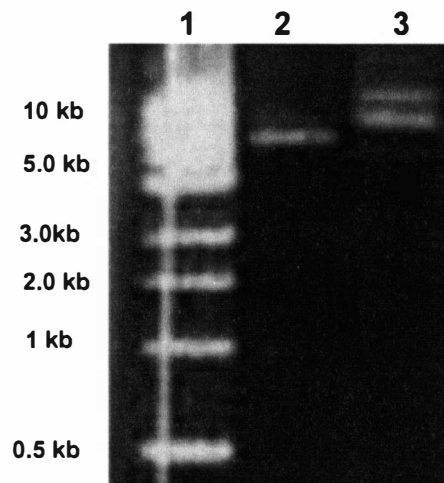
**Figure 19** 10% SDS-PAGE electrophoresis showing the purified pHAH1(C11A,C14A) lanes 2-4. Lane 1 represents the standard marker (Rainbow). The higher molecular bands (X) are due to oxidation induced dimerization.

Expression and purification of the mutated metallochaperone, pHAH1(C11A,C14A) proved to be successful. As predicted, during large scale protein expression, the protein was expressed only after induction (Figure 17). The amount of protein increased with time. Using the freeze-thaw method, pHAH1(C11A,C14A) was extracted from BL21(DE3)-*E. coli* expression cells along with a large amount of unwanted *E. coli* protein.

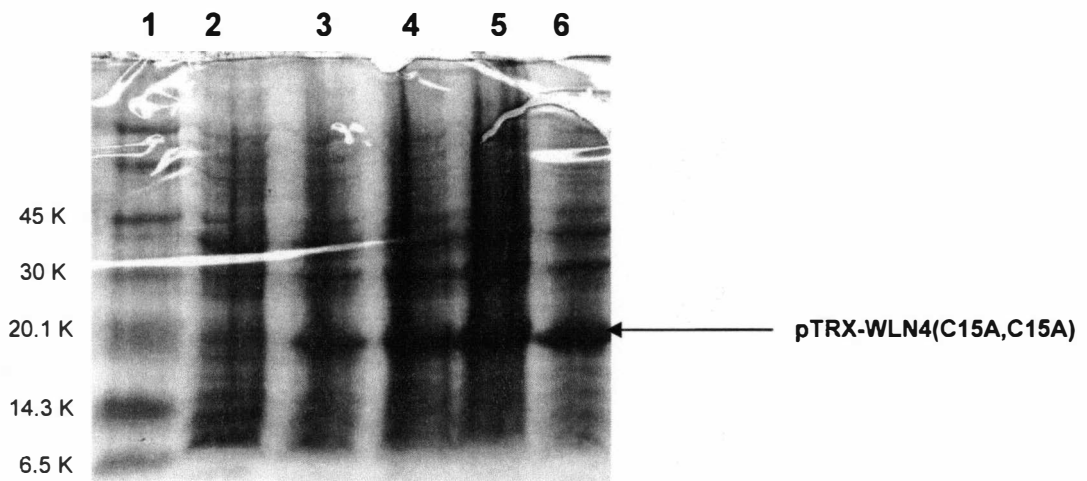
After freeze-thaw extraction, both the supernatant and pellet were analyzed by SDS-PAGE electrophoresis. The pHAH1(C11A,C14A) protein appeared to be soluble because the band was seen in the supernatant (Figure 18). The freeze-thaw extract of the protein pHAH1(C11A,C14A) was further purified with DEAE anion exchange column to isolate it from other contaminating proteins. The protein elution was monitored by the UV absorption profile at 254 nm.

### 3.4 Protein expression of pTRX-WLN4(C15A,C18A)

Site-directed mutagenesis was done to create a double mutant using the single mutant of Wilson Disease protein fourth domain, WLN4(C15A). The resultant plasmid, pTRX-WLN4(C15A,C18A) with two cysteine residues changed to alanine at positions 15 and 18 was transformed into XL-II Gold cells of *E. coli*. The plasmid was then isolated and purified (Figure 21).



**Figure 20** 1% agarose gel electrophoresis of the site-mutagenesis results of pTRX-WLN4(C15A,C18A). Lanes 2 represent the band corresponding to the 7 kb size of WLN4. Lane 3 represents the WLN4(C15A) plasmid template.



**Figure 21** 10% SDS-PAGE electrophoresis showing the initial time-course induction results of the double mutant of the pTRX-WLN4(C15A,C18A). Lane 1 represents the protein marker and Lanes 2-6 represent 4 hr induction.

Agarose gel electrophoresis was used to analyze the site-directed mutagenesis results and a band appeared corresponding to the size of the linearized full-length plasmid of 7 kb. The DNA sequence was confirmed by Retrogen (San Diego, CA).

The pTRX-WLN4(C15A,C18A) plasmid was transformed into Rosetta(DE3) *E. coli* cells and induced for protein expression in LB media containing 100 µg/L ampicillin with 1 mM IPTG and the results analyzed by SDS-PAGE gel (Figure 21).



## CHAPTER IV

### DISCUSSION

To investigate the potential copper-dependent interaction of p62 subunit of the dynactin complex with the Wilson disease protein, attempts were made to clone and express the p62 protein. The first step was to obtain the p62 gene and amplify it with the polymerase chain reaction (PCR). Cloning results were verified by DNA sequencing carried out by Retrogen. A double digestion of the plasmid by restriction enzymes BamHI and NcoI proved that the ligation worked since the mass corresponding to both the p62 DNA (700 bp) and pET 24d vector (5.1 kb) were visible on the agarose gel electrophoresis (Figure 13). The next step was to express the cloned plasmid into *E. coli* cells, Rosetta2 cells. After several expression attempts of the cloned gene the protein seemed not to express upon induction as seen in the SDS-PAGE gel (Figure 15). This could be due to protein aggregation or, during the induction, toxic metabolites of p62 protein are formed which hinder the expression. Another cloning strategy whereby we used a fusion protein to try and enhance the expression of this protein was developed. Ligation independent cloning technique (LIC) is a directional cloning technique that does not utilize use of restriction enzyme digestion. First, the p62 gene was cloned into pET42Ek/LIC which has a fusion glutathione *s*-transferase (GST-tag) and secondly in pET32Xa/LIC which has a thioredoxin fusion tag. The importance of expressing a protein using a fusion protein is to make it more soluble (LaVallie *et al.*, 1993). After obtaining the plasmid (Figure 14), the cloning was verified by the same methods mentioned above, DNA



sequencing and restriction endonuclease digestion. We then expressed the resultant p62 plasmid in *E. coli* Rosetta cells. A time-course induction was carried out and the protein induced with IPTG for protein expression which again was not successful.

Another alternative strategy to express the p62 gene was developed. Yeast expression systems are known to enhance some protein expression which may not express well in *E. coli*. *Pichia pastoris* is a methylotrophic yeast which has been developed into a heterologous protein expression system. *Pichia pastoris* combines many of the benefits of *E. coli* expression with the advantages of expression in a eukaryotic system (e.g. protein processing, folding, and posttranslational modifications). The Pichia Expression System (Invitrogen) has several advantages over other eukaryotic or prokaryotic systems which may include strong constitutive and inducible promoter, high levels of expression and eukaryotic posttranslational modifications (Invitrogen).

*Pichia pastoris* is a strain of yeast capable of metabolizing methanol as the sole carbon source. The first step in the metabolism of methanol is the oxidation of methanol to formaldehyde by the enzyme, alcohol oxidase. Expression of this enzyme, coded for by the AOX1 gene, is tightly regulated and induced by methanol to very high levels—typically >30% of the total soluble protein in the cells. The Pichia Expression System utilizes the AOX1 promoter for inducible expression or the GAP promoter for constitutive expression of the gene of interest (Hohenblum *et al.*, 2004).

We have proposed other alternative cloning strategies to enhance the expression of p62 subunit of the dynactin complex apart from the yeast strategy described above. The first technique we suggest uses a Maltose binding protein fusion technique. Maltose Binding Protein (MBP) forms part of the maltose/maltodextrin

system of *E. coli* bacteria, which is responsible for the uptake and efficient catabolism of maltodextrins (Boos & Shuman, 1998). *E. coli* maltose binding protein (MBP) is used as a fusion partner for recombinant protein expression. Because of its affinity for maltodextrins it allows for facile purification of fusion proteins by amylose affinity chromatography which uses a chemically cross-linked starch (Riggs, 2000).

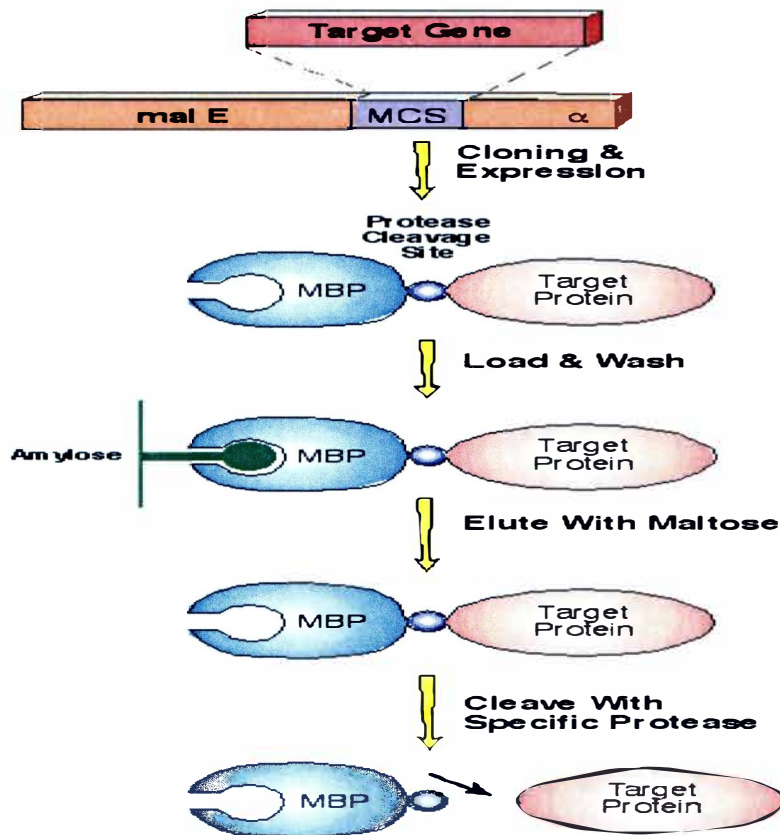
Since p62 protein has been difficult to express on its own may this technique may be used to improve its solubility once it is fused and expressed with MBP (Kapust & Waugh, 1999; Pryor & Leiting, 1997). Previous work done by Kapust and Waugh indicate that MBP is a much better solubilizing agent than either glutathione *S*-transferase (GST) or thioredoxin (TRX) fusion proteins (Kapust & Waugh, 1999). It has been suggested previously that MBP enhances the solubility of aggregation-prone proteins because it possesses chaperone-like qualities which allow it to bind reversibly to folding intermediates of its fusion partners, termed passenger proteins (Fox *et al.*, 2001). This binding temporarily sequesters them in a conformation that prevents their self-association and aggregation (Kapust & Waugh, 1999).

The purified recombinant p62 protein expressed with the MBP will be analyzed by an SDS-PAGE gel then eluted from the amylose column. The protein is then cleaved after elution by treatment with the protease called Factor Xa. Finally the pure protein is removed by passing the mixture over another amylose column where the recombinant protein simply passes through the column without binding.

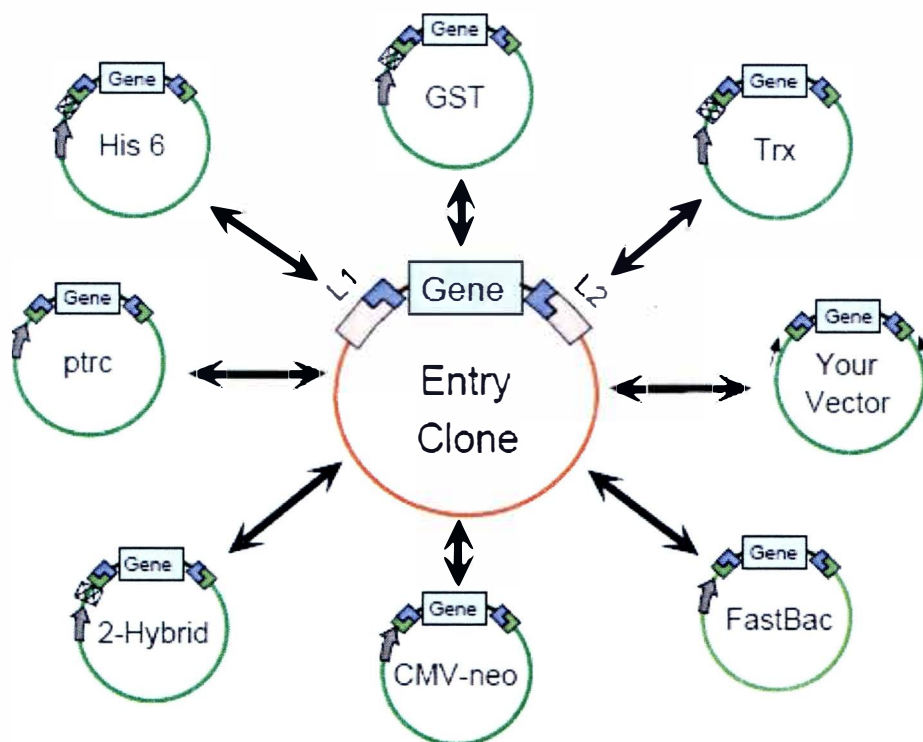
#### 4.1 Gateway cloning technology is another alternative cloning technique

Gateway cloning technology (Invitrogen) is a recent methodology that may represent another cloning strategy to enhance the expression of the p62 protein. It works by facilitating protein expression, cloning of PCR products, and analysis of

gene function by replacing restriction endonucleases and ligase with site specific recombination. The Gateway technology is rapid and utilizes a wide range of cloning vectors besides maintaining the orientation and reading frame of the transferred DNA. The first step involves inserting your gene of interest into the Gateway system by way of an entry clone. Once the entry clone has been formed it is available to express your gene in a variety of other vectors as illustrated in the diagram below (Figure 23).



**Figure 22** The illustration shows how the MBP purification works (BioLabs Inc).

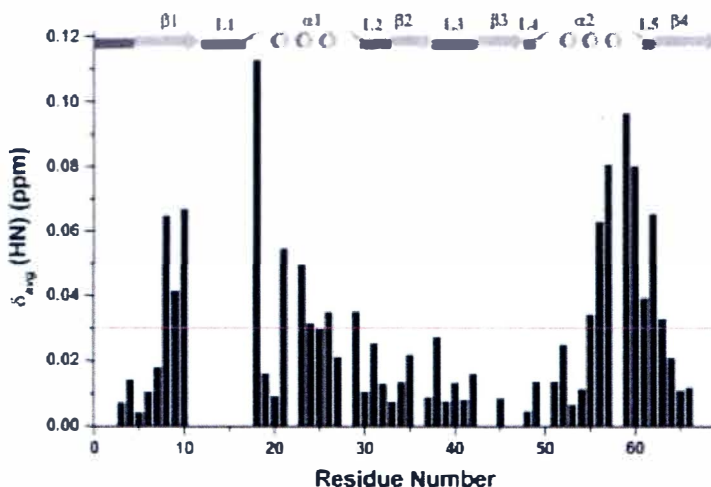


**Figure 23** An illustration of Gateway cloning technique. DNA segment (gene) is transferred from an entry clone into any number of recipient vectors (destination vectors) to generate expression clones.

The sources of your gene could be from a restriction endonuclease digestion and ligation, from a polymerase chain reaction or from a cDNA library. The generated expression clones generated are then transformed into *E. coli* cells for the p62 protein expression.

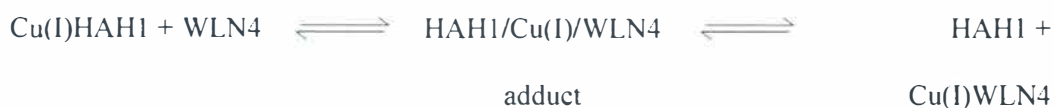
The second phase of this project involved the study of how the Atx1 human homolog (HAH1) interacts with the fourth N-terminal domain of the Wilson disease protein. A single mutant of HAH1(C11A) was obtained from our laboratory. Site-directed mutagenesis was carried out to generate the HAH1 double mutant in which two cysteine residues were mutated to alanine at positions 11 and 14. When

holoCu(I)HAH1 is titrated with apoWLN4, chemical shift changes occur in a number of residues (Figure 24) with the shift changes increasing upon addition of apoWLN4.



**Figure 24** Complex formation between Cu(I)HAH1 and WLN4 with the weighted average chemical shift differences. The secondary structure elements of Cu(I)HAH1 are reported at the top of the graph. The threshold is indicated with a horizontal red line. (Achila *et al.*, 2006).

During the copper transfer between HAH1 and WLN4 a complex is formed between the two as shown in the illustration below (Achila *et al.*, 2006). The CxxC→AxxA mutant of each protein should not form this complex, since a thiol is required for complex formation (Banci *et al.*, 2006).



**Figure 25** Complex formation between Cu(I)HAH1 and WLN4.

In native forms HAH1 forms a complex with WLN4 that allows the transfer of copper between these partners. This is due to the CxxC motif of the cysteine residues present in both. By mutating the two cysteines into alanine copper exchange is not possible between the two.

This double mutant was used to serve as a negative control of the copper transfer studies between the two proteins HAH1 and WLN4. The double mutant plasmid was confirmed by DNA sequencing. The plasmid was then expressed in BL21(DE3) *E. coli* cells (Novagen) with a time-course induction of pHAH1(C11A,C14A) in 1 mM IPTG.. The expression was successful. During the induction with IPTG, T7 polymerase then translates the mutated gene. BL21(DE3) cells were used because they contain chromosomal copy of the T7 RNA polymerase. SDS-PAGE gel, results show the induction was successful. A band is seen to intensify with time of induction up to 4 hrs (Figures 16-17).

To purify the pHAH1(C11A,C14A) a freezing and thawing cycle was carried out and after analysis using SDS-PAGE. The protein was soluble and easily extracted as it appeared to be in the supernatant (Figure 18). Mutating two cysteine residues of WLN4 at positions 15 and 18 was made possible by site-directed mutagenesis of the single mutant WLN4(C15A). The double mutant gene pTRX-WLN4(C15A,C18A) was verified by DNA sequencing and the alignment confirmed by the DNA Strider program in our laboratory. We then expressed the plasmid in Rosetta2(DE3) *E. coli* cells (Figure 21).

## CHAPTER V

### CONCLUSION

The Wilson disease protein interacts with other proteins, p62 subunit of the dynactin complex and HAH1 in a copper-dependent manner that requires cysteine residues of CxxC motif (Larin *et al.*, 1999; Lim *et al.*, 2006). The aim of this study was to clone purify and characterize p62 subunit of the dynactin complex and study its copper interaction with the N-terminal domains of the Wilson disease protein. We also aimed at generating double mutants of both HAH1 human ATX1 homolog and the fourth domain of Wilson disease protein (WLN4) by mutating two cysteine residues into alanine

We have cloned p62 into pET24d, pET41Ek/LIC and pET32Xa/LIC. We have successfully cloned, over expressed and purified pHAH1(C11A,C14A) and pTRX-WLN4(C15A,C18A). The copper interactions studies will help enhance the understanding of the best-known disorders of copper homeostasis, Wilson's disease and Menkes disease, and avail more information on the molecular mechanisms that control the distribution of copper in living cells.

We propose alternative cloning strategies for the p62 protein, such as the yeast two-hybrid expression using *Pichia pastoris*. Another proposed alternative expression technique for the p62 subunit of the dynactin complex includes the use of the Maltose binding protein fusion and also the Gateway cloning technology to enhance expression of p62 in *E. coli*.

Once the p62 protein has been expressed and purified we, intend to determine which amino acid residues are responsible for this copper-dependent interaction. We have proposed various analytical techniques including that will help achieve this goal.

These will include NMR titrations; the copper environment of p62 will be explored by metal-binding studies, XANES, and EXAFS experiments.



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