CHAPTER 2

REVIEW:
THE INVOLVEMENT OF METALLOTHIONEINS IN MITOCHONDRIAL FUNCTION AND DISEASE

2.1 INTRODUCTION

Metallothioneins (MTs) - which are small, metal binding proteins found in all eukaryotes, many prokaryotes and plants (Freisinger, 2008) - have been studied for more than 50 years. MTs have made a great number of appearances in numerous fields of study, ranging from disease to environmental (toxicological) studies (Cherian *et al*., 2003; Klaassen *et al*., 2009). Despite half a century of research, the main biological role of MTs remains unresolved. The only consensus among researchers is the diverse functionality of MTs and their involvement in a diversity of intracellular processes. In order to elucidate the main biological role of MTs, many turned their focus to the factors and chemicals that induce MT expression (Davis & Cousins, 2000). However, the list of factors and chemicals that induce MT expression is ever growing. For this reason MTs have been called '*multipurpose stress proteins*' (Andrews, 2000; Theocharis *et al*., 2003). The involvement of MTs in the function of specific organelles and cellular processes has been studied extensively and has been well reviewed (Cherian *et al*., 2003; Coyle *et al*., 2002; Giaconni *et al*., 2003; Inoue *et al*., 2009). However, a fundamental overview of the involvement of MTs with one of the most important organelles in the cell, the mitochondrion, has been lacking despite convincing evidence published over the last decade. The mitochondrion is the main production centre of energy in many eukaryotic cells and accommodate many metabolic processes, of which oxidative phosphorylation is the most commonly associated with this organelle.

However, the association of MTs with the mitochondrion is not a distinct one, as has become a common feature when evaluating the roles of MTs in general. Although MTs are known to play a part in metal homeostasis, they are also known to effectively scavenge reactive oxygen species (ROS) (Sato & Kondoh, 2002; Vallee, 1995; Vašák, 2005). Thus, not only are MTs potential chaperones for metal ions to certain enzymes in the mitochondrion, they also have the ability to function as free radical scavengers for physiologically or pathologically produced ROS. This review will summarize findings that indicate the involvement of MTs in key functions of the mitochondrion and will highlight newly formed concepts. It will be structured by presenting a brief overview of

mitochondrial function and disease, followed by structural and functional evaluation of MTs and their established or putative associations with mitochondrial functions and disease pathology.

2.2 MITOCHONDRION FUNCTION AND DISEASE

2.2.1 BIOLOGICAL IMPORTANCE AND FUNCTION

Mitochondria are complex and dynamic membrane enclosed organelles found in most eukaryotic cells, with the primary function to generate the bulk of cellular ATP. This occurs by means of oxidative phosphorylation (OXPHOS), which is precluded by the production of reducing equivalents (NADH and $FADH_2$) from carbohydrates, fatty- or amino acids. ATP production (OXPHOS) is essentially driven by the four enzyme complexes (complex I - IV), collectively called the electron transport chain (ETC), and finally produced by ATP synthase (complex V), all of which are located on the inner mitochondrial membrane (IMM). Electrons enter from NADH via complex I, or from succinate via complex II, and are transferred to ubiquinone. Complex III transports electrons from reduced ubiquinone to cytochrome *c*, and complex IV carries electrons from cytochrome *c* to oxygen. The transport of electrons is coupled to translocation of protons across the IMM into the inter-membrane space (IMS), creating a trans-membrane proton gradient that provides the driving force for ATP production by complex V. Apart from being the “power stations” of the cell, mitochondria are also involved in numerous metabolic processes including amino acid biosynthesis, lipid metabolism (both catabolic and anabolic), iron-sulphur clusters metabolism, heme biosynthesis, metal metabolism, calcium signalling, programmed cell death (apoptosis) and many other cell signalling pathways (McBride *et al*., 2006).

An important aspect to recognize is mitochondrial genetics, its effect on the function of the organelle, as well as its interplay with the nuclear genome (Reinecke *et al.,* 2009). Although each mitochondrion has several copies of its own maternally inherited circular DNA (mtDNA) as well as replication, transcription and translation machinery, these functions operate only semi-autonomously, as the majority of the factors are nuclear encoded and have to be transported into the mitochondria (Asin-Cayuela & Gustafsson, 2007). On average the human mitochondrion contains 5-10 mtDNA molecules which are 16,569 base pairs long. Each mtDNA molecule encodes 37 genes which include 2 ribosomal RNAs, 22 transfer RNAs and 13 polypeptides (Anderson *et al*., 1981) which are localised to the OXPHOS system. It is predicted that, in addition to the majority of the OXPHOS subunits, there may be as many as 1500 nuclear-encoded mitochondrial proteins (Lopez *et al*., 2000; Mokranjac & Neupert, 2009; Pagliarini *et al*., 2008), although many of these proteins have not been functionally characterized.

Interaction with the cytosol and other organelles requires a large number of carriers, transporters and channels in the two membranes. An example is the ADP–ATP carrier (ANT), which transports roughly 50 – 60 kg of ATP per day across the inner membrane of mitochondria in a human body (Reichert & Neupert, 2004). Coordination of protein biosynthesis in mitochondrial and cytosolic ribosomes, regulation of nuclear genes encoding mitochondrial proteins and regulation of protein import into mitochondria are still only vaguely understood. In order to gain better insight into the functional networks of mitochondrial proteins, the complete set must be known and therefore, mitochondrial proteomics or "mitochondriomics" is becoming a main research focus. Several studies done in recent years have addressed this aspect from different angles and resulted in the creation of different mitochondrial proteome databases to record and reveal all findings, such as MitoProteome (Cotter *et al.,* 2004), MitoP2 (Andreoli *et al.,* 2004), MitoCarta (Pagliarini *et al*., 2008), etc. However, at this stage the majority of mitochondrial proteins and their functions in the mitochondrion are still being investigated.

2.2.2 MITOCHONDRIAL DISORDERS AND OXIDATIVE STRESS

Mitochondrial disorders (or diseases) are mostly a reference to a deficiency of the OXPHOS system (Naviaux, 2004). It can, however, also result from a deficiency of other key enzymes and proteins involved in primary mitochondrial functions, or as a secondary consequence due to related pathologies, or chemically induced. The involvement of mitochondrial deficiency in degenerative diseases and ageing is a widely accepted paradigm (Wallace, 2001). The disease phenotypes associated with mitochondrial dysfunction are highly diverse and the amount of literature describing its involvement - albeit often disputing the mechanism - in human disease is overwhelming. These phenotypes include LHON, MELAS, MERRF, CPEO, Kearns-Sayre, Pearson or Leigh syndromes but also various other better known and late-onset phenotypes, including cardiovascular diseases, neurodegenerative diseases such as Alzheimer's and Parkinson's diseases, diabetes, cancer, ageing and AIDS and its treatment (Balaban *et al*., 2005; Ballinger, 2005; Fukui & Moraes, 2008; Gerschenson & Brinkman, 2004; Modica-Napolitano & Singh, 2004; Naviaux, 2004; Rolo & Palmeira, 2006).

The primary initiator of consequences of OXPHOS deficiencies, in addition to other immediate and downstream effects (reviewed by Reinecke *et al.,* 2009) is the ineffective transfer of electrons through the various subunits of the electron transport chain (complexes I – IV) and carriers (ubiquinone, cytochrome *c*) through the IMM. This leads to the accumulation of electrons and excessive leaking to molecular oxygen (O_2) to produce superoxide anion radicals ($O_{2^{\cdot}}^{-}$) which is quickly dismutated by superoxide dismutases to hydrogen peroxide (H_2O_2) (Boveris & Cadenas,

1975). Mitochondria are the main producers of superoxide and depending on the amount and source of reducing equivalents, electrons may leak from the electron transport chain at complex I and III to produce superoxide (Boveris & Chance, 1973; Liu *et al*., 2002; Turrens & Boveris, 1980) as well as from α-ketoglutarate dehydrogenase (Starkov *et al*., 2004) or complex II (Guzy *et al*., 2008). Initial *in vitro* estimations that approximately 1 – 2 % of oxygen consumed is converted to ROS under steady state levels appear to be exaggerated, and it is reported to be closer to 0.2 % *in vivo* (Staniek & Nohl, 2000; St-Pierre *et al*., 2002), which will increase under stressed conditions.

The formation and damaging effects of ROS and reactive nitrogen species (RNS) on DNA, proteins and lipids have been extensively documented (Dröge, 2002; Jones, 2008). In particular, the so-called "vicious cycle", associating increased ROS, higher mtDNA mutation rate and reduction of OXPHOS function, is widely documented, especially in *in vitro* systems (Indo *et al*., 2007). The mechanisms and consequences of putative ROS-mediated damage to mtDNA *in vivo* and in particular to what extent it contributes to mtDNA point mutations and rearrangements during diseased states and ageing is still debated (Fukui & Moraes, 2008). It is thus also doubtful if ROS has a significant effect on mtDNA under normal physiological *in vivo* conditions, but may hold significant implications to age-related degenerative diseases.

Under normal non-pathological conditions, the damaging effect of ROS is neutralized by a specialized endogenous system consisting of enzymatic, non-enzymatic- and repair components. Several proteins and metabolites, such as superoxide dismutase, glutathione peroxidase, glutathione, vitamins and other antioxidants, assist in modulating the redox balance and protection against excessive ROS formation in this highly charged environment (Cooke *et al*., 2003; Iszard, *et al*., 1995; You *et al*., 2002). As defined by Cutler *et al*. (2005), oxidative stress refers to this steady state of redox balance and its consequences, whereas increased oxidative stress results from excessive and uncontrolled ROS formation and oxidative damage. ROS, along with other regulators such as Ca^{2+} and NAD redox state, modulates expression of many of the above mentioned proteins. There is also a response in key cellular processes, such as apoptosis, to the effects of increased oxidative stress (Majima *et al.*, 1998; Reinecke *et al*., 2009). In recent years with the advent of systems biology tools, the differential expression of many other proteins has been identified to occur during mitochondrial dysfunction, including the MTs (Reinecke *et al*., 2006; van der Westhuizen *et al*., 2003). Although not recognized to be part of the mitochondrial proteome, MTs have been associated with protection against oxidative damage as well as apoptosis progression and metal ion homeostasis, all of which are downstream consequences of mitochondrial dysfunction and disorders.

2.3 METALLOTHIONEINS

2.3.1 GENERAL PROPERTIES OF METALLOTHIONEINS

MTs are small, intracellular, non-enzymatic proteins which are 61 - 68 amino acids in length with a molecular weight of 6 - 7 kDa (Coyle *et al*., 2002). MTs have high cysteine content (about 30 %) and lack aromatic amino acids (Bremner & Davies, 1975; Hunziker & Kagi, 1985; Stillman, 1995). This high sulphur content gives this protein the ability to bind 7 - 12 metal ions (depending on the valence of the metals) and hence the name – "metallothionein". The binding of different metals to the protein is what defines its tertiary structure (Rigby & Stillman, 2004) and possibly its localisation and function (Brouwer *et al*., 1992; Mididoddi *et al*., 1996; Pederson *et al*., 1998). Despite the slight differences in tertiary structure, the protein always forms two definite globular domains giving it a dumbbell-like shape (Merrifield *et al*., 2002; Romero-Isart & Vašák, 2002; Winge & Milkossy, 1982). The cysteine residues in this protein form characteristic Cys-Xaa-Cys, Cys-Xaa-Xaa-Cys and Cys-Cys sequences, where Xaa is any non-cysteine amino acid (Romero-Isart & Vašák, 2002; Stillman, 1995). These cysteine residues are externally orientated and thus exposed to the surroundings to rapidly scavenge metal ions and oxygen radicals (Rigby & Stillman, 2004). The external orientation of the cysteine residues also prevents the spontaneous formation of disulphide bridges under physiological conditions and thus save the cysteine clusters for metal binding (Rigby & Stillman, 2004). MTs can however be easily oxidized in non-physiological conditions where high levels of oxygen are available (Minkel *et al.,* 1980).

The metallothionein family consists of four major isoforms, MT-1 to MT-4, with most organisms expressing at least the first two isoforms (Moffatt & Denizeau, 1997). MT-1 and MT-2 are expressed in all major organs, especially the liver, pancreas, intestine, kidneys and the brain (Davis & Cousins, 2000). The MT-2 isoform is predominantly expressed and can account for more than 80 % of all human MT expression (Cherian *et al.*, 2003; Studer *et al*., 1987; Tan *et al*., 2005). MT-3 has seven additional amino acids to its primary structure and is mainly expressed in the brain and central nervous system (Palmiter *et al*., 1992; Yamada *et al.,* 1996). Expression of MT-3 is mostly regulated in opposite direction to MT-1 and MT-2 when exposed to the same stimulus (Carrasco *et al*., 2006; Kramer *et al*., 1996; Kim *et al*., 2003). MT-4 is the most recently discovered isoform and is expressed mainly in epithelial cells of the skin, tongue and intestinal lining (Quaife *et al*., 1994). Though these isoforms are highly homologous and share similar functional properties, there are still reported differences. However, due to the lack of pure standards and specific detection techniques, it is hard to define and distinguish between the different isoforms and their respective functions as often seen in reports. Hence, the collective term, MTs, is used which does

not discriminate between the isoforms. In cases where the specific isoforms or state of the proteins used is known these will be given in detail.

2.3.2 BIOLOGICAL IMPORTANCE AND FUNCTIONS OF METALLOTHIONEINS

MTs have generally been accepted as multifunctional stress proteins which have protective effects against various stressors (Andrews, 2000; Theocharis *et al*., 2003). Numerous reports have shown that MT expression is induced by various stressors ranging from chemicals to environmental factors, including radiation (Andrews, 2000; Coyle *et al*., 2002; Davis & Cousins, 2000; Ghoshal & Jacob, 2000; Haq *et al*., 2003; Jacob *et al*., 1999; Suzuki *et al*., 2005). From these inducers, those most studied and most relevant to this article are ROS and metals. MT expression is regulated via *cis*-acting metal responsive elements (MREs) and an antioxidant response element (ARE) in the proximal MT promoter which are responsive to a wide range of effectors, including ROS (Andrews, 2000; Ghoshal & Jacob, 2000; Haq *et al*., 2003). The addition of metals (especially heavy metals such as cadmium) to cell cultures result in increased expression of MTs which in return bind these added metals (Moltó *et al*., 2007). This also happens *in vivo* as demonstrated by Bremner & Davies (1975) which injected male Hooded Lister rats with zinc and isolated the zinc bound MTs from the livers. The addition of free radical originators, such as H_2O_2, to mouse hepacotyces, rapidly induces MT-1 expression in a dose dependent manner (Andrews, 2000). Reinecke *et al*. (2006) confirmed the increased expression of MTs in HeLa cells treated with rotenone, a respiratory chain complex I inhibitor, and *tert*-butylhydroperoxide (*t*-BHP), both of which result in increased ROS levels.

The induction of MT expression by increased levels of metals or ROS results in a negative-feedback response. As the MTs increase, they bind the excess metals or react with the free radicals to consequently decrease the (harmful) metals or ROS levels (Cattani *et al*., 1996; Min *et al*., 2005). Therefore, increased MT expression results in the protection of the cell and/or body against these stressors (Klaassen *et al*., 2009). In most studied models, MT over-expression is induced within one hour, which is sustained for up to 24 hours after the stimulus/ stressor (Cattani *et al*., 1996; Suzuki *et al*., 2005). Therefore, MTs can remain present and active in cellular functions long after the stressor (Simpkins *et al*., 1998b).

MTs have been the focus of many research reports with extensive attention to their protective effects in various situations. These reports claim that MTs protect against general stress-related conditions (Hernándes *et al*., 2000; Hidalgo *et al*., 1990; Jiang *et al*., 2005), neurological diseases (Carrasco *et al.,* 2006; Hidalgo *et al*., 2001; Yu *et al*., 2001), mitochondrial dysfunction (Reinecke

et al., 2006), xenobiotics (Coyle *et al*., 2002) and also participate in cell growth, proliferation and differentiation (Studer *et al*., 1997; Tan *et al*., 2005; Włostowski, 1993), to mention only a few examples. These diverse functions of MTs (sometimes referred to as 'secondary functions') are due to their metal-thiolate clusters (Vašák, 2005) and are considered as the macro level result (phenotype) from the work MTs do on the micro level. This work is a combination of the three primary functions or abilities of MTs, which are i) metal (zinc and copper) homeostasis, ii) detoxification of toxic heavy metals such as cadmium, mercury and silver, and iii) protection against oxidative damage by acting as a free radical scavenger. It must be noted that the use of these abilities might vary between MT isoforms (Brouwer *et al.,* 1992; Mididoddi *et al.,* 1996; Pederson *et al.,* 1998). This especially applies to MT-3, which is also known as a neuronal growth inhibitory factor and appears to function in a clearly different manner than MT-1 and MT-2 (Carrasco *et al.,* 2006; Kramer *et al.,* 1996).

2.3.2.1 Metal homeostasis

MTs have a strong affinity towards metals and consequently play an important role in metal regulation in the body. Zinc and copper is physiologically the dominant metals bound to MTs (Bühler & Kagi, 1974; Stillman, 1995), hence it is hard to distinguish between free zinc and MTs, and between their abilities/ functions. For this reason, some researchers demand better discrimination between MTs, zinc and other contributing factors. Although this is a relevant point in some sense and in certain situations, there is a general feeling that both should be studied together as they are hardly ever alone. The free zinc level in cells is very low (nM to pM range) as most of the zinc is bound to low molecular weight zinc-ligands such as MTs (Atar *et al.,* 1995; Jiang *et al.,* 1998; Maret, 2008; Outten & O'Halloran, 2001) There are also physiologically only small amounts of metal-free MTs (thioneins) in the cell (Krezel & Maret, 2007; Ma, 2005), and therefore when the term MTs is used, it refers to the metal-bound protein. Physiologically, this is Zn_7MT most of the time and secondly, Cu_{12}MT (Stillman, 1995).

MTs bind zinc with high thermodynamic stability, which is in contrast to other zinc proteins (Jiang *et al.,* 1998; Maret *et al.,* 1999). Despite this high thermodynamic stability, MTs can still participate in zinc exchange reactions which are distinctive of this protein and its involvement in zinc homeostasis. Jiang *et al.* (1998) summarized this property well: "...in MT *the protein* plays a role in the biological function of *zinc*, a paradigm quite different from that in most other zinc proteins where *zinc* plays a role in the biological function *of the protein*". MTs are responsible for zinc and copper uptake, intra- and intercellular distribution, release (when needed by enzymes) and, to a lesser extent, storage (Feng *et al.,* 2005; Maret, 2008; Rigby Duncan & Stillman, 2006; Vasák &

Hasler, 2000). Few researchers proposed that the transfer (release) of metals from MTs to other acceptor molecules require the assistance of a modulator, seeing that MTs bind these metals with higher thermodynamic stability than normal metalloenzymes (Jacob *et al.,* 1998; Jiang *et al.,* 1998). Despite this, the release of metal ions to numerous enzymes has been demonstrated, even without the assistance of these putative modulators (Feng *et al.,* 2005) which suggest protein-protein interaction and subsequently conformational changes. In addition, Jacob *et al*. (1998) found that thioneins do not remove metal ions from the catalytic site of enzymes even though it is seen as a very effective chelating agent. Therefore, when MT expression is induced, there will be minimum interference with metal ion-dependent enzymatic processes from the newly translated thioneins (Jacob *et al.,* 1998). When taking into account that induction of MT expression is mostly triggered by increased metals or ROS, this view is confirmed, as the over-expressed thioneins will react with ROS or bind the excess metals and thus not remove metals from other sources.

This ability to regulate cellular metal homeostasis also implies that MTs regulate some enzymes and transcription factors dependent on these metals and their subsequent roles in metabolism (Maret *et al.,* 1999) and DNA transcription (Cano-Gauci *et al.,* 1996; Cherian *et al.,* 2003). At least 300 enzymes in all six enzyme classes are dependent on metals such as zinc and copper (Coyle *et al.,* 2002; Vašák, 2005) and during time of need, MTs release the metal ions to the enzymes (Feng *et al.,* 2005; Vasák & Hasler, 2000). MTs also donate or accept zinc from zinc-finger transcription factors and play a role in some transcriptional responses (Cherian *et al.,* 2003; Hathout *et al.,* 2001; Vašák, 2005).

2.3.2.2 Heavy metal detoxification

The metals bound to the MTs under physiological conditions (zinc and copper) are replaced during conditions of heavy metal overdose. This is because the affinity of MTs for heavy metals, such as Hg(II), Ag(I) and Cd(II), is much stronger (Romero-Isart & Vasák, 2002; Sato & Kondoh, 2002; Stillman, 1995). Because of the higher affinity of MTs toward toxic metals, many researchers proposes that the main biological function of MTs is the detoxification of heavy metals (Kang, 1999; Stillman, 1995; Vallee, 1995) as can be seen in aquatic animals during heavy metal pollution. MTs thus protect against heavy metal toxicity (Alhama *et al.,* 2006; Vašák, 2005) such as hepatotoxicity, nephrotoxicity, hematotoxicity, immunotoxicity and bone damage (Nordberg *et al.,* 2000). Over the years, a lot of attention has been focused on the induction, binding and measurement of MTs with cadmium, as recently reviewed by Klaassen *et al*. (2009).

2.3.2.3 Free radical scavenging

Numerous cell-free *in vitro* studies on MTs have led to the discovery that MTs act as free radical scavengers for many ROS such as hydrogen peroxide, superoxide, nitric oxide ($NO^{\bullet}$) and hydroxyl ($OH^{\bullet}$) radicals, and therefore protection against the oxidative effects of these radicals (Choi, 2003; Hussain *et al.,* 1996; Kang, 1999; Quesada *et al.,* 1996). It was shown *in vitro* that MTs suppress DNA damage and the formation of 8-hydroxy-2'-deoxyguanosine in the presence of hydroxyl-radicals (Abel & de Ruiter, 1989; Min *et al.,* 2005). Since these initial discoveries, the protective role of MTs against oxidative stress has been confirmed *in vitro* with cell cultures (Lazo *et al.,* 1995; Reinecke *et al.,* 2006; You *et al.,* 2002) as well as *in vivo* (Kang *et al.,* 1997; Suzuki *et al.,* 2005; Zhou *et al.,* 2002). Transgenic mice over-expressing MTs in the heart had reduced ultra-structural changes in the nucleus after doxorubicin (DOX) treatment (Zhou & Kang 2000). Notably, it was observed in *in vitro* studies that MTs are extra-ordinarily effective free radical scavengers which can scavenge $OH^{\bullet}$ about 300 times more effectively than glutathione (GSH) (Abel & de Ruiter, 1989; Hussain *et al.,* 1996; Min *et al.,* 2005) - which is a primary scavenger of cellular ROS. Although this observation suggests that MTs can protect the cell more effectively against ROS when compared to GSH, it would depend on factors such as cellular localisation, concentration and thiol redox/ metal-bound state. The interplay between these two molecules will be discussed in more detail in Section 2.4.3.

2.3.2.4 Free radical scavenging mechanisms

Although the scavenging role of MTs is widely accepted, the mechanism by which MTs scavenge free radicals and thus act as antioxidants remains inconclusive due to certain factors. In particular, it is often believed that the scavenging ability of MTs is dependent on the metals bound to the protein. This is, however, not the case. Rather, MTs can scavenge free radicals in the thionein form (without the help of metal ions) through oxidation of its various thiol groups (Conrad *et al.,* 2000). It was shown that MTs react *directly* with superoxide anion (Hussain *et al.,* 1996), hydroxyl radical (Kang *et al.,* 1997) and hypochlorous acid (Fliss & Menard, 1992). Maret (2008) states that it is remarkable that MTs are redox proteins while other zinc proteins are considered as redox-inert because of the fact that the zinc ions always remain in the Zn(II) valence state which is not redox active in biology. It was also shown in zinc-resistant HeLa cells that thioneins protect the cells more efficiently against oxidative stress than zinc-bound MTs (Chimienti *et al.,* 2001). Therefore, it seems that MTs function as antioxidants most effectively in thionein form (without the help of metal ions).

When the protein is exposed to oxidizing agents, the SH groups are oxidized to form intra-molecular disulfide bridges, which then results in the release of the metal ions. When this happens, the structure of the protein curls and tightens (Guo *et al.,* 2005). Sometimes these disulfide bridges occur between two proteins (intermolecular) to form dimers (Hathout *et al.,* 2002). S-nitrosylation and S-glutathionylation can also lead to polymerization of MTs (Casadei *et al.,* 2008). However, there is no clear evidence that polymerization occurs *in vivo*. The oxidation of MTs in the body has no known adverse effects except for increasing free zinc levels which can have inhibitory effects on various enzymes (Maret *et al.,* 1999). Oxidized MTs (thionins) can be "restored" to thioneins by reduction agents within the cell which probably result in the binding of free metals in their direct environment. Therefore, unlike other proteins that are targeted for destruction when oxidized, or are irreversibly inactivated, MTs can buffer the dangers of free radicals and regain their original function once they are reduced (by GSH for example).

2.3.2.5 MT research models and current trends

Most of the functions attributed to MTs in vertebrates, as well as their roles in diseases, have been investigated by animal, cell culture and *in vitro* models. These include the development of gene knockout and transgenic mice for MTs (Erickson *et al.,* 1997; Klaassen & Lui, 1998; Masters *et al.,* 1994; Mickalska & Choo, 1993), MT gene knockdown (Lim *et al.,* 2009) or transformation-induced MT-null cell lines (Kondo *et al.,* 1999), as well as over-expression of MTs in mice (Erickson *et al.,* 1995; Kang *et al.,* 1997) or cell lines (Reinecke *et al.,* 2006). One problem with models that use induction of MTs is that the induction is non-specific and many of the protective effects seen can be associated with other induced cellular processes, notably antioxidant defence (Iszard *et al.,* 1995; Kang *et al.,* 1997). The antioxidant role that MTs play in these models in particular is therefore difficult to position within the existing cellular defence mechanisms. In most of these initial studies, investigations focus on specific targeted cell biological functions of expressed MTs or consequences of its deficiencies. More recently, approaches using systems biology tools such as micro-arrays have revealed novel insights but also the complexities of the role of MTs *in vivo* (Liu *et al.,* 2002; Miura & Koizumi, 2005; Penkowa *et al.,* 2006). It is clear from the diverse biological roles of MTs and their specific functions in different tissues, which is specifically evident in neurological tissue, that a systems biology approach in well-defined animal or *in vitro* models is imperative in order to better explore the role of MTs in the future. When evaluating the role of MTs in a cellular process with diverse consequences such as energy metabolism, this approach becomes all the more essential.

2.3.3 METALLOTHIONEIN LOCALIZATION

Considering the structure, size and amino acid composition of MTs, they can be considered cytoplasmic proteins, especially as they do not contain nuclear localization signals (NLS) or any other organelle targeting sequences. However, intracellular localization of MTs is as dynamic and complex as their functions are. Nuclear localization has been observed for MTs in a wide range of cells. The general size of MTs is below the size exclusion limit of the nuclear pore complex (NPC) that transports proteins via an active, signal-mediated process (Schmidt-Zachmann & Nigg, 1993). Evidence suggests that MTs, like other small molecules of similar size, might have nuclear access by means of passive diffusion through the aqueous pore which is also part of the NPC (Breeuwer & Goldfarb, 1990). However, the distinct MT sequestration patterns observed suggests some active and regulated process for its intracellular redistribution. For example, nuclear MT localization has been observed in foetal and newborn tissues of different organisms (Andrews *et al.,* 1987; Templeton *et al.,* 1985) with further redistribution to the cytoplasm within a few weeks post-partum (Cherian *et al.,* 1987; Panemangalore *et al.,* 1983; Włostowski, 1992). The nuclear localization has also been observed in human tissues characterized by rapid proliferation, especially in malignant tissues (Woo *et al.,* 1996). Moreover, nuclear-cytoplasmic MT redistribution is reported to occur in response to proliferation stimulators and also during the different phases of the cell cycle (Nagel & Vallee, 1995; Nartey *et al.,* 1987; Tsujikawa *et al.*, 1991).

Several mechanisms have been proposed for the import and retention of MTs in the nucleus. The nuclear retention of MTs has been suggested to be ATP-dependent while nuclear import requires the participation of cytosolic factors and GTPases (Nagano *et al.,* 2000; Woo *et al.,* 2000). The need for nuclear localized MTs under certain conditions may be related to the increased requirement for zinc by some metalloenzymes and transcription factors during rapid cell growth. In addition, nuclear MTs can protect cells from oxidative stress related DNA damage and apoptosis, as well as regulate gene expression during the different stages of the cell cycle (Cherian & Apostolova, 2000; Zhou & Kang 2000). However, some of the main functions associated with MTs, such as ROS scavenging and protection against apoptosis, relate more to mitochondrial than nuclear function, where metal (notably zinc) homeostasis may be considered to be a crucial function. Also, considering that the greatest source of ROS is the mitochondrion and the relatively poor diffusion properties of highly reactive ROS, it would appear sensible that MTs, as most other endogenous antioxidant elements, should also be close to this ROS production site if they are to provide an effective scavenge contribution.

Copper-bound MTs, were initially isolated from the mitochondrial fraction from the liver of newborns, and were identified as mitochondrocupreins. However, the similarities between mitochondrocupreins and MTs soon suggested that they were in fact the same protein (Rupp & Weser, 1979). The mitochondrial localization of MTs was later shown in rat liver by radioimmunoassay (Sakuria *et al.,* 1993). Subsequent studies also revealed that MTs localize to the mitochondrial IMS (Moltó *et al.,* 2007; Ye *et al.,* 2001). Since the outer membrane of the mitochondria have a ~10 kDa cut-off, it is reasonable to say that the ~7 kDa MT protein can cross the membrane freely into the IMS (Ye *et al.,* 2001). However, other entry methods are also suggested by Molto *et al*. (2007) referring to recent data from Mesecke *et al*. (2005). They suggest that MTs might be imported into the IMS through the outer membrane import and assembly machinery (MIA), which is also possibly responsible for the import of apo-cytochrome *c* and SOD1. According to Mesecke *et al*. (2005) this import pathway recognizes proteins that contain repetitive Cys motifs, which then import them via a cascade of disulfide bindings that are formed during the import process. Although this also seems a feasible explanation of entry, Molto *et al*. (2007) later question this entry method as MTs need to keep their conformation, instead of releasing all the metals, in order to have any chaperone functions. It is also suggested that MTs might follow the same import mechanism as Cox17 which also lack a mitochondrial targeting sequence and are structurally very similar to MTs (Ye *et al.,* 2001). These entry pathways seem more acceptable when considering the fact that MTs are not localised in heart mitochondria, even in MT-over-expressing transgenic mice cells, which suggest a more controlled mechanism instead of merely random diffusion (Zhou & Kang 2000).

We recently investigated the involvement of MTs in mitochondrial dysfunction in HeLa cells (Reinecke *et al.,* 2006). Previously unpublished data from this investigation reveal that MT-1B, but not MT-2A, localize exclusively to mitochondria in MT-over-expressing HeLa cells with or without any stressor interventions (Figure 2.1). All the data given allow us to consider MTs not only to be cytoplasmic and nuclear but also mitochondrial proteins in spite of their absence in mitochondrial protein databases.

Perhaps the uncertainty of their function or import into the mitochondrion makes it hard to finally conclude that MTs are also mitochondrial proteins. In addition, data from Zhou & Kang (2000) also add confusion to their relationship with the mitochondrion. They report that MTs could not be detected in mitochondria of heart cells from MT-over-expressing transgenic mice. Despite this observation, these mice still had better mitochondrial protection against DOX when compared to control mice (Ye *et al.,* 2001; Zhou & Kang 2000). Firstly, this means that MTs protect the

mitochondrion remotely in the heart, probably by preventing oxidative stress in the cytoplasm, and secondly that the localisation of MTs in the IMS is not concentration dependent (Ye *et al.,* 2001; Zhou & Kang 2000) as mentioned above. Even the incubation of isolated heart mitochondria with MTs did not result in reduced oxygen consumption or import of MTs into the IMS (Ye *et al.,* 2001). Thus, it seems that there is some kind of specific import mechanism (or transporter) allowing MTs into liver mitochondria but not into heart mitochondria (Ye *et al.,* 2001). This is certainly an area that is open for further study which might provide important evidence to the relationship of MTs with the mitochondrion, and answer the very important question: is the involvement of MTs with the mitochondrion specifically programmed or merely random?

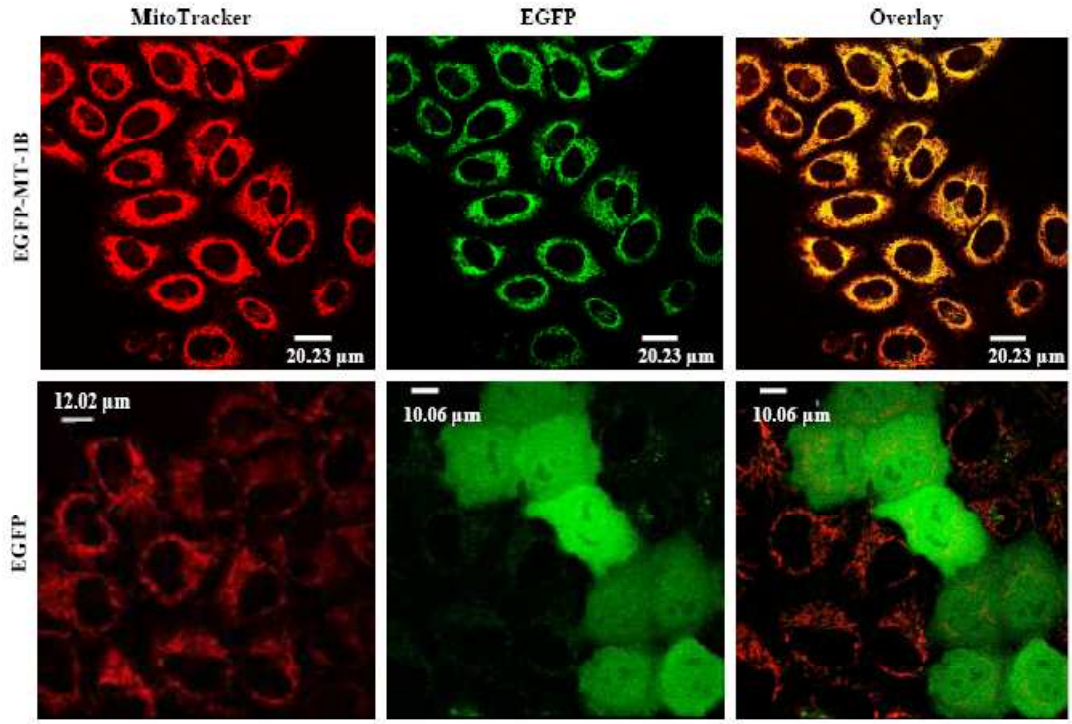

Figure 2.1: Cellular localization of EGFP-MT-1B fusion protein in HeLa cells visualized by confocal microscopy. MT-1B N-terminally fused to EGFP and pure EGFP were expressed in HeLa cells. Mitochondria were labeled with MitoTracker Red. Merged images indicate that EGFP-MT-1B fusion protein, but not EGFP itself, is localized in mitochondria.

2.4. FUNCTIONAL ASSOCIATIONS BETWEEN METALLOTHIONEINS AND THE MITOCHONDRION

The role of MTs *in vitro* and *in vivo* under normal physiological conditions is not well supported by experimental data. The role of MTs during disease states, however, is much better documented

and most studies have turned to disease states to study the role of MTs in cells, organelles and cellular processes. This is not surprising, as MTs are considered to be “stress proteins” (Andrews, 2000; Theocharis *et al.,* 2003). Therefore, the roles that MTs play in cellular processes are mainly protective to a specific pathological condition that occurs. The protective effects of MTs in various disease states, ranging from infectious to inherited diseases, have been studied extensively (Carrasco *et al.,* 2006; Hernández *et al.,* 2000; Hidalgo *et al.,* 1990; Hidalgo *et al.,* 2001; Reinecke *et al.,* 2006; Yu *et al.,* 2001). Although the mechanisms involved are not always clear, many reports do give supported hypotheses.

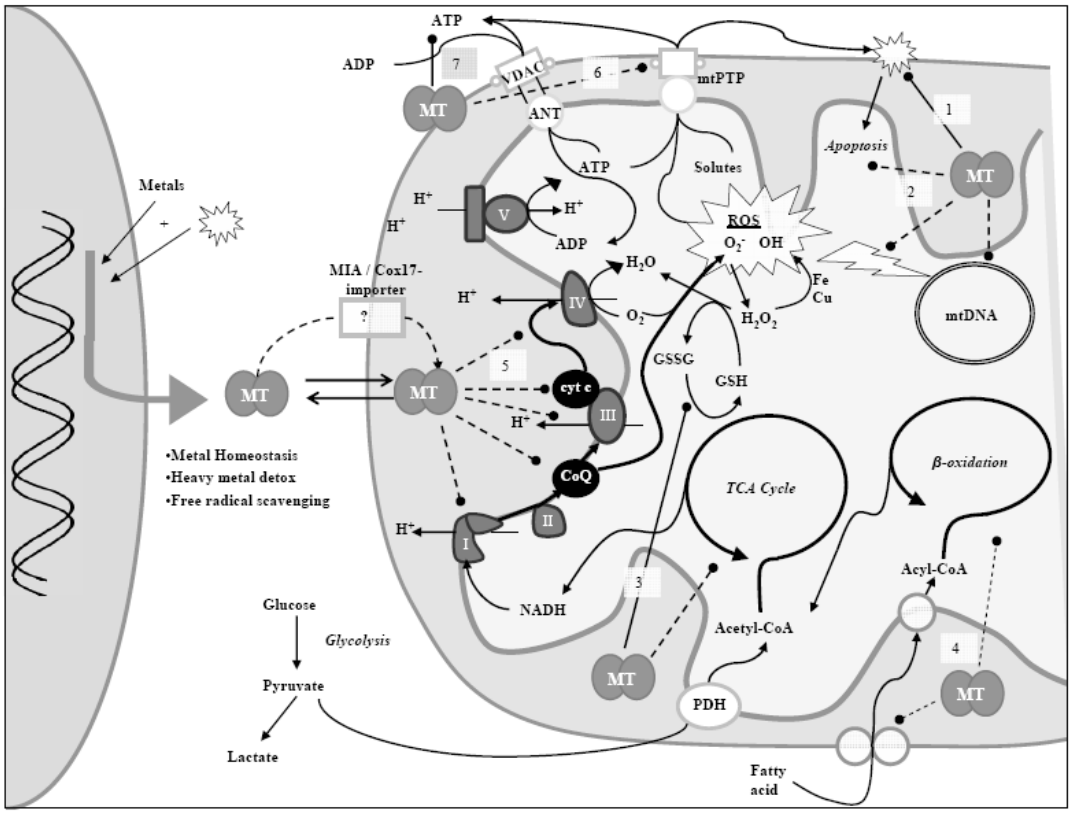

Figure 2.2: Summarized schematic presentation of the putative interactions of MTs with the mitochondrion. MTs protect the mitochondrion and mtDNA against ROS (1) and apoptosis (2). MT-glutathione redox and metal exchange cycle and involvement in enzyme regulation (3). Obese MTKO mice indicate involvement with lipid metabolism (4). Interaction of MTs with the ETC (5) and the transition pore (6). MT sequestration of ATP (7). Cyt *c*, cytochrome *c*; PDH, pyruvate dehydrogenase; TCA, tricarboxylic acid. Stippled lines indicate indirect or uncertain interactions and pathways.

While the effects of MTs in various (diseased) cellular processes have been studied thoroughly, the fundamental involvement of MTs in mitochondrial function and disease has not been studied as extensively as can be expected considering the amount of evidence that supports this involvement.

To date there are only a few research reports on which we and others have focussed to suggest the direct involvement of MTs with primary mitochondrial function. It also has to be noted that the mitochondrial disease models used thus far (where specifically targeted) were exclusively toxin induced and in our view provide at best only supporting evidence for this involvement, especially where MT expression is concerned. Also, as is the case with other models, there is limited research support for the role MTs play in mitochondrial function under normal physiological conditions, as most turn to diseased states to try and elucidate their role. In addition to the limited research support, the results obtained from these studies give conflicting evidence, especially when the role of MTs in mitochondrial function is compared to the role MTs play in mitochondrial disease. Since the mitochondrion is very complex and accommodates numerous cellular processes, of which several may involve MT function, the question can be asked: what evidence exists of this putative association and with which mitochondrial functions, if any, may MTs be directly involved? In the following sections, evidence of MT involvement with primary mitochondrial processes during physiological and disease states will be discussed and evaluated. Figure 2.2 supports this section and visually demonstrates all the putative and definite interactions of MTs with the mitochondrion.

2.4.1 METALLOTHIONEINS, ROS AND OXIDATIVE STRESS

When it comes to mitochondrial disorders, the typical view is that MTs protect cells with a mitochondrial deficiency mainly against oxidative damage and thus promote cell survival (Reinecke *et al.,* 2006). As mentioned before, the mitochondrion plays a central role in the generation of physiological amounts of ROS and RNS in a controlled environment. However, with mitochondrial disorders, this control is lost and may lead to high levels of ROS and RNS, which in return could damage other biomolecules and organelles in the cell (especially the mitochondrion itself). The increased levels of ROS induce increased MT expression (Andrews, 2000), which is believed to consequently reduce and protect against increased ROS and associated oxidative damage.

Van der Westhuizen *et al*. (2003) observed, using a micro-array approach, that the expression of various isoforms of MTs was markedly induced in various mutation associated complex I deficient fibroblast cell lines when directing the metabolism toward mitochondria-generated ATP production by changing the carbon source. The induction of MT over-expression from mitochondrial generated ROS was proven *in vitro* (Reinecke *et al.,* 2006) and *in vivo* (Kondoh *et al.,* 2001) which evidently points to a certain involvement MTs have with the mitochondrion. Suzuki *et al*. (2005) and Futakawa *et al.* (2006) also demonstrated that various mitochondrial inhibitors (inducing ROS formation) result in increased MT expression. This indicates that MTs may play a key role in

protecting the cell and organelles from increased ROS and RNS levels. This protective role was confirmed *in vitro* using rotenone-induced complex I inhibited HeLa cells as was shown that MT-2A over-expressing HeLa cells have improved cell viability, compared to wild-type cells, especially when ROS was increased with *t*-BHP (Reinecke *et al.,* 2006). This protective effect of MTs was also demonstrated *in vivo*. Treatment of MT knockout (MTKO) mice with the uncoupler 2,4-dinitrophenol caused severe liver damage in comparison with the wild-type mice (Futakawa *et al.,* 2006; Suzuki *et al.,* 2005). Although it is commonly accepted that uncouplers don't result in increased ROS formation, the administration of 2,4-dinitrophenol augmented lipid peroxidation, as measured by thiobarbituric acid reactive substances (TBARS), and MT levels. The prevention of diabetic induced oxidative damage was also observed in the hearts of MT-over-expressing mice (Cai *et al.,* 2006).

The mechanisms by which MTs protect key functions and structures in the mitochondrion are not as well investigated. Increasing levels of ROS in the mitochondrion means that those structures nearest to the production site get attacked first by these highly active species. This means that important mitochondrial structures, such as mtDNA, are always in the line of fire and suffer many onslaughts. If this is the case, complex I of the respiratory chain will most likely be affected by mtDNA damage, since seven of the 13 structural genes in mtDNA encode for polypeptides within complex I (Anderson *et al.,* 1981). Therefore, the increase in MTs and their scavenging abilities are very important for cell survival. However, there is still uncertainty over the capability of MTs to protect mitochondrial structures against oxidative damage, as the high reactivity and extremely short half-life of free radicals means that MTs must be close to the production site (Kang, 1999). Although MTs are not situated in the matrix of the mitochondrion, they do give remote protection to these structures, perhaps through the GSH/GSSG system which will be discussed later. It was also recently shown that increased ROS levels result in the reversible oxidation of certain sites on complex I which increases ROS levels even more (Taylor *et al.,* 2003). The induction of MTs and their translocation to the IMS may protect and reduce complex I. It is also apparent that MTs alter one of the nuclear encoded subunits of complex IV (CCO-Va) during oxidative stress and it is believed that the possible post-translational modification of this subunit increases its ability to utilize molecular oxygen and thus limits ROS formation (Merten *et al*., 2005). Thus, it seems that MTs protect the mitochondrion in more than one way from the damaging effects of ROS, not only by scavenging ROS, but also by preventing additional ROS formation.

Not only do MTs protect the mitochondrion from oxidative stress produced within the mitochondrion, but also from "external" oxidative stress formed by, for example, DOX (Zhou &

Kang 2000), alcohol (Zhou *et al.,* 2002) and *t*-BHP (Reinecke *et al.,* 2006). By protecting the mitochondrion from protein and lipid oxidation, the MTs protect against mitochondrial dysfunction and possible apoptosis (discussed later). During conditions of high mitochondrial oxidative stress, MTs protect the nucleus and nuclear DNA in addition to the mitochondrion and mitochondrial structures. The MTs located in the IMS of the mitochondrion might form a line of defence that can scavenge most of the ROS that leaves the organelle. In addition to this, nuclear- and cytosolic MTs would form a further line of defence for migrating free radicals (Min *et al.,* 2005). We conclude that this view of MTs promoting cell survival during mitochondrial deficient states, mainly by protecting against ROS, is well supported by experimental data, although the disease models used thus far do have limitations. However, the question remains: what, if any, involvement with mitochondrial ROS production do MTs has during normal physiological conditions?

It is well known that physiologically formed ROS participates in various signalling pathways that are crucial for cell survival (Genestra, 2007) as well as the ageing process (Balaban *et al.,* 2005). Therefore, it is tempting to suggest that MTs, which assist in the control of oxidative stress, thus participate in the ageing process (Suzuki *et al.,* 2005) and associated cell signalling pathways during normal physiological conditions. When considering this control of oxidative stress, it implies that there is (probably) an equilibrium between MTs and ROS, which therefore also enhance the "constructive" cellular roles of ROS. However, the role of MTs in normal physiological oxidative stress seems to be not that important since MTKO mice have no pertinent abnormalities associated with oxidative stress or any ageing problems. The study of metallothionein's involvement with ROS under normal *in vivo* physiological conditions, however, remains uncharted territory.

2.4.2 APOPTOSIS

Another view, which contributes to the previous discussed protective effect of MTs, is their involvement with controlled cell death (apoptosis). Apoptosis is a normal physiological occurrence that is essential for the development and survival of multi-cellular organisms. As it is a critical physiological process, it is finely regulated. A negative correlation between MT-levels and apoptosis was observed in different cells suggesting an important role of MTs in apoptosis (Shimoda *et al.,* 2003). The mitochondrion is also known to play a central part in the intrinsic pathways of apoptosis (reviewed Jourdain & Martinou, 2009; Scorrano, 2009). In addition to physiological apoptosis, there are also many pathological situations where apoptosis is over-stimulated which leads to the destruction of excessive amounts of cells. This is also the case with mitochondrial disorders and oxidative stress. Increasing levels of ROS stimulate apoptosis (Kondo

et al., 1997) in cells by releasing cytochrome *c* and activating caspase-3. Therefore, by scavenging free radicals and thus limiting ROS, MTs can reduce apoptosis (Cai *et al.,* 2006). This was supported *in vitro* by Reinecke *et al*. (2006) in complex I inhibited HeLa cells. In MT-2A over-expressing cells, caspase-3/7 activation and nucleosome enrichment was significantly lower and mitochondrial membrane potential better maintained when compared to control cells.

However, it is often questioned whether MTs reduce apoptosis merely by limiting ROS damage. Extrinsic etoposide-induced apoptosis in cells does not make use of ROS in the process and since MTs has been shown to also limit this type of apoptosis, another way of action seems possible (Shimoda *et al.,* 2003). The suggestion of other mechanisms of action might include direct participation of MTs in the apoptosis pathways such as interaction of MTs with cytochrome *c* or caspase-3. For example, the binding or modification of released cytochrome *c* can limit further apoptotic signals. Other studies have shown that certain metals such as zinc also regulate caspase-3 activity directly (Aiuchi *et al.,* 1998) which poses the question whether MTs interact with caspase-3 through zinc supply. This is also likely with p53 which has zinc incorporated into its structure. Replacement of this zinc with cadmium results in p53 turning into a 'mutant' which disrupts normal cellular growth control - a situation that is more evident in MTKO mice (Zheng *et al.,* 1996). Another observation that needs to be taken into account is that MTs reduce drug-induced apoptosis more than p38 MAPK- and cytochrome *c*-caspase 3 pathway inhibitors, which suggest that there is probably a common upstream trigger that is more effectively inhibited by MTs (Wang *et al*., 2001).

Other *in vitro* studies have also shown that MTs reduce apoptosis. Bax and p53 were measured in MTKO and wild-type mice embryonic cells and it was observed that the basal levels of these proteins were constantly higher in the MT-null cells, which suggests elevated apoptosis (Kondo *et al.,* 1997). In the same study, drug-induced apoptosis was also more severe in MT-null cells than in normal cells. Pre-treatment of the MT-null cells with zinc did not affect induced apoptosis, which not only confirms the lack of MT expression but also proves that zinc alone does not protect against apoptosis (Chimienti *et al.,* 2001; Kondo *et al.,* 1997). Also, MT-over-expressing myocytes showed remarkable resistance to DOX-induced apoptosis in comparison with wild-type cells (Wang *et al*., 2001). This reduction of drug (streptozotocin)-induced apoptosis by MTs was also demonstrated *in vivo* by Cai *et al*. (2006) in transgenic mice over-expressing MTs in the heart. These transgenic mice also had an enhanced survival rate in comparison with wild-type mice. The cell survival/ viability that is commonly seen in MTs-containing cells is thus not only due to ROS scavenging but also a result of less apoptosis. It is uncertain whether the surviving cells are

damaged and consequently promote other problems. However, even if MTs only slow the rate of cell death, it means that repair might occur in a period when the stress is reduced.

As with oxidative stress, the control of MTs on intrinsic or extrinsic apoptosis pathways appears to occur only during disease states when MTs are over expressed. MTs do not stop apoptosis completely, but rather attempt to reduce it to similar rates in normal situations. In normal physiological states, the MT levels in the cell are relatively low, as well as the ROS levels; therefore it is easy to assume that MTs do not affect apoptosis under these conditions. It is not clear whether there are perhaps other mechanisms of interaction such as *direct* participation in the apoptosis pathways, but interaction of MTs with cytochrome *c* or caspase-3 is one possibility (as mentioned). Such *direct* participation in the apoptosis pathways was also suggested by Wang *et al*. (2001).

2.4.3 THE METALLOTHIONEIN-GLUTATHIONE CYCLE

As mentioned in Section 2.2.2, the cellular antioxidant system involves several components of which glutathione is one of the most important and best characterised (Forman *et al*., 2009). Depletion of GSH (reduced glutathione) by increased oxidative stress has been shown to be a direct consequence of various secondary defects in diseases such as cardiac cell death in diabetic conditions (Ghosh *et al.,* 2005). This implies that both the preservation of GSH and control of the GSH/GSSG ratio in the mitochondrion are important to cell survival during conditions of oxidative stress. Important for this role is that glutathione is localised throughout the cell. Although glutathione is only synthesised in the cytosol, it is transported to other cellular compartments (Griffith & Meister, 1985), including the nucleus and mitochondrion. Since the concentration of glutathione is relatively high in the cytosol and mitochondria (5 – 10 mM), it can fulfil its role as antioxidant under normal physiological conditions (Kulinsky & Kolesnichenko, 2007). However, during times of oxidative stress its effectiveness is limited and this is where another key function of MTs becomes apparent.

Several studies found that MTs interact and sequester electrophilic agents *in vitro* and that there is an interaction between MTs and glutathione (Kondo *et al.,* 1997; Zaia et *al.,* 1996). Although MTs can scavenge free radicals about 300 times better than GSH, they are not used as the primary free radical scavengers in the cell due to their steady state concentrations and localization. However, this changes during conditions of high oxidative stress when MTs become the primary antioxidants as their concentrations increase significantly (Cai *et al.,* 2006; Ding *et al.,* 2002; Quesada *et al.,* 1996; Wu & Kang, 1998). GSH is consequently preserved and thus less glutathione disulfide

(GSSG) is formed in the cell (Cai *et al.,* 2006; Quesada *et al.,* 1996). Since glutathione is ubiquitously distributed it helps to protect against ROS where MTs cannot, stressing the need for its preservation and recovery. As free radicals are not selective of their targets and despite the high MT concentrations, some of the GSH inevitably gets oxidized to GSSG, especially when it is near the production site of ROS. This is where the MT-glutathione cycle plays an important role (Figure 2.3), reducing GSSG to GSH.

The reduction of GSSG is generally done by the mitochondria-localised enzyme glutathione reductase. Thus, for MTs to reduce GSSG in conditions of high oxidative stress, it would imply a similar *direct* interaction with glutathione. Work from Brouwer *et al*. (1993) supports this idea which showed that glutathione binds in a cleft in the beta domain of the MT structure. Although the exact mechanism has not been elucidated and proven that the similar interaction occurs with GSSG, it still remains an important possibility. Vice versa, when the oxidative status of the cell once more reaches physiological levels, oxidized MTs (thionins) can be reduced again as mentioned earlier. It was suggested that GSH perform this task although this reduction is not very efficient, even at high concentrations of GSH and requires selenium. The addition of selenium that has the capacity to form a catalytic selenol(ate) efficiently couples the GSH/GSSG redox pair with the MT/thionin system (Chen & Maret, 2001). However, the "protective" effect against oxidation of GSH by MTs is not accepted by all (Ferreira *et al.,* 1993) who suggested that GSH protect MTs from oxidation.

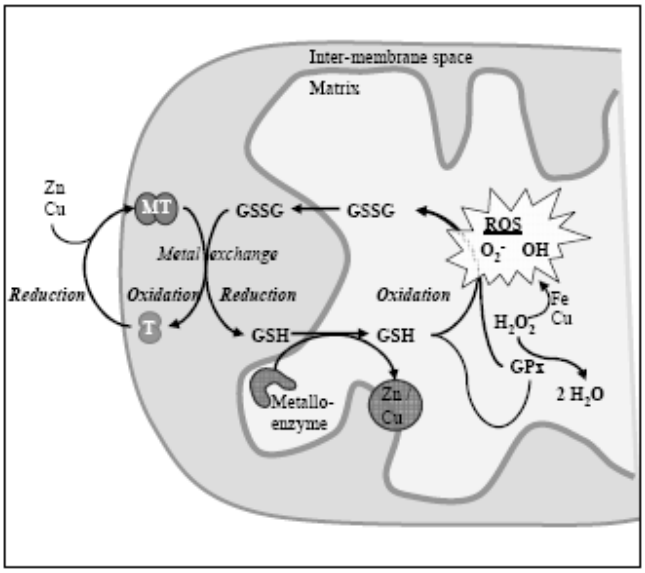

Figure 2.3: The metallothionein-glutathione redox and metal exchange cycle. Oxidized glutathione are reduced by MTs which might also result in the exchange of metal ions (Rofe *et al.,* 1996). Reduced glutathione scavenge ROS and transport metals where MTs cannot, in the mitochondrial matrix. T, Thionin; GPx, glutathione peroxidase.

Another important aspect of this interaction is the exchange of metals between glutathione and MTs. Brouwer *et al*. (1993) also propose that metals can be exchanged between these molecules which might serve as a means of delivery for metals from MTs to inter-mitochondrial enzymes. Ferreira *et al*. (1993) show that Cu(I) is effectively transported from GSH to thioneins and that this interaction can also displace Zn(II) and Cd(II) bound to MTs. It is also proposed by Maret (1994) that metals are transferred from MTs to glutathione. Thus, a two directional release of metals by these molecules seems possible but the choice/ control of direction of metal transfer remains vague. However, several reports indicate that GSSG (and thus the oxidative state of the cell) plays an important role in this metal release and distribution of MTs (Jiang *et al.,* 1998; Ye *et al.,* 2001). Although, MTs deliver metals to many acceptor proteins without the help of GSSG and the oxidative status of the cell (Costello *et al.,* 2004; Feng *et al.,* 2005; Moltó *et al.,* 2007), it is evident that MTs release metals more efficiently to metalloenzymes in the presence of glutathione (Jiang *et al.,* 1998). Taking this into account, it appears that there may be three main mechanisms by which MTs transfer metals to metalloenzymes in the presence of glutathione: Firstly glutathione can accept metal ions from MTs (Maret, 1994) and distribute them further in the mitochondrion where MTs cannot reach. Secondly, the binding of glutathione to MTs result in conformation changes which also results in the release of metal ions. And thirdly, the oxidation of the MT-Cys residues by GSSG which results in reduction to GSH. Despite the limited information, the cooperation of glutathione and MTs may well be one of the most important features of MTs and their role in oxidative stress, apoptosis and regulation of enzymes and metabolism.

2.4.4 ENERGY METABOLISM

The regulation of specific enzymes and thus metabolic pathways is a commonly accepted function of MTs as more than 300 enzymes are depended of metal ions such as zinc and copper as cofactors (Coyle *et al.,* 2002). It was also recently proposed by Molto *et al*. (2007) that MTs are involved in energy metabolism and its regulation. Moreover, several former publications provide evidence for such an association. As reported by Coyle *et al*. (2002), A.M. Rofe (unpublished data) observed that MTKO mice have, in addition to varied glycogen storage, markedly reduced hepatic ATP levels throughout the feeding cycle which indicates an altered energy state. Rofe *et al*. (1996) also gave additional supporting evidence when they reported lower blood and liver concentrations of lactate in MTKO mice compared to wild-type mice after an endotoxin intervention.

Beattie *et al.* (1998) also observed that the majority of male MTKO mice in their colony tended to be moderately obese and have a growth tempo (associated with fat deposition) that exceeds that of many other mice. The tendency to obesity implies that these mice might have a lower metabolic

rate (Coyle *et al.,* 2002). After studying the physiology and biochemistry of these mice, they reported that both *ob* gene expression and plasma leptin levels were significantly higher in the MTKO mice (similar to that of Zucker fatty rats). The livers of these MTKO mice also had a fatty appearance and high hepatic lipid levels were observed. We also made similar observations in our own MTKO mice colony (unpublished data). Beattie *et al*. (1998) further reported no changes in plasma glucose, triacylglycerol and insulin levels, but liver copper levels in MTKO mice were lower than in control mice. Although there was also no change in zinc levels in the liver (Beattie *et al.,* 1998), lower blood and hepatic lactic acid levels were recorded in these mice (Rofe *et al.,* 1996) which point to altered energy metabolism in the presence of MTs. Many other studies have shown that obesity is associated with down regulated mitochondrial oxidative phosphorylation and biogenesis (Dong et al., 2007). High-fat diet and obesity elicit enhanced ROS accumulation, mitochondrial damage and reduced mitochondrial density as well as a reduced mtDNA copy number and the crucial mitochondrial activator, PGC-1α. It has been shown that the main free fatty acid component in a high-fat diet, palmitic acid, lowers PGC-1α expression in wild-type mice cardiomyocytes. These effects (of the high-fat diet) were reduced in the presence of MTs as observed in cardiac-specific MT-over expressing transgenic mice (Dong *et al.,* 2007).

2.4.4.1 Metal homeostasis and enzyme activity

Whether MTs interact *directly* with the respiratory chain is still unclear, but not unlikely. MTs localised in the IMS may interact *directly* with complex I, III, IV, V and cytochrome *c*, but since MTs are unlikely to cross into the matrix side it is therefore not interacting with complex II and other matrix enzymes. However, MTs can interact *indirectly* with these complexes and enzymes via metal ions (Moltó *et al.,* 2007). Many of these enzymes, such as tyrosinase, cytochrome *c* oxidase, superoxide dismutase, lysyl oxidase and dopamine-beta-hydroxylase require metal co-factors such as copper, which, if not provided by MTs, result in reduced respiration and increased oxidative stress (Moltó *et al.,* 2007). The lower levels of copper detected in the livers of MTKO mice (Beattie *et al.,* 1998) suggest that one important link between altered metabolism and MTs is via the copper ion. Although iron and manganese have dominant roles in the mitochondrion, the inability of MTs to bind and transport these metals in physiological conditions disconfirms a possible link between MTs and altered metabolism via these metals (Stillman, 1995). Therefore it was proposed that MTs transport and release copper *directly* to these enzymes or *indirectly* by donating it to a copper transporter (Na+/Ca+ antiporter) (Mehta *et al.,* 2006). Another method of metal delivery to proteins within the mitochondrion might well be via the MT-GSH cycle as discussed in the previous section. Jiang *et al.* (1998) observed *in vitro* that activation of apo-sorbitol dehydrogenase through the

transfer of zinc from zinc-MTs (human MT-1 and -2) to the enzyme is semi-controlled by glutathione.

The vital role of zinc in cells suggests that control of its availability to enzymes and transcription factors is strongly regulated. Since many of the enzymes within the mitochondria also require zinc as cofactor, it has been postulated that a putative zinc transporter exists which transports zinc into the mitochondrial matrix. One possible way of entry might be via the mitochondrial Ca^{2+} uniporter (Saris & Niva, 1994). Since most zinc is bound to low molecular weight zinc-ligands (Zn_7MT for example), these ligands must release the zinc to this putative transporter. This would require *direct* interaction between the transporter and the ligand (Zn_7MT). Although there is not yet experimental evidence for such a mechanism, *direct* interaction of MTs with other zinc acceptors has been described (Costello *et al.,* 2004; Hathout *et al.,* 2001). As mentioned before, an earlier point of view was that zinc bound to MTs is released into the IMS as a result of oxidation or the low pH (Jiang *et al.,* 1998; Ye *et al.,* 2001). However, recent studies have shown that MTs have *direct* interaction with various acceptor molecules (Costello *et al.,* 2004; Feng *et al.,* 2005; Jacob *et al.,* 1998; Moltó *et al.,* 2007). Therefore, it is also possible that MTs donate zinc to some protein complexes and enzymes of the respiratory chain via *direct* interaction and thus act as chaperone for zinc (Costello *et al.,* 2004).

Several mitochondrial matrix enzymes require metal co-factors, including mitochondrial aconitase (m-aconitase), which require supply of iron (predominantly) and zinc (Dupuy *et al.,* 2005). It was shown *in vitro* with MTKO mice heart extracts that zinc-saturated rabbit MT-2 transfer zinc to m-aconitase while $ZnCl_2$ did not. As m-aconitase did not accept zinc from $ZnCl_2$, it therefore suggests a specific, *direct* interaction between MT and the enzyme (Feng *et al.,* 2005). Nevertheless, this interaction is not likely to occur *in vivo* since MTs are not located in the matrix (Ye *et al.,* 2001). Sorbitol dehydrogenase (SDH) is a cytoplasmic enzyme but is also present in the mitochondrion. When this protein is imported into the mitochondrion, it probably loses its conformation and co-factors. This is where MTs can play possible chaperone roles and thus donate zinc to the enzyme. *In vitro*, human MTs (-1 and -2) transfer one of its zinc atoms to SDH to restore the enzyme's activity (Jiang *et al.,* 1998). As mentioned before, despite the high thermodynamic stability by which MTs bind metals, they do not remove metal cofactors from the catalytic sites of enzymes, which suggest that MTs cannot inhibit enzymes via this approach (Jacob *et al.,* 1998). However, inhibition can be performed by metal donation as discussed next.

2.4.4.2 Enzyme inhibition and re-activation

The *indirect* interaction of MTs with enzymes in the mitochondrion not only includes providing metal co-factors for these enzymes, but also inhibition or re-activation of enzymes. It is well documented that the respiratory chain, and many other enzymes for that matter, is inhibited by heavy metals such as cadmium, copper and zinc (Cattani *et al.,* 1996; Dineley *et al.,* 2005; Maret *et al.,* 1999; Mehta *et al.,* 2006; Wang *et al.,* 2004; Ye *et al.,* 2001). Complex III, through interaction with the cytochrome bc_1 subunit is one of the best studied examples of Zn^{2+} inhibition (Berry *et al.,* 2000; Kleiner & von Jagow, 1972). The addition of 10 µM zinc in the form of $ZnCl_2$ to purified chicken bc_1 complex resulted in <60 % activity. The activity dropped more with increasing [zinc] and reached 20 % at 200 µM zinc (Berry *et al.,* 2000). In addition, it was later shown in intact mitochondria that a site upstream from complex III is more sensitive for zinc inhibition, and it was suggested to be the Krebs cycle enzyme, α-ketoglutarate dehydrogenase complex (Brown *et al.,* 2000). This inhibition is reversed by MTs mediated zinc sequestration (Krezel & Maret, 2007). Maret *et al*. (1999) reported that MTs (specifically rabbit MT-1) rapidly re-activate enzymes that are inhibited by zinc at what they refer to as specific 'zinc-inhibitory sites'. Thus MTs will re-activate an enzyme without taking up the metal ion in the catalytic site (Jacob *et al.,* 1998). This discrimination is probably due to the accessibility of these metals and, to a lesser extent, of their stability constants (Jacob *et al.,* 1998; Maret *et al.,* 1999). Thus, if zinc poisoning should occur, which would result in enzyme inhibition, the over-expressed thioneins will bind the excess zinc, re-activate inhibited enzymes and fight oxidative stress formed by the initial inhibition of respiratory enzymes. This proposed sequence of events supports the view that MTs predominantly have positive effects on cell function and survival.

Clearly, there is mounting evidence to recognize MTs as anti-stress proteins that protect the cell and mitochondrion. Considering current knowledge, it seems inconceivable that MTs might also have 'adverse' effects. But one must ask whether MTs are also involved in the inhibition of certain enzymes to alter metabolism. Though enzyme inhibition is wrongly associated with negative function, it is not always the case; it merely implies another way of control or regulation. Since MTs are responsible for metal transport, the existence of such an action may indeed be possible. This perspective was first shown by Simpkins *et al*. (1998a) who observed that MT-1, in the presence of calcium, reduce ADP-stimulated mitochondrial oxygen consumption. In other studies, the addition of zinc-saturated MTs to ADP-stimulated mitochondria results in the reduction of respiration (Moltó *et al.,* 2007; Ye *et al.,* 2001). Although zinc alone can inhibit the ETC (Link & von Jagow, 1995) it seems more likely that MTs play the biggest part. Molto *et al*. (2007) show that MTs can donate zinc to these sites in a controlled fashion, without metal concentration playing a role, to reduce

respiration. Therefore these results appear to contradict the often exclusively protective effect of increased MTs in the cell. Simpkins *et al*. (1998a) tried to resolve this contradictive effect by suggesting that since respiration produces superoxide and hydrogen peroxide, the suppression of it might reduce ROS formation. Even if this is true, any accumulating NADH may be re-oxidized to NAD^+ and superoxide by aldehyde oxidase (Zhou *et al.,* 2002). However, the fact that MTs regulate certain enzymes via metal co-factors while others are regulated via inhibition/ re-activation makes it a very effective controller of metabolic pathways.

Although the above *in vitro* observations are noteworthy with regard to mitochondrial function, they still may not provide a clear view of *in vivo* events. Especially when comparing the reports from Simpkins *et al*. (1998a) and Beattie *et al*. (Beattie *et al.,* 1998), the *in vitro* and *in vivo* observations appear to be contradictive. According to the *in vitro* data (Simpkins *et al*., 1998a), MTs have a lowering effect on respiration while the *in vivo* data (Beattie *et al.,* 1998) from MTKO mice suggest that the absence of MTs results in reduced energy metabolism and subsequently obesity. The little free zinc and copper in physiological normal cells implies that metal inhibition (control) can only occur through MTs and not from these metals alone. Even in metal elevated pathological conditions, such as metal poisoning, MTs donate zinc to these sites in a controlled manner without metal concentration playing a key role and consequently reduce oxygen consumption (Moltó *et al.,* 2007). In addition to the *in vivo* controversy, this view is also opposed by Jacob *et al*. (1998) who demonstrated that thionein is a particularly effective chelating agent for zinc metalloenzymes which does not inhibit enzymes by controlled donation of zinc to inhibitory sites in enzymes. Obviously this is true for thioneins but such inhibition can most probably be done by MTs. Despite this controversy, it is clear that control of the levels and location of zinc and copper in the cell and mitochondrion is important for metabolism. Several studies have investigated the role of zinc in the activity and inhibition of mitochondrial enzymes (reviews Costello *et al.,* 2004; Dineley *et al.,* 2003) and although zinc appears to be the main regulating metal, its transportation and delivery to these enzymes remains unclear as discussed before, which makes MTs the key regulators in the interplay between zinc and mitochondrial functions.

2.4.4.3 Mitochondrial permeability transition pore

In addition to the controlling effects on the respiratory chain, MTs, along with calcium and ROS, are involved in the control of the mitochondrial transition pore (mtPTP), which is an essential aspect of OXPHOS and translocation of metabolites between mitochondria and the cytosol (Simpkins *et al*., 1998a; Simpkins *et al.,* 1998b). When MT-1 was added to isolated rat liver mitochondria, the pore opened resulting in increased permeability, depolarisation of the IMM and

mitochondrial swelling (Simpkins *et al.,* 1996). The action of released metals from MTs was ruled out, but not when *directly* transferred to the pore. The addition of zinc to liver mitochondria results in the opening of this transition pore but not in heart mitochondria (Wudarczyk *et al.,* 1999), which is probably due to the fact that there are no MTs in heart mitochondria to deliver the zinc and thus cause *direct* interaction.

Increased IMM permeability and mitochondrial swelling were opposed by the addition of spermine (Simpkins *et al.,* 1998b). Like MTs, spermine is also rapidly induced and therefore the control over this pore *in vivo* might be more dependent on the ratio of these molecules in the cell. Seemingly, these results also contradict the general protective effects of MTs as they act here as uncouplers but certainly support the other results observed by Simpkins *et al.* (1998a). Corresponding with their theory to resolve the contradiction, it is known that uncouplers do not stimulate ROS formation like inhibitors of the respiratory chain (Boveris & Chance, 1973; Moltó *et al.,* 2007). Therefore, it was proposed that spermine acts as a counterbalance for MTs *in vivo,* 'controlling' both respiration and ROS formation, and that spermine counters the inhibitory effects of MTs on respiration while the MTs counter the effects of ROS production (Simpkins *et al.,* 1996) during stressed conditions of mitochondrial function. However, more recently it was shown that the Zn_7MTPA (crustacean MT) does not inhibit respiration via the opening of this pore but rather by inhibiting the enzyme complexes (Moltó *et al.,* 2007). The production of ROS confirmed ETC inhibition, as inhibitors of the ETC (and not uncouplers) increase ROS production (Boveris & Chance, 1973; Moltó *et al.,* 2007). Whether this is the same with other MTs is still unclear and therefore the above mechanism (Simpkins *et al.,* 1996) cannot be rejected at this stage.

2.4.4.4 Nucleotide complex formation

In addition to metals, phosphates can bind to MTs which results in stable MT crystals. Jiang *et al*. (1998a) determined that MTs can bind purine nucleotide triphosphates such as GTP and ATP *in vitro* which was confirmed by Maret *et al*. (Maret *et al.,* 2002). To our knowledge there is still no *in vivo* evidence for this, nevertheless, these remain important observations which can, amongst other effects, contribute to OXPHOS function regulation (Maret *et al.,* 2002) and nucleotide and MTs transport. However, the relatively low binding affinity of MTs for ATP compared to other ligands, as well as the absence of detection, creates doubt whether these complexes indeed occur *in vivo* (Jiang *et al.,* 1998a). In addition, the reason for such a complex is uncertain but several possibilities exist. Except for possible enzyme regulatory functions, it is suggested that the translocation and retention of MTs in the nucleus are ATP- and GTP-dependent (Woo *et al.,* 1996; Woo *et al.,* 2000). Also, many *in vitro* studies suggest that MTs require a certain amount of 'energy'

to release the metal ions to acceptor molecules (Jacob *et al.,* 1998; Jiang *et al.,* 1998; Kangur & Palumaa, 2001; Maret *et al.,* 2002). Whether the necessary *in vivo* 'energy' comes from ATP, oxidizing/ reducing agents or 'enzymatic-type' interaction between the molecules is not clear, but certainly would explain this type of MT-ATP interaction. It is evident that the binding of ATP to rabbit MT-2 result in conformation changes which result in the dissociation of metals (Jiang *et al.,* 1998a; Maret *et al.,* 2002). Whether this binding is a controlled action or merely accidental is another question in itself. Regarding the ATP-dependent localization of MTs, it was also proposed by several others that the conformational changes that occur when MTs bind ATP and GTP contribute to their import and retention in organelles such as the nucleus and mitochondrion (Maret *et al.,* 2002; Woo *et al.,* 1996).

2.4.4.5 Cytochrome c and Coenzyme Q

Some reports propose an interaction (*direct* or *indirect*) of MTs with the IMS electron carrier, cytochrome *c* (Mesecke *et al*., 2005; Simpkins *et al.,* 1993). This putative interaction, however, has not been studied extensively. A few reports merely mention such a relationship, masked behind the involvement of MTs in apoptosis and transport into the IMS. Since cytochrome *c* and MTs both have repetitive Cys residues, these proteins might have more in common than just their transport into the mitochondrion or relationship during apoptosis. The exchange of electrons from cytochrome *c* to MT-I, as revealed *in vitro* (Simpkins *et al.,* 1993), suggests another means of respiration reduction (Simpkins *et al.,* 1998b) even though the evidence pointing to this is not conclusive.

Another key electron transporter in the respiratory chain is the IMM localised CoQ (ubiquinol). CoQ (CoQ_{10} in humans) is the only lipid soluble antioxidant synthesized by higher organisms. In addition to its role in the respiratory chain, it also plays a role in extra-mitochondrial redox reactions. CoQ is oxidized to ubisemiquinone radical which can be converted back by a reduction reaction, either by the electron transport chain or by external means (Genova *et al.,* 2003). One possible way of reduction might occur via MTs but since CoQ is embedded in the bilayer (IMM), *direct* interaction seems impossible (and likely the reason for no such reports). Nevertheless, the elucidation of the relationship of CoQ with MTs is very important as this is a major site of electron leak (Turrens & Boveris, 1980) that results in the formation of superoxide and other free radicals. If it is possible for MTs to protect against free radicals at this point – before the formation of free radicals in the mitochondrion – it will be much more efficient in protecting the organelle and other cellular structures from oxidative damage. Work from Ebadi's group (Ebadi *et al.,* 2005) has shown a relationship between MTs and CoQ and report that MTs enhance mitochondrial function in neurons

by increasing CoQ levels. It is suggested that zinc bound MTs stimulate CoQ synthesis by stimulating the activity of lipoamide dehydrogenase (Ebadi *et al.,* 2002), a view quite remarkable as zinc is known to inhibit this enzyme (Gazaryan *et al.,* 2002).

2.4.5 NUCLEAR– AND MITOCHONDRIAL DNA TRANSCRIPTION REGULATION

The interactions between MTs and wide-ranging, but key functions of the mitochondrion and related metabolic pathways have been deduced from targeted investigations of these functions in various models. Notwithstanding, many of these interactions and their mechanisms are still inconclusive, especially for *in vivo* conditions and also with regard to MT isoforms. It gets even more complicated when the observed phenotypes (of altered metabolism for example) are not only a result of the control MTs exercise with enzyme activity but also expression. Thus an even broader interaction, that MTs are involved in, is their interaction with transcription factors and thus expression of nuclear and possibly also mitochondrial encoded genes (Cano-Gauci *et al.,* 1996; Cherian *et al.,* 2003; Hathout *et al.,* 2001). This interaction was shown to be *direct* which requires protein-protein interaction in order to transfer zinc (Hathout *et al.,* 2001). Therefore, since MTs can treat zinc-finger transcription factors in similar ways as enzymes, by supplying or removing metal ions (activation/ inhibition), it is easy to conclude that function changes associated with MTs, including mitochondrial respiration, may be a result of altered gene expression, or compounded by it. However, the data from Simpkins *et al*. (1998a) and others were obtained from *in vitro* studies where mitochondria were isolated, which eliminated this possibility in their observations.

With the recent introduction of transcriptomics technology the effect of MTs on transcriptional regulation has come to the forefront. Differential gene expression in the livers and kidneys of MTKO mice revealed that most genes that were differentially expressed were involved in energy metabolism. *Gck* (Glucokinase), *Agpat6* (lysophosphatidic acid acyltransferase), *Elovl3* (very long chain fatty acid elongase) and *Atp5a1* (ATP synthase) were among the genes that were up-regulated while *G6pc* (glucose-6-phosphatase), *Pdxk* (pyridoxal kinase) and *Entpd5* (ectonucleoside triphosphate diphosphohydrolase 5) were down-regulated (Miura & Koizumi, 2005). Previous reports have shown that *Lpl* (lipoprotein lipase), Cebpa (CCAAT/enhancer binding protein) and epididymal white adipose tissue *ob* gene (leptin) expression was also up-regulated in MTKO mice (Beattie *et al.,* 1998) although this is not confirmed in the data from Miura & Koizumi (2005). Comparing the gene expression results with previous measured metabolic differences, some correlation can be seen which supports this idea. For example, Beattie *et al*. (1998) & Rofe *et al*. (1996) report somewhat lower glycogen levels in the liver of MTKO mice that correlate with up-regulated glucokinase and down-regulated glucose-6-phosphatase expression (Miura &

Koizumi, 2005). Modifications in protein expression in wild-type versus MT-over-expressing mice were also observed prior to and after DOX treatment (Merten *et al*., 2005). When these proteins were identified with mass spectrometry, it was seen that they were involved in cellular antioxidant defence, nuclear-encoded subunits of the mitochondrial electron transport chain, enzymes involved in β-oxidation of fatty acids and glycolysis.

Hidalgo's group also reported that numerous genes are differentially expressed in MTKO and wild-type mice prior to and after external stress and diet interventions (Penkowa *et al.,* 2006). Genes involved in apoptosis and ROS protection were among those that had significant altered expression which is compatible with the known phenotype of MTKO mice. Moreover, numerous genes involved in mitochondrial function were also differentially expressed with expression much higher in MTKO mice. This is also compatible with the phenotype that suggests energy metabolism to be dysregulated in the MTKO mice. The reported mitochondria-related genes that were up-regulated in MTKO mice were *Mtif2* (translational initiation factor 2 that initiates the translation of proteins encoded by the mtDNA), *Tom20* (translocase of outer mitochondrial membrane 20 homolog that belongs to the TOM complex), *Timm8a* (translocase of the IMM 8 homologue a), *Star* (steriodogenic acute regulatory protein that mediates the intra-mitochondrial transport of cholesterol), *Slc25a1* (solute carrier family 25, member 1 or citrate transport protein which is responsible for the movement of citrate across the IMM), *Cox7a21* (cytochrome *c* oxidase subunit VIIa polypeptide 2-like), Idh3b (isocitrate dehydrogenase 3 beta) and succinate dehydrogenase complex subunit A.

Although all the mentioned genes are not encoded by the mitochondrial genome, it underlines the involvement of MTs in mitochondrial function under normal and challenged conditions. Evidence of MT regulation contribution of mtDNA transcription and replication is still limited, and although MTs are reported to have protective effects on mtDNA integrity in neural cells, *in vitro* (Ebadi *et al.,* 2005), this was linked to protection against oxidative processes. However, a recent report observed the protective effect of MTs against high-fat diet associated cardiac dysfunction where control (preventing reduced expression) of MTs on expression of peroxisome proliferator-activated receptor γ coactivator-1α (PGC-1α), which is a key regulator of mitochondrial biogenesis, was demonstrated (Dong *et al.,* 2007). This effect was also observed in the downstream factors controlled by PGC-1α, nuclear respiratory factors 1 and 2 (NRF1/2) and mitochondrial transcription factor A (mtTFA) (Dong *et al.,* 2007). These recent reports of transcriptional responses to absence of, or over-expression of, MTs significantly contribute to the scope of understanding the involvement of MTs on metabolism and in particular energy metabolism. The interplay between

nuclear-mitochondrial interactions, the signalling events involved and the role of MTs in these events need to be investigated further.

2.5. CONCLUSIONS AND FUTURE PROSPECTS

The involvement of MTs with mitochondrial functions and disease pathologies has been extensively documented in research publications over many decades. As suggested before, and as has become a feature when evaluating MT functions, these interactions are diverse and to distinguish between *direct* and *indirect* interactions is often speculative. Other limitations that complicate this evaluation are the differential expression and localization of MTs and its isoforms in tissues, challenges that still exist to measure MTs, often contradicting reports and limited *in vivo* evidence to support *in vitro* reports. Notwithstanding these limitations, we believe that the well-documented and generally accepted structural and functional properties of MTs and their gene expression responses highlighted in this review are closely associated with mitochondrial function in vertebrates. This association becomes more apparent but also more complex during pathologies of mitochondria-associated diseases, where a controlled equilibrium between metal donating and accepting (to regulate enzymes and transcriptional factors) and between scavenging and promoting ROS formation (e.g. via ETC inhibition) is encountered.

A more accurate overview of the combined interactions of MTs and cellular processes, including those of the mitochondria, will require the use of systems biology tools and well-characterised *in vitro* and *in vivo* models and perturbations. Such datasets have been published as reviewed here, but only provide a specific dimension of biological organization (transcriptome) in specific animal models. These have to be expanded to include the other dimensions of biological organization (proteome and metabolome), and for a conserved, ubiquitously expressed, but functionally diverse protein such as metallothionein, this provides an exciting challenge.