DOI 10.1515/bmc-2012-0049 — BioMol Concepts 2013; 4(2): 143–160

Review

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Metallothionein-protein interactions

Abstract: Metallothioneins (MTs) are a family of universal, small proteins, sharing a high cysteine content and an optimal capacity for metal ion coordination. They take part in a plethora of metal ion-related events (from detoxification to homeostasis, storage, and delivery), in a wide range of stress responses, and in different pathological processes (tumorigenesis, neurodegeneration, and inflammation). The information on both intracellular and extracellular interactions of MTs with other proteins is here comprehensively reviewed. In mammalian kidney, MT1/MT2 interact with megalin and related receptors, and with the transporter transthyretin. Most of the mammalian MT partners identified concern interactions with central nervous system (mainly brain) proteins, both through physical contact or metal exchange reactions. Physical interactions mainly involve neuronal secretion multimers. Regarding metal swap events, brain MT3 appears to control the metal ion load in peptides whose aggregation leads to neurodegenerative disorders, such as Aβ peptide, α-synuclein, and prion proteins (Alzheimer's and Parkinson's diseases, and spongiform encephalopathies, respectively). Interaction with ferritin and bovine serum albumin are also documented. The intercourse of MTs with zinc-dependent enzymes and transcription factors is capable to activate/deactivate them, thus conferring MTs the role of metabolic and gene expression regulators. As some of these proteins are involved in cell cycle and proliferation control (p53, nuclear factor κB, and PKCμ), they are considered in the context of oncogenesis and tumor progression. Only one non-mammalian MT interaction, involving *Drosophila* MtnA and MtnB major isoforms and peroxiredoxins, has been reported. The prospective use for biomedical applications of the MT-interaction information is finally discussed.

Keywords: CNS metal ions; metal ions and cancer; metallothionein; neurodegenerative disorders; protein-protein interaction.

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Introduction

Metallothioneins (MTs) are a family of small (<10 kDa), extremely heterogeneous proteins, sharing a high cysteine content (15–30%) that confers them an optimal capacity for metal ion coordination. After their discovery in horse kidneys by Bert Vallee in 1957 (1), MTs have been identified and characterized in most prokaryotic and all eukaryotic organisms. Besides metal ion detoxification, they have been related to a plethora of physiological events, from the homeostasis, storage, and delivery of physiological metals, to the defense against a wide range of stresses and pathological processes (tumor genesis, neurodegeneration, inflammation, etc.). It is now a common agreement among MT researchers that the ambiguity when defining MT function/s is most probably due to the fact that this question does not harbor a unique solution; on the contrary, MTs may have evolved in different organisms to fulfill diverse functions, according to the physiological and ecotoxicological needs of each situation [recent reviews and full information on MTs in refs. (2–4)]. Actually, it is also worth noting that the MT system has turned out to be polymorphic in every single organism where it has been analyzed, including either patently divergent forms, such as in yeast (Cup1 vs. Crs5 proteins) or highly similar homologous proteins, such as in mammals (MT1 to MT4 isoforms).

From a structural point of view, MTs are random coil polypeptides that only fold onto a definite 3D structure upon metal coordination, so that their 3D structure is dependent on the nature and number of the metal ions coordinated in a given metal complex. The fact of each specific MT being able to adopt different folds is highly relevant for the studies of protein interactions, as 3D is precisely the main determinant of protein-protein recognition events. Unfortunately, standard interactomics techniques have usually been applied to MT polypeptides regardless of the metallic complex they were constituting, something that may be responsible for the belated appearance of data on MT-interacting proteins in the literature. Furthermore, the same MT peptide may alternatively constitute different complexes in an organism, depending on the availability of surrounding metal ions. Hence, native

MTs are isolated as Zn(II), Cu(I), and/or mixed Zn(II)/Cu(I) forms, which exhibit high thermodynamic stability combined with kinetic lability, and the most interesting capacity in terms of biological functionality of undergoing and catalyzing metal ion exchange and/or transfer reactions with other biomolecules (5). Among all the MT peptides, the yeast Cup1 and *Drosophila* MTs (representative of the so-called Cu-thioneins), and the mammalian isoforms, all hereafter described, are the paradigmatic representatives of two different types of MTs that gather most of the data currently available on MT-protein interactions.

Cup1 [last reviewed in ref. (6)] is a 61-residue polypeptide, posttranslationally processed to a 53-residue mature form (Table 1) that is produced in response to copper overload, and which binds up to eight Cu(I) ions through metal-thiolate bonds contributed from 12 cysteine residues. The high mobility of the N- and C-terminal tails determines that Cu-Cup1 binding render different Cu(I)-to-MT stoichiometries, eventually in dimeric forms. As a consequence, elucidation of the Cu-Cup1 3D structure (Figure 1) has only been possible for a truncated Cu-thiolate core cluster, which encompasses six trigonally and two digonally coordinated Cu(I) ions (7). *Drosophila* MTs constitute a singular case among metazoan MTs, as its five MT isoforms (MtnA to MtnE) are highly similar to the yeast (*Saccharomyces cerevisiae*) Cup1 paradigmatic Cu-thionein, both in amino acid sequence and metal-handling abilities. For the model species *Drosophila melanogaster*, MTs are 40- and 43-amino-acid-long peptides, comprising 10–12 cysteines (Table 1). Their role has been related to copper homeostasis and detoxification processes both in larvae and adult flies, with special significance in the digestive tract cells (8, 9). In another scenario, the mammalian MT system includes four highly similar MT isoforms (MT1 to MT4), some of which have undergone further amplification in humans, rendering, for example, 13 *MT1* genes (10). The tissular expression patterns of the four MT isoproteins show substantial differentiation. Hence, MT1 and MT2 are ubiquitous, highly inducible isoforms that respond to a considerable number of factors – mainly metal overload and oxidative stress – and have been largely related to global homeostasis, transport, and detoxification of metals in organisms. MT3 is mainly constitutively synthesized in central nervous system (CNS) cells, having been related to neuronal growth and Zn/Cu content control and equilibrium in brain. Finally, a role for MT4 in the proliferative stratified epithelia, the only tissue where it is synthesized, has been suggested. Although in no case duplication and diversification seem to have led to a substantial differentiation and specialization of the metal-binding preferences of mammalian MTs, slightly different Zn- and Cu-binding properties have been reported for some of them (11, 12). From a structural point of view, mammalian MTs – like all vertebrate MTs – are 60- to 68-amino-acid peptides with a fully conserved Cys pattern (Table 1). Thus, their differences in metal-binding properties should be interpreted on the basis of the nature of the intercalating residues, with the exception of MT3, which encompasses an insertion of six residues near its C-terminal end, and MT3 and MT4, exhibiting a one-residue insertion in the same position of their N-terminal region. Coordination of divalent metal ions by mammalian MTs renders dumbbell-shaped complexes, including two metal clusters. The N-terminal MT half folds into the

Table 1 Amino acid sequence of the metallothionein proteins included in this review, with indication of the accession number in the UniProt-SwissProt data bank or NCBI[a].

Organism	Isoform	Sequence	SwissProt accession
M. musculus	MT1	MDPN-CSCTTGGSCACAGSCKCKECKCTSCKKSCCSCCPVGCAKCAQGCVCKGSS------EKCRCCA	P02802
M. musculus	MT2	MDPN-CSCASDGSCSCAGACKCKQCKCTSCKKSCCSCCPVGCAKCSQGCICKEAS------DKCSCCA	P02798
M. musculus	MT3	MDPETCPCPTGGSCTCSDKCKCKGCKCTNCKKSCCSCCPAGCEKCAKDCVCKGEEGAKAEAEKCSCCQ	P28184
M. musculus	MT4	MDPGECTCMSGGICICGDNCKCTTCSCKTCRKSCCPCCPPGCAKCARGCICKGGS------DKCSCCP	P47945
D. melanogaster	MtnA	MPCP-CGSGCKCASQATKGCNSCGSDCKCGGD-KKSACGCSE	P04357
D. melanogaster	MtnB	MVCKGCGTNCQCSAQKCGDNCACNKDCQCVCKNGPKDQCCSNK	P11956
D. melanogaster	MtnC	MVCKGCGTNCKCQDTKCGDNCACNQDCKCVCKNGPKDQCCKSK	Q9VDN2
D. melanogaster	MtnD	MGCKACGTNCQCSATKCGDNCACSQQCQCSCKNGPKDKCCSTKN	Q819B4
D. melanogaster	MtnE	MPCKGCGNNCQCSAGKCGGNCAGNSQCQCAAKTGAK--CCQAK	NP_001189254.1[a]
S. cerevisiae	Cup1	MFSELINFQNEGHECQCQCGSCKNNEQCQKSCSCPTGCNSDDKCPCGNKSEETKKSCCSGK	P07215

The coordinating cys residues are marked in bold. [a]Indicates NCBI accession numbers.

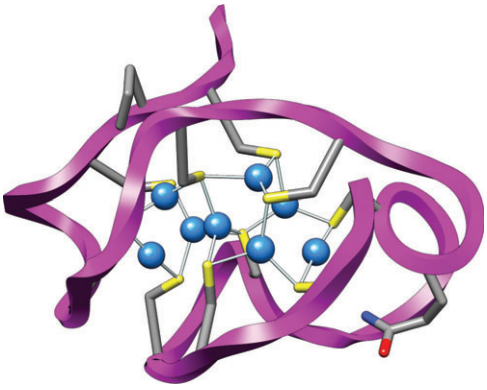

Figure 1 3D structure of the yeast *S. cerevisiae* Cu_8-Cup1 complex, showing the $Cu_8(SCys)_{10}$ cluster (PDB entry 1rju). The Cu(I) ions are shown as spheres, the Cys residues as sticks, and the S-Cu bonds as solid lines. The structure was represented using the UCSF Chimera software. Chimera is developed by the Resource for Biocomputing, Visualization and Informatics at the University of California, San Francisco, USA (100).

so-called β domain, which includes a $M^{II}_3(SCys)_9$ cluster formed by three bridging cysteine thiolates forming a six-membered ring in a distorted chair conformation, while the other six Cys act as terminal ligands, thus completing the tetrahedral coordination environment about each divalent metal ion. The C-terminal moiety forms the α domain, enclosing an $M^{II}_4(SCys)_{11}$ cluster formed by two fused six-membered rings, which can adopt either a distorted boat or a chair conformation, containing five bridging and six terminal S_{Cys}-donor atoms that fulfill the tetrahedral coordination geometry about the four divalent metal ions (Figure 2). More detailed information will be provided later in the different sections of this review, in particular for the brain MT3 isoform; however, for a recent revision on global mammalian MT structure and function, the reader is directed to (13).

Drosophila/Cup1 MTs and mammalian MTs have been the subject of the quest for interacting proteins, this considered in the broad sense of the word *interaction*, i.e., either the physical contact between two molecules or the triggering of some sort of mutual intercourse, such as ion exchange. It is our most convinced belief that interactomics should bring to bear a lot of new information about structural and functional aspects of MT research. Noteworthy, the comprehensive view of all known MT-protein interactions (Table 2) is drastically replacing the old view of these metalloproteins as inert, cul-de-sac cell deposits of undesirable metal ions with a new vision of highly dynamic cellular agents involved in such crucial events as neurotransmission, gene expression regulation, or control of neurodegenerative and neoplastic disorders.

MT-protein interactions in mammalian renal structures

In mammals, all kinds of metal ions induce high levels of MT synthesis, which then yield the corresponding metal-MT complexes that principally accumulate in the kidney and liver. This includes situations of physiological metal ion surplus, intake of toxic metal ions, and/or treatments based on metal ion drugs. It should be remembered that

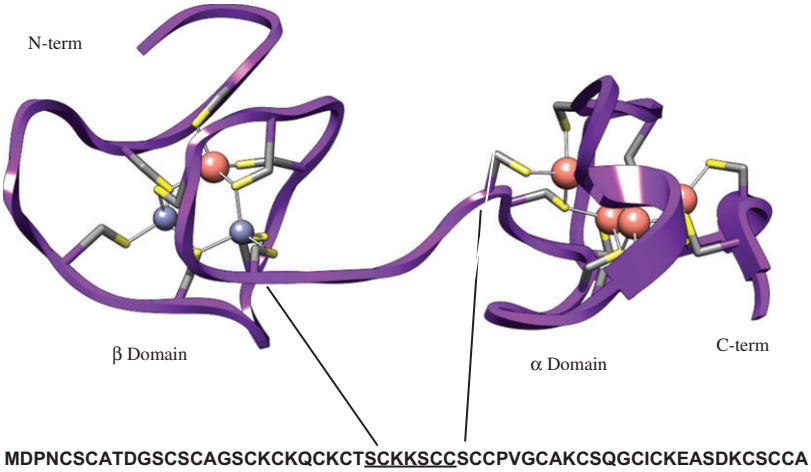

Figure 2 3D structure and amino acid sequence of the rat Zn_2Cd_5-MT2 complex, drawn with the coordinates available at the 4MT2 entry of PDB. Zn(II) and Cd(II) ions are represented as small and big spheres, respectively; the Cys residues as sticks; and the S-Cd bonds as solid lines. The underlined region corresponds to the megalin-interacting fragment. The structure was represented using the UCSF Chimera software. Chimera is developed by the Resource for Biocomputing, Visualization and Informatics at the University of California, San Francisco, USA (100).

Table 2 Summary of reported MT-protein physical interactions and metal exchange reactions.

MT isoform	Metal complex	Tissue	Interacting protein	Function	References
Mammalian MT (human and rat): physical interaction					
MT1/MT2	Cd-MT	Kidney	Megalin (LPR-2)	Tubule absorption	(16)
MT1/MT2	–	CNS	LPR-1	Neurite growth	(38)
MT1/MT2	–	Liver, CNS	Transthyretin	Aβ scavenging (diminished)	(41, 42)
MT3	–	Liver, CNS	Transthyretin	Aβ scavenging (augmented)	(42)
MT3	Zn-MT	CNS	Rab3A GTPase	Synaptic vesicle cycle	(44)
MT3	–	CNS	HSP84 (human HSP90)	Neuronal secretion	(45, 46)
MT3	–	CNS	HSP70	Neuronal secretion	(45, 46)
MT3	–	CNS	DRP2 (dihydropyrimidinase-like protein 2)	Neuronal growth	(45, 46)
MT3	–	CNS	Creatine kinase BB	Glycolytic metabolism	(45, 46)
MT3	–	CNS	β-Actin	Scaffold protein	(45, 46)
MT3	–	CNS	Exo84p (interacts with Rab3A)	Secretory vesicles exocytosis	(47)
MT3	–	CNS	14-3-3ζ	Protein signaling	(47)
MT3	–	CNS	Enolase (α in glia and γ in neurons)	Glycolytic metabolism	(47)
MT3	–	CNS	Aldolase 1 and 3	Glycolytic metabolism	(47)
MT3	–	CNS	Pyruvate kinase	Glycolytic metabolism	(47)
MT3	–	CNS	Malate dehydrogenase	Glycolytic metabolism	(47)
MT3	–	CNS	Tubulin α3	Scaffold protein	(47)
MT isoform	**Metal complex**	**Tissue/disease**	**Interacting protein**	**Function**	**References**
Mammalian MTs: metal ion swap					
MT3	Zn-MT	CNS/AD	Cu-Aβ peptide	ROS and Aβ aggregation	(48, 49)
MT3	Zn-MT	CNS/AD	Cu-Aβ peptide	Regulation of Aβ amyloid structure	(50, 52)
MT-3	Zn-MT	CNS/PD	Cu-α-synuclein	Synuclein aggregation	(58)
MT-3	Zn-MT	CNS/Prion D	Cu-PrP	ROS and PrP toxicity	(61)
MT1/MT2/MT3	Zn-MT	Systemic	Fe-ferritin	ROS and iron storage	(62)
Mammalian MTs: apoprotein metal ion exchange					
MT1, MT2	Zn-MT	Systemic	Several hydrolytic enzymes	Enzymes	(65, 66)
MT1, MT2	Zn-MT	Systemic	Sp1, TFIIIA	Transcription factors	(68, 69)
MT1/MT2	Zn,Cd-MT	Systemic	BSA	Plasma transport protein	(77)
MT1	apo-MT Zn-MT	Global cancer	p53	Tumor suppressor	(85)
MT1	Zn-MT	Global cancer	NF-κB	Tumor proliferation	(86)
MT1	Zn-MT	Prostate cancer	PKCμ	Signal transduction	(87)
MT isoform	**Metal complex**	**Tissue**	**Interacting protein**	**Function**	**References**
Drosophila melanogaster					
MtnA, MtnB	Zn-MT	–	Peroxiredoxin	Redox recycling	(88)

MTs were first identified, isolated, and characterized from equine renal cortex (1, 14). In fact, mammalian kidney is the main target of the deleterious effect exerted by xenobiotic metals (Cd, Pb, or Hg as main representatives), metals used in anticancer drugs (cisPt or V), or even an excessive load of physiological metals (Zn, Cu, and Fe). All kinds of metal ions accumulate in the proximal tubule (PT) cells, where they trigger structural damages that cause a malfunction, ending in defective reabsorption and secretion processes (15). Therefore, it is assumed that metal-MT complexes pass through the renal circuits through filtration by the glomerulus to the PT fluid, from where they are reincorporated into the PT epithelial cells. Megalin (also known as LPR2, or low-density lipoprotein-related protein 2) is a large (600 kDa) transmembrane protein acting as a multiligand receptor in a variety of epithelial, including PT, cells. Three lines of evidence demonstrate the molecular interaction between megalin and Cd-MT complexes in rat cells, which is supposed to mediate the entry of the metal-MT species into PT (16). First, direct surface plasmon resonance experiments identified divalent metal ion-dependent megalin/MT binding, at a K_d of ~10^{-4}, through a unique site; second, blockage of megalin by specific antibodies inhibited the uptake of MT or caused the displacement of already bound MT; and finally, megalin and metal-MT complexes colocalized in cultured cells, an essential requirement to support the physiological significance of *in vitro* detected protein-protein interactions. As these studies were carried out using different domains and segments of the mouse MT1 isoform,

obtained both recombinantly and synthetically, it was possible to delimit the short hinge region between the β and α MT domains (-SerCysLysLysSerCysCys-) as responsible for megalin binding (Figure 2). Hence, this work not only identified a key interaction target of metal-MT complexes in the kidney, signaling the involved protein region, but also showed that the interaction was dependent on divalent metal ion binding to MT. This is a very important information from the structural point of view, as it fully corroborates that MT interaction with a given protein is metal dependent, as it has been later on demonstrated for *Drosophila* MTs (cf. last section of the review). A very recent study (17) has confirmed the key role of megalin in metal-MT-induced nephrotoxicity, by showing that mice with inhibited megalin function exhibited a reduced renal metal content, as well as decreased kidney damage. Special efforts on this subject should contribute to develop renal metal uptake inhibition strategies, as well as to improve metal antineoplastic treatments in the near future.

MT-protein interactions in mammalian CNS

The fact that MT1, MT2, and MT3 function in mammalian CNS, both in physiological and pathological conditions, highlights the importance of their respective Zn(II) complexes in neuroprotection and neuroregeneration processes (18). On the one hand, Zn(II), together with glutamate, is a key signaling and modulating agent in synaptic vesicles, particularly in the brain; thus, MTs are the best mechanism to ensure Zn(II) homeostasis and availability in this organ. On the other hand, the broad spectrum of metal ions that cannot only be coordinated but also exchanged by MTs ensures a buffering potential of the metal ions prominent in neural tissues, i.e., Zn(II), Cu(I)/Cu(II). The formation of Fe(II)-MT complexes does not take place under physiological conditions, but it has been reported in very special situations, such as the *in vitro* Fe(II) coordination by apoMTs, or as consequence of severe health disorders (19, 20). Zinc and copper have been largely implicated in the onset and progression of several significant neurodegenerative diseases with severe socio-health impact in the current society: Alzheimer's disease (AD), Parkinson's disease (PD), Creutzfeldt-Jacob and other prion-driven spongiform encephalopathies, and amyotrophic lateral sclerosis. Different MT isoforms appear to be involved in distinct CNS physiological and pathological processes. The ubiquitously expressed MT1/MT2 have been widely associated with the prevention and healing of CNS inflammation and injuries, being mainly synthesized in astrocytes and microglia cells. On the other hand, the almost CNS-specific MT3 isoform, first identified as a growth inhibitory factor (GIF) (21), is predominant in neurons, but also present in astrocytes, choroid plexus, and other CNS cells. It appears to be mainly involved in neuronal growth and survival events, as well as in processes localized in axons and dendrites. From the wealth of data on the literature both from human studies and animal models, it becomes obvious that alteration of *MT* gene expression is a common feature among neurodegenerative disorders, although the precise MT expression pattern appears both isoform- and disease-dependent. For AD, MT1 and MT2 increased levels have been demonstrated in the hippocampus (22), and in gray and white matter astrocytes (23, 24) of brain samples, as well as in the corresponding brains of mouse models (25). For MT3, a first consensus agreed on its downregulation in AD patient brain tissues, after several studies performed both at the mRNA and protein levels (21, 26, 27). However, controversial results were later reported, indicating either non-altered (28, 29) or even increased MT3 levels in the AD brains (30). In the brain of PD patients, no change in MT1 and MT2 levels (31) has been detected, this contrasting with a marked MT3 decrease (32). Among spongiform encephalopathies, increased expression of MT2 was first characterized in scrapie-infected hamster brains (33). However, subsequent analyses in human prion diseases again demonstrated that the global scenario of MT overexpression/underexpression was far from homogeneous, apparently depending on the duration of the disease and the vicinity of the analyzed astrocytes to kuru plaques. Hence, MT1 and MT2 accumulated in the brains of short-term Creutzfeldt-Jacob patients, while MT1, MT2, and MT3 synthesis in the brain was severely diminished by a long duration of the disease, as well as by proximity of prion protein (PrP) deposits (34). Finally, it is worth noting that inflammatory neurodegenerative disorders, such as amyotrophic lateral sclerosis and multiple sclerosis, share a common pattern of MT1 and MT2 increased synthesis, in front of reduced MT3 levels (32, 35, 36). The beneficial role that MT1, MT2, and MT3 seems to produce in case of brain injury and/or disorder has been consistently confirmed using MT-overexpressing and knockout cells and transgenic organisms. Hence, MT1 and MT2 would exert an antioxidant, anti-inflammatory, and antiapoptotic function, while MT3 would be in the line of supporting neuronal survival (37). The results of all the efforts devoted to seek MT1, MT2, and MT3 partners in CNS, in an attempt to define their physiological roles and capacities, and from

there, to design neurodegeneration-targeted therapies, are analyzed in the following sections, distinguishing those that entail a physical protein interaction and those that have been detected only as metal-exchange reactions between MT and a second metalloprotein.

Protein-protein aggregates (physical interaction)

MT1 and MT2/LPR-n receptors

MT1 and MT2 were found to stimulate neurite growth through signal transduction through the megalin (LPR-2) or/and the LPR-1 (lipoprotein receptor-related protein) receptors, as blocking these receptors with antibodies before treatment abolished neurite extension (38). It is remarkable that MTs share the same receptor (megalin) in such different cell types as neurons and renal cells (cf. previous section). Several sites in the MT peptide sequence have been mapped to be responsible for the MT1 and MT2 neuritogenic properties, located in the C-terminal end of the β domain, and in both extremes of the α domain, plus two sites in each domain related to the neuronal survival promotion. Interestingly, all but the N-terminal end of the α-peptide appear to interact with receptors of the LDLR (low-density lipoprotein receptors) family (39), in accordance with the fact that these receptors bind a broad range of structurally unrelated ligands.

MT1-, MT2-, and MT3-transthyretin

Transthyretin is a homotetrameric protein that is used for the transport of thyroxine and retinol-binding protein. Systemically circulating transthyretin is synthesized and secreted by the liver; however, this protein is also highly relevant in the brain choroid plexus – the structure producing the cerebrospinal fluid – where it can represent up to one-fifth of the total protein content. It was suggested that brain transthyretin would impair amyloid-β (Aβ) peptide fibrillation and deposition by scavenging Aβ monomers, thus preventing the onset and/or progression of AD (40). Taking into account that choroid plexus cells are the main sites for both MT1/MT2 and MT3 synthesis in the CNS, and the alleged relation between MT3 and AD as a neuronal GIF, it is not surprising that a MT-transthyretin interaction was promptly suspected and detected. Hence, interaction with MT2 was identified from a standard two-hybrid assay using a human liver cDNA library (41). Further *in vitro* and *in vivo* techniques, including coimmunoprecipitation, competition binding assays (yielding a K_d of 244.8 nM for this interaction), cross-linking, protein blotting, and colocalization, corroborated the interaction and demonstrated that an MT1/MT2-transthyretin complex was indeed present in the choroid plexus and kidney tissue preparations (41). The same authors later showed that the brain MT3 isoform also bound transthyretin with similar affinity (K_d=373.7 nM), by using an equivalent methodological approach (42). However, competition-binding assays rather unexpectedly highlighted that the effect of the MT interaction on the Aβ scavenger capacity of transthyretin was totally opposite, depending on the bound MT isoform. Hence, MT2-transthyretin binding decreases the transthyretin-Aβ interaction rate, while the formation of MT3-transthyretin complexes increases transthyretin-Aβ binding, and thus enhances its Aβ scavenging potential. It was therefore specifically hypothesized that the protective effect attributed to MT3 against Aβ deposition could specifically rely on this indirect mechanism.

MT3/Rab3A GTPase

The first interaction involving an MT peptide was precisely reported for MT3 and the small G-protein Rab3a, after a yeast two-hybrid screen of a human brain cDNA library (43). Coimmunoprecipitation and protein blot experiments further confirmed this interaction in cultured mammalian cells. Rab3a is a small GTPase acting in membrane-trafficking events associated with signal transduction networks. In neurons, it specifically participates in the synaptic vesicle exocytic and endocytic cycle. As MT3 has been repeatedly proposed as an active player in the trafficking of Zn(II) ions in neurons, it was further investigated whether the MT3/ Rab3a interaction could provide any clue to better understanding the role of this MT isoform in synaptic signal recycling processes. In a very complete study, using mainly affinity precipitation and surface plasmon resonance (44), Vasak's group in Zurich determined that the MT3/Rab3a interaction, proceeding reversibly with a K_d of 2.6 μM, is dependent of specific forms of both partners, namely the GDP-charged Rab3a (but not Rab3a·GTP) and the Zn-loaded form of MT3 (but not the apo-MT3 polypeptide). The interaction region in Rab3a was localized in the C-terminal tail of Rab3a, a 30-residue unstructured segment that performs an effector-binding role, essential for intracellular membrane targeting. By integrating this information, the authors hypothesized that this interaction could serve for Zn(II) presynaptic concentration sensing. Hence, assuming that

the Rab3a·GDP complex is delivered to synaptic vesicles after GTP hydrolysis, it is feasible that Zn_7-MT3 binds to vesicular membrane-bound Rab3a at this stage of the synaptic cycle, thus enhancing the rate of exocytosis of synaptic vesicles through restructuring the Rab3a effector region. However, as the interaction only takes place at high Zn(II) concentrations, ensuring the presence of MT3 as fully loaded Zn_7-MT complex, this phenomenon would in fact constitute a sensor of Zn(II) presynaptic levels, and thus it would modulate the rate of exocytosis according to the Zn(II) concentration in the presynaptic area. Nevertheless, the alternative explanation that this interaction merely serves as Zn(II) recycling mechanism to the synaptic vesicles has also been proposed (44).

MT3/secretion multimer in CNS

The great significance of Zn(II) and MT3 in mammalian CNS also prompted Prof. Armitage's group to investigate MT3 protein interactions. The first analysis, performed by immunoaffinity chromatography and mass spectrometry proteomic identification techniques, identified five mouse proteins: heat-shock protein (hsp) 84 (mouse homologue to the human hsp 90), hsp 70, dihydropyrimidinase-like protein 2, creatine kinase, and β-actin (45). The most interesting result of this contribution was not only the identification of these five partners, but also the fact that this interaction was derived from peptides associating in a multiprotein complex. The *in vivo* existence of such a multimer was corroborated by the *in situ* immunohistochemical colocalization in the mouse brain hippocampus region of two of the involved partners (MT3 and creatine kinase) (46). More recently, the group of MT3-interacting CNS proteins has been extended to seven new members: exo84, 14-3-3ζ, α and γ enolase, aldolase C, malate dehydrogenase, ATP synthase, and pyruvate kinase (47). To propose a functional interpretation of these results, all the MT3-interacting proteins were grouped in three functional classes, those involved in transport and signaling, chaperoning and scaffolding, and glycolytic metabolism. The transport and signaling set includes components of the exocyst multiprotein complex, which plays a role in the targeting and signaling of secretory vesicles, this nicely matching with the Rab3a data presented before. The second group (chaperoning and scaffolding) comprises proteins known to participate in the guidance of neuronal growth and polarity events by providing suitable scaffolding. Finally, besides their energy-generating role, many glycolytic enzymes are known to be involved in stress tolerance responses and neuronal growth control and neurite outgrowth. A model integrating all the reported interactions was proposed on the basis that the origin of the MT3 present in neurons would be both by intracellular synthesis and by absorption from the extracellular milieu. This exogenous MT3 would be secreted by astrocytes and internalized in neurons by means of secretory complexes contributed by the identified proteins. Significantly, most of the neuronal activities of MT3 as a GIF could be explained by the inhibitory effect caused by its interaction with proteins regulating neuronal growth and differentiation, such as enolase through its role as cell surface receptor for plasminogen, or dihydropyrimidinase-like protein 2. This model would also account for the extracellular presence of MT3 in the brain, as MTs lack any signal peptide, and MT3 secretion has been determined to be independent of Zn(II) or interleukin stimulation, contrarily to what has been reported for MT1/MT2. In fact, it is compulsory to assume the presence of MT3 in the extracellular space to support its proposed role in the prevention of several neurodegenerative disorders caused by abnormal protein aggregation (cf. next sections in this review). The expression of most of the proteins reported as MT3 partners in these secretory multimers have been shown to be altered, as MT3 itself is, in AD patients, which can be considered an indirect support of the hypothesized interaction mechanisms and events (Figure 3).

Metal swap reactions

The molecular basis of three major, and most dramatic, neurodegenerative disorders currently affecting the human population are due to deposits in CNS tissues – mainly the brain – of misfolded, aggregated polypeptides and proteins: Aβ peptide for AD, α-synuclein for PD, and the PrP for spongiform encephalopathies (mad cow and Creutzfeldt-Jakob diseases). Coincidently for the three cases, the misfolding process has been associated with a lack of equilibrium of physiological metal ions (Zn and Cu), both inside and outside the different types of nerve cells, which would lead to two types of harmful consequences. On the one hand, the metal complexes of these proteins with some metal ions are more prone to aggregate and form neurotoxic plaques than with others. On the other hand, it is well known that the interaction between some redox-active metal ions and these proteins produce free radicals and reactive oxygen species (ROS), both agents closely related to CNS damage. During the past 5 years, Vasak's group, at Zurich University, has devoted its efforts to investigating the interaction between the

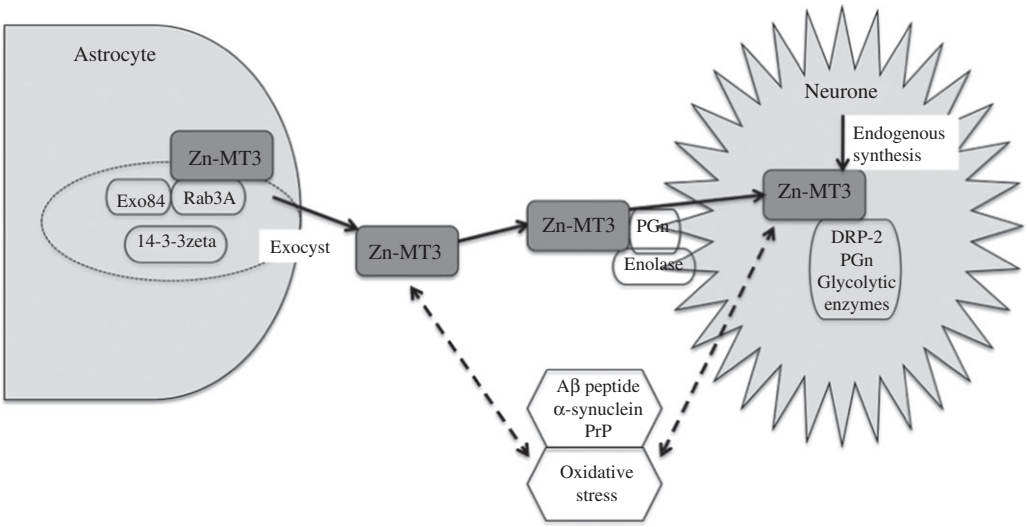

Figure 3 Schematic representation of the localization and protein interactions of the Zn-MT3 complexes reported in the astrocyte, neuron, and intracellular matrix of mammalian CNS, adapted from ref. (47).

Zn_7-MT3 complex and the Cu(I)/Cu(II) ions present either in CNS tissues or coordinated to the three potentially neurotoxic aforementioned proteins. An emerging common picture identifies Zn_7-MT3 as the source of beneficial/preventive Zn(II) transfer or Zn/Cu exchange reactions in all the cases (Figure 4).

The Aβ peptide and AD

The fibrillar deposit of Aβ aggregation plaques in the brain is accepted as one of the crucial events in AD etiology. Copper dysregulation in brain intracellular and extracellular environments has been assumed to be involved in the generation/progression of AD by binding to the Aβ peptide, thus generating Cu(II) complexes that are prone to promote both ROS formation (superoxide, hydrogen peroxide, and hydroxyl radicals) – due to the Fenton redox-active Cu(II)/Cu(I) cycle – and enhanced Aβ aggregation. As it became obvious that control of copper concentration in the brain could provide a key step in the control of AD, immediate research was aimed at MT3, the brain MT isoform *par excellence*, downregulated in AD patients (21). Isolation and characterization of native MT3 led to the identification of the peculiar Cu^{I}_{4},$Zn_{3\text{-}4}$-MT3 complexes as the common form for MT3 present in the brain, instead of the canonical Zn_7-MT3 species expected from homology to Zn_7-MT1/MT2. To understand the genesis of these native complexes, the first efforts were devoted to study the reactivity of Zn_7-MT3 with free Cu(II) ions, and it was determined that Zn_7-MT3 induced the stoichiometric reduction of four Cu(II) ions to Cu(I), with concomitant generation in its β domain of an oxygen stable Cu^{I}_{4}-thiolate cluster and two disulfide bridges (48). After the discovery of this free Cu(II) coordination ability, the potential recruiting of the Cu(II) ions bound to Aβ peptides as the result of an MT-Aβ interaction became evident. Hence, a Zn(II)-Cu(II) metal swap between the Zn_7-MT3/Cu(II)-Aβ molecules would generate Cu,Zn-MT3 complexes similar to those generated by the free Cu reaction (49), with concomitant transfer of Zn(II) to the Aβ peptides and without an apparent requirement of any further reducing agent (50). Furthermore, the capacity of Zn_7-MT3 to extract copper ions from soluble Cu(II)-Aβ complexes could be extended to already aggregated Cu(II)-Aβ, insoluble samples, this enhancing the attributed benefits of its presence in the CNS. The

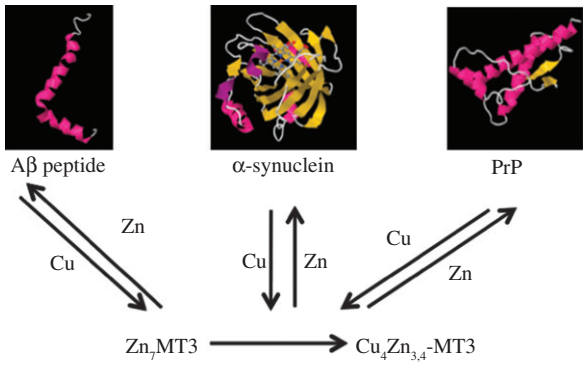

Figure 4 Metal swap reactions leading to Cu(II)/Zn(II) exchange between the Cu(II)-loaded forms of the Aβ peptide (1IYT entry in PDB), α-synuclein (1X8Q entry in PDB), and the bovine PrP (1DX0 entry in PDB), whose aggregation is responsible of the AD, PD, and spongiform encephalopathy neurodegenerative disorders, respectively. All protein structures have been represented using Jmol, an open source Java viewer available at www.jmol.org.

full investigation of the consequences of this metal swap reaction ended with the study of the fate of the Zn(II) ions liberated from the Zn_7-MT3 complexes, as it had been described that Zn(II) ions induced the quick formation of Aβ aggregates upon stoichiometric binding, and that these Aβ oligomers were the substrate for synaptic targeting and accumulation into the extracellular matrix (51). Most interestingly, the shape of the Zn(II)-induced Aβ aggregates seems to depend on the source of the Zn(II) ions, because while free Zn(II) coordination yields amorphous Aβ aggregates, the slow release of Zn(II) ions from preexisting complexes promotes the formation of amyloid-type fibrils, this observation posing an apparent paradox to the proposed beneficial role of the Zn-MT3 complexes as defenses for Aβ toxicity. Beyond these detailed analyses, it has to be noted that until very recently, no information was available about whether metal ion swap reactions required physical protein contact (i.e., a physical interaction). Recently, in 2012, it has been reported that no Aβ-metal-MT3 complex is involved in the Cu(II)/Zn(II) exchange (52), and therefore metal ion swap would not imply a specific steric recognition between both proteins. Through very precise physicochemical approaches, together with electronic microscopy, it was determined that the metal exchange takes place through free Cu(II), an interesting conclusion because it had previously been shown that intermolecular Cu(II) exchange between Aβ peptides proceeded through the formation of the corresponding ternary complexes [Aβ-Cu(II)-Aβ] (53), and that the exchange of Zn(II) from MT2 to other ligands also depended on ternary complexes (54). Finally, it is worth noting that the beneficial effects of the coexistence of Aβ and MT peptides to ameliorate the Aβ neurotoxicity has been recently shown both in astrocytes of an AD-model mouse transgene overexpressing human MT (55) and in a transgenic *Drosophila* strain that synthesizes Aβ in the eye, when human or fly *MT* genes were simultaneously induced (56).

α-Synuclein and PD

PD is another severe irreversible neurodegenerative process, which is caused by the loss of dopaminergic neurons in the substantia nigra pars compacta of the brain, as well as by the presence of fibrillar structures (Lewy bodies) in brain neurons. These amyloid fibrils are formed upon aggregation of a small protein called α-synuclein, of no definite native 3D conformation, and hypothetically related to the regulation of dopamine neurotransmission. The induction of α-synuclein oligomerization is apparently triggered by several factors, among which metal ions (Cu and Fe) seem to play a predominant role. Cu(II)-α-synuclein complexes have been demonstrated to cause α-synuclein fibrillization, and therefore they should be considered direct agents of dopaminergic neuron degeneration (57). These complexes are formed by high-affinity binding of Cu(II) at the N-terminal end of the protein, specifically to the initiator Met, the second residue (Asp), and water, yielding a tetragonal coordination geometry. In view of the similarities with the copper-induced Aβ neurotoxicity in AD, the potential effect of Zn_7-MT3 to protect against α-synuclein aggregation by Cu(II) scavenging was readily investigated (58). The results of detailed kinetic and spectroscopic approaches unambiguously demonstrated that the Zn_7-MT3 complexes are able to modulate the intrinsic reactivity of Cu(II)-α-synuclein, by inducing a Zn(II)/Cu(II) exchange reaction that involves the reduction of the Cu(II) ions to Cu(I), and the formation of $Cu^{I}_4Zn_4$-MT3 species of identical features to those reported for the Aβ peptide. This would prevent the pernicious consequences triggered by copper-containing α-synuclein complexes, such as ROS formation, dopamine oxidation, and aggregation to fibrillar structures. As in the case of Zn(II)/Cu(II) exchange in the Aβ peptide, no physical interaction has been demonstrated between both proteins. This neuroprotective role of MTs has been extended to the set of disorders classified as α-synucleinopathies, using as experimental models transgenic mice with modulated MT levels (59).

PrPs and spongiform encephalopathies

After the two previous cases, the third logical target of study of MT-induced metal swap reactions were the PrPs, which when misfolded and deposited lead to the development of spongiform encephalopathies, sadly spread to humans in the last decades through the mad cow disease. Cu(II) coordination by PrP monomers is carried out under physiological Cu concentrations, so that an antioxidant protective role can be attributed to PrP. However, excess copper, combined with the action of reducing agents, could lead to the production of ROS by Fenton reactions, Cu(II) becoming reduced to Cu(I) and the PrP protein, or some of its key residues (coordinating His, or Met), being oxidatively damaged (60). All these alterations could trigger the transformation from the safe PrP^{C} structure to the misfolded, aggregation-prone, PrP^{Sc} conformation. Cu(II) binding to PrP proceeds through different coordination complexes, mainly contributed by the histidines present in the repeat amino acid octamer PHGGGWGQ, the relative abundance of which depends on Cu(II) concentrations.

It had been previously determined that Zn_7-MT3 levels were significantly reduced in the brains of patients with Creutzfeldt-Jacob disease, this providing a first direct link between MTs and spongiform encephalopathies (34). Subsequent *in vitro* studies showed that the presence of Zn_7-MT3 abolished the capacity of Cu(II)-PrP complexes to induce the formation of ROS (61). By spectroscopic and mass spectrometry of the species formed by the coexistence of both metal-protein complexes, it became evident that the determining reaction would be the exchange of Zn(II)/Cu(II) ions induced by Zn_7-MT3, which yields inert Zn(II)-PrP complexes and Cu_4,Zn_4-MT3 species. These latter species exhibit the same features as those resulting from the interaction of Zn_7-MT3 and Cu(II)-Aβ-peptides or Cu(II)-α-synuclein, i.e., a same metal ion distribution consisting of the four incorporated Cu(I) ions bound to a partially oxidized MT3 β domain, and the four Zn(II) ions remaining in the α domain. Therefore, once again, MT3 in CNS metal homeostasis appears far from acting just as a mere reservoir of metal ions, becoming instead a key controller of redox reactions and, indirectly, of metal-induced protein damage, misfolding, and aggregation.

MT-ferritin interaction: Fe swap and the genesis of ROS

As iron in its Fe(II) reduced form is extremely toxic for organisms owing to the generation of ROS species upon oxidation, live organisms have special proteins devoted to its transport (transferrin) and storage (ferritin) in the less dangerous Fe(III) state. Apoferritin is a 24-subunit multimer that forms an internal cavity capable of accommodating up to 4500 iron atoms in the form of the mineral ferrihydrite. Narrow channels of <5 Å in diameter connect the inner chamber with the surface of ferritin nanoparticles, so that the release of iron, when and where needed in the organism, has to pass through them, by mechanisms still not well established. However, there is a wide consensus that this mobilization requires reduction to Fe(II), either triggered inside the ferritin unit by small enough reductants, able to penetrate the protein shell through its channels, or by a tunneling effect promoted from the particle surface. It has been recently reported that interaction between any of the Zn^{II}-MT complexes of the three isoforms present in mammalian CNS (MT1, MT2, and MT3) induces iron release from ferritin particles, with a concomitant oxidation of the MT thiolates that causes the release of the Zn(II) ions to the medium (62) (Figure 5). Owing to the size of the Zn(II)-MT complexes, the mechanism

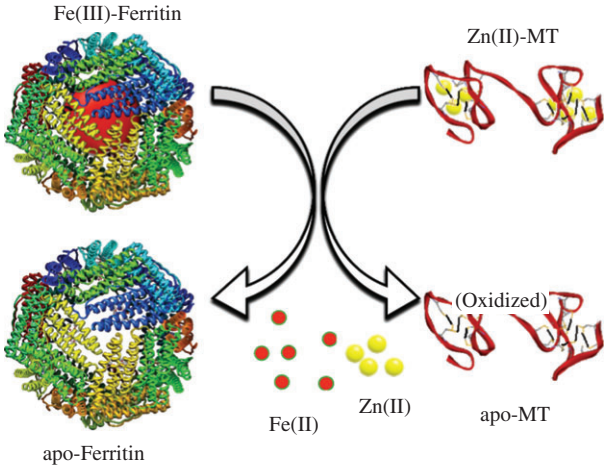

Figure 5 Schematic representation of the consequences of the interaction between iron-loaded ferritin and zinc-loaded mammalian MT1, MT2, and MT3 metallothionein isoforms.

should proceed by pumping to the ferritin cavity the electrons produced by the Zn(II)-MT oxidation. Once inside, they could trigger the reduction of Fe(III) to Fe (II), which would then diffuse to the exterior. Therefore, MTs join the small set of proteins known to interact with ferritin (α2-macroglobulin, apolipoprotein B, α-casein, and 2Fe-2S ferredoxins), all of them putatively responsible for redox reactions involving Fe(III)/Fe(II) reduction and its release.

MT and Zn-dependent-protein interaction: activating/deactivating enzymes and transcription factors

Zn(II) ions are the second most abundant metal cofactor in proteins, and the number of Zn(II) proteins in humans is supposed to amount to about 2800 [for a review on the zinc proteome, see (63)]. Zn(II) proteins belong to two main functional groups: enzymes, where Zn(II) performs either a structural or a catalytic role, and transcription factors, where Zn(II) serves to maintain the structure of the typical DNA-binding domains precisely denominated Zn-fingers. In the majority of cases, Zn(II) is tetrahedrally coordinated by four ligands, either Cys or His amino acid side chains, as found in Zn fingers and some hydrolytic enzymes. In approximately 13% of Zn(II) proteins, the metal ion is bound only by three ligands, contributed by three His, which is the situation in most common human zinc enzymes such as metalloproteinases and carbonic anhydrases (63). Demetalation of all zinc-dependent proteins invariably leads to a functional inactivation that is directly reversed by providing new Zn(II) ions. In this

scenario, and in view of the peculiar Zn(II)-MT cluster properties, which bind Zn(II) tightly (K_{Zn}=3.2×10^{13}/M at pH 7.4 for MT2) while retaining significant reactivity as Zn(II) donors (54), it is not surprising that the thionein/MT system has attracted much attention as a possible regulator of enzymatic and gene transcription activities in the cell (64). Early in 1980, it was already shown that rat liver Zn(II)-MT2 native preparations were capable of reactivating, through Zn(II) donation, a variety of previously demetalated Zn(II)-dependent enzymes, namely yeast aldolase, thermolysin, alkaline phosphatase, and carbonic anhydrase, even more efficiently than zinc salts did (65). Later on, sorbitol dehydrogenase and glycerol phosphate dehydrogenase (66), as well as caspase-3, fructose 1,6 diphosphatase, glyceraldehyde 3-phosphate dehydrogenase, aldehyde dehydrogenase, Tyr-phosphatases, and yeast enolase (67), lengthened the list of enzymes whose activity would be modulated by the thionein/Zn(II) MT shuttle cycle. Parallel results were described for the Zn(II) finger transcription factors, mainly after the study of the inhibition of DNA binding, and thus transcriptional activation capacity of Sp1 (68) and TFIIIA (69, 70) by thioneins. It is also well known that the activity of Zn(II) finger transcriptional factors is directly activated upon Zn(II) binding, with the MTF-1 (MT transcription factor) itself being a paradigmatic example (71).

After the gathered evidence that the capacity of Zn(II) transfer in both directions between Zn(II)-containing proteins and Zn(II)-MTs was able to modulate several vital cell processes, such as metabolism and gene expression (Figure 6), the mechanisms that underlie this exchange began to be investigated. From the obtained results, it is reasonable to conclude that Zn(II) transfer does not always proceed through the same molecular process. Hence, some cases, such as *Escherichia coli* alkaline phosphatase and bovine carboxypeptidase A inactivation [i.e., Zn(II) removal], required the contribution of other agents, namely oxidized glutathione or citrate (54). Alternatively, and most interestingly within the framework of this review, the rapid exchange of Zn(II) ions between Zn-MT and heart mitochondrial aconitase was revealed as fully dependent of direct interaction between the two proteins, as the exchange was absolutely abolished if a metal ion-permeable/protein-impermeable membrane separated both protein solutions (72, 73).

Systemic bovine serum albumin-MT interaction

Albumin is the predominant protein in mammalian serum, where it performs a basic function of transport of a vast variety of compounds and control of blood osmotic pressure. To this end, it reversibly binds fatty acids, bilirubin, hematin, hormones, drugs, amino acids, GSH, and both physiological (Cu^{2+}, Ca^{2+}, Na^{+}, K^{+}) and xenobiotic (Ni^{2+}, Hg^{2+}, Ag^{+}, Au^{+}) metal ions. Some authors have considered the possibility that the albumin metal transporter capacity could rely on an interaction with MTs, as extracellular MT has been shown in tissue intracellular spaces, as well as secretion and excretion fluids. While the amount of extracellular MT is very low under physiological conditions, concentrations in serum are increased by the same factors known to induce MT synthesis, i.e., metal and oxidative stress, among others, and inflammatory and injury tissular alterations. For precise information on extracellular MT, the reader is directed to a specific review in ref. (74). Both pancreatic exocrine and endocrine cells (75) and white adipose tissue (76) have been identified as precise sites for MT secretion, although MT has widely been detected in a large range of physiological fluids besides serum, such as urine, milk, bile, liver/pancreas/renal ducts, and bronchoalveolar and prostatic secretions. In view of this scenario, it was investigated whether the spectroscopic properties of bovine serum albumin (BSA) underwent any changes upon MT contact. Indeed, both the circular dichroism profile and the fluorescence

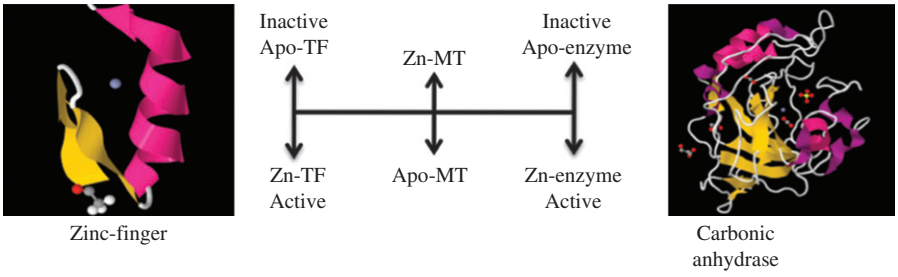

Figure 6 The possibility to load/unload Zn(II) from zinc-requiring proteins exerted by MTs confers them the role of regulators of the activity of multiple enzymes and transcription factors, here represented by a canonical Zn finger loop (1ZNF PDB entry) and the human carbonic anhydrase (2VVA PDB entry). All protein structures have been represented using Jmol, an open source Java viewer available at www.jmol.org.

signals of BSA changed upon MT addition, which was readily interpreted as a proof of the interaction of the two proteins and putatively related to the BSA metal transport and detoxification capacity (77).

MT interaction with cancer-related proteins

The possible role of MTs in tumor generation and progression, and their putative use as diagnostic and prognosis markers have been largely evidenced by their overexpression, in distinct types of human cancers. Like in the case of neurodegenerative disorders, controversial and highly heterogeneous results led to the conclusion that the increase or decrease in MT synthesis during neoplastic processes may depend on multiple factors, such as the tissue or organ affected, the state of tumor development, and/or mutations occurring in other concomitant genes. Several reviews on the subject (78–81) summarize how, on the one hand, increased MT synthesis has been detected in breast, lung, liver, pancreas, kidney, urine bladder, colon, ovary, cervix, endometrial, skin (including melanoma), prostate, testes, thyroid, salivary glands, and nasopharynx tumors as well as certain types of leukemia, and on the other hand, MT expression appears downregulated in certain types of hepatocellular, gastric, colorectal, CNS, and thyroid cancers. The biological basis of these observations has yet to be elucidated; however, there is experimental evidence of the interaction between mammalian MTs and three proteins whose mutated structure and malfunction is known to be directly related with oncogenesis: two transcription factors [p53 and nuclear factor κB (NF-κB)], and the PKCμ protein kinase signal transducer.

p53-MT interaction

The p53 tumor-suppressor is a transcription factor that becomes posttranslationally activated in cells undergoing stress conditions, this leading to the enhanced expression of genes involved in cell cycle arrest, DNA repair, ageing, and apoptosis (82). A large number of p53 mutations are known (cf. www-p53.iarc.fr), mostly consisting of missense mutations that convert the guardian p53 protein into a definite oncogenic product by means of altering its target gene repertoire, a situation that occurs in approximately half of human tumors. The wild-type p53 tetramer binds DNA through functional domains that are maintained by a structural zinc ion in each monomer. The loss of this Zn(II) ion disrupts the native p53 structure, yielding aberrantly folded domains, with non-specific, weak DNA-binding properties that lead to cell transformation. In contrast, excess zinc yields misfolded p53 monomers that end up in an irreversible p53 aggregate precipitation, and therefore also to a cell transformation phenotype owing to the absence of p53 protein function. The phenomenon of the zinc control of the p53 antitumor role has been recently reviewed in ref. (83). It is obvious that, besides the influence of single point mutations directly or indirectly affecting the tetrahedral Zn(II) binding site, contributed by three Cys residues and one His residue, all metallochaperones may influence the correct folding of p53 by either sequestering or providing Zn(II) ions. Therefore, MTs attract interest as a putative p53 function modulator. Early in 2000, it was already shown that *in vitro* interaction between apo-MT1 and p53 led to a loss of the p53 Zn(II) ions that caused its failure to bind to DNA (84). Furthermore, in this same study, cotransfection of MT1 and p53 expression vectors into mouse fibroblasts confirmed the modulation, in both senses, of the *in vivo* p53 gene expression regulation capacity, as MT1 overexpression provoked a clear loss of p53 activity as transcriptional activator – presumably through Zn(II) sequestration – while the equimolar presence of both proteins supposed an increased p53 activity – presumably due to an enhanced Zn(II) transfer to p53. Although this dual consequence of the p53-MT interaction was clearly evident, it was not until 2006 that both *in vivo* and *in vitro* standard coprecipitation procedures confirmed and characterized the physical contact between these proteins (85). Significantly, this review showed that only the apo-MT1 form, and not its Zn,Cd-MT complexes, was associated with p53, which points to the key role of the Zn(II) ion present in p53 in stabilizing this interaction, at least strongly enough to yield positive results in the interaction assays.

NF-κB-MT interaction

NF-κB is a protein complex acting as a transcription factor, which becomes activated upon translocation from the cytoplasm to the nucleus, triggering there the expression of genes mainly involved in stress and immune cell response. One of the particular roles of NF-κB is the prevention of cell apoptosis. This antiapoptotic activity may rely on the mitogenic capacity that it exerts in carcinoma cells, where it typically appears deregulated. MT had been described as a cell proliferation agent, and some work was devoted in the late 1990s to elucidate if this effect was precisely related to a possible interaction with NF-κB (86). This study showed that, in zinc-stimulated breast cancer cells, MT interacts with NF-κB, specifically with the p50

subunit of the p50/RelA NF-κB component, which would stabilize its binding to DNA, and subsequently would increase its activity as a transcriptional activator of the corresponding target genes. The molecular basis of the enhanced DNA binding remains to be analyzed; however, it is interesting that, besides modification of macromolecular structures due to the MT-NF-κB interaction, it was suggested that MT could also contribute to activate third-party transcriptional factors by Zn(II) ion supply, in perfect agreement with the results described in a preceding section of this review.

pPKCμ-MT interaction

The protein kinase PKCμ is a signal transduction protein whose synthesis is downregulated in androgen-independent prostate cancers, while it is strongly upregulated in androgen-dependent prostate cancer cells. Unexpectedly, the MT2A isoform was retrieved as a PKCμ-interacting protein in a two-hybrid yeast assay, a relation confirmed by subsequent cell colocalization results (87). The recognition region was mapped into the kinase domain of PKCμ, and more weakly to its lipid-binding domain. More specifically, the PKCμ lysine 612 at the ATP binding site, thus responsible for its kinase activity, appeared directly involved in the protein contact, which suggested that the MT interaction would decrease the PKCμ enzymatic activity in androgen-independent prostate cancers. This observation, together with the fact that the metal-binding and antiapoptotic properties (cf. section above) of MTs are generally regarded as the main cause of the resistance of transformed cells to antineoplastic treatments, make MTs specially attractive targets for novel therapeutic strategies against hormone independence and chemoresistance in certain types of cancer.

A non-mammalian study: *Drosophila* and yeast MT interactions

Drosophila MTs are the only non-mammalian MTs for which protein interaction has been investigated (88). *Drosophila* encodes five MT isoforms (MtnA to MtnE), with amino acid sequences shorter than mammalian MTs, that exhibit a marked Cu-thionein character (8, 9), both features closely resembling those of the yeast Cup1 MT. By application of the most classic protein interaction methods (two-hybrid tests and coprecipitation), it was detected that the two major *D. melanogaster* isoforms (MtnA and MtnB) interacted with the Jafrac-1 peroxiredoxin, a thiol-specific redox-recycling enzyme, also named thioredoxin-peroxidase (89). Most significantly, this interaction was shown to be absolutely metal dependent, as it was only detectable when assaying partially or totally Zn(II)-loaded MTs, while the Cu(I) complexes were absolutely inert. It is also worth noting that the interaction between the respective yeast homologues, i.e., between Cup1 MT and the Tsa1-Tsa2 thio-specific antioxidant peroxiredoxins, was also identified, and that it followed an identical metal-dependent pattern. Three hypotheses were put forward to rationalize the biological significance of this interaction. First, totally or partially oxidized Zn(II)-MT complexes may become the substrate of peroxiredoxins that catalyze their reduction, as the Cu-thionein character of MtnA, MtnB, and Cup1 implies the undermetalation of those metal-MT complexes. Second, the reaction may occur the other way round, so that oxidized peroxiredoxins could be reduced by MT. Finally, MT could serve to transfer Zn(II) ions to peroxiredoxin molecules in order to transiently stabilize thiol groups, which would be in line with several Zn transfer reactions identified for mammalian proteins (cf. following section of this review).

Conclusions and future prospects

In summary, all the information here gathered about MT-protein interactions should definitely abolish the notion of MTs as inert deposits of useless metal ions in cells and organisms, a vision that predominated in MT research for a long time. The capacity of MTs to regulate, through protein interaction and/or metal ion exchange, the number and type of metal ions present in a high variety of proteins is rapidly contributing to their acceptance as control agents in health processes such as cell metabolism and gene expression regulation, as well as active players in neurodegenerative disorders and cancer outcome. A common feature of all the reported protein-MT interactions is that they are metal-dependent processes, not only due to the biological significance of the nature of the metal ion present in the corresponding complexes but also because the metal ion coordination preferences dictate the fold of each MT polypeptide, and protein-protein interactions are basically spatial events. Protein-MT interactions fall into two distinct kinds of events: physical interactions, by forming biprotein or multiprotein aggregates, or metal swap events, probably occurring in a lapse of time too short to be detected as a physical interaction, at least with the currently available techniques. Among the first type, the most remarkable examples are the interactions

between *Drosophila* and yeast Cu(I)-thioneins and peroxiredoxins; those of the mammalian Zn(II)-MT isoforms in CNS, mainly involved in the synaptic processes; and those regulating the functionality of zinc-dependent proteins, from enzymes to transcription factors. The second type of protein-MT interactions, which consists of Zn/Cu exchange reactions, involves the CNS-specific MT3 isoform and several peptides where the aggregation is known to lead to irreversible neurodegenerative damage (AD, PD, and prion-related diseases). In all these cases, interaction of Zn(II)-MT3 with the aggregation-prone copper-loaded peptides seems to preclude their harmful effects. It is worth noting how almost all the events here analyzed identify Zn(II) ions as their main players. This enhances even more the crucial role of Zn-MTs in a wide variety of physiological and disease-related processes, both because they are involved in protein-MT interactions (this work), or thanks to the consequences of their interaction with non-protein counterparts in the control of the cell redox state, an aspect extensively investigated and reviewed by Maret's work (90–92).

Accordingly to the presented information, the therapeutic use of MTs has been approached mainly in relation to two kinds of disorders: cancer and neurodegeneration. It becomes obvious that almost all the mammalian proteins involved in MT-interaction events are related to one of these two pathological processes. However, many greater research efforts have to be still devoted to the characterization of the precise nature of these interactions in the disease onset and progression, to determine which precise intervention could lead to a therapy scheme. Unfortunately, too often, contradictory results on the association of MTs to different pathologies are reported, which precludes the design of biomedical applications unless definite data are validated. A clear example of this can be the interaction of MTs with the mitogenic factor NF-κB that seems to enhance its antiapoptotic capacity (86), while a very recent study reports that a precise mammalian MT1 isoform (MT1M), expressed in different tissues but especially in the liver, is downregulated in human hepatocellular carcinoma cells, a result that the authors explain in the light of the hypothesized MT1M capacity to block the activation of NF-κB (93). Contrarily, the beneficial contribution of MTs to the correct mammalian CNS functionality and integrity seems to gather a general consent. However, beyond the analysis of the MT partners that lie under the relation of both MT1/MT2 and MT3 in protein-aggregating neurodegenerative processes (AD, PD, or spongiform encephalopathies), the precise basis of the MT involvement in other disorders where the MT neuroprotective effect has been demonstrated (amyotrophic lateral sclerosis and multiple sclerosis among the best characterized) remains to be established [cf. the revisions (94, 95)]. Likewise, no protein interaction has been identified as responsible for the promising MT performance in cerebral ischemia (96), seizure accidents (97), or injury and burn scar formation (98, 99).

We are convinced that this field will be the subject of intense investigation in the following years, and that a great deal of the MT research that will be conducted in the near future will be centered on the detection and characterization of new protein interaction events. Then, the newly gathered information will exponentially increase the biomedical MT applicability possibilities, not only by adopting these metalloproteins as useful therapeutic molecules and vectors, but also through the consideration of all the MT-interacting proteins as targets for new drug and therapy designs.

Acknowledgements: We acknowledge the financial support of our research by the Spanish Ministerio de Ciencia e Innovación, through the projects BIO2012-39682-C02-01 (to S. Atrian) and BIO2012-39682-C02-02 (to M. Capdevila). Both authors are members of the 'Grup de Recerca de la Generalitat de Catalunya' ref. 2009SGR-1457. The figures of the three-dimensional representation of proteins have been kindly generated by Maria Guirola and Òscar Palacios.

Received July 25, 2012; accepted December 6, 2012

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