Toshiyuki Fukada · Taiho Kambe *Editors*

Zinc Signals in Cellular Functions and Disorders

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ISBN 978-4-431-55113-3 ISBN 978-4-431-55114-0 (eBook)
DOI 10.1007/978-4-431-55114-0
Springer Tokyo Heidelberg New York Dordrecht London

Library of Congress Control Number: 2014947521

Printed on acid-free paper

Springer is part of Springer Science+Business Media (www.springer.com)

Foreword

Our understanding of the roles of the essential metal zinc in health and disease processes is advancing rapidly, as is evident from the remarkable studies presented in this book on zinc signaling mechanisms. Certainly many excellent scientists have contributed to the foundations of this field, and I trust that they will not be offended if I cannot mention them all in this limited space. However, I find the pioneering studies of several clinicians and molecular biologists particularly noteworthy of mention. The clinicians Ananda Prasad and Harold Sandstead traveled to Iran in the late 1950s and 1960s and found young adults who had failed to thrive and mature and then deduced that this was due to dietary zinc deficiency (Prasad 1984). By the early 1970s, Edward Moynahan identified acrodermatitis enteropathica as a genetic disease of zinc deficiency in humans (Moynahan 1974). These findings led the American National Academy of Sciences in the 1970s to realize that humans could, in fact, become zinc deficient and that zinc deficiency could cause disease.

The molecular biologist Richard Palmiter first described the mammalian metallothionein genes and demonstrated their dramatic transcriptional induction by zinc and then created mouse models which over-express or lack metallothioneins (MT-I and II) (Palmiter 1987; Masters et al. 1994). Subsequently Walter Schaffner identified and cloned the transcription factor MTF-1, which regulates metallothionein gene transcription in response to zinc (Radtke et al. 1993). These pivotal studies paved the way for thousands of subsequent studies. Although my group provided compelling data that the unique zinc-finger domain of MTF-1 functions as a zinc sensor (Laity and Andrews 2007), the structural basis for that mechanism remains to be resolved in detail. Nonetheless the concept that substantial changes in "available" zinc in higher eukaryotic cells and organisms are sensed by the cell was fundamental to our understanding of zinc biology and zinc homeostasis mechanisms. We now understand that zinc fluxes modify kinase signal transduction cascades and control the localization and stability of several zinc transporters. Using the MT over-expressing or knockout mice created by Richard Palmiter, we presented some of the first evidence that the mouse metallothioneins provide a biologically important labile pool of zinc (Dalton et al. 1996; Andrews and Geiser 1999). These proteins are now considered to function as zinc buffers. Richard Palmiter's contribution did not end with the metallothioneins.

He subsequently cloned the first mammalian zinc efflux transporter (ZnT1; Slc30a1), described the ZnT gene family, and created mouse models that lacked ZnTs (Palmiter and Findley 1995; Palmiter and Huang 2004). His ZnT3 knockout mouse model has been and continues to be employed in hundreds of neurobiology studies (Cole et al. 1999).

Another fundamental advance in the field was the identification of the first ZIP family member IRT-1 in Mary Lou Guerinot's laboratory (Eide et al. 1996). In collaboration with David Eide they showed that *Saccharomyces* ZRT zinc transporters (Zhao and Eide 1996) and *Arabidopsis* IRT1 iron transporters belong to a structurally related family of metal ion transporters, thus the acronym **Z**rt-**I**rt-like **P**roteins (Guerinot 2000). The ZIP proteins are found in all eukaryotes, and orthologues are found in bacteria. Since the identification of this family of metal ion transporters, there have been hundreds of publications on their structure, regulation, and functions. Pioneering studies by Jane Gitschier (Wang et al. 2002) and Sebastien Kury (Kury et al. 2002) identified *Zip4* mutations in patients with acrodermatitis enteropathica about 30 years after the description of this devastating zinc deficiency disease by Moynahan (1974). Among the 14 known ZIP family members, we now have mouse knockout models of over half of these genes. My group created mouse knockout models of *Zip1* through *Zip5* which includes mouse models of acrodermatitis enteropathica (Kambe et al. 2008; Dufner-Beattie et al. 2007; Geiser et al. 2012, 2013). Our studies revealed that expression of the *Zip4* gene in intestinal enterocytes and embryonic visceral endoderm in mice is essential for viability and that the loss of function of this gene causes a rapid shift from anabolic to catabolic metabolism in the animal accompanied by a devastating loss of intestinal integrity and impaired stem cell differentiation.

As you will see when you read this book, the field of zinc biology has matured rapidly in the past decade. The current availability of zinc-sensing fluorescent probes, zinc-transporter genes, and expression vectors, antibodies (still a weak point), and genetic mouse models allows investigators to probe mechanistic aspects of zinc metabolism in great depth. Evidence for functions of zinc and specific zinc transporters in several diseases has emerged, including functions in cancer as well as in normal growth and development. Studies of structure–function relationships in zinc transport proteins are rapidly progressing, and an active field of investigation involves understanding the biophysics of zinc–protein interactions in regulatory proteins and the multiple mechanism of cellular and organismal zinc sensing. We can look forward to many exciting and novel findings in this field over the next few years.

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Preface

More than five decades ago, Dr. Ananda S. Prasad discovered zinc (Zn) as the essential trace element for human life. Zn deficiency was the first discovery in Zn imbalance-related abnormality that causes growth retardation, immunodeficiency, hypogonadism, and neuronal and sensory dysfunctions. Human diseases including cancer, diabetes, osteoporosis, dermatitis, and auto-immune and neurodegenerative diseases have been shown to be associated with abnormal Zn status. Investigations of the biological roles of Zn, however, had been challenging because Zn compounds are normally colorless, and the natural status of Zn is stable as a divalent cation, unlike other bioactive metals such as iron and copper.

Until now, there have been at least four issues that advanced our knowledge about the significant roles of Zn in physiology and diseases. First: bioinformatics, which revealed that approximately 10 % of all proteins in humans may bind with Zn. Second: genetic approaches using animal models and human genetics, which contributed to demonstrating the physiological roles of Zn in cells, tissues, and the whole body. Third: investigation of Zn transporters and metallothioneins in vitro and in vivo, which provided a variety of information on the importance of Zn transportation within and between cells, which led us to the fourth issue: Zn indeed acts as a signaling factor like calcium, called "Zn signaling". Because this is a quite new field, we were motivated to introduce the current status of the study of Zn signaling and to review the whole scheme of this area to date.

The present book overviews up-to-date information on the study of Zn signaling, describing not only the essence of Zn signaling including its history, the molecular analysis of the structures and functions of Zn transporters and metallothioneins, and detection techniques for Zn signals, but also the involvement of Zn signaling in physiology and disease status as in brain function, immunity, inflammation, skeletogenesis, diabetes, and cancer. Besides the introduction of new insights in the study of Zn signaling, this book aims to address the many unsolved problems in the field. For this reason, we made a great effort to furnish educational contexts that will provide great introductions for students, young scientists, and clinical personnel. These contexts can also be valuable references for the pioneers and aficionados among researchers involved with Zn. So that all these goals would mesh, we as editors invited contributions from investigators who are world leaders in this field.

We believe the publication of this book is timely for reviewing the nature of Zn signaling, in which there is growing evidence that Zn signals regulate intra- and extracellular events leading to biological homeostasis, as all the authors will discuss. Also, we are confident that readers will find the book valuable for teaching, lecturing, and other outreach activities that can help make known to the public the importance of Zn itself. Finally, we express our heartfelt thanks to the splendid contributions of all authors, which will lead us to our goal.

Yokohama, Kanagawa, Japan — Toshiyuki Fukada, Ph.D.
Kyoto, Japan — Taiho Kambe, Ph.D.

Contents

Chapter 1
Introduction: "Zinc Signaling"–The Blossoming Field of Zinc Biology

Taiho Kambe

Abstract Zinc plays an indispensable role in life. It regulates a great number of protein functions including transcription factors, enzymes, adapters, receptors, and growth factors as a structural or catalytic factor. Recently, a further function of zinc has received extensive interest and attention because of its potential importance as a signaling mediator. Zinc plays a dynamic role as an extracellular and intracellular signaling factor, which enables communication between cells in an autocrine, paracrine, or endocrine fashion, conversion of extracellular stimuli to intracellular responses, and regulation of various intracellular signaling pathways. These zinc functions are recognized as "zinc signaling," which has critical roles in physiology, and thus their imbalance can cause a variety of problems with regard to human health. This book extensively reviews the field from the basic aspects to the crucial roles of zinc signaling in biological processes, discussing the future directions and questions at both the molecular and the physiological level.

Keywords Zinc • Zinc homeostasis • Zinc signaling

1.1 Introduction

There are two types of zinc ions in the body. One is protein-binding zinc, which functions as a structural component and enzyme cofactor to stabilize and functionalize proteins. Proteome analyses estimate that approximately 10 % of proteins encoded in the human genome have potential zinc-binding motifs (Andreini et al. 2006). The other is labile zinc, which means "free" zinc ions. The amount of free zinc ions in the cytosol maintained is extremely low and is estimated to be less than nanomolar concentrations, while it is high in some kind of zinc containing vesicles such as insulin granules and synaptic vesicles. (Kambe et al. 2004; Maret 2011). Recent studies have revealed exciting information about the dynamic roles of free zinc ions in a great number of biological processes (Frederickson et al. 2005; Hirano et al. 2008; Sensi et al. 2009; Haase and Rink 2009, 2014; Fukada et al. 2011), which has highlighted the signaling functions of zinc, generally called "zinc signaling" (Fig. 1.1).

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T. Fukada, T. Kambe (eds.), *Zinc Signals in Cellular Functions and Disorders*,
DOI 10.1007/978-4-431-55114-0_1

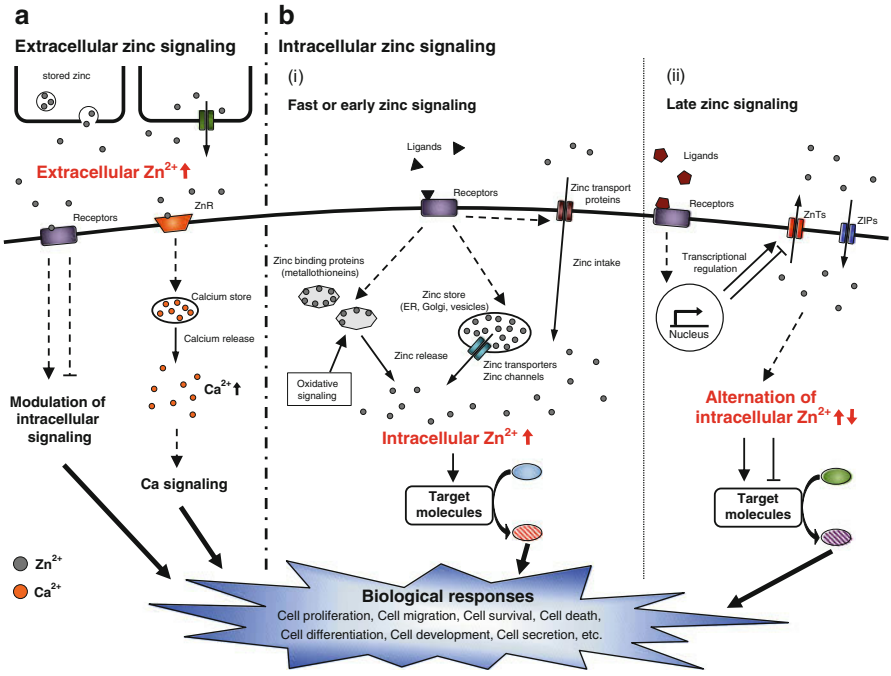

Fig. 1.1 Schematic diagrams of zinc signaling. Zinc signaling is caused by alternation in zinc concentrations in both the extracellular and intracellular milieu and can be divided into several types. **a** "Extracellular zinc signaling" takes place in extracellular milieu such as the synaptic cleft. In extracellular zinc signaling, zinc, which is released from cells via exocytosis of vesicular zinc or zinc efflux across the plasma membrane by zinc transport proteins, acts as a ligand of receptors expressed on the cellular membrane of target cells. One example is the ZnR (zinc receptor) signaling that activates calcium (Ca) signaling in different types of cells, and another example is α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) or *N*-methyl-D-aspartate (NMDA) receptor signaling, which can modulate intracellular signaling in postsynaptic cells. **b** "Intracellular zinc signaling" is the result of extracellular stimuli via their receptors, which generally can be divided into two groups depending on the timescale in which it operates. *i* "Fast or early zinc signaling" occurs within seconds or a minute after the extracellular stimulus. In fast or early zinc signaling, intracellular free zinc is influxed from the extracellular space or released from intracellular zinc stores, such as the endoplasmic reticulum (ER), Golgi, and some vesicles, by zinc transport proteins, including ZIPs (Zrt, Irt-like proteins), AMPAR, and L-type calcium channels. Furthermore, zinc is released from cytosolic zinc-binding proteins such as metallothionein (MT), which can be triggered by oxidative stimuli. The increased intracellular free zinc functions as an intracellular second messenger as does Ca. *ii* "Late zinc signaling" occurs in the timescale of hours. In late zinc signaling, extracellular stimuli induce transcriptional regulation of zinc transport proteins including ZIPs and ZnTs (Zn transporters). This regulation can contribute to the alteration in intracellular zinc concentrations and zinc distribution across the cellular membrane, leading to regulation of downstream signaling pathways. All types of zinc signaling modulate a wide variety of biological responses such as cell proliferation, cell migration, cell survival, cell death, cell differentiation, cell development, cell secretion

Zinc signaling occurs via alterations in zinc concentrations in both extracellular and intracellular sites. It contributes to multifarious physiological and pathological processes, and the mechanisms underlying zinc signaling events have begun to be elucidated at the molecular level. Thus, it is very timely to review the extensive functions of zinc signaling. This book has 15 chapters that give a comprehensive understanding of the current knowledge of zinc signaling in physiology and pathogenesis. As an introduction to the book, zinc signaling is briefly overviewed from several different perspectives.

1.2 "Extracellular" Versus "Intracellular"

Zinc signaling can fall into two classes from the perspective of the location where it occurs: one is extracellular zinc signaling, and the other is intracellular zinc signaling (Fig. 1.1). The former has been extensively investigated in synaptic transmission in the central nervous system (CNS) (see Chaps. 4, 8, and 9), where zinc released in synaptic clefts from presynaptic neurons can modulate neurotransmitter functions via a number of receptor channels and transporters on postsynaptic neurons through its zinc-binding sites (Frederickson et al. 2005; Sensi et al. 2009). Besides synaptic transmission, zinc released with insulin from pancreatic β cells reduces insulin secretion from the β cells and suppresses insulin clearance in the liver (see Chap. 13). Thus, extracellular zinc signaling mediates communication between cells in an autocrine, paracrine, or endocrine fashion. For sensing extracellularly released zinc, zinc receptor (ZnR), which is identical to G protein-coupled receptor 39 (GPR39), is important (see Chap. 6).

Intracellular zinc signaling, which plays a role in a great number of cell types, has been extensively investigated in immune cells (see Chaps. 10 and 11). In the cytosol, zinc functions as a second messenger of extracellular signals similarly to Ca. The "zinc wave," which has been identified as zinc release from the perinuclear area including the endoplasmic reticulum (ER) in antigen-stimulated mast cells (Yamasaki et al. 2007), is well known (see Chap. 5). Release of zinc from proteins with zinc-thiol/disulfide coordination such as metallothionein (MT) is thought to contribute to intracellular zinc signaling (see Chaps. 2 and 4). In intracellular zinc signaling, a number of molecular targets have been identified, including protein tyrosine phosphatases (PTPs), phosphodiesterases (PDEs), caspases, and kinases (Huber and Hardy 2012; Wilson et al. 2012). In oocytes, zinc is taken up during the final stage of maturation, and then released into the extracellular milieu on fertilization, which is named the "zinc spark." Zinc spark decreases intracellular zinc content, thereby contributing to regulation of cell signaling (see Chap. 15).

1.3 "Membrane Transport Protein-Mediated" Versus "Cytosolic Protein-Mediated"

Alteration of zinc concentrations initiates zinc signaling; therefore, zinc transport proteins have critical roles in this process (see Chap. 3). ZIP (Zrt, Irt-like protein) and ZnT (Zn transporter) transporters are the major two zinc transport proteins and thus have crucial roles in zinc signaling (see Chaps. 12 and 14). Both transporters are localized to the plasma membrane and membranes of the intracellular compartments, which indicates that zinc is mobilized between the extracellular space or the lumen of intracellular compartments and the cytosol across cellular membranes. In addition to ZIPs and ZnTs, it has been shown that other membrane transport proteins are involved in triggering zinc signaling (see Chaps. 3 and 5).

An alternative mechanism of zinc signaling is by zinc release from proteins with oxidation-sensitive zinc-binding sites in the cytosol, which is triggered via oxidative signaling (see Chap. 2). However, zinc released by oxidative stress can be involved in deleterious signaling pathway (see Chap. 4). Most likely, cooperative regulation of zinc signaling by zinc transport proteins and cytosolic proteins such as MT is required for crucial roles in physiology.

1.4 "Fast" and "Early" Versus "Late"

Extracellular stimuli can increase or decrease the cytosolic concentration of labile zinc within minutes or several hours (Fig. 1.1). Thus, intracellular zinc signaling can also be classified into several classes according to the timescale in which it acts (see Chap. 10). Zinc signaling occurring within minutes is termed "fast" or "early" zinc signaling. Fast or early zinc signaling does not need transcription of proteins (reviewed by Hirano et al. 2008; Haase and Rink 2009, 2014; Fukada et al. 2011). Fast zinc signaling occurs within seconds to a minute, and early zinc signaling, for example, a "zinc wave," occurs within minutes after triggering; in both cases, zinc ions serve as a type of second messenger (see Chaps. 5 and 10). The other type of zinc signaling, which requires transcription of zinc transport proteins including ZIP and ZnT transporters, occurs on a timescale of hours. This type of zinc signaling is termed "late" zinc signaling. In late zinc signaling, local zinc homeostasis is changed in zinc concentrations and distribution by changes of expression levels of zinc transport proteins in response to various stimuli such as cytokines and lipopolysaccharide (Kambe 2013). Thus, late zinc signaling is important as homeostatic control mechanisms.

1.5 Conclusions

The increasing body of evidence clearly reveals that zinc signaling is essential for life, but it is also clear that recent findings represent the very tip of the iceberg. Zinc signaling is essential for human physiology, and its dysfunction likely causes health deterioration and diseases including cancer, inflammatory diseases, diabetes, and neurodegenerative diseases, as shown in this book. There are still many questions and ongoing discussions in the field of zinc signaling. However, novel techniques, such as genetically encoded fluorescent sensors (see Chap. 7), will contribute to clarifying those questions and discussions, similarly to the case of small fluorescent probes, which have greatly contributed to detecting and monitoring both extracellular and intracellular zinc signaling (Nolan and Lippard 2009).

This book is the first one to extensively review zinc signaling. Readers will enjoy each chapter with interest and find that zinc signaling definitely goes mainstream!

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Chapter 2
Molecular Aspects of Zinc Signals

Wolfgang Maret

Abstract Zinc ions (Zn^{2+}) have chemical properties that make them ideally suited to carry biological information in intracellular and intercellular communication. Such zinc signaling has much in common with calcium signaling in terms of fast binding in coordination environments of proteins, but there are also important differences between the two metal ions. Biological control with zinc occurs at much lower metal ion concentrations. Zinc ions bind with higher affinity and hence dissociation rates are slower, resulting in longer-lasting biological effects. Selectivity of coordination environments is different as zinc employs oxygen, nitrogen, and sulfur donors from ligands whereas calcium binds almost exclusively to oxygen donors. Zinc and calcium ions are redox inert but sulfur donors in zinc/thiolate coordination environments confer redox activity, thereby linking zinc metabolism and redox metabolism. In humans, 24 zinc transporters and more than 12 metallothioneins exert precise control over cellular zinc homeostasis, cellular redistribution, and transients of zinc ions that are used for biological regulation. Zinc ions are stored in subcellular compartments and released by different stimuli. Rises in cytosolic Zn^{2+} concentrations target proteins and affect a variety of cellular processes, such as phosphorylation signaling and gene expression. Zinc signaling complements and interacts with calcium signaling and redox signaling and is an integral part of the cellular signal transduction network. It has fundamental importance for health and disease.

Keywords Zinc • Zinc buffering • Zinc homeostasis • Zinc muffling • Zinc signaling

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T. Fukada, T. Kambe (eds.), *Zinc Signals in Cellular Functions and Disorders*,
DOI 10.1007/978-4-431-55114-0_2

2.1 Historic Perspective: The Emerging Role of Zinc Ions in Cellular Control

Through its catalytic and structural functions in about 3,000 human proteins, zinc is associated with about 10 % of all proteins and affects most aspects of cellular biology (Andreini et al. 2006; Rink 2011). The concept that zinc has regulatory functions in multicellular organisms enhances its importance even further. The concept is recent but rooted in rather early reports. One relates to the transcription factor MTF-1 (metal regulatory transcription factor-1), which senses increased cellular zinc concentrations and mediates zinc-dependent gene expression (Günther et al. 2012). The other is the discovery of autoradiographically detectable zinc in certain parts of the brain (hippocampus), which was found to be *zinc ions* (Zn^{2+}) stored in a yet to be defined chemical form in synaptic vesicles of specific zinc-rich neurons and secreted into the synaptic cleft when these neurons are stimulated. Acting like a classical neurotransmitter in these specialized neurons, the zinc ions affect presynaptic and postsynaptic physiology (Frederickson et al. 2005; Sensi et al. 2009; Takeda et al. 2013). These discoveries expanded into fields other than neurobiology when zinc ions were detected autoradiographically in other organs and tissues (Danscher and Stoltenberg 2005). It is now known that cells sequester zinc ions in cellular vesicles ("zincosomes") and other organelles and that this is a way for the cell to store zinc and to make it available for specific processes. This aspect is different from iron metabolism, where ferrous ions are oxidized to ferric ions and stored in the protein ferritin. In addition to the specialized neurons, a variety of cells secrete zinc ions by vesicular exocytosis (Box 2.1). This process differs from merely exporting zinc, which is an activity of zinc transporters in every cell. The presence of subcellular pools of zinc ions was verified and thus became a much more general issue. Investigations then began to address the functional significance of the zinc ions that are not bound to proteins.

Box 2.1 Cells Secreting Zinc Ions

- Pancreatic β cells and acinar cells
- Prostate epithelial cells (tubuloacinar cells)
- Mammary gland epithelial cells
- Epididymal epithelial cells
- Paneth cells (crypts of Lieberkühn)
- Somatotrophic cells (pituitary gland)
- Osteoblasts
- Platelets
- Mast cells
- Granulocytes, neutrophils
- Fertilized oocytes

Numerous experiments demonstrated that changing zinc in cells by either adding zinc ions extracellularly or by removing them with chelating agents affects multiple signaling pathways (Beyersmann and Haase 2001). These observations gain new significance as they mimic the events that occur when transients of zinc ions are induced intracellularly. There has been an initial focus on immune cells (Hirano et al. 2008; Haase and Rink 2014), but the field is rapidly moving into investigations of other cell types (Fukada et al. 2011; Taylor et al. 2012a). In particular, with the advent of fluorescent chelating agents ("probes") for detecting cellular zinc ions and their fluctuations it became known that zinc ions are intracrine, paracrine, and perhaps even endocrine signals. With this abbreviated account of the origins of the field, three principles are now recognized for the ways cellular zinc ions are made available for signaling functions:

1. Vesicular exocytosis
2. Opening of gated channels on vesicular/organellar stores and the plasma membrane
3. Modification of zinc/thiolate coordination environments in proteins

The first two principles involve zinc in vesicles whereas the third involves zinc in proteins.

In this chapter, I discuss the molecular framework that makes signaling with zinc ions possible. To understand zinc signaling, it is critical to address and define the meaning of the terms "zinc ions" and "zinc signals." These terms are used with ambiguity in the literature. Biologists tend to refer to "zinc," but what they mean is chemically the zinc ion. Zinc(II) (Zn^{2+}) is the only valence state of zinc in biology. Implied in signaling are free zinc ions that are not bound to proteins. Zinc in proteins is also in the form of zinc ions but tightly bound and hence not free. This distinction is the only basis for the term "free," and the term does not refer to an absence of coordinating ligands. There is presently no method to examine the chemical speciation of zinc at the low concentration of free zinc ions in the cytosol or in organelles, and therefore it is not a foregone conclusion that the main species is the hydrated ion $Zn(H_2O)_6^{2+}$. Spectroscopic analysis of zinc in cellular vesicles, where the free zinc ion concentrations are considerably higher, demonstrated interactions with ligands other than water (Wellenreuther et al. 2009). In vesicular exocytosis, zinc ions with their ligands move to an environment with a different pH value. Whether ligand exchange occurs after exocytosis is not known. Free zinc ions may have different ligands and concentrations in the cell ("i", intracellular), outside the cell ("e", extracellular) and in organelles ("o", organellar), and therefore the concentrations of zinc ions in the different pools are referred to as $[Zn^{2+}]_{i,e,o}$. An alternative to low molecular weight complexes of zinc ions being diffusible signals would be that zinc signaling occurs via protein-mediated zinc transfer without the ion ever being free (Fig. 2.1). Such interprotein associative zinc transfer has been observed in vitro (Maret et al. 1997). However, aside from metallothioneins, metallochaperones for such zinc delivery to another protein are not known. Given the large number of structurally diverse zinc proteins, associative zinc transfer is unlikely to be the general case for zinc redistribution as it would require too many

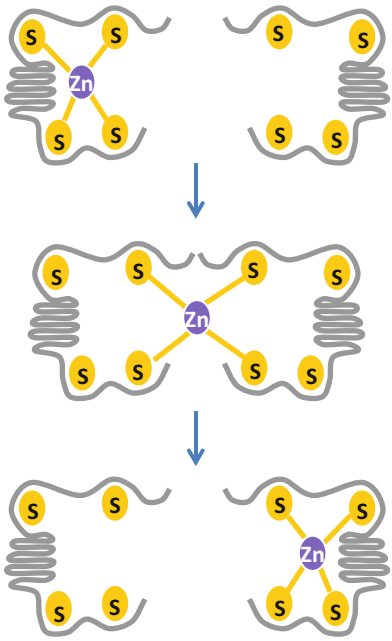

Fig. 2.1 Zinc/thiolate coordination environments as conduits for zinc transfer. Zinc ions can be transferred between two proteins by swapping ligands (associative mechanism). Zinc ions are never free in this reaction. Sulfur donors are particularly well suited for such reactions

chaperones for recognition of all the different zinc proteins, in contrast to copper, the most competitive ion in the Irving–Williams series (see following), which is controlled by chaperones. The fact that free zinc ion concentrations and fluctuations can be measured and that they have functions at rather low concentrations makes them not negligible and supports the role of dissociative mechanisms in zinc signaling. Such mechanisms of redistribution do not exclude the participation of other, low molecular weight ligands as such ligands have been shown to accelerate the rate of zinc removal from proteins (Jacob et al. 1998; Chong and Auld 2007).

Under which conditions zinc ions become "zinc signals" also needs further consideration as the term zinc signaling is being used quite broadly. I suggest that one uses the analogy with the classic signaling metal ion Ca^{2+} to define zinc signaling (Table 2.1) and then focuses on the differences between the two ions, namely, characteristics of zinc such as its binding to sulfur and its higher affinity for proteins and other ligands. As for calcium signaling, the term zinc signaling should be used for fast processes, induced transients of zinc ions, and subsequent binding to an effector.

$$\text{Physiological stimulus} \rightarrow Zn^{2+} \rightarrow \text{effector}$$

This sequence does not exclude MTF-1 from participating in zinc signaling, but it is the rapid activation of MTF-1 as the direct result of a zinc signal rather than the slower consequence of this activation resulting in changes in mRNA and protein abundance.

Table 2.1 Comparison of calcium and zinc signals

Ca^{2+}		Zn^{2+}
Dissimilar:	*Similar*: Subcellular stores involved in release Buffers and mufflers Plasma membrane receptor	*Dissimilar*:
Outward gradient		Inward gradient
O coordination environments		O, N, and S coordination environments
e.g., calmodulin (O chemistry)		e.g., metallothionein (S chemistry)
		Chemical mechanism of release
Short duration of effects		Long duration of effects
Transients >100 nM		Transients >100 pM
Mostly activation of processes		Mostly inhibition of processes

One finds terms such as labile zinc, mobile zinc, or rapidly exchanging zinc in the literature. These terms imply different characteristics of zinc or lack a chemical definition altogether. In fact, they may prevent understanding the molecular basis of zinc ion signals. I believe it is best practice to use one operational definition such as free zinc to refer to zinc ions that are not bound to proteins, albeit with the notion that the coordination environment of the zinc ions is not defined. How strongly proteins bind zinc and how zinc is buffered determine the concentrations of these free zinc ions.

2.2 Control of Zinc Ions

2.2.1 Zinc Buffering

The concepts of free zinc and zinc signaling are intricately linked to metal buffering. Metal buffering is treated as is pH buffering. In the same way that one defines a pH value (hydrogen potential, $pH = -\log [H^+]$), one defines a pZn value (zinc potential, $pZn = -\log [Zn^{2+}]$). pH is determined by the pK_a of the buffering acid–base pair (1), and pZn is determined by the pK_d of the buffering ligand(s) (2):

(1) $pH = pK_a + \log ([A]/[AH])$
(2) $pZn = pK_d + \log ([L]/[ZnL])$

For the most part, the zinc buffering ligands in biology are the coordinating side chains of proteins (Asp, Glu, His, Cys). The dissociation constants (pK_d) for *cytosolic* zinc proteins are picomolar, $pZn = <9$ and >12 (Maret 2004a; Krężel and Maret 2008), although some may be lower, as discussed later. Thus, on the

basis of equilibrium constants, the concentrations of free zinc ions should also be picomolar. One could argue that biomolecules other than proteins contribute to zinc buffering. Many biomolecules bind zinc (free amino acids, carboxylic acids, peptides such as glutathione, ATP, etc.). However, these biomolecules do not bind zinc as tightly as proteins and hence it is unlikely that they contribute significantly to zinc buffering, at least not in binary or 1:1 complexes. To examine this point, the analogy to pH buffering is again helpful. A buffer with a different pK_a value contributes to a buffer at a given pK_a only if higher concentrations compensate for the differences in pK_a values. As we are concerned with logarithmic scales, one pK_a unit would need to be compensated by one order of magnitude in concentrations. Thus, ligands with different pK_d values would contribute to zinc buffering only at much higher concentrations. For zinc-binding biomolecules with lower pK_d values, such as those just mentioned, high concentrations would be required to contribute to physiological zinc buffering. An equally important consideration is how strongly zinc is buffered: this is *not* an issue of the pZn value but an issue of the *buffering capacity at the particular pZn where zinc is buffered*.

2.2.2 Zinc-Buffering Capacity

There is considerable uncertainty in the literature regarding cellular zinc-buffering capacity because it is often assumed to be high as a result of the many ligands that *potentially* bind zinc. It is not the buffering capacity that keeps the free zinc ion concentrations low but the affinity of the ligands. The buffering capacity determines how resistant the pZn is to change.

It is often remarked that "zinc is tightly/strongly buffered" when investigators mean that there is very little free zinc. The low free zinc ion concentrations are a consequence of the high-affinity binding sites in proteins, not the buffering capacity. Tight and weak buffering refers primarily to the buffering capacity, *which is not high as it allows pZn fluctuations to occur*. We determined the cellular buffering capacity at the physiological pZn to be only about 10 % of the total zinc ion concentrations of a few hundred micromolar (Krężel and Maret 2006). This finding has important consequences as it means that some coordination sites with high affinity for zinc are not occupied at physiological pZn and can bind zinc when free zinc ion concentrations increase. These sites determine physiological zinc buffering. Overall, the zinc-buffering capacity of all the ligands is high, but it is not relevant. Zinc is (and must be) buffered at a particular physiological pZn, and only the buffering capacity of the ligands that control this pZn is important. If the buffering capacity were high at physiological pZn, zinc signaling could not occur as any zinc signal would be quenched immediately. Changes in pZn are necessary for zinc signaling to occur. Thus, the cytosolic zinc-buffering capacity and the physiological pZn are major determinants for the role of zinc ions in signaling.

2.2.3 The Role of Zinc Transporters: Muffling

Transporters bring zinc ions into the cell and into cellular compartments or export them from the cell and from these compartments. Transport processes also contribute to biological metal ion buffering and have been referred to as muffling in the calcium field (Thomas et al. 1991). Muffling zinc ions increases the cellular zinc-buffering capacity because it removes an excess of cytosolic zinc ions (Colvin et al. 2010). Thus, what we see as buffering in a cell is a combination of (thermodynamic) buffering by ligands and (kinetic) muffling by transporters. The contribution of transporters in muffling depends on their transport characteristics and the capacity of intracellular stores. Very little quantitative information is available to define these parameters that determine the timescale, frequency, and amplitudes of zinc signals.

Computational approaches in combination with experimental data have been used to model muffling and zinc transients in neurons (Colvin et al. 2008). It became evident that a steady-state buffer model is not sufficient to explain how cells handle extracellular zinc loads, which can mimic cellular transients of zinc ions. A cellular protein that binds zinc and delivers it to a store was required. One molecule that satisfies the required characteristics is metallothionein (MT). A dual role in buffering and muffling zinc, that is, transporting zinc for the delivery to a store, resolves the issue that the concentrations of MTs are generally not high enough to account for the overall cellular zinc-buffering capacity.

This discussion explains why changing the cellular zinc muffling and buffering capacities and hence the pZn but not the total zinc concentration allows zinc redistribution.

2.2.4 Quantitative Measurements

A quantitative approach is needed to define signaling with zinc ions. Total cellular zinc concentrations are rather high, that is, a few hundred micromolar. However, the free zinc ion concentrations (pZn) are picomolar, about six orders of magnitude lower. Free zinc ion concentrations have been estimated and reported in two classic articles. One considers that zinc inhibits muscle phosphoglucomutase, a magnesium enzyme, with high affinity for zinc in vitro (Peck and Ray 1971). For the enzyme not to be zinc inhibited in muscle, the free zinc ion concentration must be less than 32 pM (Ray 1969). It is noteworthy that this consideration merely sets a limit on the enzyme being fully active but it leaves the possibility that free zinc ion concentrations are indeed higher than 32 pM in muscle tissue and modulate the activity of this enzyme. In the other article, free zinc ion concentrations of 24 pM in erythrocytes have been determined (Simons 1991). One expects pZn values in this range on the basis of the affinity of zinc proteins for zinc. With the advent of new molecular probes for zinc ions, that is, chelating agents that become highly fluorescent when binding zinc, picomolar values of free zinc were confirmed in different cell lines,

although the reported values vary between tens and hundreds of picomolar (Krężel and Maret 2006; Bozym et al. 2006; Vinkenborg et al. 2009; Qin et al. 2011). Zinc sensors based on the zinc enzyme carbonic anhydrase measure 5–10 pM, but other protein sensors as well as low molecular weight probes measure hundreds of pM (see table 1 in Maret 2013a). Any probe or sensor that is brought into the cell to measure free zinc contributes to zinc buffering and *lowers free zinc ion concentrations*. We have measured this effect as a function of different probe concentrations and applied an extrapolation to a zero probe concentration to correct for the additional buffering of the probe, and indeed found higher free zinc ion concentrations in the absence of the probe (Krężel and Maret 2006). Some investigators refer to the zinc ions that the probes measure as labile zinc. When extrapolation is applied, the zinc measured cannot be labile because there is no probe to remove zinc from other ligands. The term labile zinc should not indicate that zinc bound weakly to proteins coexists with tightly bond zinc in the same cellular compartment. The free zinc ion concentration determined in the absence of a probe is simply the result of existing equilibria. Tightly bound zinc can be kinetically labile, however. For example, MT binds zinc tightly but the sulfur ligands allow fast exchange with other zinc/thiolate coordination environments (Maret et al. 1997). Measured cellular pZn values indicate that predictions based on zinc-binding affinities of proteins are valid and that there is no buffering increment from other biomolecules that would lower the free zinc ion concentration further.

Although consensus is building regarding cellular (cytosolic) pZn values and zinc-buffering capacity, pZn values in different subcellular compartments are just beginning to be defined. Estimates are 0.9 pM for the endoplasmic reticulum (ER), 0.2 pM for the Golgi (Qin et al. 2011), 0.2 pM for the mitochondrial matrix (McCranor et al. 2012), and 0.14 pM for mitochondria (Park et al. 2012). However, there is a fundamental issue with these concentrations as well as those given for small structures such as erythrocytes. Taking the proton as an example, calculations demonstrate that there are limits to volumes and concentrations where there is essentially no longer a single free ion (Bal et al. 2012a). In other words, given the same concentrations for a larger volume such as the cell and for a much smaller volume such as an organelle leads to a seemingly unrealistically small number of ions in the latter.

Few data exist on extracellular free zinc ion concentrations. Experimental estimates are about 200 pM for horse blood plasma (Magneson et al. 1987), suggesting there is virtually no gradient between extracellular and intracellular free zinc. With higher total intracellular (>100 μM) than extracellular (<10 μM) zinc concentrations, one wonders about the source of energy to transport zinc into the cell against this gradient. However, recent examination of human blood plasma revealed free zinc concentrations of about 500 nM (Maarten Merkx, personal communication). This finding has important implications as it allows zinc binding to serum albumin, which has an apparent zinc-binding constant of about 100 nM (Ohyoshi et al. 1999; Bal et al. 2012b). Such zinc ion concentrations are also in the range for free zinc to be considered as the substrate of zinc transporters. For example, zebrafish ZIP1 has a K_m value for zinc of less than 500 nM (Qiu et al. 2005).

The necessity of buffering zinc in cells with resulting picomolar free zinc ion concentrations becomes evident when considering the properties and the control of

other essential metal ions. A fundamental principle in inorganic chemistry, the Irving–Williams series (Irving and Williams 1948), describes relative binding affinities of divalent transition metal ions and the free metal ion concentrations resulting from binding equilibria. For the cell to keep the biochemistry of metal ions separated from each other, every metal ion needs to be controlled in a specific range of pM. Without such control, the more competitive ions such as zinc and copper would bind to sites that need to be occupied by the less competitive ions. There is no free ranging of metal ions over concentrations needed for the control of other metal ions. According to the Irving–Williams series, the affinity of the biologically relevant divalent metals ions to the same ligands is $Mn < Fe < Co < Ni < Cu > Zn$; that is, zinc is the most strongly bound divalent ion after copper. Thus, for the binding equilibrium

$$P + Zn^{2+} \rightarrow ZnP + 2H^{+}$$

with picomolar affinities of the proteins (P), picomolar free zinc ion concentrations result. There is a scarcity of data about how greatly the affinities of zinc proteins for zinc differ. If there were sites with more loosely bound zinc, there would be considerable dissociation of zinc, loss of function, and interference with the functions of other metal ions. There is no evidence for a hierarchy in such a way that some proteins yield their zinc under zinc deficiency to preserve the functions of other, potentially more essential zinc proteins. Some proteins bind zinc more strongly, with femtomolar affinities for zinc (Sikorska et al. 2012). The reason for this could be that at very low protein concentrations, such as for low abundance zinc proteins, zinc would dissociate if the binding were not strong enough, thereby compromising the function of the protein.

First principles also relate free zinc ion concentrations to biological events. It has been estimated that it would take about 14 h for a zinc enzyme to obtain its zinc at a concentrations of 1 pM free zinc and with a comparatively fast on-rate (Heinz et al. 2005). Clearly, if free zinc concentrations were so low—as they appear to be in some cellular compartments (see earlier)—they could not serve as a source of zinc for enzymes unless low molecular substances enhance the transfer rates (see foregoing) (Heinz et al. 2005). Measured free zinc ion concentrations in the range of a few hundred picomolar, however, are sufficient for association rates on a biologically relevant timescale, in the range of seconds. Of course, this argument also applies to zinc signaling: Zinc ion concentrations need to be at least a few hundred picomolar to affect proteins sufficiently rapidly.

Zinc sites of most zinc proteins seem to be fully occupied with zinc. It would be a significant waste of energy to synthesize a large protein and then not have the zinc available for function. How the synthesis of a zinc protein and the supply of zinc are coordinated and how, when, and where zinc proteins acquire their zinc is not known. There is no experimental evidence that mononuclear sites in zinc proteins are regulated by zinc ion fluxes. The evidence is less clear for enzymes with a second, co-catalytic zinc. These enzymes could be regulated through zinc ion fluctuations. Zinc regulation must occur above the free zinc ion concentrations

that keep zinc proteins saturated. Regulation also requires mechanisms that restore the steady-state free zinc ion concentrations after transients have occurred.

Several investigators attempted to quantify *global* fluctuations of intracellular free zinc ion concentrations. The fluctuations are a few hundred picomolar above the steady-state concentrations of free zinc ions and may reach about one nanomolar (Table 2 in Maret 2013a). For example, when cells are deprived of extracellular zinc, they have a way of mobilizing zinc ions intracellularly (Li and Maret 2009). Also, synchronized rat pheochromocytoma (PC12) cells increase their free zinc ion concentrations at two stages during the cell cycle (Li and Maret 2009). Generally, fluctuations have been measured in different states of a cell and rarely in real time. Hence, the time period over which changes develop and persist is largely unknown.

However, *local* free zinc ion concentrations are expected to be significantly higher. They could establish microdomains akin to those in calcium signaling. Induced local zinc ion transients seem to be fast acting not to act pleiotropically or change gene expression. Although the transients are likely short lived, the effects are expected to be long lasting. Again, first principles support this prediction. With high affinity for zinc and fast on-rates, the corresponding off-rates must be slow. For example, the half-life for zinc dissociation from carbonic anhydrase with a pK_d of 11.4 (pH 7.0) is about 250 days (Ippolito et al. 1995). Of course, carbonic anhydrase is not thought to be regulated by zinc. Because proteins that are targets of fluctuating zinc ions have affinities that are not significantly lower than those of genuine zinc proteins (see following), the off-rates, and hence the duration of zinc signals, are still of the order of many hours or even days. Thus, zinc signaling is expected to elicit long-lasting effects, which is in contrast to calcium signaling. Overall, this interpretation is consistent with zinc ions being inhibitory for cellular functions (Williams 1984) and being involved in long-term adjustments of the state of a cell, such as growth, differentiation, and survival.

Genuine zinc ion signals are to be distinguished from global changes to a different steady-state concentration of free zinc ions (pZn). For example, growth-arrested, proliferating, differentiating, and apoptotic intestinal epithelial cancer cells (HT-29) all have different pZn values (Krężel and Maret 2006), which seem to be the consequence of adjustments in buffering rather than changes in total zinc concentrations. If changes of total zinc were responsible for such adjustments, unreasonable large fluctuations in the range of hundreds of micromolar zinc would be required. Global adjustments of buffering can be brought about by changes of redox potentials that may affect specific redox pairs coupled to zinc binding and release. About 30 % of the zinc-buffering capacity at physiological pH depends on sulfur (thiol) donors and therefore is redox sensitive (Krężel et al. 2007). Changing the expression of zinc homeostatic proteins such as ZnT1 and MT, which are controlled by MTF-1, also changes zinc buffering. The dynamics of the MT pool is an example of how zinc buffering and pZn are interrelated. In different states of HT-29 cells, concentrations of total zinc, free zinc, metallothionein, buffering capacity, and the redox state are correlated (Krężel and Maret 2006). Changed buffering increases or decreases free zinc ion concentrations, and this is a cause for long-term adjustments. Indeed transcriptomics, and

more recently proteomics, investigations have shown extensive changes in protein expression profiles in response to zinc added to growth media or zinc removed by chelating agents.

2.3 Molecular Mechanisms of Proteins Involved in Cellular Zinc Homeostasis

In contrast to proteins that use zinc as a permanent cofactor, the proteins involved in regulating zinc (zinc transporters, metallothioneins, and MTF-1) and in being regulated by zinc have mechanisms for moving zinc and binding it reversibly in sites with coordination dynamics (Maret and Li 2009; Maret 2011a, 2012). MTF-1 has six zinc fingers (C_2H_2 coordination of zinc: C = Cys, H = His) for DNA recognition, but it is neither entirely clear how they are involved together with a metal-reponsive activation domain in zinc sensing nor whether free zinc ions are sensed in the cytosol before MTF-1 translocates to the nucleus (Laity and Andrews 2007; Günther et al. 2012). Three-dimensional (3D) structures of MTF-1 or any of the 24 mammalian zinc transporters [10 members of the ZnT family (SLC30A) and 14 members of the Zip family (SLC39A)] have not been reported and therefore there is virtually no insight into the transport and sensing mechanisms. However, detailed information about the sequences, biological regulation, and genetics of these transporters is available (Fukada and Kambe 2011). The number of zinc transporters is remarkable as homeostatic mechanisms for other metals ions such as iron and copper rely on only a few transporters. None of the mammalian zinc transporters uses ATP as a source of energy. The *Escherichia coli* Yiip protein, which belongs to the ZnT family, functions as a Zn^{2+}/H^+ antiporter and has served as the only model for human ZnTs (Lu and Fu 2007). It has three different zinc-binding sites, all of which use oxygen (Asp/Glu) and nitrogen (His) donors; one is located between the transmembrane helices and one at the interface between the transmembrane domain and the cytoplasmic domain. The third site is binuclear and located between the dimer interface of the cytoplasmic domains. It is thought to be a sensor site of cytoplasmic zinc ion concentrations. Once zinc is bound at these sites, a conformational change of the protein occurs and triggers zinc transport. At present, it is not known whether transporters contribute directly to maintaining physiological pZn. If they do, they would need to have K_m values in the picomolar range, many orders of magnitude lower than their experimentally observed values. Alternatively, the substrates could be proteins that deliver zinc to the transporters by an associative mechanism. Which zinc complexes are the substrates for these transporters and which ligands receive zinc on the opposite side of the membrane is not known.

About a dozen human MT genes are expressed and participate in the control of cellular zinc homeostasis (Li and Maret 2008). Their zinc coordination involves exclusively zinc/sulfur (thiolate) interactions. They have been thought to store zinc, and there is an extensive literature about their possible functions as cellular antioxidants. At least two chemical properties of MTs and the molecular biology

of MT gene expression support a dynamic role in zinc metabolism. One is that MTs have different binding constants for the seven zinc ions. The zinc affinities of human MT-2 are in the range where zinc regulation takes place, allowing MTs to transport, accept, and donate zinc ions dependent on cellular conditions (Maret 2011b). Although four zinc ions are bound with affinities similar to those of other zinc proteins, two zinc ions bind less tightly and one zinc ion only with nanomolar affinity, making MT a protein that is not saturated with zinc at physiological pZn (Krężel and Maret 2007). These properties would allow MT to buffer zinc exactly in the range where such buffering is required. The second property is that MTs are redox-active zinc proteins. Zinc itself is redox inert and remains Zn^{2+}, and hence zinc proteins were generally not considered to be redox proteins. However, the oxidation of the sulfur donors of the cysteine ligands of zinc causes zinc dissociation whereas the reduction of cystines (disulfides) to cysteines (thiols) generates zinc-binding capacity (Maret and Vallee 1998). This property establishes a cellular redox cycle that links redox changes and the availability of zinc from MT and other proteins with zinc/thiolate sites (Chen and Maret 2001; Maret 2009). Because of the redox activity of the sulfurs, MTs are indeed "antioxidants"; however, any discussion of an antioxidant function needs to consider that MT is oxidized when it reacts with oxidants and releases zinc ions that are potent effectors at very low concentrations, and that the released zinc itself can have pro-antioxidant and pro-oxidant functions depending on its concentrations (Maret 2008a).

2.4 Stimulation of Zinc(II) Ion Fluctuations

This section focuses only on the three basic mechanisms of zinc release. The signaling pathways and proteins triggering or controlling zinc release are treated in considerable depth in other chapters of this book.

2.4.1 Vesicular Exocytosis and Paracrine Signals

Zinc transporters ZnT3, ZnT8, and ZnT2 load cellular vesicles with zinc in the brain, pancreatic β cells, and mammary epithelial cells, respectively, for exocytosis of zinc ions. Neither the concentration of zinc in the vesicles (millimolar?) nor the local concentration of released zinc (submicromolar?) is exactly known. Exocytotic vesicles have slightly acidic pH favoring free zinc ions, but once the zinc is exocytosed it is in an environment of higher pH, which favors zinc binding to proteins. Zinc ions secreted from neurons diffuse over a distance of 100 μm, and the signal is eliminated within tens of seconds (Ueno et al. 2002). This investigation demonstrates the significance of the spatiotemporal characteristics of extracellular zinc signals and makes it an issue over which space and time measured zinc ion concentrations are averaged. Owing to their high affinity for proteins, free zinc ions