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Preface

The global public health relevance of environmental Cd^{2+} toxicity is steadily increasing. Intensified industrial and agricultural activities have enhanced Cd^{2+} disposal and, once released into the environment, Cd^{2+} cannot be degraded. Moreover, there was a recent and significant paradigm shift regarding the causes of Cd^{2+} exposure. Rather than focusing on the rare cases of acute intoxication or on occupational exposure (Cd^{2+} being among the Top 20 hazardous substances [1], various public health measures have been implemented to control and prevent Cd^{2+} toxicity in occupational settings), the real challenge for human populations in the twenty-first century is chronic, low (i.e., at concentrations barely exceeding the "natural" environmental Cd^{2+} levels) Cd^{2+} exposure (CLCE). Indeed, CLCE has become a significant health hazard for $\sim 10\%$ of the world population that increases morbidity and mortality [2]. CLCE damages multiple organs in humans and other mammalian organisms by causing nephrotoxicity, osteoporosis, neurotoxicity, genotoxicity, teratogenicity, or endocrine and reproductive defects [3].

Nowadays, CLCE largely originates from dietary sources and cigarette smoking [4]. Modern agriculture globally uses Cd^{2+} containing phosphate fertilizers to increase the efficacy of harvests. Plants, including tobacco, accumulate Cd^{2+}, which is passed on to animals and man in the food chain. Smoking is now established as a major cause of chronic health issues, such as cardiovascular diseases and cancer. Large parts of the population do not refrain from smoking and evidence is accumulating that Cd^{2+} in tobacco smoke takes a share in the development of smoking-associated chronic diseases.

Cd^{2+} is stored in various organs with a half-life of several decades [3]. This happens, because cytosolic Cd^{2+} induces the expression of detoxifying molecules that form a complex with the metal ion and thereby reduce its toxic effects. The major detoxifying tool of the cell for Cd^{2+} complexation is metallothionein (MT), a cysteine-rich metal-binding protein that has the capacity to bind both physiological Zn^{2+} ions and toxic Cd^{2+} ions through the thiol group of its cysteine residues with very high affinity (K_D for Cd^{2+} of $\sim 10^{-14}$ M) [5]. But this apparently beneficial effect is a two-edged sword because the "inactive" Cd^{2+} complexes represent an endogenous source of high concentrations of potentially toxic Cd^{2+}. The kidneys

and liver are the major organs that store Cd^{2+} over a lifetime. Together they contain ~85% of the Cd^{2+} body burden, and more than 60% is found in the kidneys in the age range of 30–60 years [6]. The urinary excretion of Cd^{2+} is assumed to mirror chronic exposition to Cd^{2+} and increases with age and body burden; and smokers have higher urinary excretion than nonsmokers (reviewed in [7]).

Cd^{2+} has no known role in physiological processes. Hence, as a nonessential metal ion, it has to compete with essential metal ions to be taken up. Chapter 1 summarizes membrane transport proteins and receptors for Cd^{2+} and Cd^{2+} complexes that bind and transport toxicologically relevant concentrations of these Cd^{2+} species. Once in cells, Cd^{2+} displaces essential ions from intracellular macromolecules and/or binds to essential sites of biomolecules (e.g., SH groups) that disrupts cellular functions and may lead to cell death and disease. Chapter 2 details with great care the molecular interactions of Cd^{2+} with proteins, which involve metal substitution reactions with many Zn^{2+}-proteins that may perturb protein functions with ensuing toxicity, or contribute to Cd^{2+} inactivation (as with its reaction with Zn^{2+}-MT). Furthermore, Cd^{2+} can also substitute for Ca^{2+} in cellular signaling. Chapter 3 is devoted to the interactions of Cd^{2+} with signaling molecules, with an emphasis on Ca^{2+} signaling and thiol-dependent redox systems as well as the cross-talk between both pathways, and its impact on downstream levels of second messengers, growth, and transcription factors. Besides signaling molecules, mechanical force through change in organelle shape or direct membrane contacts mediate interorganellar communication, which is crucial to orchestrate correct cell behavior and adaptive stress responses. Cd^{2+} effects on organelle structure and function are the topic of Chap. 4, with a particular emphasis on disruption of organelle physiology in vertebrates, including mitochondrial dysfunction, stress response in the endoplasmic reticulum, altered nuclear architecture and chromatin organization, lysosomal damage, disruption of autophagy flux, and interference with vesicle trafficking. Although Cd^{2+} is not capable of catalyzing *Fenton* chemistry in biological systems, it does initiate reactive oxygen species (ROS) formation indirectly by depleting endogenous redox scavengers, inhibiting anti-oxidative enzymes, blocking the mitochondrial electron transport chain, and/or displacing redox active metals, such as Fe^{2+} or Cu^{2+} from their carrier proteins [8] and thereby triggers cell death [9]. Moreover, Cd^{2+} can also substitute for Zn^{2+} in many enzymes and transcription factors which may account for some of its biological effects [10], including damage to the genome [11]. The volume terminates with Chap. 5 in which the impact of Cd^{2+} on genomic stability is pointed out. Cd^{2+} is a class-1 carcinogen because it interacts indirectly with DNA as a consequence of elevated ROS levels, interferes with major DNA repair systems, as well as inactivates tumor suppressor functions by targeting proteins with Zn^{2+}-binding structures. This may cause genomic instability and promote tumor initiation and progression.

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Contents

Chapter 1
Membrane Transport Proteins and Receptors for Cadmium and Cadmium Complexes

Frank Thévenod

Abstract Cadmium (Cd^{2+}) is a non-essential divalent metal ion without physiological function in animal cells. For toxicity to occur, Cd^{2+} must first enter cells by utilizing physiological transport pathways for essential divalent metal ions, such as Fe^{2+}, Zn^{2+}, Cu^{2+}, Ca^{2+}, or Mn^{2+}. 'Free' Cd^{2+} ions and Cd^{2+} ions bound to small organic molecules are transported via ion channels, carrier proteins or ATP hydrolyzing pumps, whereas metalloproteins are internalized by receptor-mediated endocytosis (RME). This review describes Cd^{2+} transport (influx/efflux) pathways that were validated by electrophysiology (e.g. patch clamp), $^{109}Cd^{2+}$ flux, inductively coupled plasma mass spectrometry, atomic absorption spectroscopy, Cd^{2+}-sensitive fluorescent dyes, specific ligand binding, and ligand internalization assays that are ideally studied in heterologous expression systems. Convincing evidence has been obtained for Cd^{2+} permeation for Ca^{2+} channels at toxicologically relevant concentrations ($Ca_V3.1$, CatSper) TRP channels (TRPA1, TRPV5/6, TRPML1), solute carriers (DMT1, ZIP8, ZIP14, system (b0, + AT)) and RME of Cd^{2+}-protein complexes (Lipocalin-2 receptor). The carrier OCT2 mediates Cd^{2+} influx and MATE1/2 and the ATPase ABCB1 Cd^{2+} efflux at high, toxicologically irrelevant Cd^{2+} concentrations. L- and N-type voltage-, ligand-gated, store-operated Ca^{2+} channels, CFTR, connexins and the transporter ferroportin-1 are not permeated by Cd^{2+}. More experimental evidence is needed for the mitochondrial Ca^{2+} uniporter, the ATPase ABCC1 and the transferrin receptor 1. Although the receptor megalin: cubilin mediates RME of Cd^{2+}-metallothionein complex at high, but toxicologically irrelevant concentrations, its in vivo Cd^{2+}-protein–ligand complexes still need to be identified. A stringent methodology is mandatory to prove additional Cd^{2+} transport pathways instead of propagating unsubstantiated speculations.

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1.1 Introduction

Cd^{2+} enters the body primarily through the lungs and the gastrointestinal (GI) tract (reviewed in [1]): The absorption of Cd^{2+} from the lungs is more effective than that from the gut, however, Cd^{2+} absorption from the GI tract represents the main route of Cd^{2+} exposure in non-smoking populations. When Cd^{2+} reaches the blood, it mainly binds to albumin and to other thiol-containing high- (HMWP) and low-molecular weight plasma proteins (LMWP), as well as to blood cells. The blood level of Cd^{2+} reflects recent Cd^{2+} exposure with a half-life of 2–4 months [2]. It ranges between 0.1 and 2 μg/l (~2–18 nM) depending on the populations studied [3, 4].

Cd^{2+} has similar bioinorganic chemical properties as divalent essential metal ions (Me^{2+}) (for a detailed account see [5]), and Cd^{2+} complexes with endogenous biological molecules are analogous to these Cd^{2+}-free molecules. To describe these properties, the term 'ionic and molecular mimicry' has been coined [6]. For toxicity to occur, Cd^{2+} must first compete with essential Me^{2+}, such as Fe^{2+}, Zn^{2+}, Cu^{2+}, Ca^{2+}, or Mn^{2+}, for uptake by cells. The hydrophilic Me^{2+} permeates lipophilic cellular membranes by using intrinsic transport (entry or exit) pathways. 'Free' Me^{2+} and hydrophilic complexes of Me^{2+} with small organic molecules (e.g. amino acids, organic anions or small peptides) are transported via ion channels, carrier proteins or ATP hydrolyzing pumps, whereas Me^{2+}-proteins complexes, i.e. metalloproteins, are internalized by receptor-mediated endocytosis (RME). Several candidate Cd^{2+} transport pathways have been summarized in a review published in 2010 [7]. But meanwhile significant progress in the field has been made and an update is necessary.

To avoid confusion and to clarify the aim of this review, 'transport' by a membrane protein is defined by the methodology used to assess transport. Hence, Cd^{2+} 'transport' can only be proven if directly demonstrated by electrophysiological techniques (e.g. patch clamp), $^{109}Cd^{2+}$ flux, inductively coupled plasma mass spectrometry (ICP-MS), atomic absorption spectroscopy, or Cd^{2+}-sensitive fluorescent dyes, to name a few (particularly for channels, carriers and pumps). RME of Cd^{2+}-protein complexes needs to be validated both, by specific interaction (e.g. ligand binding and competition) and ligand internalization (e.g. by combining microscopy of fluorescent ligands, cell viability assays and/or RNA interference, in addition to measurements of Cd^{2+} accumulation). Ideally, these methods need to be tested in a heterologous expression system. This 'conservative' and 'reductionist' approach is mandatory to highlight the relevance of proven transport pathways for Cd^{2+} toxicity and to prevent the perpetuation of elusive speculations.

In contrast, the use of pharmacological inhibitors as a sole tool is insufficient evidence to prove the involvement of specific proteins for Cd^{2+} transport because of limitations due to overlapping inhibitor specificity. At first sight, the use of genetically modified animals or animals with a loss-of-function mutation appears to be the gold standard to prove Cd^{2+} transport. Yet, compensatory upregulation of other Me^{2+} transporters may mitigate the results obtained, leading to wrong conclusions (see, for example, [8]). Neither are the multitude of studies useful that correlate expression of an arbitrary selection of transporters in tissues and cells with Cd^{2+} transport

(e.g. [9]), Cd^{2+} toxicity (e.g. [10]) or Cd^{2+} resistance (e.g. [11]). The latter point needs to be underscored because Cd^{2+} interferes with a multitude of complex biological processes. For instance, Cd^{2+} triggers a variety of pro- and/or anti-apoptotic signalling cascades (see [12]), which activate transcription factors and target genes, in particular, survival genes, that may induce Cd^{2+} resistance without concomitant Cd^{2+} extrusion/transport. For example, Cd^{2+} upregulates the drug resistance pump ABCB1 [13], and it has been subsequently postulated that ABCB1 extrudes Cd^{2+} from cells, thus decreasing toxicity [14]. Yet additional studies showed that ABCB1 interferes with pro-apoptotic sphingolipid signalling pathways activated by Cd^{2+}, and no coupling of $^{109}Cd^{2+}$ to ABCB1 could be demonstrated [15].

1.2 Channels and Pores

1.2.1 Ca^{2+} Channels

Depending on the gating mechanism, Ca^{2+} channels can be categorized into five major groups: (1) voltage-gated Ca^{2+} channels VGCC (or Ca_v), (2) channels gated by physical and chemical factors (transient receptor potential (TRP) channels), (3) store-operated channels (SOC), (4) endogenous ligand-gated channels (LGC) and (5) intracellular second messenger-gated channels (SMGC). Because Ca^{2+} and Cd^{2+} have similar ionic radii, permeation of Ca^{2+} channels by Cd^{2+} has been suggested as a mechanism of entry in cells [16], although Cd^{2+} is a potent blocker of Ca^{2+} channels, in particular of VGCC [17].

Ad 1. The strongest evidence for permeation of Ca^{2+} channels by Cd^{2+} has been obtained for VGCC. Depending on their voltage activation these channels are classified into low (LVA), intermediate (IVA) and high voltage activated (HVA) Ca^{2+} channels.

'T-type' ($Ca_v3.1$-3) Ca^{2+} channels are LVA Ca^{2+} channels that are important for the repetitive firing of action potentials in cells with pacemaker activity such as cardiac muscle cells, and in neurons in the thalamus of the brain, but are also found in liver, bone, endocrine system, vascular smooth muscle and kidney [18, 19]. $Ca_v3.1$ (also known as α_{1G}) channels are suitable for Cd^{2+} transport because they have a substantial window current near the resting membrane potential at which the driving force for Me^{2+} entry is high, and they are ~twofold less selective for Ca^{2+} than L-type Ca^{2+} channels [18]. This suggested that Cd^{2+} may have an increased chance of permeating these channels, even in the presence of competing Ca^{2+}. Lopin et al. [20] provided the first evidence for permeability of these channels to Cd^{2+} by whole-cell patch clamp recording of Cd^{2+} currents in human embryonic kidney (HEK293) cells stably transfected with $Ca_v3.1$. The magnitude of the Cd^{2+} current amounted to 5–17% of Ca^{2+} current. Incubation with radiolabeled $^{109}Cd^{2+}$ confirmed $Ca_v3.1$-mediated Cd^{2+} uptake with an independent methodology. These observations have been recently confirmed [21]. Considering the relatively high permeability of $Ca_v3.1$

channels for Cd^{2+} at low nanomolar (!) concentrations [20], these channels are a likely entry pathway for Cd^{2+} into all tissues expressing $Ca_v3.1$ channels [18, 19], including the kidney, and could thus contribute to the in vivo renal toxicity of Cd^{2+}.

Many reports have postulated Cd^{2+} uptake by L- and N-type VGCC in excitable and non-excitable cells (e.g. reviewed in [16]). Yet no direct evidence for Cd^{2+} permeation of these channels has been provided by electrophysiological and/or radiotracer techniques. On the contrary, Cd^{2+} potently blocks N- and L-type Ca^{2+} channels with an IC_{50} of ~0.3–2 μM [17, 22], which contrasts to T-type Ca^{2+} channels (IC_{50} of~500 μM) [23].

What is the explanation for these differences in the permeability properties and inhibitory potency of Cd^{2+} between different VGCCs? VGCCs have an exceptional Ca^{2+} selectivity, which is largely determined by the presence in their selectivity filter of a Ca^{2+} binding site, formed ideally by four glutamate residues (EEEE motif) within the pore. Interestingly, T-type Ca^{2+} channels possess two aspartates instead of glutamates (EEDD motif) in their Ca^{2+} selectivity filter which makes them less Ca^{2+}-selective, more Cd^{2+} permeable and less Cd^{2+} sensitive among VGCCs (reviewed in [24]). Recent electrophysiological analysis of heterologously expressed T-type Ca^{2+} channels and three pore mutants (DEDD, EDDD, and DDDD) supports the hypothesis that Cd^{2+}-sensitivity and -permeability are related to the absolute number of D in the Ca^{2+}-selective filter [21]. It thus predicts that Cd^{2+} permeation of L- and N-type Ca^{2+} channels with an EEEE Ca^{2+} selectivity motif is poor.

CatSper is a sperm-specific VGCC channel that plays an essential role in male fertility and displays four aspartic acids (DDDD) in the Ca^{2+}-selective filter motif [25]. Using the fluorescent indicator FluoZin-1 to measure Cd^{2+} concentration, a recent study has provided preliminary evidence for Cd^{2+} influx in human spermatozoa [21], which could impact on fertility, although additional evidence by patch clamp measurements of sperm cells is needed.

Ad 2. The TRP superfamily of cation channels consists of about 28 TRP channels that are grouped into seven subfamilies: the five group 1 TRPs TRPC, TRPV, TRPM, TRPN and TRPA, and two group 2 subfamilies TRPP and TRPML [26]. TRP channels play critical roles in sensory physiology and are activated by a variety of sensory stimuli. They are located mostly in the plasma membrane (PM) of cells and are relatively non-selectively permeable to cations, including Na^+, Ca^{2+} and Mg^{2+}. The impact of trace metal ions, including Cd^{2+}, on TRPs has been recently reviewed [27]. There is little evidence for permeation by Cd^{2+}, with a few exceptions summarized in the next paragraphs that may have a putative impact on Cd^{2+} toxicity in relevant target tissues.

TRPA1 is the only member of the family of TRPA channels in mammals. It is a nonselective cation channel that operates as a promiscuous chemical nocisensor in nociceptive sensory neurons, as well as in other sensory cells, including epithelial cells [28]. Using the Cd^{2+} indicator Leadmium Green in neurons of mouse dorsal root ganglia, Cd^{2+} was shown to permeate TRPA1 channels [29]. Intraplantar injection of Cd^{2+} induced pain-related behaviors that were largely attenuated in $TRPA1^{(-/-)}$ mice [29]. The permeability of TRPA1 channels to Cd^{2+} may, therefore, have pathophysiological importance.

TRPV5 (ECaC1) and TRPV6 (CaT1) are two major Ca^{2+} transport pathways that maintain systemic Ca^{2+} homeostasis [30]. TRPV5 and TRPV6 are the most Ca^{2+} selective (P_{Ca}:P_{Na} > 100) of mammalian TRPs. TRPV5 is mainly expressed in the distal convoluted (DCT) and connecting tubule of the kidney where it is the major hormonally regulated pathway for renal Ca^{2+} reabsorption; TRPV6 is an important Ca^{2+} entry pathway in the duodenum and the placenta [30]. When these channels were heterologously expressed in HEK293 cells, Cd^{2+} at micromolar concentrations permeated human (h) TRPV5 and TRPV6 in fluorescence imaging experiments with the Me^{2+}-sensitive dye Fura-2 or the calcium-insensitive dye Newport Green [31, 32]. Using whole-cell patch clamp measurements, the amplitude of the current carried by Cd^{2+} was 30–50% of that of Ca^{2+}, and overexpression of hTRPV5 or hTRPV6 sensitized HEK293 cells to Cd^{2+} toxicity [31, 32]. Hence, TRPV5 and TRPV6 may play a role in Cd^{2+} uptake and toxicity, e.g. in the DCT, duodenum and placenta, especially under low Ca^{2+} dietary conditions, when these channels are maximally upregulated.

TRPM6 and TRPM7 belong to the TRPM subfamily of TRP proteins [33]. TRPM7 is ubiquitously expressed, and TRPM6 is expressed in intestinal and renal epithelial cells where they have been implicated in cellular and systemic Ca^{2+} and Mg^{2+} homeostasis. TRPM6 and TRPM7 have been suggested as candidates for Cd^{2+}-mediated toxicity [34, 35], yet the ability of Cd^{2+} to permeate both channels is almost the lowest of all Me^{2+} tested [36, 37].

The only known intracellular TRP channel that is permeated by Cd^{2+} is TRPML1 (mucolipin-1/MCOLN1). It is an intracellular ubiquitously expressed late endosomal and lysosomal nonselective cation channel belonging to the TRPML subfamily of TRP proteins [38]. Mutations in the human TRPML1 gene cause mucolipidosis type IV disease characterized by the lysosomal accumulation of lipids and soluble substances [38]. Using the patch clamp technique and radioactive tracers, Dong et al. [39] showed that TRPML1 (and TRPML2) is an endosomal/lysosomal Fe^{2+} release channel whose transport is enhanced at low pH, indicating that TRPML1 extrudes Fe^{2+} from these acidic compartments into the cytosol. The relative Me^{2+} permeability of TRPML1 channels is Ba^{2+}>Mn^{2+}>Fe^{2+}~Ca^{2+}~Mg^{2+}>Ni^{2+}~Co^{2+}~Cd^{2+}>Zn^{2+}≫Cu^{2+} (at pH 4.6). The physiological role of TRPML channels in endosomal/lysosomal trafficking of endocytosed metal–protein complexes (such as transferrin (Tf)) [40] as well as autophagy [41] suggests that they could contribute to Cd^{2+} toxicity following RME of Cd^{2+}-protein complexes [42] and to Cd^{2+}-induced disruption of autophagy [43].

Ad 3. Store-operated Ca^{2+} channels (SOCs) are activated by depletion of endoplasmic reticulum (ER) Ca^{2+} elicited by hormones and neurotransmitters and are responsible for a Ca^{2+} current named CRAC (Ca^{2+} release-activated Ca^{2+} current) mediated by Stim proteins in the ER and Orai channels in the PM carrying CRAC (for a review, see [44]). CRAC channels are amongst the most Ca^{2+}-selective channels known (P_{Ca}/P_{Na} > 1000), similar to VGCCs. A ring of (likely) four glutamates (E106) at the external mouth of the pore functions as the Ca^{2+} selectivity filter [44]. Based on these molecular attributes, it is unlikely that Cd^{2+} permeates CRACs.

Ad 4. Cd^{2+} permeation of LGCs has been suggested for the highly Ca^{2+}-permeable NMDA receptor that belongs to the ionotropic glutamate receptor (iGluRs) family [45]: Following activation of the channel by depolarization, Cd^{2+} influx was apparently detected in rat cerebellar granule neurons loaded with the Me^{2+}-sensitive dye Fura-2 (which binds equally well free cytosolic Ca^{2+} and Cd^{2+}) that could be reversed by addition of TPEN, a membrane-permeant Cd^{2+} chelator [46]. However, the authors did not consider that Cd^{2+} may have activated a G-protein coupled 'Cd^{2+} receptor' resulting in increased cytosolic Ca^{2+} [47], whose activation may have been terminated when extracellular Cd^{2+} was chelated by TPEN.

Ad 5. No evidence is available that intracellular SMGC are permeated by Cd^{2+}.

1.2.2 Mitochondrial Calcium Uniporter (MCU)

Mitochondria buffer cytosolic [Ca^{2+}] and thereby regulate cellular processes controlled by intracellular [Ca^{2+}], e.g. Ca^{2+}-binding proteins, kinases, etc. Ca^{2+} also enters the mitochondrial matrix via a MCU in the inner mitochondrial membrane (IMM) to positively regulate oxidative metabolism. Excessive mitochondrial Ca^{2+} entry consequent to stress stimuli causes opening of a mitochondrial permeability transition pore and the release of pro-apoptotic factors which lead to cell death [48] (see also Chaps. 3 and 4). By patch clamping mitoplasts, Clapham et al. [49] identified a Ca^{2+} selective channel in the IMM with biophysical and pharmacological characteristics of the MCU and that binds Ca^{2+} with extremely high affinity ($K_D \leq 2$ nM). More recently, several groups identified *ccdc*109*a* as the gene encoding the pore-forming subunit of MCU that forms a protein complex with its regulatory subunits MICU1-3 and EMRE, and purified protein reconstituted in planar lipid bilayers displayed the electrophysiological properties of MCU (reviewed in [50]). Using isolated rat kidney cortex mitochondria, Lee et al. [51] showed that Cd^{2+} (2–50 μM) induces mitochondrial swelling via the Ru360-blockable MCU. Using mitoplasts and the Cd^{2+}-sensitive fluorescent indicator FluoZin-1, they also showed that Cd^{2+} enters the mitochondrial matrix [51]. Subsequent studies have confirmed those results in rainbow trout liver mitochondria [52, 53]. Hence Cd^{2+} may permeate the MCU directly with a deleterious impact on respiration, ATP production, and Ca^{2+} homeostasis and leading to the release of pro-apoptotic factors [51, 54]. Direct evidence, e.g. by patch clamp technique and/or $^{109}Cd^{2+}$ radiotracer experiments would be useful to substantiate the role of MCU in Cd^{2+}-induced mitochondrial toxicity. How Cd^{2+} crosses the outer mitochondrial membrane (OMM) is unknown.

1.2.3 Cystic Fibrosis Transmembrane Conductance Regulator (CFTR/ABCC7)

The cystic fibrosis transmembrane conductance regulator (CFTR) belongs to the ATP binding cassette (ABC) family of integral membrane proteins. It is mainly located in the apical membrane area of salt transporting tissues, such as secretory epithelia and exocrine glands, where it functions as a cAMP-dependent Cl^- channel required to control ion and fluid homeostasis on epithelial surfaces [55]. Mutations in the CFTR gene cause cystic fibrosis, the most common autosomal recessive disease in the Caucasian population. Similar to other members of the ABC protein family (see 1.4.2.), CFTR mediates glutathione (GSH) export from cells [56]. It has been suggested that CFTR mediates efflux of Cd^{2+}-GSH complexes from renal cells [57], however, no experimental proof has been provided.

1.2.4 Gap Junction-Forming Connexins

Gap junctions, found in the PM of animal cells, consist of clusters of closely packed pairs of transmembrane channels, the connexons, through which small molecules, e.g. nucleotides and ions, diffuse between neighboring cells [58]. The connexons consist of homo- or heterohexameric arrays of connexin proteins (Cx), comprising about 20 isoforms with distinct channel properties [59]. Because cigarette smoke extract containing Cd^{2+} induced opening of connexons in cultured rat L2 lung epithelial cells expressing Cx43 [60], it was speculated that Cx43 connexons mediate Cd^{2+} uptake and toxicity [61]. Yet no evidence for Cd^{2+} flux through connexons has been provided so far. On the contrary, Cd^{2+} likely inhibits gap junctional intercellular communication through various mechanisms [62].

1.3 Solute Carriers (SLCs)

1.3.1 Divalent Metal Transporter 1 (DMT1/DCT1/NRAMP2/SLC11A2)

The divalent metal ion transporter protein DMT1 (divalent metal transporter 1) is a Me^{2+} transporter, in particular Fe^{2+} transporter, that is energized by an H^+ electrochemical potential gradient while Fe^{3+} is excluded [63]. DMT1 is ubiquitously expressed, especially in the proximal duodenum, red blood cells, macrophages, but also in the kidneys [63]. Typically in enterocytes, DMT1 is expressed in the apical PM, where it mediates Tf-independent Fe^{2+} absorption into the organism (reviewed in [64]). Alternatively, DMT1 is located intracellularly, typically in

erythrocyte precursors or macrophages, but also in epithelial cells (reviewed in [65]). In these cells, DMT1 is localized to endosomes and lysosomes that are formed during RME of metal–protein complexes (e.g. Tf or metallothionein (MT). Vacuolar-type H^+-ATPases acidify endosomes and lysosomes which induces dissociation of the metal ion and—after enzymatic reduction, if required—allow DMT1-mediated co-transport of Me^{2+}, along with H^+ into the cytosol. Recently, evidence has been obtained for the localization of DMT1 in the OMM, which suggests that mitochondrial DMT1 is an entry pathway for Fe^{2+} and other Me^{2+} utilized by mitochondria [66].

Studies using a combination of voltage clamp, radiotracer and fluorescence assays in *Xenopus* oocytes or transfected HEK293 cells have established that hDMT1 is capable of transporting Cd^{2+} as efficiently as Fe^{2+} (K_m ~1 μM) [67, 68]. Because of its relatively high affinity to Cd^{2+}, DMT1 may be a key transporter involved in Cd^{2+} toxicity. Cd^{2+} may be taken up from the duodenum into enterocytes via apical DMT1. A link between Cd^{2+} uptake (and toxicity) and DMT1 expression has been proposed under conditions of disturbed iron homeostasis [69, 70], because under iron deficiency DMT1 is upregulated in the duodenum and other organs (e.g. kidney and liver) [63].

In the kidney, which is strongly affected by chronic Cd^{2+} toxicity [1, 2], DMT1 is expressed in late endosomes and lysosomes of the proximal tubule (PT), but not in the PM [71], which is compatible with the observation that knockdown of DMT1 in PT cells reduces Cd^{2+} toxicity induced by RME of Cd^{2+}-MT [72] (see, however, 1.5.2. for a critical discussion of the data). There is in vivo experimental evidence indicating that free Cd^{2+} is also taken up via apical DMT1 by distal segments of the rat nephron [73]. The localization of DMT1 in endosomes, lysosomes and mitochondria also suggests that DMT1 may contribute to Cd^{2+} uptake and subsequent disruption of the function of these organelles (see Chap. 4).

1.3.2 Zip8/Zip14 (SLC39A8/A14)

The Zrt, Irt-related proteins 8 (ZIP8/SLC39A8) and 14 (ZIP14/SLC39A14) belong to the ZIP (SLC39) family of metal ion import proteins (reviewed in [74]). ZIP8 is abundantly expressed in lung, placenta, testis and kidney whereas ZIP14 is most abundantly expressed in liver, heart duodenum and pancreas [75] (reviewed in [74]). At the cellular level, ZIP8 and ZIP14 are likely localized in the PM as well as in subapical endosomes and lysosomes [74].

Initially described as zinc transporters, ZIP8 and ZIP14 mediate the uptake of a variety of essential Me^{2+} (e.g. Zn^{2+}, Fe^{2+}, Mn^{2+}) transporters with affinities varying between 0.5 and 20 μM [75–77]. The abundant expression of ZIP8 in the lung suggests that it plays a role in the pulmonary absorption of Cd^{2+} [78]. Several studies showed that heterologous expression of ZIP8 mediates the accumulation and toxicity of Cd^{2+} (Km of ~0.5–0.6 μM) [77]. In a heterologous expression system, ZIP14 has a high affinity towards Cd^{2+} (Km ~0.1–1.1 μM depending on the splice variant tested)

[76]. In the same study, overexpression of ZIP14 decreased the survival of cells exposed to Cd^{2+}, indicating that ZIP14 contributes to Cd^{2+} toxicity. Hence, ZIP8 and ZIP14 are two likely candidate transporters for uptake of Cd^{2+} at sub-micromolar concentrations.

1.3.3 Organic Cation Transporter 2 (SLC22A2) and Multi-antimicrobial Extrusion (MATE) Proteins (SLC47A1/A2)

OCT2, a member of the poly-specific organic cation transporters (OCT/SLC22A), is located at the basolateral membrane (BLM) of renal PT and transports various organic cations from the interstitial space into PT cells [79]. There is long-standing evidence that Cd^{2+} is taken up at the basolateral surface of PT cells [80], and Cd^{2+} uptake has recently been shown to take place via hOCT2 [81] and rabbit OCT2 [82]. However, a K_m of ~50 μM for Cd^{2+} transport by hOCT2 [81] suggests that the in vivo toxicological relevance of this transporter is low.

Multi-antimicrobial extrusion proteins, also known as multidrug and toxin extrusion/multidrug and toxic compound extrusion (MATE/SLC47A) proteins are drug/sodium or proton antiporters which are expressed in the apical membrane of hepatocytes and renal PT cells (reviewed in [83]). HEK-293 cells overexpressing hMATE1, hMATE2-K, a splice variant of MATE2, and mouse Mate1 were used to study the cellular transport of Cd^{2+} that was determined by ICP-MS [84]. Cells overexpressing MATEs showed a 2–4 fold increase of Cd^{2+} uptake with a Km of ~100 μM hMATE1 extruded preloaded Cd^{2+} and decreased Cd^{2+}-induced cytotoxicity, suggesting that MATEs protect against Cd^{2+} toxicity. However, similarly to OCT2, hMATE1 and hMATE2-K are unlikely to be toxicologically relevant in vivo because of their low affinity for Cd^{2+}.

1.3.4 Amino Acid/Cystine Transporter (SLC7A9/SLC3A1)

Due to its high affinity for SH groups, Cd^{2+} in the extracellular space is likely bound to thiol-containing molecules, such as the amino acids L-cysteine (Cys), L-homocysteine (Hcy), N-acetylcysteine, GSH, and proteins. Low-molecular-weight thiol-S-conjugates of Cd^{2+} have been hypothesized to be taken up by epithelia as mimics of L-cystine and/or L-homocystine (i.e. the oxidized dimer form of the respective amino acid) via specific amino acid transporters [85]. However, experimental evidence is scarce. Recently, lumen-to-cell transport and cellular accumulation of the Cys or Hcy-S-conjugates of Cd^{2+} (Cys-S-Cd^{2+}-S-Cys, Hcy-S-Cd^{2+}-S-Hcy) were studied in isolated, perfused rabbit kidney PT segments [86]. Based on competition of transport with L-cystine, the data suggest that Cys-S-Cd^{2+}-S-Cys and Hcy-S-Cd^{2+}-

S-Hcy at sub-micromolar concentrations are substrates of one or more amino acid transporters for luminal absorption of L-cystine in the kidney. A likely transporter is a system (b0, + AT) (SLC7A9/SLC3A1) of cationic amino acid transporters that mediate apical influx of cystine and dibasic amino acids in the renal PT (reviewed in [87]) and hence could contribute to PT Cd^{2+} toxicity.

1.3.5 Ferroportin-1 (FPN1/SLC40A1)

Ferroportin-1 (FPN1/SLC40A1) is the sole cellular iron exporter described so far [88]. Consistent with its assigned function in body iron homeostasis, mammalian FPN1 is expressed in the BLM of duodenal enterocytes, and in the PM of splenic and hepatic macrophages. The expression is also high in the BLM of human placental syncytiotrophoblasts, which suggests a role of FPN1 in Fe transfer to the fetal circulation [89]. In rat kidney, FPN1 is expressed in the BLM of PT [90]. Recently, human FPN1 was expressed in *Xenopus* oocytes and was equally well permeated by microinjected radioactive Fe^{2+}, Co^{2+}, and to some extent Zn^{2+}, but not by Cd^{2+}, Cu^{2+} or Mn^{2+} [91]. Hence, FPN1 may promote Cd^{2+} nephrotoxicity by not permitting Cd^{2+} efflux from PT cells. However, it remains unclear how Cd^{2+} that is taken up via apical DMT1 by duodenal enterocytes (see 1.3.1.) exits the duodenal epithelia to enter the circulation.

1.4 ATPASES

1.4.1 Multidrug Resistance P-Glycoprotein (MDR1/ABCB1)

P-glycoprotein (ABCB1), a member of the superfamily of ABC transporter proteins, can confer multidrug resistance (MDR) by actively extruding structurally unrelated, hydrophobic amphiphilic and cationic drugs from cells [92]. In epithelial cells of the gastrointestinal tract, liver, kidney, and capillaries of the brain, testes, and ovaries, ABCB1 acts as a barrier to the uptake of xenobiotics, and promotes liver and renal excretion of drugs and xenobiotics into the bile and urine. Decreased toxicity to Cd^{2+} was demonstrated in kidney PT cells overexpressing ABCB1 [13]. The observed ABCB1-dependent resistance to Cd^{2+} toxicity was found to be due to ABCB1-dependent extrusion of pro-apoptotic sphingolipids ceramides from cells [15]. However, ABCB1-dependent trans-epithelial Cd^{2+} transport (~1 μM $^{109}Cd^{2+}$) was also postulated in ABCB1 expressing kidney PT and intestinal cell monolayers cultured on permeable filters [14, 93]. Yet, ABCB1-independent trans-cellular transport pathways and Cd^{2+}-induced disruption of para-cellular junctions were not considered [94]. To clarify this issue, $^{109}Cd^{2+}$ efflux was investigated as a function of ABCB1 expression in MDCK cells and MDCK cells permanently overexpressing

ABCB1 [15]. A substrate of ABCB1, rhodamine 123+, showed increased kinetics of efflux from ABCB1-MDCK cells that were abolished by the ABCB1 blocker PSC833, yet efflux of 10 μM $^{109}Cd^{2+}$ was neither affected by the expression level of ABCB1, nor by PSC833 or the inhibitory ABCB1 antibody UIC2, thus proving that ABCB1 does not transport low micromolar Cd^{2+} concentrations. However, it cannot be excluded that high micromolar concentrations of Cd^{2+} are transported by ABCB1 [95].

1.4.2 Multidrug Resistance-Associated Protein 1 (MRP1/ABCC1)

The multidrug resistance protein 1 (MRP1) encoded by ABCC1 was originally discovered as a cause of MDR in tumour cells. The substrate specificity of MRP1 is broad and includes many organic anion conjugates of structurally unrelated endo- and xenobiotics. The antioxidant GSH and the pro-inflammatory cytokine cysteinyl leukotriene C_4 are physiological substrates of MRP1 (reviewed in [96]). MRP1 is expressed in epithelial tissues, such as liver and kidney, as well as in endothelia of the blood–brain barrier and in brain [96]. It has been suggested that MRP1 can also extrude Cd^{2+}-GSH complexes [97]. Yet, although equilibrium Cd^{2+} concentrations of mussel and zebrafish tissues expressing MRP1 and exposed to low micromolar Cd^{2+} concentrations were increased by the MRP1 inhibitor MK571 [98, 99], Cd^{2+}-GSH flux by MRP1 was not demonstrated. Moreover, Cd^{2+} concentrations are increased in MRP1 knockout zebrafish tissues [99], which complicates the interpretation of those data.

1.5 Receptors

1.5.1 Lipocalin-2 Receptor (Lip2-R/SLC22A17)

Neutrophil gelatinase-associated lipocalin (NGAL [human]/siderocalin/24p3 [rodent]) or lipocalin-2 (Lip2) is secreted by neutrophils [100] and binds Fe^{3+} through association with bacterial siderophores. It hence plays an important role in antibacterial innate immunity [101]. Lip2 is also secreted by epithelia, however, its function there is less clear. It has been proposed that Lip2 stimulates epithelial growth and differentiation, and promotes repair and regeneration of damaged epithelia, e.g. during acute kidney injury [102], via mammalian siderophores [103, 104].

A receptor for Lip2 (Lip2-R/SLC22A17) has been cloned [105] (MM ~60 kDa) whose affinity for Lip2 is ~1000× higher (K_D ~90 pM) [106] than that of megalin (K_D ~60 nM) [107] (see 1.5.2.). Lip2-R protein is expressed in epithelial tissues, including the kidney and intestine [105]. Lip2-R is expressed apically in rodent

kidney DCT and collecting duct (CD) (mainly inner medullary CD) [108], and in human and rodent distal intestine (ileum, colon) [109]. In a heterologous expression system, i.e. Chinese hamster ovary (CHO) and cultured mouse DCT cells expressing Lip2-R, Lip2-R internalized sub-micromolar concentrations of fluorescence-labelled Tf, albumin, and MT (K_D of ~100 nM) and their uptake was blocked by 500 pM Lip2 [108]. Moreover, Cd^{2+}-MT caused cell death of both cell lines expressing Lip2-R that could be rescued by 500 pM Lip2 [108]. In cultured human Caco-2 BBE intestinal cells, Lip2-R mediated uptake, toxicity and transcytosis of Cd^{2+}-MT and Cd^{2+}-phytochelatins (e.g. PC_3) [109, 110].

Hence, it is possible that Lip2-R operate as a high-affinity receptor for RME of Cd^{2+}-MT and other Cd^{2+}-protein complexes in the distal nephron to initiate or enhance nephrotoxicity. In the intestine, Lip2-R could contribute to apical internalization and transcytosis of intact plant or animal Cd^{2+}-loaded proteins and peptides (e.g. Cd^{2+}-MT and Cd^{2+}-PC_3), thus contributing to systemic Cd^{2+} toxicity.

1.5.2 Megalin: Cubilin

Megalin is a 600 kDa single transmembrane-domain receptor protein belonging to the low-density lipoprotein receptor family. Megalin-dependent endocytosis occurs in cooperation with the 460 kDa receptor cubilin [111]. The megalin: cubilin receptor complex is expressed primarily in luminal PM of polarized absorptive epithelia, including the kidney PT, the intestine and the placenta [111]. In the kidney PT, the megalin: cubilin receptor complex binds and endocytoses various protein ligands filtered by the renal glomeruli, including α1- and β2-microglobulin, albumin, Tf, MT, NGAL, etc. (reviewed in [112]), thus preventing their loss into the urine. Endocytosed proteins are delivered to endosomes, and the receptors are recycled to the apical membrane whereas ligands are transferred to late endosomes/lysosomes for protein degradation [112].

Cd^{2+} that enters the body through the GI tract is thought to be taken up from the plasma by the liver. However, which Cd^{2+} species enter liver cells, using which entry pathways, and which respective roles hepatocytes and Kupffer cells play in this process has been poorly investigated (for further discussion, see [113]). In the liver, Cd^{2+} induces synthesis of the protein MT (molecular mass ranging from 3.5 to 14 kDa), which binds and detoxifies Cd^{2+} due to its very high affinity for Cd^{2+} (K_D of~ 10^{-14}M) [114]. It has been suggested that a small proportion of liver Cd^{2+}-MT is released into blood plasma as the cells in which Cd^{2+} is sequestered die off, either through normal turnover or due to Cd^{2+} injury, and redistributes to the kidney. Although no experimental proof exists that Cd^{2+} redistributes from the liver to the kidney via circulating Cd^{2+}-MT, this hypothesis prevails in the literature (reviewed in [113]).

Cd^{2+} in the circulation may be more or less tightly bound to various proteins [114–116]. Whereas low-molecular weight proteins (LMWP), e.g. microglobulins and MT, easily cross the glomerular barrier (cut-off of ~80 kDa), only a small proportion of high-molecular weight proteins (HMWP), e.g. albumin and Tf, are filtered by the glomerulus, and all these proteins may be reabsorbed in the PT via megalin:cubilin [112].

According to current dogma, Cd^{2+}-MT delivered into the primary urine is the major source of renal Cd^{2+} because it is 'avidly' reabsorbed by PT cells via megalin-mediated endocytosis [42], trafficked to acidic late endosomes/lysosomes [117] for proteolytic degradation of MT, and Cd^{2+} extruded into the cytosol via DMT1 (and possibly TRPML1) (see 1.2.1. and 1.3.1.). This process may cause PT toxicity if cells have to handle high amounts of endocytosed Cd^{2+}-MT [42, 72]. But during chronic exposure to low (or 'normal') Cd^{2+}-MT concentrations, adaptive processes may be activated, e.g. upregulation of detoxifying MT for long-term storage of cytosolic Cd^{2+}. Cd^{2+} accumulation in the PT (and storage as Cd^{2+}-MT) is likely further promoted by the absence of an efflux pathway for cytosolic Cd^{2+} into the extracellular space because basolateral FPN1 in PT cells does not transport Cd^{2+} (see 1.3.5.).

However, this model relies on in vivo and cell culture studies with high nanomolar or micromolar Cd^{2+}-MT concentrations (reviewed in [118]) that are far above the (Cd^{2+}-)MT concentrations encountered in vivo of 05–5 nM depending on the methodology and the populations studied (discussed in [119]). Furthermore, the K_D of megalin for MT (~100 μM) [120] is ~10^5-times higher than the plasma concentrations of (Cd^{2+}-)MT. Moreover, MT k.o. mice chronically exposed to oral Cd^{2+} also develop PT toxicity [121]. Finally, kidney pathology from Cd^{2+}-MT injections differs from that induced by chronic oral Cd^{2+} exposure [122]. It is therefore likely that other filtered proteins are more relevant ligands of megalin: cubilin for PT Cd^{2+} accumulation and toxicity (apart from other pertinent Cd^{2+} uptake pathways). Microglobulins, albumin and Tf also bind Cd^{2+} and other divalent metal ions [115, 116, 123] and reach concentrations in the ultrafiltrate near their K_D for megalin: cubilin (reviewed in [118]). Although Cd^{2+} exhibits relatively low affinities to these proteins (K_D of ~10^{-6}M) compared to MT (see above), their concentration in the ultrafiltrate near their K_D for megalin (see [118] for details) paired with the multiplicative effect of continuous glomerular filtration makes them more likely candidates for chronic Cd^{2+} toxicity via megalin: cubilin in the PT. To sound a note of caution, though, two recent in vivo studies showed that chronic low or moderate Cd^{2+} exposure in rats disrupts PT megalin: cubilin-mediated protein endocytosis (but without signs of overt PT damage) [124, 125], which may limit the importance of megalin: cubilin for chronic Cd^{2+} toxicity in the PT. This may also be the case for mechanisms of Cd^{2+} toxicity in other tissues expressing megalin: cubilin (e.g. intestine and placenta) [111].

1.5.3 Transferrin Receptor 1 (TfR1)

Plasma iron circulates bound to Tf, which has two specific high-affinity binding sites for Fe^{3+}. In normal plasma, Tf is ~30% saturated with Fe^{3+} (holo-Tf) [126], thus leaving a considerable binding capacity for other metal ions. The internalization of plasma holo-Tf is a major mechanism for Fe assimilation by cells [127]: Holo-Tf interacts with a ubiquitous cell-surface Tf receptor (TfR1), a transmembrane glycoprotein composed of two disulfide-linked monomers. Each monomer binds one holo-Tf molecule creating a holo-Tf-TfR1 complex which enters the cell by RME into endosomes and lysosomes. In endosomes, Fe^{3+} is reduced to Fe^{2+} by an oxidoreductase activity named 'Steap' (six transmembrane epithelial antigens of the prostate), which together with endosomal acidification promotes Fe^{2+} release; Fe^{2+} is transported out of the endosomes/lysosomes through DMT1 and/or TRPML1 (see 1.2.1. and 1.3.1.) whereas apo-Tf and TfR1 return to the cell surface for reuse [127].

Because Tf is not saturated with Fe^{3+}, it can transport other metal ions from the bloodstream to tissues [128], including Cd^{2+} [115]. Indeed, sequential binding of two Cd^{2+} to Tf occurs with a K_D of ~10^{-6}M for the first Cd^{2+} ion and a K_D of ~10^{-5}M for the second Cd^{2+} [115, 129], which makes it likely that TfR1 mediates Cd^{2+} uptake from the circulation to induce cellular toxicity through the ubiquitous Tf-TfR1 pathway of cellular iron delivery. Yet, so far, experimental evidence for cellular Cd^{2+} uptake and toxicity via TfR1 is lacking.

1.6 Summary, Conclusions and Outlook

In recent years, convincing experimental evidence has been provided for the transport of sub-micromolar or low micromolar concentrations of Cd^{2+} and Cd^{2+} complexes through several channels, carriers and receptors (See Fig. 1.1). Hence, Cd^{2+} permeates several Ca^{2+} channels, e.g. T-type Ca^{2+} channels, and several TRP channels (TRPA1, TRPV5/6, TRPML1). Among the SLCs, ZIP8/14, the amino acid transporter (b0, + AT) and DMT1 are relevant candidate entry pathways mediating Cd^{2+} toxicity. DMT1 is also expressed in intracellular organelles (lysosomes and mitochondria) where it may disrupt their function. In contrast, the SLCs OCT2 and MATE1/2-K and the efflux pump ABCB1 transport Cd^{2+}, but at concentrations that are too high to have in vivo relevance. The Lip2 receptor binds and endocytoses Cd^{2+}-MT with a K_D of ~100 nM, whereas megalin binds Cd^{2+}-MT with a K_D of ~100 μM with no in vivo significance. Hence, the Cd^{2+}-protein complexes that are ligands of megalin in vivo remain to be identified. The influx of Cd^{2+} into the mitochondrial matrix via the MCU, of Cd^{2+}-Tf into cells via the TfR1 receptor, and efflux of Cd^{2+}-GSH via MRP1 await further proof by future studies that should also unravel additional transporters mediating Cd^{2+} toxicity.

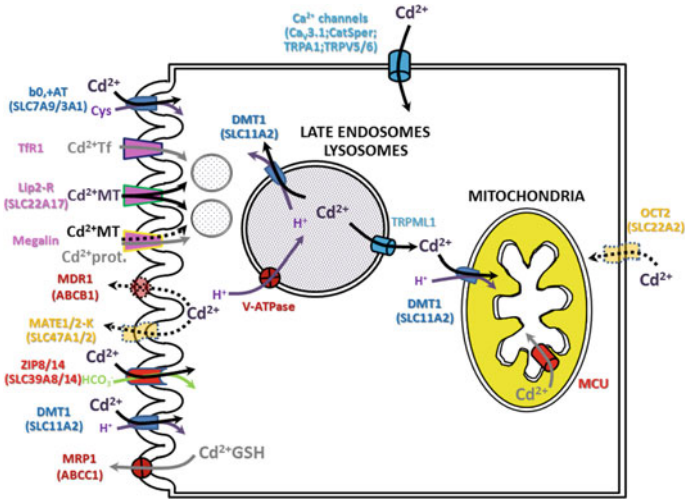

Fig. 1.1 Transport proteins for Cd^{2+} and Cd^{2+} complexes. Although drawn in a prototypical polarized epithelial cell, transport proteins are also expressed in non-polarized cells (e.g. immune cells, neurons or cardiac cells). Full black lines indicate proven pathways, broken black lines and symbols designate Cd^{2+} flux at high concentrations with no toxicological relevance. Full grey lines specify likely transport routes for Cd^{2+}/Cd^{2+} complexes that require additional experimental evidence. For further details, e.g. abbreviations, refer to the text

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Chapter 2
The Chemical Biology of Cadmium

Eric Lund, Susan Krezoski and David Petering

Abstract Cadmium is a major toxic element with important and long-recognized consequences for human health. As a result, Cd^{2+} has been subjected to thousands of toxicological studies that reveal its complex and wide-ranging impacts on cellular processes. Nevertheless, as with other toxic metals, it has been difficult to extend this research to the molecular level, where Cd^{2+} binds to particular molecules and initiates mechanisms of cell injury. This chapter sets forth a framework for considering the molecular interactions of Cd^{2+} with biomolecules, principally proteins. The paradigm is developed that at the proteomic level, Cd^{2+} may undergo metal exchange reactions with many Zn-proteins. In some cases, protein functions are perturbed and toxicity results. In others, as with its reaction with Zn-metallothionein, Cd^{2+} is inactivated. In all cases, the important role of adventitious binding of Cd^{2+} and Zn^{2+} within the proteome is emphasized. These ideas are given specificity in research into the molecular involvement of Cd^{2+} in nephrotoxicity and carcinogenesis. Examples of the chemical participation of Cd^{2+} in cell signaling are described. These include the activation of MTF-1 transcription factor and the stimulation of complex apoptotic pathways that may depend on the initial production of reactive oxygen species. With little information that Cd^{2+} interacts directly with proteins involved in the trafficking or function of other metals (Fe, Cu, Ca), the chapter concludes with a discussion of analytical methods to determine the speciation of cadmium within the proteome and particularly the zinc proteome.

2.1 Introduction

Human exposure to chemical forms of cadmium has paralleled global industrial expansion for most of its 200 hundred year history as a recognized element [1, 2]. As chemical and engineering applications rapidly increased, major commercial uses

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of cadmium resulted in acute occupational exposure of workers to cadmium [3]. Environmental contamination ensued as well and the general public experienced both overt and subtle health effects from contact with cadmium [3]. In the 1950s, for example, reports from Japan revealed widespread bone and kidney damage resulting from consumption of rice laced heavily with cadmium that had been dumped by a mining operation into a river that was used as an agricultural source of water [4].

In the same time frame, halfway around the world, researchers were studying the basic metallo-biochemistry of cadmium. They resorted to horse kidney as a source of cadmium (Cd) in an effort to isolate Cd-binding biomolecules [5]. A single protein was discovered that sequestered the preponderance of cadmium in the organ. Because of its large cysteine-sulfhydryl content, the protein was named metallothionein (MT) [6]. Shortly thereafter, scientists determined that the deliberate exposure of rodents to Cd^{2+} resulted in the de novo synthesis of Cd-MT [7, 8].

Such early reports set the direction of cadmium toxicological research for decades to follow. The demonstration that Cd^{2+} induces the synthesis of MT and that this protein binds almost all of the Cd^{2+} entering cells sparked the hypothesis that MT protects cells from the toxicity of Cd^{2+} [7]. Is this hypothesis correct? How does cadmium stimulate MT synthesis? What are the chemical properties that facilitate preferential cellular binding of Cd^{2+} to MT? Is MT a normal constituent of cells and what might be its function? For decades, these and related questions preoccupied researchers in the field of cadmium toxicology. As scientists focused on metallothionein and how it afforded protection against cadmium-induced cell injury, they largely ignored how cadmium actually causes toxicity.

According to a myriad of more recent papers, Cd^{2+} causes a multitude of deleterious changes in organ and cell-specific functions and related, underlying biochemical pathways [9–12]. In the context of this chapter, understanding the basis of cadmium toxicity requires comprehending how Cd^{2+} as a metal ion reacts chemically with key sites within the complex environment of the cell to initiate molecular changes that result in toxicity. Reaching this level of knowledge remains a formidable challenge. How metal ions in general, let alone Cd^{2+}, interact with their cellular surroundings is not well established. Even solidifying an understanding of the cellular roles of metallothionein continues to frustrate researchers. As a result, the approach here will be to place what is known within a conceptual scaffold that is aimed at facilitating new research to establish the chemical basis of cadmium toxicity.

2.2 Inorganic Biochemical Framework for Cd^{2+} Toxicology

Cadmium as Cd^{2+} is an inorganic cation that can form complexes with organic ligands [13]. Existing in the single, stable oxidation state, Cd^{2+} does not participate in oxidation–reduction reactions. As such, it can only react with and directly perturb biomolecules that contain metal binding sites that are comprised of one or more metal binding ligands (L) (Reaction 2.1):

$$Cd^{2+} + nL \rightleftarrows Cd\text{-}L_n \quad \left(K_{eq},\ k_1,\ k_{-1}\right) \tag{2.1}$$

Of interest in Reaction 2.1 are the nature and number of ligands in the binding site; the structure and properties of Cd-L complexes; the equilibrium constant of the reaction (K_{eq}); and the rate constants for formation (k_1) and dissociation (k_{-1}) of the complex. In terms of the contribution of Reaction 2.1 to the toxicology of Cd^{2+}, one needs to know whether the formation of Cd-L compromises its functionality or otherwise impacts the availability of Cd^{2+} to bind to toxicologically important molecules. Changes in structure and reactivity resulting from the formation of Cd-L offer insights into the mechanism by which Cd^{2+} alters cellular biochemistry. The equilibrium constant provides information about the stability of the complex alone or in the presence of other cellular ligands that compete to bind Cd^{2+}, and, therefore, about the possibility that Cd-L can exist in the cell. In a complementary way, the rate constants, particularly k_{-1}, inform whether Cd^{2+} bound to Cd-L may become available for timely interaction with other, competing ligands in the environment through a dissociative process.

Cadmium resides just below zinc in family 2B of the periodic table. Because of this relationship, the chemical properties of the two elements significantly overlap [13]. Both form complexes with ligands involving functional groups that contain oxygen (O), nitrogen (N), and sulfur (S). A large variety and large concentration of potential binding sites for Cd^{2+} exist within cells. The exterior surface of aqueous proteins is decorated with amino acid side chains with affinity for metal ions, including carboxylate (O), imidazole (N), thiolate (S), and amino (N) groups [14]. The tripeptide, glutathione comprises another large pool of metal binding ligands that might react with Cd^{2+} [15]. Thus, it is anticipated that cellular constituents as a whole display substantial, nonspecific affinity for Cd^{2+}. Having a larger atomic number than Zn^{2+}, Cd^{2+} displays a stronger preference for sulfhydryl (S) based or soft ligands and less for harder ligands such as carboxyl (O) and imidazole (N) groups [13].

Besides the presence of many adventitious binding sites for metal ions, numerous metalloproteins populate cells. For instance, it is estimated that mammalian cells contain about 3000 Zn-proteins (Zn-P) [16, 17]. Generally, the presence of Zn^{2+} either contributes to the catalytic mechanism of action at enzyme active sites or acts as a determinant of protein conformation [18]. In the latter role, Zn^{2+} might provide thermodynamic stabilization for the structure and/or serve as a critical factor for correct folding. Considering the similarity of Cd^{2+} and Zn^{2+}, P may also exhibit substantial affinity for Cd^{2+}:

$$Cd^{2+} + P \rightleftarrows Cd\text{-}P \quad \left(K_{eq},\ k_1, k_{-1}\right) \tag{2.2}$$

As in Reaction 2.1, knowledge of equilibrium and rate constants is critical for assessing the possible significance of any particular example of this reaction.

With this background in mind, one can pose a general pathway for the trafficking of Cd^{2+}, including its transport across membranes, intracellular distribution within any given compartment, and final sites of localization [19]. Figure 2.1 serves as a starting point for rationalizing what is known about Cd^{2+} speciation in relation to

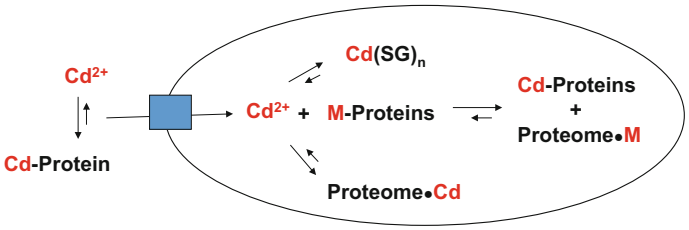

Fig. 2.1 General pathway of trafficking of Cd^{2+} within cell compartments. M, native metal in metalloprotein, M-Protein; GS, glutathione; box represents transporter connecting compartments

toxicity. Modes of transport are reviewed elsewhere in this monograph [see Chap. 1]. Once in the cell, Cd^{2+} associates transiently with adventitious proteomic binding sites and possibly with glutathione. Some of these Proteome · Cd sites may be toxicologically significant. However, the underlying hypothesis expressed in Fig. 2.1 is that Cd^{2+} ultimately undergoes metal ion exchange with metalloproteins to generate Cd-Proteins, in which Cd^{2+} substitutes for M^{m+} at native binding sites to form structures with modified biochemical properties. Because of the close relationship between Cd^{2+} and Zn^{2+}, the predominant class of hypothetical reactions involves Zn-Proteins [19]. The plausibility of the hypothesis is abundantly supported by structural studies of Zn-Proteins, in which Zn^{2+} is replaced by NMR-active $^{113}Cd^{2+}$ in order to gain detailed information about the nature of the metal binding site [20].

Large numbers of nonspecific sites within the cell that bind Zn^{2+} and, thus, can potentially bind Cd^{2+} (Pro_1, Pro_2, Pro_3, … Pro_n, or the sum of all these proteins, ΣPro_n) [19, 21–24]. After Cd^{2+} reacts with Pro_1, the product, $Pro_1 \cdot Cd$, may transfer its metal ion to other protein ligands through successive association and dissociation steps as in Reaction 2.2. Eventually, Cd^{2+} attains a distribution among ΣPro_n consistent with the equilibrium and rate constants of all of the participating reactions. Alternatively, direct ligand substitution reactions occur, in which metal exchange between $Pro_1 \cdot Cd$ and Pro_2 occurs directly without the dissociation of Cd^{2+} from P_1:

$$Pro_1 \cdot Cd + Pro_2 \rightleftarrows Pro_2 \cdot Cd + Pro_1 \quad \left(K_{eq},\ k_1,\ k_{-1}\right) \tag{2.3}$$

Generalizing, one recognizes that through the operation of Reactions 2.2 and 2.3, intracellular Cd^{2+} might readily be distributed among many proteins, $\Sigma Pro_n \cdot Cd$. Based on its chemical properties, one may surmise that Cd^{2+} associates preferentially with sites in ΣPro_n that contain sulfhydryl ligands. On this basis, Cd^{2+} might also coordinate to glutathione that features a cysteine thiol as one of its ligands.

Figure 2.1 illustrates the hypothesis that Cd^{2+} ultimately finds it way into specific metalloprotein binding sites (M-Proteins). Concentrating on Zn-Proteins ($\Sigma Zn\text{-}P_n$) as the primary target, Cd-Proteins might result from the reaction of Cd^{2+} with such targets. But in light of Reaction 2.3, they may also be produced in metal exchange reactions:

$$\Sigma\text{Zn-P}_n + \Sigma\text{Pro}_n \cdot \text{Cd} \rightleftarrows \Sigma\text{Cd-P}_n + \Sigma\text{Pro}_n \cdot \text{Zn} \quad (2.4)$$

The intent of the following sections is to use this abstract picture of the potential cellular trafficking of Cd^{2+} as a framework for organizing and understanding a range of experimental results in the literature. In the process, unresolved questions will be highlighted. Finally, considering the sheer number of possible reactions and products summarized in Reactions 2.4, a major analytical chemical problem emerges, namely, how to measure the real intracellular distribution of Cd^{2+}. Potential solutions to this issue will also be considered.

2.3 Metallothionein and the Proteome: Reactions with Cd^{2+} and Zn^{2+}

Comprehension of the chemical basis of cadmium toxicity begins as it did historically with metallothionein [25, 26]. After exposure of organisms or cells in culture to Cd^{2+}, the great majority but not all of the cellular Cd^{2+} is gathered into newly synthesized MT over time and remains there under steady-state conditions of protein biodegradation and resynthesis [27]. Numerous experiments have demonstrated that the presence of MT shields cells from the toxicity of Cd^{2+} [28, 29]. Most clearly, MT-null mice are considerably more sensitive to Cd^{2+} than are wild-type animals [25, 28, 30, 31]. Two features of this widely observed protective mechanism require explanation. What chemical characteristics of MT and other ligands in the cell explain the dramatic localization of Cd^{2+} in MT? And, how does some Cd^{2+}, either $\text{Pro}_n \cdot \text{Cd}$ or Cd-P_n, escape capture by MT?

Mammalian MT presents itself as four major isoforms, I–IV [33]. The first two have been found ubiquitously in organs and tissues throughout the body, including kidney. The others have a much narrower distribution. MT I and II, the subjects of this chapter, are small, 60 amino acid proteins, each containing 20 cysteine residues identically placed within the sequence [25, 26, 32]. Their side chain sulfhydryl groups provide MT with an enormous capacity to bind metal ions and up to 7 Cd^{2+} and/or Zn^{2+} per molecule.

A sophisticated nuclear magnetic resonance (NMR) study of induced liver MT isolated from rats injected with NMR-active $^{113}Cd^{2+}$, showed that the protein, $^{113}Cd_5$, Zn_2-MT-II, exists as a 2-domain structure [33]. Each domain bears a metal-thiolate cluster, M_4, S_{11} or M_3, S_9, with Cd^{2+} primarily in the 4-metal cluster and Zn^{2+} in the 3-metal cluster. Every metal ion is bound to four thiolate groups with some of them serving as bridging ligands between metal ions, knitting the cadmium ions together into clusters (Fig. 2.2). Further structural characterization of this protein by X-ray crystallography and Cd_7-MT by NMR spectroscopy demonstrated that the clusters constitute the interior structures that organize the folding of each domain about them (Fig. 2.3) [34, 35]. Moreover, in the folded conformation with the polypeptide backbone and side chains folded around the clusters, both thiol groups and metal ions

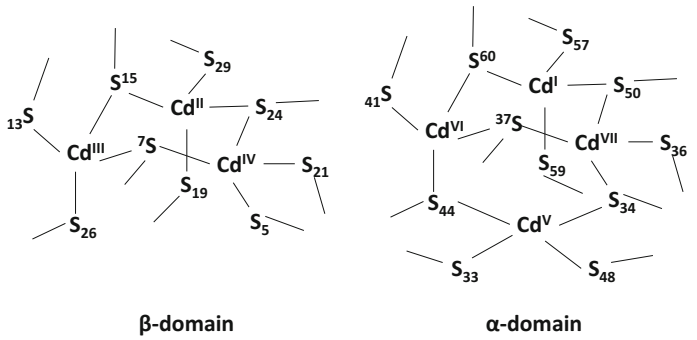

Fig. 2.2 Cd^{2+}-thiolate clusters in MT: Cd_3S_9 (β) and Cd_4S_{11} (α). Roman numerals refer to numbering of ^{113}Cd-NMR peaks and numbers to the sequence position of the cysteine residues [25, 33–35]. Shown with permission [25]

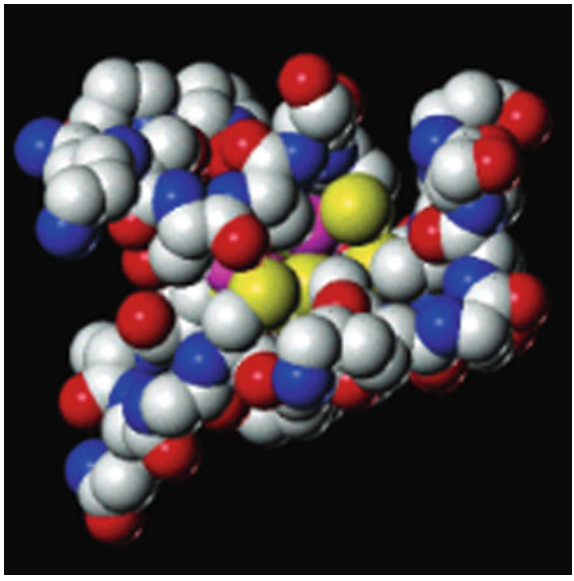

Fig. 2.3 Space-filling peptide folding about Cd_4S_{11} cluster. Shown with permission [37]

retain significant exposure to the solvent [25, 34, 36]. In turn, this property offers other molecules potential access to the clusters for direct reaction.

An early paper demonstrated that Cd^{2+} binds ca. 10^3 times more strongly to the protein than does Zn^{2+} in keeping with its stronger affinity for sulfhydryl ligation [37]. Recent measurements of the stability constants ($K_{Zn\text{-}MT}$) of Zn^{2+} in MT fall in the 10^{11-12} range [38, 39].

By inference, Cd^{2+} binds with constants ($K_{Cd\text{-}MT}$) in the range of 10^{14-15}. These numbers alone suggest that MT should be able to compete robustly with other cellular metal binding sites to sequester Cd^{2+}.

Recent experiments have inquired into the affinity of Zn^{2+} for nonspecific proteomic binding sites [21, 24]. Utilizing a fluorescent probe to compete with the Proteome for Zn^{2+}, two classes of sites were revealed [24]. The manifold of stronger binding sites involves thiolate ligands and displays an average stability constant for Zn^{2+} at pH 7 of 10^{10} [40, 41]. Moreover, their cellular concentration ranges in the hundreds of micromolar. Even if Cd^{2+} interacts with the Proteome with a larger average stability constant than Zn^{2+} ($K_{Pro \cdot Cd}$), it is probable that the reaction between newly synthesized, metal free, apo-MT with $\Sigma Pro_n \cdot Cd$ would generally favor the formation of Cd_n-MT (stoichiometry unspecified).

$$\text{Apo-MT} + \Sigma Pro_n \cdot Cd \rightleftarrows Cd_n\text{-MT} + \Sigma Pro_n \quad K = K_{Cd\text{-}MT}/K_{Pro \cdot Cd} \tag{2.5}$$

As MT is induced and newly synthesized, might the protein compete for Zn^{2+} bound as Zn-Proteins (Reaction 2.6), considering that measured stability constants for Zn-Proteins range between 10^9 and 10^{12} [18, 42]?

$$\text{Apo-MT} + 7\text{Zn-Protein} \rightleftarrows Zn_7\text{-MT} + 7\text{apo-Protein} \tag{2.6}$$

The answer seems to be "no" or only to a small extent, based on two observations. First, apo-MT or MT lacking its full complement of metals (MT with less than 7 Zn^{2+} and/or Cd^{2+} ions bound to it) is commonly detected under various physiological and pathological conditions in a variety of cell types, sometimes in large concentration [43–45]. This pool of metal-unsaturated sites has been described as a "thermodynamic" sink for Zn^{2+} that should compete for metal ion bound to Zn-proteins. Nevertheless, MT that is undersaturated with metal ions coexists with Zn-Proteins. Second, when the reactants of Reaction 2.6 are deliberately mixed in vitro, little or nothing occurs in terms of product formation [46]. Because at least some Zn-Proteins should react with apo-MT on the basis of their relative stability constants for Zn^{2+}, there must be a kinetic barrier to this type of reaction. Another intriguing feature of unsaturated metallothionein is that it can be present in large concentrations without inducing the transport of additional Zn^{2+} into the cell to constitute Zn_7-MT. This situation stands in contrast to the uptake of Zn^{2+} that accompanies the induction of MT by Cd^{2+} (see below).

A possible explanation for the widespread observation of unsaturated MT is that the reverse of Reaction 2.6 takes place in cells: Zn_n-MT serves as a source of Zn^{2+} for newly synthesized apo-Zn-proteins.

$$Zn_n\text{-MT} + \text{n apo-Protein} \rightleftarrows \text{Apo-MT} + \text{nZn-Protein} \tag{2.7}$$

For example, reports show that Zn_n-MT can donate Zn^{2+} to apo-carbonic anhydrase [39, 47]. As indicated above, the Proteome also contains a large concentration of sites that bind Zn^{2+} with high affinity ($\Sigma Pro_m \cdot Zn$). Zn^{2+} from this source is also kinetically available for donation to apo-carbonic anhydrase [41]. Thus, it may be that MT is simply the most prominent of the group of proteins that participate in Zn^{2+} trafficking and together may supply Zn^{2+} to apo-Zn-Proteins (Reactions 2.8 and 2.9)

$$Zn_n\text{-MT} + \Sigma Pro_n \rightleftarrows \text{Apo-MT} + \Sigma Pro_n \cdot Zn \tag{2.8}$$

$$\Sigma Pro_n \cdot Zn + n \text{ apo-Protein} \rightleftarrows \Sigma Pro_n + \text{nZn-Protein} \tag{2.9}$$

The distribution of M^{2+} in M_n-MT, where M is Zn^{2+} or Cd^{2+} and $n \leq 7$, has been investigated. Initially, studies seemed consistent with the sole presence of M_4- and M_7-MT, as well as apo-MT [48]. That is, only fully occupied clusters are stable. However, more recent experiments utilizing highly sensitive electrospray mass spectrometry document that when MT is titrated with n moles of Zn^{2+} or Co^{2+} or the experiment is conducted in the presence of the competitive ligand apo-carbonic anhydrase, the full range of metal to MT stoichiometries (0–7) is observed [39, 49–52]. In contrast, titration with Cd^{2+} involves concerted formation of the cadmium-thiolate clusters above pH 7.1 and increasing contributions from sequential reaction as the pH is lowered [53].

A puzzling feature of all cadmium metallothioneins isolated from cells or organs is that they contain both Cd^{2+} and Zn^{2+} [33]. Indeed, in an analysis of Cd^{2+} and Zn^{2+} distribution during the exposure of mouse kidney cortical cells to Cd^{2+}, mixed metal, Cd, Zn-MT begins to appear within 3 h of time zero and continues to grow in concentration [54]. Inquiry into the mechanism of cluster-specific formation of Cd_n, Zn_{7-n}-MT has shown, surprisingly, that the native distribution of the two metals among the seven binding sites results from a complicated metal ion exchange process between Cd_7-MT and Zn_7-MT [55]:

$$n\text{Cd}_7\text{-MT} + (7-n)\text{Zn}_7\text{-MT} \rightleftarrows 7\text{Cd}_n,\ \text{Zn}_{7-n}\text{-MT} \tag{2.10}$$

Whether this mechanism applies when the overall MT pool is unsaturated with Cd^{2+} and Zn^{2+} remains to be defined. The source of Zn^{2+} to constitute Zn_7-MT appears to be extracellular because induction of MT by Cd^{2+} raises the total intracellular concentration of Zn^{2+} and does not seem to compete for protein-bound Zn^{2+} as in the reverse of Reaction 2.7 [56].

A persistent question related to the routine formation of Cd_n, Zn_{7-n}-MT is why cells exposed to Cd^{2+} seemingly make more MT than is necessary to gather all of the invasive metal ion into its structure. In Reaction 2.10, Cd^{2+} tends to concentrate in the 4-metal cluster and Zn^{2+} in the 3 metal cluster [55]. As a result, it has been hypothesized, the protein retains its capacity to donate Zn^{2+} to other sites (Reaction 2.7) even as it also sequesters Cd^{2+} [57].

The X-ray crystallographic analysis of Cd_5, Zn_2-MT reveals two molecules of MT per unit cell that are oriented in opposite directions such that the M_4S_{11} and M_3S_9 clusters are juxtaposed and amino acid sidechains in the vicinity of the clusters can interact [34]. Cd_7-MT also dimerizes in solution and ^{13}Cd NMR spectra reveal perturbation of the Cd_4, S_{11} cluster in the holoprotein but not in the isolated C-terminal domain, consistent with head–tail orientation of the dimer [36]. Together, these findings suggest that metal ion exchange is mediated by a dimer intermediate which facilitates cluster–cluster interaction and shifts Cd^{2+} into the 4-metal domain and Zn^{2+} into its proximate 3-metal domain.

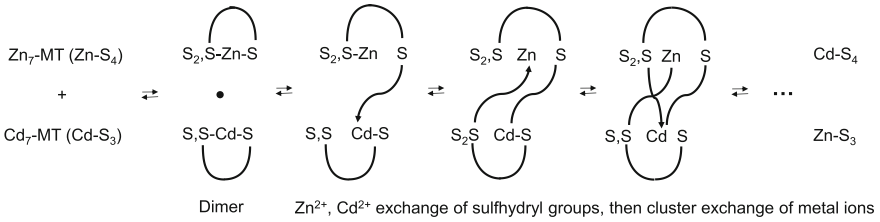

Fig. 2.4 Proposed mechanism of inter-protein metal exchange involving two molecules of metallothionein

Figure 2.4 illustrates a plausible mechanism of metal ion exchange between Cd^{2+} ($Cd\text{-}S_3$) and Zn^{2+} ($Zn\text{-}S_4$) sites located on different molecules of MT. Two MT molecules dimerize. The docked metal clusters undergo metal-thiolate bond dissociations linked with subsequent attack of the newly free sulfhydryl groups on a metal ion in the other cluster. These reactions progressively exchange metal ions between the two clusters. This is a remarkable reaction because two sites with metal ions that are fully saturated with ligands undergo progressive reaction. Neither structure has free metal binding ligands that can facilitate the exchange process.

Reaction 2.10 requires that intracellular Zn_7-MT be formed at some point. It might serve as the precursor of Cd_7-MT according to Reaction 2.11.

$$Zn_7\text{-MT} + 7\text{Pro} \cdot \text{Cd} \rightleftarrows Cd_7\text{-MT} + 7\text{Pro} \cdot \text{Zn} \tag{2.11}$$

Here, the overall equilibrium constant is the ratio $(K_{Cd\text{-}MT}K_{Pro\cdot Zn})/(K_{Zn\text{-}MT}K_{Pro\cdot Cd})$. Both the relative strength of binding of Cd^{2+} and Zn^{2+} to Pro as well as the strongly favorable affinity of Cd^{2+} versus Zn^{2+} for MT $(K_{Cd\text{-}MT})/(K_{Zn\text{-}MT})$ play roles in determining whether Reaction 2.11 is energetically feasible. Interestingly, the titration of Zn_7-MT with Cd^{2+} instead of Pro · Cd produces intermediate species of Cd_n, $Zn_{7\text{-}n}$-MT that differ markedly in the detailed location of metal ions compared with those generated in Reaction 2.10 [55, 58].

The principal hypothesis underlying Fig. 2.1 is that upon entrance into cells, Cd^{2+} eventually displaces Zn^{2+} from key Zn-Proteins (Reaction 2.4), producing an array of Cd-proteins with altered function. In a general test of the hypothesis, the properties of Reaction 2.4 have been directly assessed. Whole Proteome, including the complete manifold of Zn-Proteins, was titrated with Cd^{2+} and the amount of products, $\Sigma Cd\text{-}P_n$ and $\Sigma Pro_n \cdot Zn$, measured [59]. Surprisingly, Cd^{2+} is able to mobilize much of the Zn^{2+} associated with Zn-Proteins, resulting in proteomic binding of both metal ions. Presumably, Cd^{2+} replaces Zn^{2+} in Zn-Proteins and the latter then associates with nonspecific proteomic binding sites.

The product mixture of Reaction 2.4 was reacted with enough apo-MT to coordinate all of the cadmium ion [59]. Cd^{2+} is preferentially transferred to apo-MT together with some Zn^{2+}:

$$\Sigma Cd\text{-}P_n + \Sigma Pro_m \cdot Zn + \text{apo-MT} \rightleftarrows \Sigma P_n + \Sigma Pro_m + Cd_n, Zn_m\text{-MT} \tag{2.12}$$

The reaction is driven energetically by the much larger affinity of apo-MT for Cd^{2+} than for Zn^{2+}, described above. In contrast to the lack of reaction of apo-MT with the complement of constitutive Zn-Proteins (ΣZn-P_n), Reaction 2.12 indicates broad reaction of apo-MT with Cd^{2+} bound in native protein sites normally occupied by Zn^{2+}.

Apo-MT was replaced in Reaction 2.12 by Zn_7-MT [59]. In response, the product distribution changes such that as Cd^{2+} moves into the pool of MT, a similar amount of MT-bound Zn^{2+} shifts into the protein fraction, possibly restoring Zn^{2+} to its native binding sites and functional activity to the corresponding proteins:

$$\Sigma\text{Cd-P}_n + \text{Zn}_7\text{-MT} \rightleftarrows \Sigma\text{Zn-P}_n + \text{Cd}_n, \text{Zn}_{7-n}\text{-MT} \tag{2.13}$$

The potential of Zn_7-MT to restore Zn^{2+} and functionality to cadmium-altered proteins is a long-standing hypothesis [57]. Two model reactions support this attractive idea: Zn_7-MT carries out metal ion exchange with a Cd-substituted zinc-finger protein, Tramtrak, and Cd-carbonic anhydrase [60, 61].

An important characteristic of Cd^{2+} intoxication is that as Cd^{2+} reaches the kidney, it largely accumulates there as Cd_n, Zn_{7-n}-MT and remains bound in this form in the steady state of its degradation and resynthesis [27]. One imagines that as metallothionein is hydrolyzed into its constituent amino acids, Cd^{2+} becomes available transiently for redistribution (Fig. 2.1). If so, the apparent maintenance of Cd^{2+} bound to MT may involve continual reacquisition of Cd^{2+} from Cd-Proteins and ΣPro $\cdot$ Cd.

This dynamic picture of the continual turnover of Cd_n, Zn_{7-n}-MT feeds the hypothesis that Cd^{2+} becomes toxic when the concentration of Cd^{2+} within cells exceeds the storage capacity of MT as it undergoes steady-state degradation and resynthesis [62]. In this situation, Cd^{2+} spills out of the MT pool into other, sensitive sites. Until that point, the hypothesis maintains that all of the cellular Cd^{2+} is irreversibly bound to MT in an innocuous form. This is not the reality, however. In studies involving both animals and cells exposed to modest concentrations Cd^{2+}, a small fraction of non-MT-bound Cd^{2+} has been detected in the presence of Cd_n, Zn_{7-n}-MT, even though the sites occupied by Zn^{2+} should be available to exchange with Cd^{2+} [54, 56]. It is this pool of residual Cd^{2+} that may initiate cell injury.

The concentration of non-MT Cd^{2+} observed in some of these experiments may be estimated by comparison with the concentration of intracellular Zn-Proteins. The approximately 3000 Zn-Proteins (ΣZn-P_n) contribute hundreds of micromolar Zn^{2+} to the cell [63]. Assuming a value of 250 μM Zn^{2+}, one study places the pool of potentially toxic Cd^{2+} at about 10 μM and that of MT-bound Cd^{2+} many times larger [56]. Several sections below address what is known about members of the non-MT group of proteins.

It is evident that Cd-Protein complexes can exist in the presence of Cd_n, Zn_{7-n}-MT. Is it possible that this small pool of non-MT-bound Cd^{2+} is derived from Cd_n, Zn_{7-n}-MT apart from the residual concentration of ΣCd-P_n that exists in the equilibrium established in Reaction 2.13? In particular, do redox reactions that oxidize its sulfhydryl groups labilize bound Cd^{2+}? Zn_7- and/or Cd_7-MT do undergo reaction with oxidants such as 5,5′-dithio-bis(2-nitrobenzoate) and glutathione disulfide

(GSSG), nitric oxide, and hydrogen peroxide [64–68]. However, the rate of oxidation with GSSG is unreasonably slow and NO prefers to react with proteomic and GSH pools of thiol groups (NO) [65, 68]. To the extent that cellular MT exists as a dimer, its reactivity may be substantially diminished in comparison with that observed under typical in vitro conditions [69]. Thus, whether such reactions occur in cells is unclear.

2.4 MTF-1: Reactions with Cd^{2+} and Zn^{2+}

Cells respond to Cd^{2+} and Zn^{2+} by upregulating the synthesis of metallothionein. The molecular switch that mediates this process is the transcription factor, metal regulatory transcription factor 1 or MTF-1, that binds selectively to metal response elements (MREs) located in the promoter region of MT and other genes [70, 71]. Extensive attention has been devoted to clarifying the mechanism by which Zn^{2+} influences gene expression. The metal-responsive part of the protein resides in its DNA binding region and is comprised of six tandem cys_2, his_2 (C_2H_2) zinc fingers. Normally, such structures are folded and functional only in the presence of Zn^{2+} (Reaction 2.9) [72, 73].

$$n\mathrm{Zn}^{2+} + \mathrm{MTF\text{-}1}_{\mathrm{inactive}} \rightleftarrows \mathrm{Zn}_n\text{-}\mathrm{MTF\text{-}1}_{\mathrm{active}} \quad \left(n \leq 6,\ \mathrm{K}_{\mathrm{eq}}\right) \tag{2.14}$$

According to this simple reaction, Zn^{2+} reversibly activates MTF-1 depending on its concentration, which varies based on the availability of extracellular Zn^{2+} to the cell.

An in vitro experiment using cell lysate as a source of MTF-1 required the addition of micromolar Zn^{2+} to stimulate MTF-1 · MRE binding [74]. At first, this result suggests that the critical stability constants (K_{eq}) controlling the formation of Zn_n-MTF-1 lie in the range of 10^6. However, measurement of the equilibrium constants for the association of Zn^{2+} with each of the zinc-finger domains of MTF-1 demonstrates that they are much larger, on the order of 10^{10-11} [75]. The latter makes sense when one realizes that the actual reaction involved in MTF-1 activation begins with Zn^{2+} bound to the Proteome as Pro · Zn,

$$\Sigma\mathrm{Pro}_n \cdot \mathrm{Zn} + \mathrm{MTF\text{-}1} \rightleftarrows \Sigma\mathrm{Pro}_n + \mathrm{Zn}_n\text{-}\mathrm{MTF\text{-}1} \tag{2.15}$$

Because Zn^{2+} is associated with high affinity sites in the Proteome ($K_{eq} \sim 10^{10}$), MTF-1 must, itself, strongly coordinate Zn^{2+}. In retrospect, the cell lysate experiment above is consistent with this view, recognizing that most of the added Zn^{2+} binds to cellular ligands other than MTF-1 [75]. The key point to appreciate is that the balance between MTF-1 and Zn_n-MTF-1 depends not only on the total concentration of mobile Zn^{2+} that has entered the cell but upon the relative stability constants of ΣPro_n and MTF-1 for Zn^{2+} and their cellular concentrations.

How does Cd^{2+} upregulate MTF-1? The initial hypothesis that Cd^{2+} successfully substitutes for Zn^{2+} in Reaction 2.14 proved incorrect. In fact, Cd^{2+} inactivates

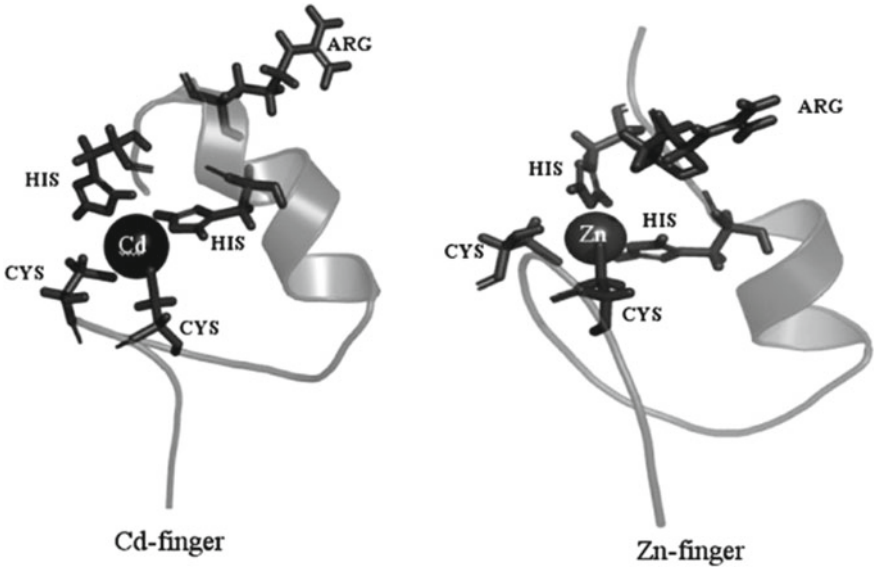

Fig. 2.5 NMR structural comparison of a C_2H_2 zinc-finger domain from transcription factor IIIA with Cd^{2+} or Zn^{2+} bound. Shown with permission [79]

MTF-1 [76]. This result can be rationalized with the results of a study comparing the DNA binding activity of Zn- and Cd-containing zinc-finger domains related to transcription factor IIIA (TF-IIIA) and related three-dimensional structures. As with MTF-1, Cd^{2+} prevents specific interaction of the zinc fingers with the internal control region of the 5S ribosomal RNA gene [77]. The perturbation of the peptide structure by Cd^{2+} is subtle but powerful (Fig. 2.5) [78, 79]. C_2H_2 zinc-finger domains display a ββα series of secondary structures with the β-sheet providing the two thiolate ligands for Zn^{2+} and the α-helix, the two imidazole nitrogens [80, 81]. The amino acid side chains of the helix serve as the DNA recognition elements. Both Zn- and Cd-peptides exhibit similar conformational folds. However, the helix in the latter structure is slightly perturbed because of the larger ionic radius of Cd^{2+} versus Zn^{2+} (92 vs. 74 pm) and does not present its amino acid side chains properly to the base-pair edges in the major groove of cognate DNA (note arginine in Fig. 2.5). Complementary non-covalent bonding interactions between peptide and base pairs cannot be made optimally and specific DNA-protein association is lost.

The mechanism of inactivation in a protein with multiple, tandem zinc fingers may be more drastic. In its reaction with Zn_9-TF-IIIA, Cd^{2+} displaces Zn^{2+} and binds with smaller stoichiometry, Cd_{4-5}-TF-IIIA [77]. Possibly, the original one for one replacement of Zn^{2+} by Cd^{2+} is followed by a rearrangement of the Cd-ligand configuration in a single finger to favor Cd-C_4 binding involving two adjacent fingers:

$$2\ \text{Cd-}(C_2H_2) \rightleftarrows \text{Cd-}C_4 + 4\ \text{His} + Cd^{2+} \tag{2.16}$$

If this were to happen, any semblance of the original finger domain structure would be destroyed.

An alternative hypothesis to explain the activation of MTF-1 by Cd^{2+} proposed that the apo-transcription factor is switched on by Zn^{2+} released from Zn-MT during its reaction with Cd^{2+} [82]. Indeed, upon mixing Cd^{2+} with a lysate containing inactive MTF-1 and added Zn_7-MT, functional Zn_n-MTF-1 is generated as Cd^{2+} displaces Zn^{2+} from MT making it available for reaction with apo-MTF-1. In this reaction mixture, MTF-1 competes with ΣPro_n for Zn^{2+} as in Reaction 2.15. Nevertheless, the concentration of Zn-MT may be vanishingly small in some cells and not available to act as a source of Zn^{2+} upon reaction with Cd^{2+}. Thus, the more general hypothesis is that Zn^{2+} from $\Sigma Zn\text{-}P_n$ activates MTF-1 when it exchanges with Cd^{2+} as in Reaction 2.4.

This extension to the hypothesis was tested in an experiment in which Cd^{2+} was introduced into LLC-PK_1 cells at time zero via a membrane permeant Cd-pyrithione complex (Lund et al. unpublished information). Cd^{2+} becomes localized rapidly in the proteome as illustrated in Fig. 2.1 and hypothetically distributes itself into Zn-Proteins as well as nonselective proteomic sites. No Cd_n-MT is observed consistent with the very low constitutive concentration of MT in these cells. During the subsequent 24-h period, de novo synthesis of metallothionein protein shifts most of the Cd^{2+} from the proteome into MT and also coordinates some extracellular Zn^{2+} to form Cd_n, Zn_{7-n}-MT. This experiment demonstrates that MT can be induced after the rapid binding of Cd^{2+} to the proteome without initial displacement of Zn^{2+} from Zn-MT. Mobilization of Zn^{2+} from $\Sigma Zn\text{-}P_n$ by added Cd^{2+} is thought to drive the formation of Zn_n-MTF-1. In addition, subsequent transfer of Cd^{2+} from the proteome to MT presumably involves either Reaction 2.12 or 2.13. Remaining to be resolved in the complexity of this model of activation is how MTF-1 initially escapes reaction with Cd^{2+} after it enters cells, becomes disseminated within the proteome, and reacts with $\Sigma Zn\text{-}P_n$. Lacking Zn^{2+} and having high affinity for Cd^{2+} because of its cysteinyl sulfhydryl groups, apo-MTF-1 would seem to be a preferred site of reaction with cadmium ion.

2.5 Reactions of Cd^{2+} with Metallothionein in Relation to Animal Toxicity

Epidemiological studies clearly link Cd^{2+} with chronic kidney failure and bone disease [3, 11, 29, 62, 83]. For many years, efforts to mimic human nephrotoxicity utilized a rodent model in which large amounts of Cd^{2+} were injected intraperitoneally to rapidly elicit overt kidney injury [28–31]. From such experiments, general agreement arose that the induction of metallothionein synthesis protects animals from the toxicity of Cd^{2+}. Wild-type rodents experience less toxicity than MT-null animals; animals pre-induced to synthesize MT or carrying constitutively expressed MT transgenes are likewise more resistant to Cd^{2+} than unmodified control rodents.

These studies also reached the unexpected conclusion that cadmium metallothionein (Cd-MT) is the proximate agent that damaged the kidney [84–86]. Following injection, Cd^{2+} concentrates in the liver, stimulates the synthesis of MT, and coincidentally damages hepatic cells causing them to passively release a wave of Cd-MT into the plasma. Upon reaching the kidney, the 6 kDa MT protein is filtered into the tubule, where it is resorbed and degraded. The large concentration of Cd^{2+} mobilized within tubule cells causes massive nephrotoxicity.

Whether this model is relevant to the human situation of prolonged oral intake of small amounts of Cd^{2+} is doubtful because of the focus on acute toxicity and the use of the unnatural route of Cd^{2+} administration by injection. The observation that MT-null rodents injected with Cd^{2+} still develop the same kidney disorder in the absence of the capability to make hepatic Cd-MT strongly argues against the primacy of Cd-MT circulating in the plasma as the initiator of nephrotoxicity [87]. Moreover, other studies in which rats were exposed orally to smaller concentrations of Cd^{2+} indicate that Cd_n,Zn_{7-n}-MT produced in the liver is stable and does not supply measurable Cd^{2+} to the kidney over time (Fowler and Petering, unpublished information). Finally, in kidney cortical cell culture exposed to Cd^{2+} and Cd-MT, equal concentrations of Cd^{2+} provided by the two sources of Cd^{2+} cause indistinguishable toxic effects [88]. Thus, it seems likely that Cd^{2+} entering plasma from the intestine after oral intake is, itself, filtered and resorbed in the kidney tubule, leading to toxicity.

2.6 Reactions of Cd^{2+} with Zn-Proteins in Relation to Nephrotoxicity: Zn_3-SP1

Chronic kidney disease develops slowly in humans and animals following long-term exposure to Cd^{2+} [89]. It is characterized as a Fanconi Syndrome, in which tubular cells lose their capacity to resorb nutrients such as glucose, amino acids, and phosphate from the plasma glomerular filtrate [90]. In an effort to understand the chemical basis for the disruption of glucose resorption, primary mouse kidney cortical cells, rich in proximal tubular cells, were incubated with low micromolar Cd^{2+} for 24 h [91]. Sodium-dependent glucose uptake mediated by two transporters, SGLT 1 and 2, is inhibited in a concentration-dependent manner without altering viability and DNA or protein synthesis.

$$\text{Glucose}_{\text{out}} + m\text{Na}^+_{\text{out}} \rightleftarrows \text{Glucose}_{\text{in}} + m\text{Na}^+_{\text{in}} \tag{2.17}$$

The kinetics of transport are consistent with a loss of functional transporters. Importantly, intracellular ATP, Na^+ and K^+ concentrations are normal in Cd^{2+} treated cells, indicating that neither the sodium gradient supporting active transport, nor the sodium-potassium ATPase that establishes the gradient or oxidative phosphoryla-

tion that supplies ATP to drive the formation of the ion gradient are compromised by Cd^{2+}.

These results point to a direct impact of Cd^{2+} on the synthesis or degradation of SGLT 1 and/or 2 protein. Inquiry into the integrity of these two processes in Cd^{2+} exposed cells revealed that the concentrations of the mRNAs for SGLT 1 and 2 are substantially reduced due to a selective inhibition of their synthesis [92]. Investigating the origin of this effect, the promoter regions of each gene, sglt 1 and 2, were cloned and shown to contain multiple GC-rich binding sites selective for binding the transcription factor Sp1, a common zinc-finger structure using the C_2H_2 metal chelation motif [93].

$$Zn_3\text{-Sp1} + GC_{site} \rightleftarrows Zn_3\text{-Sp1} \cdot GC_{site} \tag{2.18}$$

Expression of the luciferase gene driven by these promoters or the Sp1-specific cytomegalovirus promoter is downregulated in the presence of Cd^{2+}.

The inhibitory effect of Cd^{2+} on Sp1 was further demonstrated by showing with the chromosomal immunoprecipitation assay that Cd^{2+} reduces in vivo binding of Sp1 to cognate DNA (GC_{sites}) in the promoters of sglt 1 and 2 [93]. Furthermore, direct in vitro assay shows that the specific DNA binding activity but not the concentration of Sp1 is severely depressed in Cd^{2+} treated cells.

Another study focusing on the impact of Cd^{2+} on DNA repair reached a similar conclusion, namely, that Cd^{2+} downregulates the synthesis of 8-oxoguanine glycosylase, a DNA repair enzyme, by inhibiting the positive transcriptional activity of Sp1 required for its gene expression [94]. In both studies, it was hypothesized that Cd^{2+} competes successfully with Zn^{2+} bound to Sp1 to generate Cd_n-Sp1 and, in the process, inactivates the protein toward selective DNA complexation:

$$Zn_3\text{-Sp1} + 3Cd^{2+} \rightleftarrows Cd_3\text{-Sp1} + 3Zn^{2+} \quad (K_{eq}) \tag{2.19}$$

The properties of this reaction have been probed using recombinant human Zn_3-Sp1 [95, 96]. First, the equilibrium properties of (K_{eq}) of Reaction 2.18 were determined after simultaneously mixing a range of concentrations of Cd^{2+} and Zn^{2+} with Zn_3-Sp1. The reaction displays a small preference for Cd_3-Sp1 with $K_{eq} \sim 10$. As more Cd^{2+} shifts the reaction to the right, the extent of the product's reaction with GC_{site} diminishes (Reaction 2.18). But Reaction 2.19 is also reversible; increasing the concentration of Zn^{2+} shifts it to the left and elevates the concentration of Zn_3-Sp1 and $Zn_3\text{-Sp1} \cdot GC_{site}$ in Reaction 2.18.

This experiment was also conducted by mixing Zn_3-Sp1 first with Cd^{2+} and then after a short incubation time with Zn^{2+}. In this case, large concentrations of Zn^{2+} fail to reverse the inhibition of DNA binding imposed by Cd^{2+}. These results were interpreted in terms of a two-step reaction sequence in which Cd^{2+} initially displaces Zn^{2+} as in Reaction 2.19 and then undergoes an irreversible rearrangement to produce highly stable Cd_n-Sp1′:

$$Cd_3\text{-Sp1} \rightleftarrows Cd_n\text{-Sp1}' + (3\text{-}n)Cd^{2+} \tag{2.20}$$

In Reaction 2.19, Cd^{2+} probably substitutes for Zn^{2+} with minimal change in the conformation of the three fingers of Sp1 as described above for the reaction of Cd^{2+} with another finger peptide [78]. In contrast, Reaction 2.20 may drastically unfold and rearrange the standard C_2H_2 conformation into one in which Cd^{2+} binds to three or four sulfhydryl groups located within adjacent fingers (see Reaction 2.16) [77]. As portrayed, this interconversion results in the formation of a highly stable Cd^{2+}-Sp1 complex.

To approximate the in vivo situation, proteome would also need to be present. In that case, the relative affinities of Cd^{2+} and Zn^{2+} for proteomic binding sites (ΣPro_n) might influence whether Cd_n-Sp1 formed. This condition was modeled using Cd-EDTA in place of Cd^{2+} [96]. Like Cd^{2+}, Cd-EDTA effectively inhibits Zn_3-Sp1 even though EDTA binds Cd^{2+} with high affinity ($K_{eq} = 1.4 \times 10^{13}$, pH 7) (Reaction 2.21) [97]:

$$\text{nCd-EDTA} + Zn_3\text{Sp1} \rightleftarrows Cd_n\text{-Sp1}' + \text{nZn-EDTA} + (3\text{-}n)Zn^{2+} \tag{2.21}$$

From an energetics standpoint, the high affinity of EDTA for Zn^{2+} ($K_{eq} = 8.6 \times 10^{12}$, pH 7) balances its similarly large stability with Cd^{2+} [97]. As a result, the strongly favorable formation of Cd_n-Sp1′ potentially resulting in Cd-S_4 coordination drives the reaction toward products. Reaction 2.21 involves metal ions that are fully coordinated with ligands. In this situation, like that shown in Fig. 2.5, the metal exchange mechanism is complicated but apparently kinetically feasible.

Reactions 2.19 and 2.20 or 2.21 take place slowly or not at all if Zn_3-Sp1 is present in the reaction mixtures as $Zn_3\text{-Sp1} \cdot GC_{site}$ [96]. Specific DNA binding protects the transcription factor from reaction with Cd^{2+} or a variety of ligands including EDTA, which readily inactivate Zn_3-Sp1 in the absence of GC_{site} [98]. This protective effect was not observed when the protein was associated with nonspecific DNA. Thus, in the cellular context, it is likely that a pool of Zn_3-Sp1 not GC_{site}-bound is reactive with Cd^{2+} and that the dynamics of selective DNA-protein interactions plays a role in the details of inhibition (Reactions 2.22 and 2.23).

$$Zn_3\text{-Sp1} + DNA_{\text{non-specific}} \rightleftarrows Zn_3\text{-Sp1} \cdot DNA_{\text{non-specific}} \tag{2.22}$$

$$Zn_3\text{-Sp1} \cdot DNA_{\text{non-specific}} + GC_{site} \rightleftarrows Zn_3\text{-Sp1} \cdot GC_{site} + DNA_{\text{non-specific}} \tag{2.23}$$

Thus, as Cd^{2+} reacts with Zn_3-Sp1 or $Zn_3\text{-Sp1} \cdot DNA_{nonspecific}$, Reaction 2.23 shifts toward the left, reducing the concentration of active $Zn_3\text{-Sp1} \cdot GC_{site}$.

Zn_3-Sp1 is one of nearly 1000 Zn-finger proteins with C_2H_2 ligands [16]. It seems unlikely that Cd^{2+} targets only Zn_3-Sp1 and a few other proteins described below, considering the structural similarity of zinc binding sites that these proteins share. If there is selectivity, factors such as differential disposition of zinc-finger proteins between free, and DNA bound as described in Reactions 2.22 and 2.23 may contribute. In addition, the possible exchange of Zn^{2+} associated with Zn_n-MT with Cd^{2+} bound to cadmium-substituted proteins (Reaction 2.13) may play a role in limiting the steady-state localization of Cd^{2+} among these cellular binding sites.

The kinetics of reaction and trafficking of Cd^{2+} with primary mouse kidney cortical cells reveal that Cd^{2+} enters the proteome beginning at time 0 and stimulates MT mRNA synthesis beginning at 1 h before it begins to bind to newly synthesized MT protein after 3 h [54]. Some Cd^{2+} remains bound to the proteome throughout 24 h of observation even after MT begins to be synthesized as Cd_n, $Zn_{(7-n)}$-MT, consistent with the downregulation of glucose transport that also begins at 3 h and continues even in the presence of a growing pool of MT. In comparison, pre-induction of Zn_7-MT before exposure to Cd^{2+} prevents its toxicity. Evidently, Cd^{2+} partitions into protein binding sites that are important for toxicity in the absence of a significant pool of endogenous MT. Such reactions may cause toxicity directly as with Sp1 because the cadmium-containing products can coexist with a growing pool of MT. Alternatively, they may set in motion pathways of toxicity that cannot be reversed by subsequent sequestration of Cd^{2+} by MT.

2.7 Reactions of Cd^{2+} with Zn-Proteins in Relation to Carcinogenesis

Cd^{2+} has been designated as a human carcinogen [99]. The proposal that toxic metal ions might target zinc-finger domains as a basis for their cancer causing properties was set forth shortly after the first Zn-finger protein (C_2H_2), transcription factor IIIA (TFIIIA), was described [80, 100, 101]. A number of reports have demonstrated negative effects of Cd^{2+} on the properties of Zn_9-TFIIIA as described above [77, 102].

Downregulation of the DNA nucleotide excision repair protein, Zn-XPA (C_4 ligation) by substitution of Cd^{2+} for Zn^{2+} coupled with subtle conformation changes within XPA offers a mechanistic hypothesis to explain some facets of the carcinogenicity of cadmium [103–108, and Chap. 5]. The much larger affinity of Cd^{2+} than Zn^{2+} for sulfhydryl groups immediately makes Cd^{2+}-Zn^{2+} exchange a plausible reaction. Likewise, the deleterious impact of Cd^{2+} on p53 dependent regulation of cell division suggests the potential substitution of Cd^{2+} for Zn^{2+} in p53 as in Reaction 2.4 [108, 109]. Because Zn^{2+} binds to p53 through C_3H coordination, its conversion to Cd-p53 is also strongly favored.

2.8 Reactions of Cd^{2+} Related to Apoptosis

Many studies of the toxicity of Cd^{2+} focus on cell death as the endpoint. Besides frank necrosis at higher concentrations of Cd^{2+}, programmed cell death or apoptosis is initiated by Cd^{2+} at lower concentrations [12]. The latter outcome results from the stimulation of pathways of cell signaling, eventually activating proteins such as caspases that directly cause cell death [9, 11, 12]. The multiplicity of proteins

involved in such pathways challenges the effort to pinpoint relevant molecular targets of Cd^{2+}. In fact, there is no consensus about the cadmium binding sites that contribute to apoptosis.

Enhanced reactive oxygen species (ROS) and/or elevated intracellular Ca^{2+} are commonly documented in cells exposed to lethal concentrations of Cd^{2+} [11 and Chaps. 3 and 4]. Thus, it has been hypothesized that the role of Cd^{2+} in apoptosis is located at the front end of the process, either initiating the production of ROS or stimulating the release of Ca^{2+} from endoplasmic reticulum or mitochondria. To unravel cause and effect in this process, studies of the kinetics or time dependence of the observed component reactions of the whole process need to be conducted. In so doing, the ordering of steps along the pathway can be assigned. In one of a few such reports, the timed appearance of these steps is as follows [110]:

$$\begin{aligned} &Cd^{2+}_{t=0\,h} \rightarrow ROS_{t=0.75\,h} \rightarrow Ca^{2+}\text{elevation}_{t=1.5\,h} \sim \text{Mitochondria membrane} \\ &\quad \text{potential collapse}_{t=1.5\,h} \rightarrow \rightarrow \text{Apoptosis} \end{aligned} \tag{2.24}$$

According to this sequence, ROS, including the production of species such as superoxide anion, hydrogen peroxide, and hydroxyl radical, may be the primary event stimulated by Cd^{2+}.

An alternate approach to establish cause and effect has treated cells exposed to Cd^{2+} with agents that either destroy ROS such as the membrane permeable reducing agent, *N*-acetyl-cysteine, or bind elevated concentrations of Ca^{2+} such as 1,2-Bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid tetrakis(acetoxymethyl ester) or BAPTA-AM. This compound is readily accumulated by cells and hydrolyzed internally to BAPTA. Inhibition of apoptosis caused by such agents supports the role of ROS and/or Ca^{2+} in the apoptotic sequence [111, 112]. However, the impact of each agent on both ROS production and Ca^{2+} mobilization must be examined in tandem in order to probe whether elevation of ROS or Ca^{2+} is the primary initiator of apoptosis in relation to exposure to Cd^{2+} [113].

There are questions about how BAPTA-AM inhibits the pathway to cell death [114]. Generally, to test the involvement of a rise in Ca^{2+} in apoptosis, the fate of control cells treated with Cd^{2+} has been compared with that of cells preincubated with BAPTA-AM before the addition of Cd^{2+}. BAPTA, the intracellular form of the chelating agent, binds Ca^{2+} at pH 7 with a stability constant of 9.1×10^6 [115]. In contrast, Zn^{2+} is coordinated with a larger stability constant of 1.3×10^8, such that putative spectral measurements of cellular Ca^{2+} with BAPTA include a contribution from the formation of Zn-BAPTA due to the successful competition of BAPTA for cellular Zn^{2+}. Considering that Cd^{2+} binds three orders of magnitude more strongly than Zn^{2+} to an analog of BAPTA, EGTA (ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid), it seems likely that BAPTA intercepts Cd^{2+} as it is transported into cells and before it can initiate the death program that, otherwise, might include the elevation of cytosolic Ca^{2+} [97]. This putative scenario is similar to the repeated

observation that pre-induction of MT protects cells from the toxicity of subsequent exposure to Cd^{2+} [54].

Turning to the question of the critical binding sites for Cd^{2+} in Reaction 2.24, it is recognized that Cd^{2+} is not redox active. As such, the chemical explanation for its stimulation of ROS must lie elsewhere. It has been suggested that Cd^{2+} binds to the thiol group in glutathione (GSH) and prevents it from serving as a reductant that deactivates ROS [116]. But the small concentration of non-MT-bound Cd^{2+} is incommensurate with the large concentration of GSH in cells (1–10 mM) and, thus, cannot bind and directly exert an impact on a significant fraction of this pool of sulfhydryl groups [54, 56]. In addition, the small stability constant of Cd-SG ($10^{4.9}$), and $Cd(SG)_2$ ($10^{7.2}$) at pH 7.2 indicate that GSH cannot compete effectively with protein binding sites for Cd^{2+} [117]. Speciation of Cd^{2+} in kidney, for example, does not include measurable association with GSH [56]. Lastly, contrary to a reduction of free GSH by Cd^{2+}, it is commonly observed that exposure of cells to Cd^{2+} increases the concentration of GSH. This results from an elevation in GSH synthesis, driven by the MTF-1 dependent upregulation of glutamate-cysteine ligase, a constituent enzyme in the synthetic pathway to GSH [118, 119].

A plausible explanation for the observed production of ROS is that Cd^{2+} inhibits mitochondrial oxidative phosphorylation. Early papers reported that Cd^{2+} blocks ATP synthesis in isolated mitochondria [120, 121]. An important extension of this work was the demonstration that several Cd-ligand complexes, including Cd-EDTA, with large stability constants for Cd^{2+} at pH 7 are effective as inhibitors of electron transport and oxidative phosphorylation in bovine heart mitochondria [122]. This finding revealed that high affinity mitochondrial binding sites can effectively compete with these ligands for Cd^{2+}. In turn, cadmium binding obstructs electron transport, directly or indirectly, causing the reduction of intermediates along the electron transport chain. The reduced metal centers then react with oxygen, starting its rapid conversion to ROS.

Zn^{2+} also acts as a strong inhibitor of mitochondrial electron transport [123]. One site of reaction appears to reside in the cytochrome bc_1 complex, where there are several sites involving imidazole and carboxyl groups that coordinate Zn^{2+} [124]. Cd^{2+}, too, disrupts electron transfer in the bc_1 structure but binds with substantially lower affinity than Zn^{2+} [124]. These properties suggest the possibility that the exchange of Cd^{2+} for Zn^{2+} in Zn-Proteins ($\Sigma Pro_n \cdot Zn$ in Reaction 2.4) liberates Zn^{2+} which secondarily inhibits electron transport.

Reactive oxygen species are known to cause widespread cellular damage by oxidizing vulnerable classes of biomolecules. In light of the established deleterious impact of Cd^{2+} on zinc-finger proteins, Cd^{2+} might act on them through the intermediate agency of ROS to oxidize their sulfhydryl ligands. For example, according to Reaction 2.25, the direct reaction of hydrogen peroxide with the thiol ligands of zinc-finger peptide domains would liberate Zn^{2+} from its

$$H_2O_2 + 2\ RS^- \rightleftarrows RSSR + 2\ HO^- \tag{2.25}$$

sulfhydryl ligands and result in the destruction of the functional domain conformation [125]. Experiments with Zn_3-Sp1 and Zn-p53 demonstrate that exposure to ROS does, in fact, inactivate both proteins [125–128]. In contrast, MTF-1 exposed in a nuclear extract to large concentrations of H_2O_2 in the presence of Zn_7-MT gains capacity to bind to its DNA metal response elements as Zn^{2+} is released from oxidized MT [82]. Evidently, as documented in other experiments, hydrogen peroxide reacts preferentially with the sulfhydryl groups of MT and perhaps other thiol pools, leaving MTF-1 in its reduced state to bind Zn^{2+} released from metallothionein [66].

It is possible that Cd^{2+} reacts both directly (binding) and indirectly (ROS) with protein zinc-thiolate binding sites. During the reaction of Cd^{2+} with primary mouse kidney cortical cells discussed above, Zn_3-Sp1 DNA binding activity is downregulated without causing cell death or altering intracellular ATP concentration [91]. The presence of an intact oxidative phosphorylation system inferred from these results demonstrates that at least in this instance, mitochondrial dependent ROS generation is probably not involved in Cd^{2+} toxicity.

Inquiry into the mechanistic pathway by which cadmium causes apoptosis has not included the evaluation of the role of Cd^{2+}-dependent induction of metallothionein synthesis. In principle, the presence of MT with its abundance of thiol groups might afford protection against oxidative stress either by sequestering intracellular Cd^{2+} or by inactivating reactive oxygen species as discussed above. Nevertheless, the results indicate that whatever upregulation of MT does occur is not sufficient to prevent apoptosis. Probably, the kinetics of MT induction are too slow to disrupt the key reactions involving Cd^{2+} that lead to cell death (e.g. stimulation of ROS production in Reaction 2.24). If later steps do not directly require Cd^{2+}, then the cascade cannot be halted by MT once it is set in motion. In agreement with this general hypothesis, studies of the time dependence of inhibition of Na^+ dependent glucose cotransport by Cd^{2+} show clearly that downregulation of *SGLT* transporter mRNA synthesis occurs before metallothionein mRNA and protein appear and persists after MT is synthesized [54].

2.9 Reactions of Cd^{2+} with Zn-Proteins in Relation to Signal Transduction

Cd^{2+} modulates a variety of signaling pathways in different cells [9, 11, 12, 114]. It is likely that many of these are activated after Cd^{2+} stimulates the production of reactive oxygen species. In addition, some may be directly impacted through binding of Cd^{2+} to sites that have yet to be identified. Hypothetically, they are anticipated to contain multiple sulfhydryl groups and possibly bound Zn^{2+}.

Isoforms of membrane-bound protein kinase C (PKC) represent an example of a protein involved in metabolic control that may undergo direct reaction with Cd^{2+}. Upregulation of PKC is commonly observed in cells exposed to cadmium [129–132]. The regulatory C1 domain of PKC contains two cysteine-rich subdomains (C1A,

C1B) that each coordinate two zinc ions [133, 134]. In its inhibited state, amino acid residues at the N-terminus of the C1A subdomain associate with the kinase active site, forming a pseudo enzyme-substrate complex that physically prevents catalytic interaction of PKC with true substrate proteins [135]. PKC agonists such as diacyl glycerol or phorbol myristate bind to the C1 domain, adding hydrophobic character to its surface and favoring dissociation of the regulatory domain from the active site and binding to membrane [135]. This sequence of reactions activates PKC to phosphorylate target proteins involved in signal transduction.

Each Zn^{2+} within PKC exists in a separate ligand coordination environment of three cysteinyl sulfhydryl groups and one histidinyl imidazole nitrogen [133, 134]. According to one study, Zn^{2+} is required for proper folding of the C1 domain [136]. Recognizing the large affinity of Cd^{2+} for thiol groups, it is hypothesized that Cd^{2+} displaces Zn^{2+} from each subdomain and forces the rearrangement of its conformation as a highly favorable Cd-S_4 site is formed [137]:

$$2\ \text{Zn-N, S}_3 + \text{Cd}^{2+} \rightleftarrows \text{Cd-S}_4 + 2\ \text{Zn}^{2+} + \text{Residual ligands} \tag{2.26}$$

The substitution of Cd^{2+} for Zn^{2+} then activates PKC in the absence of agonists as the regulatory domain loses its inhibitory conformation. Surprisingly, Zn^{2+} and Cd^{2+} bind to C1 with similar affinities, making it less likely that PKC is a preferred site of reaction of Cd^{2+} [139].

Other experiments have demonstrated that PKC is activated by exposure to reactive oxygen species [139, 140]. The cysteine-rich domains of the protein may be attractive targets for reaction between oxidants and their constituent sulfhydryl groups. In principle, Zn^{2+} coordinated to thiol groups should suppress such reactions. But, for example, the sulfhydryl ligands of Zn_7-MT are unusually reactive with oxidants such as hydrogen peroxide and that may also be the case for PKC [66].

2.10 Interactions of Cd^{2+} with Iron, Copper, and Calcium

The metal ion exchange chemistry described for Cd^{2+} and Zn-Proteins conceivably might take place with other metalloproteins (M-Proteins) where M=$Fe^{2+,3+}$, $Cu^{1+,2+}$, or Ca^{2+} [141]. Apart from the well-described impact of Cd^{2+} on calcium homeostasis, few investigations have reported intracellular antagonism between Cd^{2+} and other metals [Chap. 3]. Nevertheless, one might hypothesize that iron-sulfur cluster proteins that utilize sulfhydryl ligands are potential targets of reaction with Cd^{2+} [142]. Similarly, chaperone-based trafficking of copper may also interact with Cd^{2+}, recognizing that some of the intermediate transfer proteins use multiple thiolate ligands to coordinate Cu^{1+} [143].

Binding of Cd^{2+} to proteins involved in Ca^{2+} trafficking has been hypothesized as one mechanism by which Cd^{2+} impinges on Ca^{2+} signaling [144]. This reaction can be considered in terms of Reaction 2.27, where ΣP_n represent proteins that coordinate Ca^{2+}:

$$\Sigma\text{Ca-P}_n + \Sigma\text{Pro}_n \cdot \text{Cd} \rightleftarrows \Sigma\text{Cd-P}_n + \Sigma\text{Pro}_n \cdot \text{Ca} \quad (2.27)$$

Ca^{2+} strongly prefers to bind to oxygen ligands provided by carboxyl, alcohol, or carbonyl groups. Thus, a key regulatory protein calmodulin binds 4 Ca^{2+} ions with ligand sets that include three aspartate or asparagine residues, one glutamate side chain, a peptide carbonyl group, and a water molecule [145]. These sites coordinate Ca^{2+} with stability constants at pH 7 of about 10^{6-7} M^{-1} (149). By contrast, Cd^{2+} associates principally with sulfhydryl and nitrogen ligands [13]. Considering the plethora of such ligands binding sites in cells, symbolized by $\Sigma\text{Pro}_n \cdot \text{Cd}$ in Reaction 2.27, it is unclear whether any Cd-P_n species would be energetically favored over $\Sigma\text{Pro}_n \cdot \text{Cd}$ as Cd^{2+} distributes in cells.

2.11 Analytical Methodology to Detect Cd-Proteins

Studies of the intracellular binding sites of Cd^{2+} described above have revealed some structures, principally Zn-proteins, that interact with Cd^{2+} and, in so doing, are functionally perturbed. Such findings arise out of hypothesis-driven research that intentionally leads to the identification of particular proteins as loci of reaction of Cd^{2+}. For instance, the interaction of Cd^{2+} with Zn_3-Sp1 followed from the interest in the downregulation of sodium-dependent glucose transport into kidney cortical cells [93, 95, 96]. Sp1 is a generic C_2H_2 zinc-finger protein. Would not the thousand or more proteins with similar zinc binding motifs also compete to coordinate Cd^{2+} [16]? Moreover, the potential of Cd^{2+} to displace Zn^{2+} broadly from Zn-proteins (Reaction 2.4) has been demonstrated in vitro [59]. Might this also occur in vivo? These questions raises a general concern about the need for robust methods to evaluate the distribution of Cd^{2+} and other metal ions such as Zn^{2+} within the proteome.

A strategy to accomplish this analysis is to separate the proteome on a solid support, such as polyacrylamide gel, and then search for metals using a laser to raster across the gel and ablate (laser ablation, LA) the gel and its contents into an inductively coupled plasma mass spectrometer (LA-ICP-MS) for identification of resident metals [147]. One problem with the method lies at the front end with the protein separation methodology [147]. High-resolution separation methods result in protein denaturation and loss of bound metal ions as in sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE).

Recently, a modification of SDS-PAGE, called native SDS-PAGE or NSDS-PAGE, has been shown to provide refined separation of proteins with retention of their native properties including bound metal ions [148]. Early experiments suggest that many zinc proteins can be resolved and observed using NSDS-PAGE in combination with LA-ICP-MS [148]. In recent, unpublished experiments, the operation of Reaction 2.4 has been under investigation in the in vitro reaction of Cd^{2+} with individual proteins within fractions of the Zn-proteome.[1] As illustrated in Fig. 2.6,

[1] Lund and Petering (2018) unpublished information.

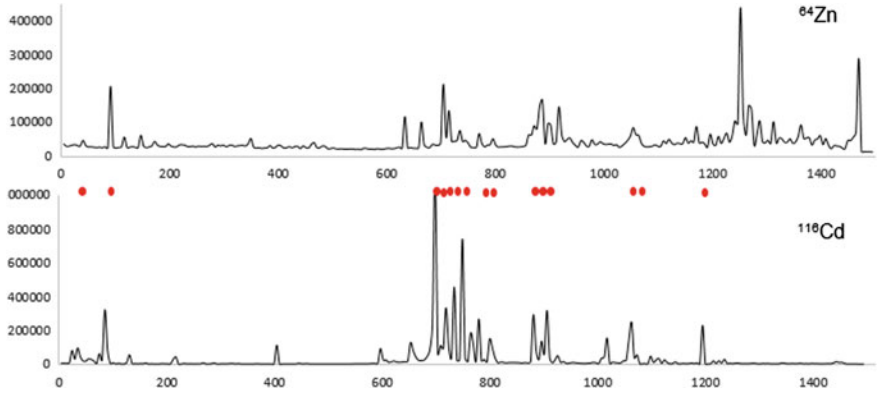

Fig. 2.6 LA-ICP-MS of a DEAE-HPLC fraction (1 of 50) of LLC-PK$_1$ cell supernatant treated with Cd^{2+} in a 1:1 ratio with Zn^{2+} in the fraction and separated by NSDS-PAGE. Y-axis: counts/sec. X-axis: distance along the gel. 1400 is 0.7 of total length. Dots (filled red circle) designate coincident ^{64}Zn and ^{116}Cd peaks

after incubation with Cd^{2+}, many of the Zn-proteins contain Cd^{2+} as well, a condition that would result from the exchange of Zn^{2+} for Cd^{2+} in these proteins.

Initial in vivo experiments using these methods show at the resolution of individual proteins that exposure of LLC-PK$_1$ cells to Cd^{2+} leads to the appearance of many bands of non-MT-bound Cd^{2+}, a significant fraction of which contain both Zn^{2+} and Cd^{2+}. Identification of cadmium-containing proteins using a combination of NSDS-PAGE and LA-ICP-MS should provide a new, powerful approach to understanding the chemical biology of cadmium.

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Chapter 3
Interactions of Cadmium with Signaling Molecules

Douglas M. Templeton and Ying Liu

Abstract Cadmium has no known function in higher organisms, which have evolved in an essentially cadmium-free environment until the last several hundred years of industrial activity. Thus, cadmium's interactions with biological molecules are generally fortuitous and determined by its inorganic chemistry. In biological systems, it exists exclusively in the Cd^{2+} state as the only ionized form, and it is somewhat unique in showing properties both of a Ca^{2+} mimic (by virtue of its ionic radius) and of a "soft" sulfur-binding ion (with polarizable *d* electrons). We review here the interactions of Cd^{2+} with cellular signaling systems; these are broad and non-specific, and result in interactions with both Ca^{2+} signaling and thiol-dependent redox systems, sometimes with ambiguous consequences. The chapter focuses on interactions more than consequences, as the latter are often very complex in origin, but can sometimes be simplified by collecting some of the interactions that have been observed. We discuss some of the general effects of Cd^{2+} on cellular Ca^{2+} levels, with signaling implications, and also some of the major interactions of Cd^{2+} with Ca^{2+} binding sites in proteins. A good part of our discussion is of effects of Cd^{2+} on signaling pathways through kinase activation, phosphatase inhibition, modulation of second messengers, and effects on levels of growth factors and transcription factors. It will be seen that there is a lot of empirical data here that are only partially understood on the basis of Cd^{2+} chemistry. Cadmium regulation of thiol-dependent redox chemistry is also discussed, and some new directions in redox sensing are suggested.

3.1 Introduction

Cadmium is well established to have no known function in the biology of higher organisms, and thus its interactions with them can be expected to display primarily adverse effects as a consequence of chemistry that has not been exploited for biolog-

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ical function in evolution. This is certainly true of Cd^{2+} ion in its interactions with cell signaling systems. Whether it is stimulating or suppressing kinase or Ca^{2+} signaling pathways, favoring cell death, or promoting unwanted cell survival, exposure to Cd^{2+} generally elicits detrimental responses. An exception may be redox-sensing systems that respond to Cd^{2+} with potentially protective outcomes, but even here the responses are not always of ultimate benefit.

Cadmium exists in biological systems only as the Cd^{2+} ion, given its Group 12/*d*10*s*2 electron configuration and unfavorable ionization potentials for other oxidation states. It is a very "soft" ion on all scales such as those of Pearson or Klopman, reviewed by Kinraide [1] who reports an observed softness, σ_{con}, of 0.42 for Cd^{2+} (compare 0.03 for Cr^{3+}, 0.07 for Ni^{2+}, 0.46 for Pb^{2+} and 1.16 for Hg^{2+}), and so prefers soft ligands such as sulfur. Similar scales classify Ca^{2+} as "hard", born out by its preference for O-rich centers. Nevertheless, Cd^{2+} has an ionic radius very close to that of Ca^{2+} (Cd^{2+} 97 pm, Ca^{2+} 99 pm, or 109 pm vs. 114 pm crystal ionic radii), giving similar charge/radius ratios (Ca^{2+} = 0.202 e/nm, Cd^{2+} = 0.206 e/nm), and determining that these ions are able to exert strong electrostatic forces on biological macromolecules [2]. Thus, Cd^{2+} is found substituted into many Ca^{2+}-binding centers that would not otherwise be predicted by its soft nature, and Cd^{2+} samples a variety of possible chemical environments in the cell and has actions that impinge upon diverse cellular signaling processes. Although Cd^{2+} concentrations in the cytoplasmic compartment remain low (e.g., in the 1–3 pM range in cell culture experiments [3], and thereafter buffered by other cellular components such as metallothionein), the higher affinity of typical Ca^{2+}-binding sites for Cd^{2+} (e.g., with Fura-2 the K_d of Cd^{2+} is 0.0011 nM compared with 134 nM for Ca^{2+}) allows favorable competition of Cd^{2+} and Ca^{2+} [3].

This chapter will first discuss interactions of Cd^{2+} with Ca^{2+} second messenger and Ca^{2+} buffering homeostatic systems, then its effects on kinase phosphorylation and phosphatase-dependent dephosphorylation, and finally its impact on thiol-based defense mechanisms. Along the way, we will see examples of promotion of cell death and cell survival, interactions with systems that manage reactive oxygen species (ROS), and a variety of effects on cytoskeletal remodeling (Fig. 3.1). However, a comprehensive review of the multiple documented effects of Cd^{2+} on cell death, ROS, or the cytoskeleton is not attempted.

3.2 Cadmium Interactions with Calcium Signaling Pathways

3.2.1 Effects of Cd^{2+} on Cytosolic Ca^{2+} Levels

As noted above, the physical properties of Cd^{2+} promote its association with Ca^{2+}-binding sites, and a focus of this chapter will be with the effects of Cd^{2+} binding to such sites in signaling proteins. However, similar considerations pertain to Ca^{2+}

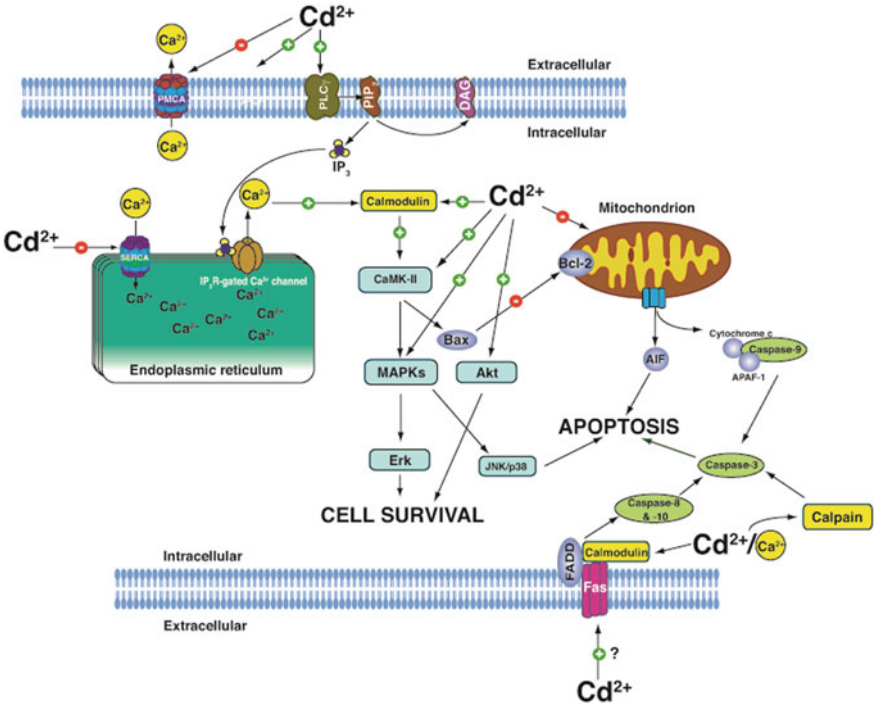

Fig. 3.1 Some pathways involving Cd^{2+} in apoptotic cell death and cell survival. The scheme is based in part on [39], where some additional details may be found. Some color-coding is included: Ca^{2+} and Ca^{2+}-dependent proteins (yellow), kinases (blue), caspases (green), other apoptotic components (shaded blue-gray), membrane components (white font). Abbreviations not defined in the text: AIF—apoptosis-inducing factor; APAF-1—apoptotic protease-activating factor 1; DAG—diacylglycerol; FADD—Fas-associated protein with death domain; Fas—factor for apoptotic signaling; GPCR—G protein-coupled receptor; PMCA—plasma membrane Ca^{2+} ATPase

transporters and more kinetically labile states of Ca^{2+}, and these are also potential targets of Cd^{2+}. Brief mention should be made of the effects of Cd^{2+} on the important second messenger role of cytosolic Ca^{2+} levels, $[Ca^{2+}]_{cyt}$. These can be affected by Cd^{2+} through interactions with Ca^{2+} channels and transporters (discussed by Thévenod elsewhere in this book). Of note, the SERCA-type ATPase involved in resequestration of cytosolic Ca^{2+} in the endoplasmic reticulum may be inhibited by Cd^{2+}, with the short-term result that elevated $[Ca^{2+}]_{cyt}$ persists, but in the longer term this Ca^{2+} is lost to the exterior and intracellular stores become depleted [4].

Fluorimetric studies confirm effects of Cd^{2+} on $[Ca^{2+}]_{cyt}$. Cadmium causes a transient increase in $[Ca^{2+}]_{cyt}$ through several mechanisms, and transient elevations in $[Ca^{2+}]_{cyt}$ of several hundred nM to >1 μM occur either by influx through ligand- or voltage-gated ion channels, or by release from intracellular stores [5]. Because Ca^{2+} is a major second messenger involved in multiple signaling cascades, intracellular $[Ca^{2+}]$ is tightly regulated, and basal levels of Ca^{2+} are usually kept below

100 nM through cooperation of ion channels, ion exchangers, and ATPase pumps. Among the latter are the plasma membrane Ca^{2+}-ATPase (PMCA) and sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) that actively pump Ca^{2+} out of the cytoplasm. Low-affinity/high-capacity intracellular buffering proteins such as calreticulin and calsequestrin (see below) are also involved in Ca^{2+} homeostasis. In addition, upon receptor stimulation, inositol trisphosphate (IP_3) is produced and releases Ca^{2+} from the endoplasmic reticulum through IP_3 receptor-gated channels, or via ryanodine receptors on the sarcoplasmic reticulum. Both duration and amplitude, and possibly frequency of changes in $[Ca^{2+}]_{cyt}$ [6, 7], as well as subcellular location of the stimulus, determine outcomes such as muscle contraction, metabolic activity, cell division, cell motility, and apoptosis [8, 9], and so Cd^{2+} can potentially interfere with all of these processes simply by altering $[Ca^{2+}]_{cyt}$.

Questions have been raised about the accuracy of data on $[Ca^{2+}]_{cyt}$ measured in the presence of Cd^{2+}, because of the potential interference of Cd^{2+} with interaction of Ca^{2+} with Ca^{2+}-sensitive fluorophores. However, it is now believed that newer aequorin-derived Ca^{2+} fluorophores are relatively free of Cd^{2+} interference and give reliable $[Ca^{2+}]_{cyt}$ measurements at concentrations of Cd^{2+} below 15 μM [10]. On the other hand, conventional Ca^{2+} fluorophores can be used to measure cytosolic Cd^{2+} relatively free from Ca^{2+} interference because of their much higher affinity for Cd^{2+} and the relative selectivity of Cd^{2+} chelators to suppress a Cd^{2+}-based signal [11]. Under these circumstances, Cd^{2+} added to culture medium at 5–20 μM was found to increase $[Ca^{2+}]_{cyt}$ to about 250 nM in renal mesangial cells over 8 h, while $[Cd^{2+}]_{cyt}$ was stable at about 2 pM, independent of added Cd^{2+}. It is proposed that available $[Cd^{2+}]_{cyt}$ is buffered by interactions with proteins (e.g., metallothionein) as more Cd^{2+} is added [11].

Cadmium stimulates IP_3 production in skin fibroblasts [12], and at concentrations above 10 μM it increases $[Ca^{2+}]_{cyt}$ in both human hepatic and hematopoietic cells after 6 h [13]. Calcium-dependent phospholipase C (PLC) is the major enzyme generating IP_3, and a PLC inhibitor diminished the Cd^{2+}-evoked increase in $[Ca^{2+}]_{cyt}$ in *Xenopus* oocytes [14], supporting the view that Cd^{2+} activation of PLC may mediate this rise of $[Ca^{2+}]_{cyt}$. However, not all cells (e.g., rat aortic smooth muscle cells, rat embryo fibroblasts, and human epidermoid carcinoma cells) respond to Cd^{2+} in this manner [12]; Cd^{2+}-stimulated release of Ca^{2+} from intracellular stores is thus cell-specific. In cells where Cd^{2+} does induce Ca^{2+} release from intracellular stores, it is unlikely to do so by direct action at intracellular sites, as the response is rapid and occurs before much Cd^{2+} has been taken up by the cell. Evidence that Cd^{2+} can exert an effect on $[Ca^{2+}]_{cyt}$ by an extracellular or cell–surface interaction is provided by the observation that cells preloaded with the cell-permeable Cd^{2+} chelator, *N*,*N*,*N*′,*N*′-*tetrakis*-(2-pyridylmethyl)ethylenediamine (TPEN), prior to Cd^{2+} treatment show only a slight inhibition of Ca^{2+} release from intracellular stores [12]. This has led to speculation that Cd^{2+} is acting through a cell-surface receptor whose identity has yet to be determined, but is thought to be reversibly blocked by Zn^{2+} [15], to bind a lectin [16], and to be a G-protein-coupled receptor [17].

Cadmium inhibits Ca^{2+}-ATPase, thus preventing cellular Ca^{2+} efflux. It has been shown that Cd^{2+} is a non-competitive inhibitor of the erythrocyte Ca^{2+}-ATPase [18,

19]. Cadmium-mediated elevations of intracellular Ca^{2+} can also occur through SERCA pump inhibition, but prolonged exposure to Cd^{2+} leads to depletion of the substantial endoplasmic reticulum Ca^{2+} pool and results in decreased $[Ca^{2+}]_{cyt}$ [4].

While it is clear that Cd^{2+} generally increases $[Ca^{2+}]_{cyt}$, with multiple ramifications for Ca^{2+}-based second messenger signaling, exposure of cells to Cd^{2+} has also been implicated in Ca^{2+} depletion [20]. Cadmium decreases intracellular Ca^{2+} stores through binding to thiol groups on a specific cell surface protein of the *Xenopus* oocyte [14], and also of human embryonic kidney cells [17], although the identity of the protein has yet to be determined. Cadmium has also been shown to deplete Ca^{2+} stores over longer periods of exposure (>24 h) [20]. It seems that while Cd^{2+} is able to initiate rapid rises in $[Ca^{2+}]_{cyt}$, at least in part through stimulation of IP_3 production, it eventually leads to decreased $[Ca^{2+}]_{cyt}$ as Ca^{2+} is lost from the cell.

Thus, Cd^{2+} can interfere with $[Ca^{2+}]_{cyt}$ through mechanisms that include inhibition of uptake, efflux, and resequestration. Displacement of Ca^{2+} from other binding sites, as is proposed for Cd's interactions with Cu^{2+} and $Fe^{2+/3+}$ [10, 21, 22] is also likely, but not well documented for Ca^{2+}. More direct effects of Cd^{2+} on signaling arise from direct interactions with Ca^{2+} signaling molecules.

3.2.2 Direct Effects of Cd^{2+} on Ca^{2+}-Binding Proteins

3.2.2.1 Calmodulin

The Ca^{2+}-binding protein, calmodulin, is central to integrating cytosolic signaling. Calmodulin has four EF hand Ca^{2+} binding motifs, and occupancy of at least two of these sites with Ca^{2+} results in a conformational change that exposes a hydrophobic patch on the protein. In this "active" form, calmodulin interacts with a number of signaling molecules, and although it lacks kinase activity and cell specificity itself [8, 23], it enhances the activity of its signaling partners. These partners include adenylate cyclase, Ca^{2+}-ATPase, myosin light chain kinase (MLCK), Ca^{2+}/calmodulin-dependent protein kinases (CaMKs), calmodulin-dependent phosphatases (CaMPs), and phosphodiesterase (PDE) [5].

The Cd^{2+} ion can interact with calmodulin, and thus affect downstream calmodulin targets, in several ways. Displacement of Ca^{2+} from CaM by Cd^{2+} results in Ca^{2+} mobilization and an increase in $[Ca^{2+}]_{cyt}$ [24]. However, Cd^{2+} binding can itself activate calmodulin, and thus increase its interaction with downstream targets. Calmodulin activation by several ions, including Cd^{2+}, is directly correlated with the similarity of their ionic radii to that of Ca^{2+} [24, 25]. Calmodulin has two high-affinity binding sites, each with a K_d of 4.5 μM for Cd^{2+}. Binding of Cd^{2+} and Ca^{2+} induce similar conformational changes in the protein, and promote a similar interaction between calmodulin and PDE [26, 27]. However, the consequence of Cd^{2+} binding is more complex than binding of Ca^{2+}; Cd^{2+} alone does not activate PDE to the maximal level, but the effects of low amounts of Ca^{2+} are potentiated in the

presence of Cd^{2+}, indicating that the similarity of the ionic radii of Cd^{2+} and Ca^{2+} alone does not determine this effect [27].

These early experiments were performed in vitro using purified calmodulin. More recent work implicates a functional role of Cd^{2+}-activated calmodulin in vivo. Liu et al. [28] showed that the calmodulin antagonist, W-7, suppresses mitochondrial-mediated apoptosis in osteoblasts exposed to 2 μM Cd^{2+} for 3 h, but were unable to distinguish direct effects of Cd^{2+} on calmodulin from secondary activation of calmodulin by increased $[Ca^{2+}]_{cyt}$. However, another calmodulin antagonist, trifluoperazine, was shown to block phosphorylation of both CaMK-II and myosin light chain kinase as soon as 30 s after exposure of mesangial cells to 0.5 μM Cd^{2+} [29]. This early response to a low $[Cd^{2+}]$, before re-equilibration of $[Ca^{2+}]_{cyt}$ is expected to occur, supports a direct effect of Cd^{2+} on calmodulin.

3.2.2.2 Cadherins

Cadherins are transmembrane proteins involved in adherens junctions. They contain an extracellular Ca^{2+}-binding domain, a transmembrane domain, and an intracellular domain that binds to catenins and to the actin cytoskeleton [30, 31]. This latter interaction places the cadherins among molecules involved in outside-in signaling. Calcium ions have an important role in maintaining the structural stiffness of cadherins, with higher extracellular Ca^{2+} levels helping to maintain adherens junctions. The epithelial protein, E-cadherin, is an extracellular target of Cd^{2+} [32]; Prozialeck has shown that Cd^{2+} can displace Ca^{2+} from E-cadherin, causing activation of downstream signaling cascades. Based on the time course of the response, it is probable that Cd^{2+} is acting on the extracellular domain of E-cadherin to produce these results, where Cd^{2+} can compete with Ca^{2+} for its binding sites [33]. Competition of the Ca^{2+}-binding sites by Cd^{2+} results in loss of cell–cell adherens junctions, a similar effect to that seen following Ca^{2+} depletion [34]. Cadmium can also alter the localization of E-cadherin, neuronal cadherins and β-catenin in proximal convoluted tubule cells [35]. Cadmium increases E-cadherin processing through γ-secretase in a breast cancer cell line [36].

The roles of calmodulin and CaMK-II in altering cadherin structure are unclear. Cadherin function is primarily mediated through interactions of its extracellular domains with proteins of similar structure. However, it has also been shown that IQGAP1, an intracellular E-cadherin-binding protein and member of the Rho GTPase family, associates with both the actin cytoskeleton and calmodulin, and calmodulin competes with IQGAP1 for its E-cadherin binding site [37], potentially linking the functions of calmodulin and E-cadherin. Given the link between calmodulin/CaMK-II and other structural proteins, they may have a significant but undetermined role in affecting adherens junction signaling, with Cd^{2+}-sensitive targets both inside and outside the cell.

3.2.2.3 Calpains

Among Ca^{2+}-binding proteins known to be affected by Cd^{2+} are the calpains. Calpains are Ca^{2+}-activated cysteine proteases that cleave many substrates, including some components of the apoptotic pathways. They have been implicated in both cleavage and inhibition of caspases [38]. Their targets also include the pro-apoptotic factors Bax and Bid, and they also function in mediating degradation of DNA. As Ca^{2+}-binding proteins, they are potentially affected directly by Cd^{2+}, but this has not been demonstrated [39]. The number of functional Ca^{2+}-binding sites on calpain is not known with certainty, although at least two functional Ca^{2+} sites have been identified [40]. There is at present no data on binding constants of other metals with calpain.

Cadmium activates calpains, thus contributing to a mitochondrial-independent pathway of apoptosis. In human embryonic kidney cells Cd^{2+}-dependent calpain activation results in N-terminal cleavage of Bax protein and its insertion into the mitochondrial membrane leading further to caspase-3 activation [17]. This effect was dependent on phospholipase C activation accompanied by a rise in $[Ca^{2+}]_{cyt}$ after a 24 h exposure to Cd^{2+}. Activation of calpains has also been implicated in the apoptotic mechanism initiated in lung epithelial fibroblasts by Cd^{2+} [41]. On the other hand, treatment of Chinese hamster ovary cells with 4 μM Cd^{2+} for 24 h produced a necrosis attributed to calpain-induced mitochondrial membrane damage, accompanied by a separate, independent pathway that appeared to involve ROS production and NF-κB inhibition [42]. Ceramide, a component of the structural backbone of sphingolipids/spingomyelin, plays a role in intracellular signaling as a second messenger [43, 44]. Calpain activity has been linked to increased ceramide production in response to Cd^{2+} in renal proximal tubule cells, at least in part through stimulation of de novo synthesis [43, 45]. Cd^{2+}-initiated $[Ca^{2+}]_{cyt}$ overload was marked by enhanced calpain protein levels that were blocked by modulators of $[Ca^{2+}]_{cyt}$ in rat proximal tubule cells in a context of Cd^{2+}-induced autophagy [46]. An active calpain-1 fragment was detected in rat proximal tubule cells treated with 1.25 μM Cd^{2+} for 12 h [47].

3.2.2.4 Cytoskeletal Proteins

Cadmium has multiple effects on the cytoskeleton (Fig. 3.2), and through them upon signaling, some discussed below in appropriate sections. Because a multiplicity of cytoskeletal proteins and factors modifying cytoskeletal dynamics are Ca^{2+}-dependent, it might be expected that many of Cd^{2+}'s effects in this area are mediated through direct protein interactions. Direct evidence of this, as with several of the proteins discussed above, is available in somewhat older literature [48]. Actin has a high affinity (~10^{-9} M) Ca^{2+}/Mg^{2+}-binding site [49–51] in addition to several lower affinity Ca^{2+} sites that are important for nucleation of the actin monomer to initiate polymerization to filamentous actin [49]. A mutation in the Ca^{2+}-binding EF hand of *Dictyostelium* actin interfered with regulation of actin filament crosslinking and cytoskeletal functioning in cell size and growth [52]. A number of studies have

demonstrated disruption of actin filaments in cultured cells, e.g., in renal epithelial cells [53, 54], mesangial cells [3, 11, 55, 56] and vascular smooth muscle cells [11].

When monomeric actin was allowed to polymerize in the presence of Mg^{2+}-ATP in vitro, actin filament formation was increased by Cd^{2+}, but only at 100 μM and higher, and there was no effect on depolymerization [3]. At even higher concentrations (250–500 μM) Cd^{2+} increased the rate of polymerization above the effects of Ca^{2+} and Mg^{2+}, and at the highest concentration (500 μM Cd^{2+}) Ca^{2+} was displaced from its high-affinity site and nucleation was enhanced [57]. Upon supplementation of the polymerization reaction mixture with extracts from Cd^{2+}-treated cells, however, the rate of polymerization was decreased and depolymerization was increased, reminiscent of the disruption of filaments seen in vivo [3]. Thus, it seems reasonable to conclude that effects of Cd^{2+} on disruption of filamentous actin in cultured cells may be secondary to effects on $[Ca^{2+}]_{cyt}$, or perhaps to effects on other actin-regulatory proteins, rather than direct binding to actin. In lung fibroblasts, low concentrations of Cd^{2+} (~1 μM) increased actin polymerization [58], potentially contributing to a fibrotic response.

Actin polymerization is regulated by a myriad of proteins, many of them Ca^{2+}-dependent [59–62]. Exposing mesangial cells to Cd^{2+} influenced actin-binding activity of protein(s) with molecular weights of approximately 90 kDa and 45 kDa, differentially in cytosolic and cytoskeletal-associated fractions [3]. The indication is that Cd^{2+} may not act directly on the cytoskeleton, but affects the expression and (or) activity of proteins that may regulate the dynamic state of actin. One such protein is gelsolin, a protein that binds to both actin monomer—favoring nucleation—and actin filaments—to sever them. Binding of gelsolin to actin monomer is cooperative and Ca^{2+}-dependent [63, 64], and in cultured mesangial cells Cd^{2+} favors translocation of gelsolin to the cytoskeletal compartment and colocalization with actin [65]. While this is not proven to be a direct effect of Cd^{2+}, it is noteworthy that Cd^{2+} can substitute for Ca^{2+} at a Ca^{2+}-binding site that is associated with gelsolin stabilization and activation [66].

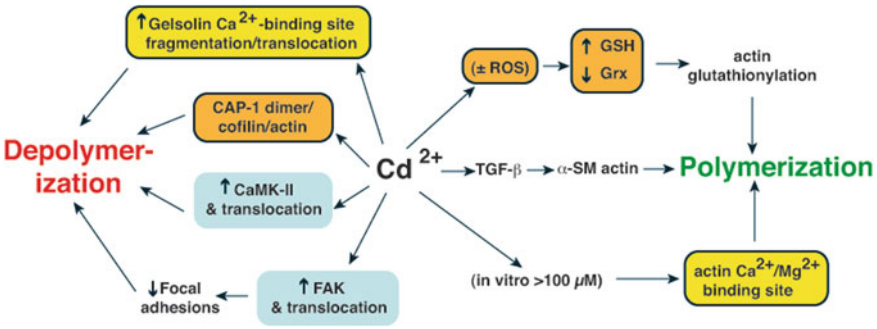

Fig. 3.2 Influences of Cd^{2+} on the actin polymerization/depolymerization equilibrium. Colored boxes are yellow—Ca^{2+}-dependent pathways; blue—kinases; orange—redox pathways. All abbreviations are defined in the text

3.3 Cadmium's Effects on Kinases, Growth Factors and Second Messengers

At the center of cadmium's ability to influence cellular signaling pathways seems to be its ability to activate a number of protein kinases. In some cases this will be due to effects mediated through Ca^{2+} or calcium-binding sites (such as the activation of CaMKs by Cd/calmodulin), but the scope of Cd^{2+}-stimulated kinase activity is much broader, and we should also consider activation of upstream kinases as well as the inhibition of kinase-deactivating phosphatases. A major impact of Cd^{2+} on cell signaling is mediated through activation of the mitogen-activated protein kinases (MAPKs).

3.3.1 Mitogen-Activated Protein Kinases

The MAPKs are a family of kinases central to orchestrating cellular processes that are activated by upstream MAPK kinases and their kinases, denoted as various MEK/MKKs (the acronyms referring to MAPK Erk kinase, or simply MAP kinase kinase) and MEKK/MKKKs, respectively. In cells of higher animals, major players are the extracellular-regulated kinases (Erks), p38 kinase, and the Jun kinase (Jnk). In general, Erks are activated by growth factor receptors and stimulate cell proliferation, whereas Jnk and p38 are responsive to genotoxic agents and stresses such as oxidative stress, radiation, and inflammatory cytokines [67–71]. Cadmium is a known regulator of each of the Erk, p38 and Jnk pathways [10, 22, 29, 68, 72, 73], albeit with differential effects in different cell types. Cadmium (1.5 μM for up to 60 min) activated Erk1/2 and p38, but not Jnk, in chicken hepatoma cells [74]. Higher concentrations of Cd^{2+} (>100 μM for 3 h) persistently activated all three kinases in human lung carcinoma cells [68, 75], whereas at lower concentrations Erk activity was decreased and Jnk was only transiently increased, with no effect on p38 [75]. Activation of p38 was confirmed at 100 μM Cd^{2+} in rat brain tumour cells, but was absent at 60 μM Cd^{2+}, whereas the opposite was true of Erk1/2 [76].

Early observations of MAPK activation by Cd^{2+} addressed issues of mitogenic effects and proto-oncogene activation. The rapid induction of the proto-oncogene c-*fos* at 30 min by exposure of renal mesangial cells to 10 μM Cd^{2+} [77] raised the possibility that upstream Ca^{2+}-dependent events, such as activation of protein kinase C or CaMK might be involved. However, measurements of both these kinases under circumstances where c-*fos* transcription was increased independent of their activation pointed to another mechanism, and suppression of the effect by the Erk inhibitor, PD98059, demonstrated a role for the MAPKs [77]. Activation of MAPKs as early as 5 min after Cd^{2+} exposure subsequently delineated a rapid protein kinase C-independent pathway [78].

All three MAPK pathways are activated by Cd^{2+} in cultured mesangial cells, and this cell line has been instructive [79–81]. In general, in these experiments

mesangial cells are made quiescent by starvation in low-serum conditions for 48 h to minimize basal activity of kinases, prior to treatment with $CdCl_2$ in serum-free conditions. Erk phosphorylation and kinase activity are significantly increased very quickly—by 15 min of treatment with as little as 0.1 μM Cd^{2+}—and subsequently decline by 30 min [79]. With 10 μM Cd^{2+}, a second peak of activation occurs at 8 h. This biphasic pattern is also characteristic of Jnk activation. This biphasic activation of both Erk and Jnk with 10 μM Cd^{2+} stimulates c-*fos* induction, with an early phase of kinase activity peaking at 15 min that seems to account for rapid oncogene activation, and a later phase sustained beyond 4 h [79] that may be involved in later effects on cell death. Suppression of Erk activation by PD98059 implicates MEK1/2 in Cd^{2+}-dependent Erk activation. Dominant-negative inhibition of upstream MKK and MEKK activators of Jnk (SEK1 and MKK7, respectively) localized the effect of Cd^{2+} on Jnk and c-*fos* expression to the MEKK member MKK7, and showed that Jnk activation in response to cadmium is mediated by MKK7 in preference to SEK1/MKK [80]. Activation of the third MAPK, p38, was observed following a 6 h treatment of mouse mesangial cells with 10 μM Cd^{2+} [81]. A comparatively low concentration of $CdCl_2$ (0.5 μM) that activated Erk at 15 min in rat mesangial cells also increased phosphorylation of the epidermal growth factor receptor (EGFR) at 5 min of treatment, and activated the EGFR-dependent PI3 kinase/Akt pathway at 15 min [29].

While these earlier studies addressed mitogenic activity through oncogene activation, interest in this phenomenon soon shifted to Cd^{2+}-dependent cell death. Whereas Erk is generally anti-apoptotic, the stress-activated kinases Jnk and p38 are thought to facilitate apoptosis in cells subjected to a variety of environmental stresses. However, a number of exceptions to this rule have been documented [71], and indeed p38 inhibition increases the level of apoptosis in TNF-α-treated mesangial cells [82]. Two MKKs, MKK4 and MKK7, are upstream activators of Jnk that respond differentially to environmental stresses. The apoptotic death ligand TNF-α activates Jnk through MKK7 [71]; MKK7 is also responsible for increased Jnk activity in Cd^{2+}-treated mesangial cells as noted above [80]. While 10–20 μM Cd^{2+} was sufficient to sustain phosphorylation of Erk, Jnk, and p38 at 6 h, inhibition of p38 activity was linked to suppression of apoptotic death [81]. However, as might be expected, other signals were involved in both caspase-dependent and independent apoptosis in these experiments, including ROS, mitochondrial membrane instability, and poly(ADP-ribose) polymerase cleavage [81].

Although activation of the major MAPKs has been inconsistently linked to apoptosis in a variety of cell culture systems (nicely summarized in [10]), more recent experiments have strengthened the link. Western blots consistently show increases of the phosphorylated forms of the MAPKs after Cd^{2+} treatment. (It might be mentioned here that, whereas in the older literature it was important to show increased kinase activity against specific substrates, commercial antibodies developed over the last couple of decades against specific sites of phosphorylation are of sufficient quality to justify inferring activation by Western blot.) For instance, significant increases in phophorylation of Erk1/2, p38, and Jnk in human osteoblasts after a 30 min exposure to 10 μM Cd^{2+} led to apoptosis in these cells [83]. Inhibition of each kinase

reduced apoptosis, whereas interference with apoptosis by caspase inhibitors did not affect kinase phosphorylation, implying kinase activation preceded, and was not secondary to, initiation of the apoptotic program. This was confirmed in rat osteoblast cultures with 2 μM Cd^{2+} increasing levels of all three phosphorylated kinases by 2 h with inhibition of their activity blunting apoptosis [84]. Very low concentrations of Cd^{2+} (<0.5 μM) inhibited endothelial nitric oxide synthase and reduced endothelial nitric oxide production with attenuation of tumor angiogenesis, but at 5–10 μM stimulated angiogenesis through activation of Erk, Jnk and p38 (reviewed in [85]). At even higher levels (>10 μM Cd^{2+}), activation of p38 resulted in endothelial cell procaspase-3 cleavage, apoptosis, and inhibition of tumor angiogenesis.

Activation of MAPKs by Cd^{2+} is of central importance in Cd^{2+} signaling, but the mechanisms are still unclear. Direct activation seems unlikely, but involvement of activation of upstream kinases, second messengers, and inhibition of phosphatases are among potential mechanisms that can be considered.

3.3.2 Ca^{2+}/Calmodulin-Dependent Kinase

One of the more upstream kinases that appears to be crucial in Cd^{2+} signaling is CaMK. This calmodulin-dependent kinase is actually a family of multifunctional serine/threonine kinases that undergo autophosphorylation in response to Ca^{2+}-charged calmodulin, and remain in the activated (phosphorylated) state after Ca^{2+} levels subside, serving as a memory of the Ca^{2+} signal until deactivated by protein phosphatases [86, 87]. CaMK-II has been the most widely studied in general, and indeed in the context of Cd^{2+} exposure. The CaMK-IIβ isoform regulates actin assembly by binding monomeric actin [88, 89] while the δ isoform is implicated in signaling events that control cell migration and proliferation in vascular smooth muscle and renal mesangial cells [90, 91]. However, the δ isoform also targets actin filaments, and has been found to stabilize them and enhance filament extension [92]. The rapid activation of CaMK-II by Cd^{2+} (e.g., a burst in CaMK-II phosphorylation by 30 s after exposure of mesangial cells to 0.5 μM $CdCl_2$ [29]) can be accounted for by rapid activation of calmodulin, probably via substitution of Cd^{2+} for Ca^{2+} (see above).

Documented consequences of Cd^{2+}-dependent activation of CaMK-II include both apoptosis and effects on cytoskeletal dynamics, and much evidence suggests the two processes are related. Cadmium disrupts the balance between actin polymerization and depolymerization in mesangial cells, and inhibiting CaMK-II offers partial protection against this effect [93]. After 6 h exposure to 10 μM Cd^{2+}, suppression of caspase-independent apoptosis was achieved with a variety of CaMK-II inhibitors [94]. Although early activation of CaMK has also been suggested to activate MAPKs downstream, Erk inhibition offered no protection against apoptosis in this scenario [94]. The CaMK inhibitor KN93 preserved the structure of cortical actin and stress fibers [93] and of actin filament-anchoring focal adhesions [95] after Cd^{2+} treatment. CaMK-IIδ associates with monomeric actin in mesangial cell cytosol, but translocates to the actin filaments after Cd^{2+} treatment, in a dose-dependent manner

that requires CaMK phosphorylation [95]. Overexpression of CaMK-IIδ in mesangial cells followed by isolation of cytoskeletal fractions showed a decreased rate of actin polymerization upon Cd^{2+} treatment that was sensitive to CaMK inhibition, whereas silencing CaMK-II with siRNA negates this effect, and in fact produces a cytoskeletal fraction with an increased capacity to enhance actin polymerization in an in vitro assay [91, 95].

3.3.3 Other Kinases

While Cd^{2+} thus has profound effects on cell signaling through the major MAPK and CaMK-II pathways, it also exerts effects through other downstream or independent pathways.

Phosphorylation of the epidermal growth factor receptor (EGFR) on its activating site Tyr845 was rapidly stimulated by low-dose Cd^{2+} (0.5 μM, 5 min) in mesangial cells [29]. The EGFR is a known activator of PI3 kinase [96, 97], and an EGFR inhibitor suppressed downstream activation of the IP_3 kinase substrate Akt/protein kinase B [29], an anti-apoptotic survival pathway. Cadmium-dependent phosphorylation of the oncogene c-*src* Tyr416 at 30 s was also implicated as a very early activator of this survival pathway [29]. Renal proximal tubule cells treated with 10–20 μM Cd^{2+} for 1–6 h showed increased phosphorylation of Akt and a downstream transcription factor target, FOXO3a, inactivation of which by phosphorylation is associated with cell survival [98]. Neuronal cell cultures treated with a much higher concentration of Cd^{2+} (200 μM) for 3 h showed a decrease in Akt phosphorylation that was attributed both to inhibition of the antioxidant glutaredoxin 1 (Grx1; see below) by Cd^{2+}, and to increased association of Akt with protein phosphatase PPA2 [99]. Recently, Li et al. [100] showed that 20 μM Cd^{2+} increased phosphorylation of Akt at 30 min that was sustained at 6 h in lung fibroblasts. This was accompanied by phosphorylation of the cell cycle regulator cdc2 at 2 h, and downstream phosphorylation of the intermediate filament, vimentin [100]. A 24 h exposure of neuronal cells from rat cerebral cortex to 5–20 μM Cd^{2+} for 24 h showed a concentration-dependent phosphorylation of Akt and one of its downstream targets, mammalian target of rapamycin (mTOR) [101]. Prolonged exposure (9–15 weeks) of human lung adenocarcinoma cells to 10–20 μM Cd^{2+} induced a proliferative phenotype with increased cell motility and epithelial-mesenchymal transition markers, through an insulin-like growth factor-1 receptor/Akt/Erk pathway [102]. The effects were partially decreased by Notch1 silencing [102].

Focal adhesions represent a molecular assembly of several proteins including paxillin, vinculin, α-actinin, and focal adhesion kinase (FAK); FAK is a non-receptor protein tyrosine kinase involved in focal adhesion formation. Both the intracellular environment and the integrity of the actin scaffold determine the assembly and disassembly of focal adhesions [103]. Upon integrin clustering, FAK becomes autophosphorylated at Tyr397, resulting in recruitment of Src kinases that phosphorylate other tyrosine residues leading to subsequent activation of several downstream signaling

cascades [104]. Thus, they play an important role in regulating intracellular signaling, and it is significant that they are regulated by Cd^{2+}. Exposure of mesangial cells to 10 μM Cd^{2+} for 6 h causes disruption of focal adhesions with loss of both molecules from the assembly [105]. Inhibition of CaMK-II is protective against loss of FAK from focal adhesions, and cytoskeletal-disrupting agents promote its loss, but the effects of Cd^{2+} on FAK are not restricted to secondary disruption of the focal adhesion. Cadmium stimulates a rapid (within 30 s) site-specific autophosphorylation of FAK on Tyr925 and promotes translocation of the phosphorylated FAK to the cell membrane [105]. While CaMK-II helps to preserve the FAK content of the focal adhesions, its inhibition does not affect FAK phosphorylation [105]. In assessing the role of Cd^{2+}-dependent changes in the cytoskeleton on a metastatic phenotype in cultured breast cancer cells [106], it was noted that both c-*src* and FAK were phosphorylated by 1 μM Cd^{2+} as early as 15 min. In contrast to phosphorylation on FAK Tyr925, activation of FAK by phosphorylation of Tyr397 was observed, possibly accounting for phosphorylation of its downstream target, Rac 1 [106]. Cytoskeletal reorganization with continued increased expression of β-catenin was observed when 3 μM Cd^{2+} treatment was continued for 4 weeks.

3.3.4 Transforming Growth Factor β

Transforming growth factor β (TGF-β) signals through its heterodimeric receptors to regulate a variety of cellular processes, notably those involving fibrosis, increased extracellular matrix synthesis, and angiogenesis. The signals are transduced through a collection of SMAD proteins. Cadmium has recently been shown to increase SMAD2/3/4 signaling in fetal lung fibroblasts at <1 μM, with effects on increasing α-smooth muscle actin (indicative of a myofibroblast phenotype) and fibronectin [107], providing a potential link between Cd^{2+} and fibrosis [58]. Treatment of trophoblasts with 25 μM Cd^{2+} for 24–48 h resulted in increased expression of genes coding TGF-β1, TGF-β receptors, and miRNAs regulating SMAD1 and SMAD2 [108]. Further epigenetic analysis of Cd^{2+}-treated trophoblasts indicated increased TGF-β signaling and decreased cell migration [109].

3.3.5 Second Messengers

Direct effects of Cd^{2+} through Ca^{2+}-dependent second messenger pathways, such as calmodulin and ceramide, and substitution or interference with Ca^{2+} itself, have been discussed above, but effects of Cd^{2+} on other second messengers are mentioned briefly here as another process by which Cd^{2+} affects cell signaling. Under various conditions of exposure, both in animals and cell cultures, Cd^{2+} has been shown to have differential effects of either increasing or decreasing cell/tissue levels of cAMP [10]. The fact that some forms of adenylyl cyclases are activated by, and others inhibited

by, Ca^{2+}-calmodulin may account for some of this variability [10, 110]. A decrease in Leydig cell viability with an $IC_{25} = 24$ μM Cd^{2+}, accompanied by decreased progesterone production, was attributed in part to a significant decrease in cellular cAMP levels [111]. Increased levels of both cAMP and cGMP may arise in some circumstances from Cd^{2+}-inhibition of cyclic nucleotide phosphodiesterases [112]. In contrast, NO levels and NO synthase activity seem to be consistently decreased by Cd^{2+} [10], implying a Cd^{2+}-mediated decrease in cGMP and NO signaling. A similar effect was observed in a model of developing chick embryo, where teratogenic effects of Cd^{2+} on vascular development was attributed to decreased NO-cGMP signaling and partially abrogated by an exogenous NO donor [113].

Phospholipase C (PLC) can be activated by Cd^{2+} through its interaction with a G protein-coupled receptor, leading to phosphoinositol breakdown and the generation of the second messenger, inositol trisphosphate (IP_3) [17]. Cadmium may thus potentially cause an increase in IP_3 signaling, which activates IP3 receptors on the membrane of the endoplasmic reticulum, causing Ca^{2+} release from the endoplasmic reticulum into cytoplasm [17]. On the other hand, Cd^{2+} may inhibit Ca^{2+}-dependent PLC activity, leading to defective IP_3 production [114, 115]. And, a more general effect of Cd^{2+} on membrane phospholipid synthesis may indirectly affect substrate availability for IP_3 synthesis [116]. Ambiguous effects of Cd^{2+} on PLC may account for the variable effects of Cd^{2+} on protein kinase C (reviewed in [10]), as the production of IP_3 accompanies the generation of diacylglycerol, an activator of major protein kinase C isoforms.

3.3.6 Phosphatases

A general mechanism by which Cd^{2+} may increase the activity of multiple kinases is by increasing ROS which in turn oxidize thiol groups on kinase-regulating phosphatases [117, 118]. It is possible that Cd^{2+} also poisons phosphatase thiols directly. Protein tyrosine phosphatese (PTPs) are ROS-sensitive targets that play an important role in signal transduction. Metal cations are potent inhibitors of PTP1B, and in in vitro experiments using recombinant PTP1B, Cu^{2+}, Zn^{2+}, and Cd^{2+} inhibit PTP1B at nM concentrations [119]. This inhibition occurs when the enzyme is in the open conformation with the catalytic cysteine exposed. The dual-specificity phosphatase 4 (DUSP4)/MAPK phosphatase 2 has an active cysteine in its catalytic site that is a redox-sensitive. Exposure of endothelial cells to 100 μM Cd^{2+} overnight increases the ratio of oxidized to reduced glutathione (GSSG/GSH) and increases degradation of DUSP4, leading in turn to sustained p38 and ERK1/2 activation, and apoptosis [120].

3.4 Cadmium and Redox Signaling

3.4.1 Thiol-Based Antioxidant Molecules

Reactive oxygen species play a number of roles in cells, as toxic prooxidants that damage proteins, DNA and lipids to disrupt cellular structure and enhance necrosis, apoptosis, and autophagy. They also function as important signaling molecules. It is well established that Cd^{2+} increases ROS levels in many cells, and often this accounts for its toxic effects, but here we are interested in Cd^{2+}'s interactions with antioxidants that may increase ROS-dependent signaling. The most abundant antioxidant in cells is glutathione, which cycles between reduced thiol (GSH) and oxidized disulfide (GSSG) states. Glutathione is present in mM concentrations in some tissues (notably liver) and maintains a ratio of GSH/GSSG of 20–100 under normal circumstances (reviewed in [121]), thus providing a robust barrier against oxidative stress. Concentration of the GSH thiol is comparable to, or greater than, all protein thiols combined [122], and so it is an obvious buffer of their redox state. This should be kept in mind when considering Cd^{2+}'s effects on exposed thiols in phosphatases (see above) and kinases (e.g., Akt [99]), and effects on transcription factors [e.g., Nrf2 and HIF-1α (see below)].

After several decades of studies on cadmium-glutathione structures, it seems finally resolved that the speciation of cadmium in this system involves the 1:2 $Cd(GS)_2$ and 1:1 Cd(GS) complexes as major components [121]. Given that concentrations of available Cd^{2+} in the various cell culture models probably do not exceed a few pM [3], direct interactions of Cd^{2+} and GSH may not seem to be important. However, a caveat to this is the potential availability of Cd^{2+} to susceptible thiols by exchange from sites of storage—such as Cd-metallothionein—or attraction from Cd^{2+} sinks in Ca^{2+}-buffering proteins. Initial decreases in GSH levels in response to Cd^{2+} exposure, whether from Cd–GSH interaction or, more likely, increased oxidative stress, can nevertheless lead to a rebound increase in total glutathione levels as a consequence of induction of the regulatory enzyme of glutathione synthesis, glutamate-cysteine ligase (γ-glutamylcysteinyl synthase, EC 6.3.2.2) [123], and possibly through activation of Nrf2 (see below) [124]. Exposure of proximal tubule cells to 1–30 μM Cd^{2+} for 24 h caused a significant decrease in GSH and an increase in GSSG/GSH ratio, with induction of both subunits of glutamate-cysteine ligase [125].

3.4.2 Glutathionylation Reactions and Glutaredoxin

Protein modification and functional modulation by addition of glutathione to amino acid side chains is a major redox-sensitive regulatory pathway in cells. Protein *S*-glutathionylation is a posttranslational modification resulting from formation of a mixed disulfide between a protein cysteinyl residue and GSH. Glutathionylation occurs in response to oxidative stress and may be a mechanism to protect proteins

from irreversible oxidative damage [57, 126]. However, protein glutathionylation can also occur in the absence of exogenous oxidative stress, and may represent a mechanism for redox regulation of protein function and activity [127]. Thus, it has been shown to regulate several proteins, including nuclear factor kappa B (NF-κB), protein tyrosine phosphatase-1B (PTB1), and actin [128–130].

Actin is one of the major cellular proteins that is glutathionylated [130, 131]; actin glutathionylation is constitutive and increases under conditions of oxidative stress [132] and ischemia-reperfusion injury [133]. Actin glutathionylation has been observed in haemocytes treated with 5 μM Cd^{2+} [134], and increased actin glutathionylation in cultured mesangial cells occurred with Cd^{2+} in a concentration-dependent manner that was maximal with 2 μM Cd^{2+} and decreased back to basal levels at higher concentrations [135]. Glutathionylation of actin [130, 131, 136] decreases the efficiency of actin polymerization, shifting the dynamic equilibrium between monomeric and filamentous actin towards depolymerization, resulting in disorganized actin filaments at the cell periphery [131, 137].

Some unique cadmium-sulfur biochemistry may occur in Cd^{2+}-induced glutathionylation reactions. Although the precise mechanism of actin glutathionylation in vivo is still uncertain, there is evidence for the occurrence of a sulfenic acid intermediate [126] rather than a thiol-disulfide exchange mechanism [131] suggested earlier. Both mechanisms depend on the redox status of the cell, but Cd^{2+}-dependent ROS production does not lead to increased actin glutathionylation in mesangial cells [135], bringing into question the suggestion that oxidative stress is primarily responsible for increased protein *S*-glutathionylation [132, 133]. Further evidence that actin glutathionylation is not always dependent on levels of intracellular ROS is provided by the observation that while treatment of squamous carcinoma cells with exogenous H_2O_2 increased actin glutathionylation, EGF produced a rise in intracellular ROS accompanied by enhanced deglutathionylation [130]. Because Cd^{2+} causes rapid activation of the EGFR in mesangial cells (see above, [29]), this signaling pathway may also play a role in establishing steady state levels of actin glutathionylation upon Cd^{2+} exposure. Further, Dailianis et al. [134] have implicated various kinases, including PI3 kinase and protein kinase C, in Cd^{2+}-mediated actin glutathionylation in mussel haemocytes—kinases previously shown to be activated by Cd^{2+} [29]. Therefore, ROS-independent actin glutathionylation by Cd^{2+} may occur via activation of different signaling cascades as well as by changes in redox state.

Glutaredoxins (Grx) are members of a family of thioltransferase oxidoreductases that catalyze the reduction of glutathionylated protein mixed disulfides [127, 138]. They are susceptible to non-specific inhibition by Cd^{2+}, resulting in an increase in the level of glutathionylated proteins [129, 136, 139]. The Grx catalytic reaction, by its nature, contributes to the equilibrium between glutathionylated and deglutathionylated proteins in a cell. In addition, the Cd^{2+}-mediated increase in total glutathione concentration may shift the equilibrium towards a more glutathionylated state, consistent with the observation, for example, that Grx activity directly correlates with the glutathionylation status of NFκB [140]. Inhibition of Grx activity by Cd^{2+} in T cell-derived cells lines (IC_{50} ~ 10 μM, or ~1 μM with isolated enzyme components) correlated with inhibition of protein deglutathionylation and progression to apoptosis

[141]. Grx was also inactivated in aortic endothelial cells by a high concentration of Cd^{2+} (200 μM for 3 h in the presence of serum) that led to increased glutathionylation of endothelial nitric oxide synthetase and decreased NO signaling [142]. On the other hand, actin glutathionylation in Cd^{2+}-exposed mesangial cells was maximal at 2 μM Cd^{2+}, a concentration that did not inhibit Grx [135], suggesting a mechanism independent of Grx.

Thioredoxin (Trx) is a small redox-active, ubiquitous protein component of the redox defense system of all organisms. In common with a number of redox-active proteins, including Grx, it contains a Cys-x-x-Cys motif that renders it potentially susceptible to metal ion binding [143]. It is mentioned here in the context of the glutaredoxin/thioredoxin redox signaling system; in contrast to Grx (see above), which is only reduced by GSH that is in turn regenerated by glutathione reductase (EC 1.8.1.7), oxidized Trx is reduced by its own enzyme, thioredoxin reductase (EC 1.8.1.9). The main isoforms of thioredoxin, Trx1 and Trx2, were shown to be differentially oxidized by different metals, and differentially in comparison to GSH. Thus, Cu^{2+}, Fe^{3+}, and Ni^{2+} preferentially oxidized GSH with little effect on Trx1/2 under circumstances where the Trx protein was susceptible to As^{3+} species, Hg^{2+} and Cd^{2+} [144]. Nuclear translocation and accumulation of redox-active Trx1 was apparent in HeLa cells treated with 0.5 μM Cd^{2+} for 1 h [145].

3.4.3 Redox Sensors

Much attention relevant to Cd^{2+} signaling has focused on the role of thiol-containing proteins and transcription factors as redox sensors or switches [122, 146–148]. Cysteine is underrepresented in proteins, overrepresented in harmful genetic mutations, and highly conserved where it occurs [149], pointing to a critical role of Cys that has been attributed in part to the range of Cys thiol redox potentials in the biological range (say 0–400 mV) that positions Cys to be partially oxidized and sensitive to reductants [122]. Redox signaling and redox-sensing thiols have been distinguished based on whether thiol-disulfide exchange takes place in the context of a biochemical reaction (a redox switch [150]), or more generally in the context of integrating signals in many pathways [122]. Thus, GSH can be considered to be a redox-sensing compound, and integration of signals is at least in part due to glutathionylation reactions of multiple proteins, in addition to its action as a general reducing agent. Wang et al. [151] have defined redox sensing based on oxidation of protein thiols and the subsequent conformational changes. A major sensing system of hypoxia and oxidative stress is provided by the reciprocal activity of the HIF-1α (hypoxia-inducible factor 1α)–VHL (von Hippel–Lindau protein) and Nrf2 (nuclear factor erythroid 2-related factor 2)–Keap1 (kelch-like ECH-associated protein 1) systems, respectively, that signal compensatory gene expression, complemented by the more ambiguous responses to oxidative stress of NF-κB. Cadmium affects each of these systems.

3.4.3.1 Nuclear Factor Erythroid 2-Related Factor 2 (Nrf2)

Nrf2 is a basic leucine zipper transcription factor that binds to antioxidant response elements (AREs) upregulate transcription of many genes related to antioxidant defense, such as catalase, heme oxygense-1, NAD(P)H quinone oxidoreductase, and glutamate-cysteine ligase [124]. Nrf2 is located in the cytoplasm bound to an actin-tethered protein, Keap1. Under normoxic conditions, the Keap1-Nrf2 complex is rapidly degraded by the ubiquitin proteasome pathway [124]. However, oxidants, including ROS, target redox-sensitive cysteine thiols in Keap1, resulting in its dissociation from Nrf2 and allowing its translocation to the nucleus [152]. In hepatoma cells, Cd^{2+} (50 μM, 30 min) stabilizes Nrf2 against proteasomal degradation and leads to protective heme oxygense-1 synthesis [153]. Nrf2 is activated by Cd^{2+} in mouse embryonic fibroblasts (MEF), and $Nrf2^{-/-}$ MEF cells are more susceptible to 1 μM Cd^{2+} at 6 h than their wild-type counterparts [154]. In renal proximal tubule cells, 2.5 μM Cd^{2+} increases Nrf2 levels at 12 h, accounting for a presumed cytoprotective effect [155], and activation of Nrf2 is reported to protect an NRK rat kidney cell line against Cd^{2+}-induced apoptosis [124]. Most activators of the Nrf2/Keap1 system target cysteine residues Cys151, Cys273 and Cys288 in Keap1. However, activation by Cd^{2+} (as well as Zn^{2+} and peroxide) appears to be independent of these three cysteines [152]. Thus, the mechanism by which Cd^{2+} activates Nrf2 is not yet fully understood. Keap1 also contains thiol-bound Zn^{2+}, which also regulates cysteine reactivity to regulate Nrf2, and it was suggested earlier that Cd^{2+} may activate Nrf2 through affecting Zn^{2+}-thiol binding [156].

3.4.3.2 Hypoxia-Inducible Factor-1

The hypoxia-inducible factor subunit 1α (HIF-1α) is maintained in an oxidized state under normoxic conditions, by the action of a prolyl hydroxylase. This results in maintenance of the hydroxyproline-containing protein in the cytoplasm, and targeting for degradation by the proteasome through interaction with the Von Hippel–Lindau protein (VHL). Under hypoxic conditions, the unoxidized protein dissociates from VHL and translocates to the nucleus, where it combines with the constitutively expressed HIF-1β subunit to form an active heterodimeric transcription factor that is involved in responses to hypoxia such as transcription of erythropoietin and vascular endothelial growth factor (VEGF). Primary regulation of HIF-1α signaling, then, is through enzymatic proline hydroxylation [157, 158]. Cadmium (5–20 μM during 4 h of hypoxia) prevents HIF-1α activation in hepatoma cells by enhancing its proteasomal degradation, independent of oxidative stress [159]. A Cd^{2+}-dependent increase in HIF-1α was confirmed to occur in immortalized human bronchial epithelial cells by 2 h (5 μM Cd^{2+}), and in conjunction with activation of Erk, Akt, and VEGF contributed to transformation and morphological changes characteristic of angiogenesis [160]. In the study of Notch signaling and the Erk/Akt pathway mentioned above [102], HIF-1α stabilization was also noted, but attributed to inhibition of proteasomal degradation by Cd^{2+} rather than by proline oxidation.

3.4.3.3 Nuclear Factor-κB

Once thought to be the central sensor of oxidative stress, considerable doubt was cast on this role by observations that NF-κB is, in some cases, susceptible to down-regulation/deactivation by ROS [161]. Now realized to play roles in a number of transcriptional responses to stressors, inflammation and cancer, to mention a few factors, it is certainly important in responding to oxidative stress and metal ions [162, 163]. NF-κB was implicated in protection of rat proximal tubule cells against ROS-dependent apoptosis induced by 10 μM Cd^{2+} in 5% serum, in part through upregulation of P-glycoprotein multidrug resistance protein [164]. ROS-dependent activation was demonstrated in murine osteobasts treated with 2.5 μM Cd^{2+} for 24 h in serum-free medium [165]. On the other hand, 5 h of Cd^{2+} treatment of mouse proximal tubule cells (20 μM in serum-free medium) diminished basal and inducible NF-κB levels that potentiated apoptosis [166], a difference that has been attributed in part to a greater sensitivity of the mouse cell line compared to rat proximal tubules [43]. Nuclear translocation of Trx1 with low-dose Cd^{2+} in HeLa cells noted above [145] was accompanied by increased nuclear translocation and activation of NF-κB, and this effect was abrogated by a Trx1 dominant-negative mutant [145]. In endothelial cells of the renal glomerulus, 4 μM Cd^{2+} (up to 24 h in 10% serum) increased NF-κB activity by degrading its inhibitory protein, I-κB [167]. This appeared to protect the cells against Cd^{2+}-induced apoptosis, because an inhibitor of NF-κB given with Cd^{2+} increased apoptotic death. Thus, on balance, NF-κB appears to be increased by moderate Cd^{2+} concentrations, and to offer some protection against possible ROS-mediated pathways of cell death.

3.4.3.4 Adenylyl Cyclase-Associated Protein 1

The adenylyl cyclase-associated protein 1 (CAP-1), first identified as a component of the yeast adenylyl cyclase complex [168], facilitates activation of adenylyl cyclase by Ras [169] and modulates actin filament formation [170]. Although CAP-1 does not regulate cAMP in animal cells, it has a conserved function regulating actin dynamics [171, 172]. Liu et al. recently showed that treatment of renal mesangial cells with 5–20 μM Cd^{2+} over 6 h induced a disulfide-bonded dimer through a conserved Cys29 residue of the protein [173]. The effect was independent of increased ROS production and was not achieved with the other redox-active metals, Fe^{3+}, Fe^{2+}, or Cu^{2+}, but could be demonstrated with the disulfide-crosslinking agent diamide, and in some cells by treatment with peroxide. Occurrence of the dimer increased CAP-1's association with the actin-binding protein cofilin, and Cd^{2+}-induced dimer formation increased the formation of a CAP1–cofilin–actin filament complex. Silencing CAP-1, and overexpression of a CAP-1 mutant lacking Cys29 (incapable of dimerization in response to Cd^{2+}), both increased cell viability and provided some protection of actin filaments against Cd^{2+} [173]. It was proposed that CAP-1 may be a novel thiol-based redox sensor of Cd^{2+} that alters actin dynamics.

3.4.3.5 Iron-Regulatory Protein 1

The iron-regulatory protein 1 (IRP-1) is another candidate for a novel Cd^{2+}-dependent redox sensor. IRP-1 regulates cellular Fe^{3+}/Fe^{2+} homeostasis by interacting with iron-responsive elements in mRNAs coding for a number of proteins such as ferritin, transferrin receptor, aminolevulinate synthase and divalent metal transporter-1 [174–176]. IRP-1 also contains an iron-sulfur [Fe_4-S_4] cofactor with a labile Fe atom; when the cluster is intact, it acts as a cytosolic aconitase, but the labile Fe^{2+} also serves in sensing cellular iron levels and oxidative stress [177]. This labile Fe^{2+} is a potential target for disruption by ROS and displacement by other metal ions, and its replacement with Cd^{2+} to give a [Fe_3Cd-S_4] structure restores cytosolic aconitase activity [21]. Further suggesting a link between cellular iron levels and energy metabolism, IRP-1 has ATP-hydrolyzing activity [178]. Cadmium induces aggregation of IRP-1, both in vivo and in vitro [179]. Titration of the recombinant protein with Cd induces polymerization and aggregation, accompanied by conformational changes. Polymerization induced by Cd was associated with translocation of the protein to the cytoskeletal compartment, a process suppressed both by antioxidant treatment and by inhibition of CaMK-II [179]. Thus, IRP-1 is a multifunctional protein involved in iron, energy, and oxygen metabolism that is a potential target for Cd^{2+} by virtue of its redox-sensitive iron–sulfur cluster.

3.5 Conclusions

Cadmium interacts with biological systems as the Cd^{2+} ion, and influences cell signaling in a multitude of ways. This chapter has given an overview of these influences that is necessarily far from comprehensive. Nor have we examined in any detail the consequences of Cd^{2+} signaling for cell death or survival, or the large field of cytoskeletal dynamics. The focus has been on effects in cell culture systems where individual signaling pathways can be dissected and observed. These effects are generally seen in the $[Cd^{2+}] = 1–20\ \mu M$ range, with occasional reports mentioned of lower (down to 0.05 μM) and higher (greater than 100 μM, requiring the presence of serum to keep Cd^{2+} in solution and maintain cell viability) concentrations. Cadmium ion behaves as a Ca^{2+} mimic, but also as a soft ion with a propensity for thiol groups. While we have attempted to classify major effects as acting through Ca^{2+} and Ca^{2+}-binding proteins, kinases and their associated growth factors and second messengers, and redox-thiol-sensitive and redox-sensing proteins, there is a complex cross-talk among these various pathways that must always be kept in mind when a specific effect of Cd^{2+} is reported.

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Chapter 4
Cell Organelles as Targets of Cadmium Toxicity

Wing-Kee Lee

Abstract Ever increasing environmental cadmium presence consequent of industrial activities is considered a health hazard and is closely linked to deteriorating global health status as general animal cadmium exposure expands from cigarette smoke and ingestion of foodstuffs sourced from heavily polluted hotspots to widespread contaminated air and water, including cadmium-containing microplastics found in household water. Cadmium exerts myriads of cellular perturbances based on its abilities to directly interact with macromolecules and to mimic or displace essential physiological ions. Cell organelles are membrane-bound structures that form complex tightly regulated compartmentalized networks with specialized functions which are fundamental to life. Interorganellar communication is mediated either by release of signaling molecules, mechanical force through change in organelle shape or direct membrane contacts and is crucial to orchestrate correct cell behavior and adaptive stress responses. In this chapter, cadmium effects on organellar structure and function will be reviewed with particular consideration to disruption of organelle physiology in vertebrates. Mitochondrial dysfunction (electron transport chain, mitochondrial membrane potential, permeability transition), mitochondrial dynamics, intralumenal homeostasis and stress response in the endoplasmic reticulum, altered nuclear architecture and chromatin organization, lysosomal expansion, instability and membrane permeabilization, autophagic flux, and disruption of vesicle trafficking will be discussed in the context of cadmium.

4.1 Introduction

Cell organelles are characterized by a lipid membrane boundary creating distinct structures and a microenvironment optimal for specialized organellar function, such as storage, e.g., lipids in lysosomes, protein synthesis in endoplasmic reticulum

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(ER), ATP synthesis in mitochondria, internalization of extracellular signals and signal transduction through the endo-/lysosomal pathway, and cargo trafficking and degradation in autophago(lyso)somes [1]. Interorganellar communication is essential for cell function, behavior, and adaptation to extracellular cues therefore organelles cannot and do not exist as single entities but rather as an extended interactive network [2]. In emphasis, the ER is an extension of the nuclear membrane, the ER has intimate contact with mitochondria and plasma membrane (PM), trafficking vesicles fuse with multiple organelles as well as maturing into different vesicles, and existing organelle membranes are used to generate new membrane-bound structures.

Lipid bilayers are essential to organelle function providing structural separation, appropriate membrane curvature, optimal lipid microenvironment to functionalize membrane proteins, maintaining intraorganellar homeostasis, and dictating fusogenicity. Lipid composition varies between organelles albeit differing ratios of phospholipids, cholesterol and sphingolipids are found across all organelles. Cadmium uptake and transport mechanisms into the intracellular space have been well elucidated in comparison to cadmium effects on the lipid bilayer. Using liposomes to mimic erythrocyte outer leaflet membranes, cadmium interacts preferentially with phosphatidylethanolamine, but not with cholesterol [3], causing tighter lipid packing and increased membrane rigidity without changes in lateral organization (reviewed in [4]). In conjunction with cadmium-induced altered sphingolipid metabolism [5, 6], membrane-associated cellular processes, such as transport or second messenger signaling, as well as organelle structure could be modulated by cadmium interactions with membrane lipids.

4.2 Cadmium and Mitochondria (Fig. 4.1)

Mitochondria form the central hub of bioenergetic metabolism through ATP synthesis. Comprising of a relatively permeable outer mitochondrial membrane (OMM), a highly selective inner mitochondrial membrane (IMM), the intermembrane space (IMS) and the matrix core, electron donors are formed through the citrate/Krebs cycle and fed into the IMM-located electron transport chain (ETC) to drive ATP production. Energy released from electron shuttling generates a matrix-directed electrochemical H^+ gradient necessary to fuel F1-F0 ATP synthase-mediated conversion of kinetic energy to chemical energy, which is stored into phosphoanhydride bonds in the ATP molecule during oxidative phosphorylation (OXPHOS). Energy is released in exergonic hydrolysis reactions by ATPases to power energy-dependent cellular processes. Damaging reactive oxygen species (ROS), usually superoxide anion ($O_2^{\bullet -}$) or hydrogen peroxide (H_2O_2), are generated as byproducts of single electron escape from the ETC, OXPHOS, and matrix biochemical reactions but are detoxified by antioxidants, such as glutathione or ROS-metabolizing enzymes.

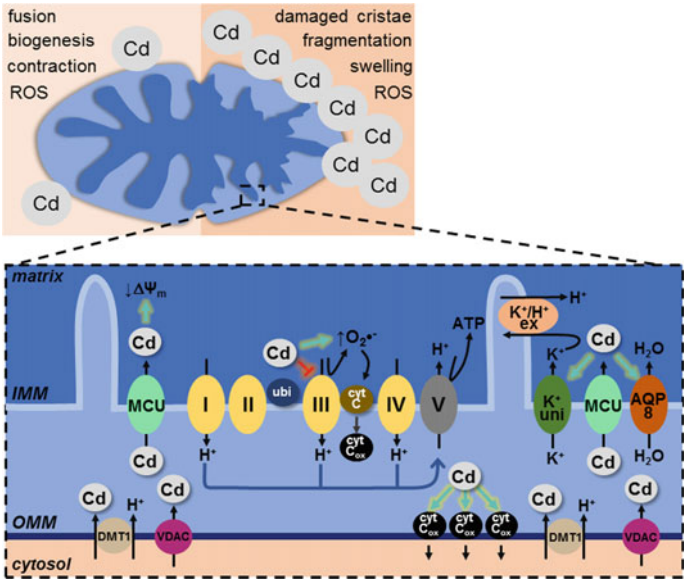

Fig. 4.1 Cadmium effects on mitochondria. Low cadmium-induced mitochondrial fusion, contraction, and biogenesis are elicited by mild oxidative stress and engage adaptive responses. High cadmium leads to mitochondrial damage, such as cristae loss, fragmentation, swelling, and high ROS. Cadmium putatively permeates the outer mitochondrial membrane (OMM) via VDAC or DMT1 and the inner mitochondrial membrane (IMM) through the mitochondrial calcium uniporter (MCU). From within the matrix, cadmium blocks complex III, increasing superoxide anion which oxidizes cytochrome c (cytC to $cytC_{ox}$) facilitating its release. Cadmium also activates aquaporin-8 (AQP8) for swelling and K^+-cycling for contraction as well as dissipating mitochondrial membrane potential ($\Delta\psi_m$). See Sect. 4.2 for further details

4.2.1 Mitochondrial Membranes

The IMM and OMM function together to maintain intramitochondrial spaces with defined ionic and proteinaceous composition that are highly regulated by a multitude of channels and transporters mainly expressed in the selective IMM.

The lipid microenvironment functionalizes resident proteins. Mitochondrial membranes contain relatively low amounts of cholesterol compared to the PM. CdAc (2 mg/kg/day, i.p. 7–30 days) increases cholesterol at the expense of certain phospholipids (phosphatidylethanolamine, phosphatidic acid) in mitochondrial membranes derived from rat liver [7] and brain [8] albeit with no change in membrane fluidity. Intriguingly, sphingomyelin is also increased [8]. Sphingomyelin and cholesterol are key components of lipid rafts, specialized membrane domains, wherein recognition and transporting proteins are activated, and both lipids are well documented to be augmented in tumor cells [9]. In fact, increased cholesterol has been suggested to underlie the metabolic switch from OXPHOS to anaerobic glycolysis in cancer-

ous cells [10] and could form part of cell alterations initiated during the process of cadmium carcinogenesis [11, 12].

Cardiolipin, a mitochondrial phospholipid, is localized exclusively in the IMM, whereupon the ETC shuttling and proapoptotic protein, cytochrome c (cytC), is tightly bound by electrostatic interactions. Oxidized cardiolipin serves two purposes: release of cytC from the tightly bound pool into the IMS and translocation from the IMM to OMM to facilitate cytC leakage into the cytosol. Cadmium weakly interacts with cardiolipin, increasing membrane rigidity and liposome aggregation [13], and could possibly promote cytC release in apoptotic signaling [14] (Fig. 4.1) through interference with electrostatic interactions and membrane biophysical properties.

Invaginations of the IMM called cristae serve to increase surface area for permeability and ETC proteins and bring ETC complexes into closer proximity for efficient electron transfer and ultimately, greater capacity for ATP synthesis. Numerous reports using transmission electron microscopy (TEM) evidence deleterious effects of cadmium on mitochondrial cristae, such as their reduction in number and their shortening, in various animal systems [15–19] and has been correlated with reduced expression of cytochrome c oxidases (COX), essential components of ETC complexes, indicating compromised mitochondrial function [20].

4.2.2 Mitochondrial Dynamics

As cellular energy demands fluctuate, mitochondria adapt through redistribution within the cell, trafficked to the sites of high metabolic demand, and undergo fusion and fission/fragmentation. Fusion events occur during high energy demands as well as during stress conditions to mitigate cell stress responses and limit the number of damaged mitochondria by mixing them with healthy mitochondria. Conversely, fission quality controls mitochondrial health, helping to sort out dysfunctional/damaged mitochondria, and usually precedes cell death execution [21]. Mitochondrial fragmentation is induced by high cadmium in the brain [22] but also by low cadmium in cultured liver cells [23]. Unexpectedly, yeast mitochondrial fusion mutants were more resistant to cadmium [24] suggesting that fusion could a preliminary step to mitochondrial fission and elimination.

To cope with demand, chronic stress and high metabolic demand can induce mitochondrial biogenesis, which is partially regulated by the transcription factors peroxisome proliferator-activated receptor (PPAR) and PPAR coactivator 1 (PGC-1), members of the nuclear co-regulator family [25].

In cultured renal proximal tubule cells (RPTCs), PPARγ and mitochondrial DNA (mtDNA) were augmented by 1–10 μM $CdCl_2$ for 24 h and correlated with glutathione loss and low rates of apoptosis [26]. In contrast, 30 μM $CdCl_2$ attenuated PPARα, PGC-1β, and mtDNA, despite sustained PPARγ increase, and was associated with apoptotic markers. Similarly in subtoxic subchronic $CdCl_2$-treated Fischer rats (1 mg/kg/day, s.c., 2 weeks), PPARα, and mtDNA significantly increased whereas glutathione was unchanged compared to saline-treated controls [26], further

confirming mitochondrial biogenesis as part of an adaptive mechanism to chronic oxidative stress by cadmium. Conversely, PGC-1α activity was turned off (assessed by its acetylation status), mtDNA content and mitochondrial mass were reduced in hepatocellular carcinoma cells exposed to <10 μM $CdCl_2$ for 12 h [27]. Despite increased ROS, and even at nontoxic $CdCl_2$, no adaptive responses involving mitochondria were recorded. This discrepancy can only be explained by the different cell models used, that is, noncancerous versus cancerous cells, which likely harbor divergent antioxidant status and could result in cadmium-induced generation of ROS subspecies to varying degrees.

4.2.3 Permeation into Mitochondria

Mitochondrial transport of cadmium is reviewed in depth in the chapter "*Membrane Transport Proteins and Receptors for Cadmium and Cadmium Complexes*".

The most abundant OMM protein called porin or voltage-dependent anion channel (VDAC), which permits molecules of up to 5 kDa, is thought to mediate passage of molecules into mitochondria, however, recent evidence revealed the presence of the divalent metal transporter (DMT1), which transports cadmium, in the OMM where it is thought to regulate mitochondrial iron levels [28]. Conversely, the IMM is equipped with an array of transport proteins that tightly regulate movements across the membrane. Ion movement requires uniporters, symports, and antiports, such as the mitochondrial calcium uniporter (MCU) and K^+/H^+ exchanger, whereas small nucleotides and nascent proteins require the adenine nucleotide transporter (ANT) and TIM/TOM complex, respectively. Due to their similar ionic radii, calcium and cadmium can imitate each other at recognition sites [29]. To this end, cadmium permeation of the IMM via the MCU has been demonstrated in isolated kidney or liver mitochondria using MCU inhibitors ruthenium red, Ru360 or La^{3+} [30–33].

4.2.4 Electron Transport Chain

The ETC comprises five multimeric complexes (CI–CV) localized in the IMM wherein electrons are shuttled from the multivalent metal core of one complex to the next, helped by ubiquinone and cytC on either side of CIII, generating energy for shunting of protons from the matrix to the IMS. Consequently, a proton-motive force and a mitochondrial membrane potential ($\Delta\psi_m$) is created across the IMM. CV, the F1-F0 ATP synthase, uses the energy stored in the H^+ gradient to drive its turbine to form ATP.

Mitochondria are the major site of ROS production as a consequence of electron shuttling, in particular, CI and CIII produce highly reactive superoxide anions ($O_2^{\bullet -}$). Cadmium is well evidenced to increase ROS levels either by affecting mitochondrial function, ROS-producing/metabolizing enzymes, or negatively targeting antiox-

idants, such as glutathione. In an elegant study in isolated mitochondria, cadmium inhibited ETC complex activities (CIII > CII ≫ CIV > CI) where CIII was maximally inhibited by ~75% at 20 μM $CdCl_2$ that could be reversed by EDTA [34], corroborating an earlier study wherein electron transfer from ubisemiquinone to cytochrome b_T, a component of CIII, is blocked by 30 μM $CdCl_2$ [35]. Cadmium targets CIII through competitive binding at the zinc binding site, preventing electron transfer and resulting in increased superoxide [34]. Since the oxidative status of cytC appears to be prerequisite for its transfer to a loosely bound pool [36], which is then ready for liberation in apoptosis signaling, ROS production by cadmium in the immediate vicinity of cytC makes for a favorable mechanism by enhancing cytC apoptogenicity (Fig. 4.1).

4.2.5 *Mitochondrial Membrane Potential ($\Delta\psi_m$)*

Consequent to the ETC, $\Delta\psi_m$ is generated where the matrix is negatively charged compared to the IMS. Dissipation of $\Delta\psi_m$ indicates mitochondrial dysfunction, has been well documented for cadmium [11, 14, 27, 31] and could be linked to ETC block [34, 35].

4.2.6 *Mitochondrial Permeability Transition (mPT) and Permeability Transition Pore (PTP)*

Apoptotic stimuli, such as calcium or ROS, can induce the IMM to undergo permeability transition such that water and solutes can freely pass into the matrix, concluding with an increase in matrix volume, consequent of osmotic pressure, and swelling of mitochondria. With sufficient matrix expansion, the IMM, with its larger surface area, causes the OMM to disrupt culminating in the release of proapoptotic factors, such as cytC, from the IMS and mitochondrial dysfunction. PTP formation at IMM-OMM contact sites, permitting solutes up to 1500 Da across the IMM, is thought to underlie mPT. Once thought to be comprised of OMM VDAC, IMM ANT, and matrix cyclophilin D, apoptosis execution was observed despite genetic deletion of VDAC [37] or ANT [38], interrogating the molecular composition of PTP and interpretation of studies using ANT modulators bongkrekic acid or atractyloside.

Does cadmium induce PTP opening? Numerous light scattering studies have reported PTP participation in cadmium cell death signaling using isolated mitochondria from rodent liver or kidney and monitoring mitochondrial volume/swelling [30, 32, 33, 39]. Considering the current literature, wherein VDAC and ANT as PTP components has been challenged [37, 38], it seems even more important to draw conclusions only from modulation of cyclophilin D, which has escaped molecular scrutiny. In fact, ablation of calcium-induced mitochondrial swelling and cytC

release in cyclophilin D-null mice leaves no doubt to its contribution to PTP [40]. The ineffectiveness of cyclosporine A (CsA), a cyclophilin D inhibitor, on cadmium-induced mitochondrial swelling [30, 32, 39] indicates that the PTP is not a ubiquitous mitochondrial swelling mechanism elicited by cadmium. Rather, the opening of aquaporin-8 by cadmium permits water influx into the matrix to cause swelling [30].

4.2.7 Mitochondrial Volume Dynamics

In response to cellular energetic demands, mitochondria do not only undergo fusion and fission but can also swell and contract through monovalent cation cycling to regulate chemical reactions [41]. Swollen mitochondria exhibit decreased β-oxidation, Krebs cycle activity, and respiration and can be made to contract by ATP, ADP, Mg^{2+}, or potassium cyanide, depending on the swelling stimulus. Furthermore, it has been proposed that mitochondrial shape changes through swelling serve as mechanical signals to communicate with other cell organelles [42].

Isolated rat kidney cortex mitochondria, suspended in KCl buffer and energized with rotenone/succinate, undergo swelling followed by contraction completed within 2 min after 5–20 μM $CdCl_2$ addition [31]. Nonenergized mitochondria swell but do not contract. Using pharmacological inhibitors, swelling was MCU- and K^+-influx-dependent but PTP-independent while contraction was $\Delta\psi_m$-independent and mediated by action of the K^+/H^+-exchanger, which depended on the ETC-generated pH gradient across the IMM [31] (Fig. 4.1). Transient limited mitochondrial swelling by nontoxic cadmium doses may represent a mechanical signal to neighboring organelles as part of an adaptive stress response and may precede mitochondrial fusion/fission, damaged mitochondria removal by mitophagy, temporary switches in energy metabolism, and altered expression of mitochondrial proteins.

4.3 Cadmium and ER

The ER is an expansive and highly dynamic network, maintaining contacts with all other organelles and could be regarded as the delegator, by sensing signals and giving instruction in cellular responses. Through mitochondria-associated membranes (MAMs), the ER directs calcium flux to and from mitochondria in addition to dictating and aiding mitochondrial fission [43]. Furthermore, ER membranes supply autophagophore formation [44]. ER-PM contacts mediate store-operated calcium entry through STIM1/Orai and maintain lipid homeostasis at the PM, for instance, during second messenger signaling (reviewed in [45]).

Primary ER functions are mRNA translation, protein folding, some protein modifications, and lipid synthesis that demand an oxidizing and calcium-rich environment. Protein folding is prone to errors therefore several quality control and

damage-limiting mechanisms protect from potential stress elicited by large amounts of misfolded proteins. ER-resident chaperones (GRP78/BiP, GRP94) occupy ER stress sensor proteins, maintaining them in an inactive state, and bind polypeptides to aid protein folding as well as offering refolding opportunities, if mistakes are made. Should these refolding endeavors prove unsuccessful, unfolded proteins are directed to ER-associated degradation (ERAD) machinery that results in cytosolic proteasome-driven destruction. Increased unfolded protein load shifts ER chaperone distribution, such that ER stress sensor proteins become unoccupied, and initiates the unfolded protein response (UPR), which initially delays cell damage by reducing mRNA translation but subsequently engages cell death-promoting pathways, culminating in upregulation of proapoptotic GADD153/CHOP and caspase-12 activation [46].

4.3.1 Intralumenal Homeostasis

Folding a polypeptide into its tertiary conformation requires formation of disulfide bridges. The intralumenal oxidizing environment of the ER is optimal for this process; but too oxidizing or too reducing results in aberrant disulfide bridge formation and thus, malformed protein structure. It is not yet clear as to exactly how the ER maintains correct redox balance though glutathione could be involved [47] and targeting by cadmium affects ER lumen redox status, increases misfolded proteins, and initiates the UPR.

High ER lumenal calcium is maintained by a pump-leak system, whereby the thapsigargin-sensitive sarco-/endoplasmic reticulum calcium ATPase (SERCA) actively transports calcium back into the ER lumen following passive leakage into the cytosol. Using aequorin-based probes, cadmium diminishes SERCA activity without effect on leakage that results in ER calcium depletion and evokes the ER stress response in parallel with the mitochondrial apoptosis pathway suggesting interorganellar communication through calcium [48].

4.3.2 ER Stress

ER stress is initiated by decreased intralumenal calcium and oxidative stress that contribute to the accumulation of unfolded proteins, activating UPR and ERAD. When ER stress sensor proteins PERK, IRE1, and ATF6 are non-chaperone bound, they become active. The PERK-eIF2α-ATF4 pathway blocks further mRNA translation to prevent ER overloading so the cell has time and capacity to attempt correction of misfolded proteins. In acute and prolonged ER stress, IRE1 activation of XBP1 mRNA splicing and ATF6 truncation in the Golgi culminate in upregulation of proapoptotic genes [46].

Amongst heavy metals, cadmium is particularly effective in inducing ER stress [49], which has been well documented in various models (reviewed in [11, 14]), wherein ER chaperones (GRP78, GRP94, and BiP), all UPR arms, and CHOP are upregulated. In mammalian cells, it appears that ROS/reactive nitrogen species (RNS), specifically superoxide anion ($O_2^{\bullet -}$) or peroxynitrite ($ONOO^-$) [50], or calcium precede ER stress and UPR, however, caution should be taken when potential cadmium chelators, such as BAPTA, N-acetylcysteine, and Fura-2, are used. In contrast, ER accumulation of cadmium in yeast elicits UPR but not through inhibition of disulfide bond formation [51]. Analogous to autophagy, mild ER stress by cadmium triggers the PERK-eIF2α-ATF4 pathway and precedes signs of damage [49], acting in a protective manner, whereas major ER stress recruits the prodeath UPR arms culminating in CHOP.

4.3.3 ER Restructuring

Swollen, disorganized and damaged rough ER is caused by cadmium in various models, including insect cells (66 μM $CdCl_2$, 24 h) [17] and rat liver (0.84 mg/kg, i.p. CdAc, 48–96 h) [16]. The effect of cadmium on distribution of physical ER contacts has not yet been investigated.

4.4 Cadmium and the Nucleus

Genetic material in the nucleus is partitioned from the cytosol by the nuclear membrane, consisting of outer and inner membranes and an underlying nuclear lamina that surrounds the nuclear matrix, which comprises cytoskeletal proteins and nuclear sap, acting as a support framework for intranuclear macromolecules. Nuclear pores span both nuclear membranes and permit communication and translocation of gene regulatory molecules between the cytosol and nucleus albeit in a regulated manner.

The central subcellular localization of the nucleus poses a spatial hurdle for cadmium as it must cross the PM followed by the cytosolic minefield to encounter the nucleus. Atomic absorption spectroscopy evidenced cadmium uptake into nuclei, plateauing at ~1 nM [52], and radioactive cadmium (^{109}Cd or ^{115}Cd) data evidence nuclear cadmium, driven by diffusion, within and peaking at 1 h of exposure [53, 54], weakly bound, and subsiding over time. Upregulation of cytosolic cadmium-binding sites shifts equilibrium towards the cytosolic compartment wherein cadmium is complexed [54], strongly suggesting that cadmium-induced effects from within the nucleus are short-lived and dependent on the level of cadmium-sequestering proteins in the extranuclear compartment. Thus, primary cadmium exposures probably trigger genetic and epigenetic changes from within the nucleus as its journey encounters less hurdles whereas secondary and repeated cadmium exposures will have less impact

because augmented intracellular cadmium-binding sites sequester cadmium before it can reach the nucleus [55].

Genomic instability and DNA mutational effects by cadmium will be discussed in the chapter "*Cadmium and its Impact on Genomic Stability*".

4.4.1 Nuclear Architecture

Ultrastructural transmission electron microscopy (TEM) studies of cadmium-exposed animal tissues and cell lines generally indicate dilated nuclei, dilated or ringed nucleoli, nuclear indentation and aberrant chromatin condensation [19, 56, 57], accumulation of RNA storage perichromatin granules (PG) at the nucleolar edge [19, 58], and micronuclei formation [19, 59, 60], wherein damaged chromosomes reside and indicates chromosome instability. Isolated nuclei evidenced millimolar cadmium-induced redistribution of lamin A, but not lamin B, from nuclear periphery to across the nuclear matrix [61] and could represent adaptive stiffening of the nuclear lamina in response to mechanical signals [62], such as mitochondrial swelling. Incidentally, large holes in the nuclear membrane after cadmium [58] could be a result of increased membrane rigidity [4] causing the nuclear membrane to become fragile and brittle, and therefore prone to breakages.

4.4.2 Chromatin Organization and Epigenetics

Chromatin comprises repeating units called nucleosomes and describes DNA wound around and condensed by histone proteins, influencing the accessibility of a gene. Histone proteins are susceptible to modifications, for example, acetylation or methylation, and can affect gene transcription through changes in DNA winding/unwinding and masking or exposing sites for transcription. Low cadmium (1 μM $CdCl_2$) treatment causes changes in chromatin structure [58] and appearance of perichromatin granules (PGs) [58–60], which are storage sites for newly synthesized heterogeneous nuclear RNA, including pre-mRNA, indicating its maturation and export are impaired by cadmium. How can cadmium affect gene transcription when mRNA processing is hindered? A possible explanation is biphasic responses concerning RNA: low/acute cadmium augments whereas high/chronic cadmium attenuates RNA synthesis and mRNA activity, probably due to interactions with zinc-dependent enzymes, such as RNA polymerase [51]. Further, topoisomerase IIα, which alters the topological state of nucleic acids, is redistributed in cadmium-exposed K562 cells [63] and could affect gene transcription.

Epigenetics describes inheritable changes in gene expression without modifications to the DNA sequence and is strongly influenced by environmental factors [64]. Histone modification and DNA methylation have been implicated in protection against cadmium toxicity as well as in malignant transformation (summarized

in [11]), whereas cadmium effects on noncoding RNAs, namely microRNAs, are currently emerging (reviewed in [65]). Recently, monomethylation of histone H3 at K27 (H3K27me1) was decreased by IC_{25} $CdCl_2$ in mouse embryonic stem cells and was associated with prolonged mitosis, decreased population doublings, and compensatory increased total histone protein production. Importantly, daughter cells inherited these alterations [66]. In cultured lung cells, carcinogenesis-associated histone methylation marks H3K4me3 and H3K9me2 were elevated by $CdCl_2$ ($\leq$2.5 μM, 6–48 h), which inhibited histone demethylases [67]. In transformation studies with 2 μM $CdCl_2$, H3K4me3 and H3K9me2 were elevated at 4 weeks but no longer at 20 weeks suggesting transient changes in histone methylation is sufficient to bring about persistent genetic changes associated with transformation [67].

4.4.3 Nuclear Matrix Proteins

The nuclear matrix and perichromatin space contains a plethora of regulatory proteins that maintain the composition of the nuclear sap, execute signaling cues, govern turnover of proteins, and modulate gene transcription.

DNA fragmentation is the penultimate step in the apoptotic signaling cascade, prior to apoptotic body formation and engulfment by macrophages, and is executed by a Ca^{2+}/Mg^{2+}-dependent endonuclease that cleaves DNA at intranucleosomal linker regions [68]. In isolated bovine liver nuclei, cadmium activates the endonuclease and induces DNA laddering only at low concentrations (<10 μM free Cd^{2+}), corroborating apoptosis is associated with low cadmium, but also potently inhibited DNA laddering induced by calcium [69]. The same group reported cadmium blockade of nuclear calcium ATPase, resulting in lower nuclear calcium [52], therefore, it is unclear whether the cadmium effects are consequent of nuclear calcium uptake inhibition or direct endonuclease blockade.

Activation of nuclear protein kinase C (PKC) by phorbol esters is potentiated in the presence of cadmium by ~2-fold [70]. Astonishingly, PKC binding to nuclear proteins is enhanced by 0.1 nM free Cd^{2+} compared to 1 nM free Zn^{2+} in rat liver nuclei [70]. These observations could affect the phosphorylation status of histones and therefore chromatin organization. Lastly, it was proposed that inhibition of 8-oxo-dGTPase by CdAc (20 μmol/kg/bw, s.c., 2–48 h) in rat testis may lead to incorporation of promutagenic 8-oxo-2'-deoxyguanosine, promoting carcinogenesis, but time course inconsistencies question a causal relationship [71].

4.4.4 Transcriptional Regulation

Typically, activated cytosolic transcription factor protein is stabilized and shuttles to the nucleus wherein transcription is initiated after promoter binding. A multitude of factors determines whether a gene is "switched on" or "turned off" such

as nuclear abundance and activating modifications of transcription factor protein, levels of accessory regulatory proteins, accessibility of response elements, and the presence of metal ions, in particular zinc. Extensive reports in the literature evidence altered gene expression upon cadmium exposure that involves classical transcription factors, such as nuclear factor kappa B, AP-1, c-Myc, and Nrf2 (reviewed in [14]). It is important to note that most of these studies allude to regulation by upstream signaling pathways initiated by cadmium, and not a direct effect. However, nuclear export of transcription factors [72] or mRNA [73] can also be affected by cadmium. Additional putative mechanisms are displacement of zinc (reviewed in [74]), changes in nuclear architecture that prevent access to response elements, and modification of accessory transcription complex proteins or transcriptional machinery.

To date, only a single study has identified the direct participation of cadmium on transcription factor activity. The tumor suppressor gene p53 is often quoted as the guardian of the cell, overseeing genotoxic and non-genotoxic-induced stress responses, primarily by DNA quality control, and is mutated in over 50% of cancers. In a set of elegant experiments, Meplan et al. utilized recombinant wildtype p53 and conformation-specific antibodies to show that cadmium induces a conformational change in p53, dissipating its DNA binding capacity, preventing activation of p53 target genes [75], and thus contributing to cadmium-induced carcinogenesis.

4.5 Cadmium and Lysosomes (Fig. 4.2)

Cargo internalized into the cell by endocytosis, macrocytosis or phagocytosis as well as cargo from within the cell, taken up into autophagosomes (see Sect. 4.7.1), are trafficked to the lysosome for degradation by acidic digestive hydrolase enzymes followed by expulsion or recycling.

4.5.1 Lysosome Maturation

Lysosomes originating from the endocytic/endolysosomal pathway have been best studied. Early endosomes (EEs) bud off the PM and can either return to the PM as a recycling endosome (RE) or enter the late endosomal (LE) pathway. LEs are acidified to pH ~ 6 by the vacuolar H^+-ATPase (V-ATPase) and acquire lysosomal proteins through fusion with lysosome-targeting mannose-6-phosphate transport vesicles from the trans-Golgi. Further acidification of lysosomes to pH ~ 4.5 depends on V-ATPase driven accumulation of protons in the intralysosomal space in concert with extrusion of a counterion. The final step in lysosome maturation is activation of acid phosphatase. In rat RPTCs, cadmium inhibits the V-ATPase and perturbs the endocytic trafficking pathway culminating in proteinuria [76] (see Sect. 4.6.1). Intriguingly, micromolar cadmium inhibits phosphomannose isomerase by competition with the substrate mannose-6-phosphate [77] thereby increasing its

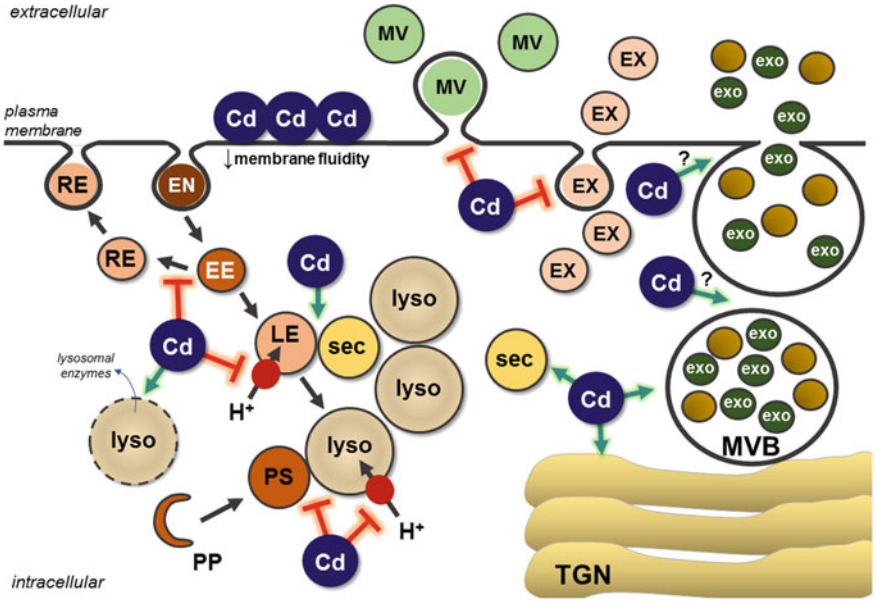

Fig. 4.2 Cadmium effects on vesicular trafficking. Through molecular mimicry, cadmium inhibits calcium-dependent processes, such as regulated exocytosis (EX) and microvesicle (MV) formation. Blockade of the vacuolar H^+-ATPase prevents late endosome (LE) and lysosome (lyso) acidification and thus endocytosis (EN). Further, cadmium prevents recycling endosome (RE) formation from early endosomes (EE) or RE exocytosis. Cadmium expands the trans-Golgi network (TGN) and lysosome number, possibly due to increased secretory vesicles (sec) containing lysosomal proteins. Lysosomal membrane permeabilization by cadmium could lead to cell death. Autophagopore (PP) formation precedes autophagosomes (PS), which are prevented from fusing with lysosomes by cadmium and hindering autophagy. Cadmium could be involved in mobilizing multivesicular bodies (MVB) during exosome (exo) secretion. See Sects. 4.5–4.7 for further details

levels and promoting lysosome biogenesis. To this end, increases in heteromorphous lysosome size and number by cadmium have been observed using TEM [17, 18] and identified by neutral red uptake and acid phosphatase staining [17], as well as fluorescent dye labeling of acidic compartments [78]. Lysosomal system expansion could be resultant of: (1) lysosomal homotypic fusion and fission; (2) inhibition of lysosomal trafficking; (3) inhibition of RE formation forcing endosomes into lysosomal maturation; and (4) increased delivery of lysosomal proteins from Golgi to LEs to drive lysosomal maturation. Enlargement of the Golgi apparatus by cadmium [19, 79] could contribute to increased lysosome biogenesis, however, how cadmium affects these processes is unknown (Fig. 4.2).

4.5.2 Lysosomal Membrane Permeabilization (LMP)

Loss of lysosomal membrane integrity results in leakage of lysosomal digestion enzymes and consequent lysosomal cell death. LMP is most likely caused by alterations in lysosomal membrane lipid composition since sphingomyelin increases lysosomal instability and LMP [80]. In RPTCs, defective lysosomes were observed with 5 μM $CdCl_2$ after 6 h as assessed by decreased lysosomal LAMP1 and cathepsin B [5], suggesting cadmium induces LMP. Furthermore, increased sphingomyelin levels were associated with increased membrane fluidity, which could alter protein interactions with the lysosomal membrane, such that they are degraded and leaked out. Cadmium-induced LMP has also been indicated by loss of acidic compartment labeling [78, 81], leakage of lysosomal DNase II in necrotic endothelial cells [78], and selective leakage of β-glucuronidase but not acid phosphatase, which was inhibited by cadmium, from isolated lung lysosomes [82].

4.6 Cadmium and Vesicular Trafficking (Fig. 4.2)

Through endocytosis, transcytosis, and exocytosis, intracellular trafficking vesicles exist in many different interconnected pools and are multifunctional, such as enabling the cell interior to communicate with the external environment, directing cargo to its final destination, regulating transmembrane proteins, such as receptors and transporters, and regulating lipid balance.

4.6.1 Endocytosis

Invagination and budding from the membrane with the aid of clathrin or caveolin during endocytosis results in the formation of EEs that develop into REs or into acidic LEs and subsequently, lysosomes in a GTPase-dependent manner.

Cadmium impairs endocytosis. In the RPTC, which is the primary site of cadmium accumulation and has high endocytic turnover at its apical brush border membrane (BBM) because of its role in mass reabsorption, cadmium exposure diminished receptor-mediated endocytosis of fluorescently labeled ligands. In opossum kidney (OK) proximal tubule cells, FITC-albumin receptor binding, B_{max}, and uptake were maximally attenuated by 100 μM $CdCl_2$ for 1 h, whereas no effect was seen on fluid-phase endocytosis. In more intricate studies in a rat model (2 mg/kg/day s.c. $CdCl_2$, 14 days), BBM was damaged and isolated BBM vesicles harbored ~40% less V-ATPase protein expression and bafilomycin-sensitive ATPase activity [76]. Since acidification is essential for endocytic trafficking, cadmium inhibition of vesicular acidification would impair endocytosis. Indeed, using BBM vesicles from non-treated rat kidneys, 10 μM $CdCl_2$ significantly attenuated bafilomycin-sensitive

ATPase activity, quenching of acridine orange (which accumulates in acidic compartments), and uptake of FITC-dextran [76]. Furthermore, cadmium-metallothionein exposed rats exhibited redistribution of apical membrane proteins, such as megalin and Na^+/H^+-exchanger, into vesicles suggesting that REs are also perturbed by cadmium inhibition of vesicular acidification [83].

In addition to fluid-phase and receptor-mediated endocytosis, cells with high rates of exocytosis, such as developing, endocrine, secretory epithelial, and nerve cells, must retrieve exocytosed membrane by compensatory endocytosis to prevent significant changes in membrane surface area. Compensatory endocytosis in response to triggered exocytosis appears to be calcium-dependent thus cadmium inhibits the influx of extracellular calcium and, in turn, compensatory endocytosis in sea urchin embryos [84].

4.6.2 Secretory Vesicles and Exocytosis

Secretory vesicles derived from the trans-Golgi network migrate along microtubules to the PM for exocytosis, whereby their contents are either incorporated into the PM or released into the extracellular space.

Cytosolic calcium increase is prerequisite to stimulated exocytosis. In addition to neutralizing opposing surface negative charges between the cell and vesicle membranes, calcium is required for activation of membrane fusion proteins, such as synaptotagmin, which mechanically pull the membranes together. Based on the interplay of calcium and cadmium [29], an effect of cadmium on exocytosis is assumed. In fact, isolated single synaptotagmin C2 domains, which harbor calcium-binding sites, do not associate with lipid membranes when complexed with cadmium, in contrast to full-length synaptotagmin containing multiple C2 domains, indicating the avidity of C2 domains [85]. However, the association of cadmium-complexed full-length synaptotagmin with lipid membranes was shallower than when calcium was used [85] and could have an impact on the fusogenicity of membranes during exocytosis events.

4.7 Cadmium and Other Cell Organelles (Fig. 4.2)

4.7.1 Autophagosomes

During autophagy, the mechanism of self-digestion that fuels that cell during periods of starvation, membranes are pinched off from intracellular organelles, such as the ER or mitochondria, and used to form double-membraned autophagosomes [86], which link cytosolic LC3 to phosphotidylethanolamine. Lipidated LC3, also known as LC3-II, serves as an anchorage point for autophagosomal chaperones that direct

cargo destined for degradation but is also degraded itself. Actually, LC3 degradation is a measure of autophagic flux. Autophagosomes engulf cytosolic constituents in either a targeted (ERphagy, mitophagy) or nontargeted (macroautophagy) manner and subsequently fuse with lysosomes to generate acidic autophagolysosomes, wherein cargo is degraded by lysosomal enzymes.

Without a doubt, cadmium induces autophagy. However, the outcome is disputed. The self-preservation function of autophagy has led to the hypothesis that cadmium autophagy is protective but use of the multi-target autophagy inducer rapamycin hampers correct interpretation [87]. Nevertheless, numerous studies have reported that autophagy protects against cadmium toxicity (reviewed in [14, 88]). Autophagy has been observed in kidney cortex of rats exposed to sublethal cadmium (0.3 mg/kg/bw $CdCl_2$, i.p. 1–5 days) suggesting autophagy as a stress or protective response [89]. Unfortunately, the contribution of autophagy in protection against kidney damage by cadmium was not investigated. In cell culture studies, cadmium-induced morphological changes and LC3-II were reversed using 3-methyladenine but not bafilomycin A1 [89]. The authors suggested cadmium inhibits the V-ATPase (see above and [76]), thus accounting for the lack of effect by bafilomycin A1, but indicate cadmium hinders both autophagosome formation and autophagic flux.

More recent evidence clearly shows that cadmium negates autophagy execution. Colocalization of LAMP proteins with LC3 was significantly inhibited by cadmium (2.5–50 μM, 12–24 h) [90, 91], whereas 3-methyladenine and Atg5 siRNA had no effect on LC3-II, p62 or cadmium-induced loss of cell viability [91]. Moreover, cadmium increased lysosomal pH and lysosome biogenesis-related genes and decreased protease activity [91]. Melatonin reversed detrimental lysosomal effects by cadmium, but melatonin, a powerful antioxidant, also binds cadmium [92]. In RTPCs, 5 μM $CdCl_2$ induces transient protective autophagy within 3 h (↓LC3-II/p62) but disrupts autophagic flux at 6–8 h (↑LC3-II/p62) [5]. Conversely to previous reports, rapamycin was ineffective against cadmium cell death despite inducing autophagy [5, 87]. Low cadmium stress-induced autophagy delayed the onset of apoptosis, which could be reversed by autophagosome–lysosome fusion inhibition, whereas accrual of cadmium stress decreased the effectiveness of lysosomal inhibitors on LC3-II/p62, and increased membrane fluidity and instability of isolated lysosomes [5]. These changes elicited by cadmium could alter the fusion capacity of lysosomes, thereby preventing autophagy execution, which is in agreement with other reports [90, 91]. Blockade of autophagic flux by cadmium concurs with decreased prosurvival signaling as well as with accumulation of autophagosomes resulting in cell death [93].

4.7.2 Extracellular Vesicles

Communication between cells can occur through secreted extracellular vesicles that pass on information with mRNA/microRNA, proteins and signaling molecules. Microvesicles (MVs) are derived from PM budding whereas exosomes are produced

in multivesicular endosomes. Exosomes are enriched in tetraspanins, flotillin and lipids, including sphingomyelin and cholesterol [94], and tend to be smaller in size (40–100 nm) than MVs (100–1000 nm).

Cadmium has not been documented to affect exosome formation or secretion though it would be a plausible hypothesis since cadmium increases ceramide [6, 95] which promotes exosome release [96], and cadmium-containing cigarette smoke induces the release of ceramide-rich exosome-containing microparticles from lung endothelial cells [97]. Budding of the PM in MV formation is calcium-dependent thus, unsurprisingly, cadmium prevents MV secretion of glutamate from rat pineal gland cells [98].

4.7.3 Peroxisomes

Surrounded by a single membrane, peroxisomes are characterized by the presence of H_2O_2-producing and -degrading enzymes, present in virtually all eukaryotic cells and largely associated with oxidative status [99]. They execute similar biochemical reactions as those found in mitochondria though the enzymatic machinery employed by each organelle is entirely different. Peroxisomes do not possess an ETC, therefore electrons from $FADH_2$ are passed onto O_2 to form H_2O_2. Though H_2O_2 is metabolized to water and oxygen by intraperoxisomal H_2O_2-metabolizing enzymes, of which catalase is the most abundant, this is insufficient to prevent H_2O_2 release into the cytosol where it could serve as a messenger or cause imbalance in cellular oxidative status.

Peroxisomes have been largely overlooked in cadmium toxicity in spite of their well-described ROS/RNS generating capacity probably due to their functional similarity to mitochondria (see Sect. 4.2). Only a single study has examined the effect of cadmium directly on peroxisomes. In yeast, 1 mM $CdCl_2$ for 24 h inhibited cell growth but increased peroxisome number and catalase activity [100]. Despite catalase's detoxifying function, oxidative stress has been associated with peroxisome proliferation because H_2O_2-producing enzymes are augmented by >10 times over increase in H_2O_2-metabolizing enzymes [101] suggesting that cadmium-induced peroxisome proliferation and catalase activity would contribute to oxidative stress. In RTPCs, 10–50 μM $CdCl_2$ increased catalase activity (Lee, W. K. and Thévenod, F., unpublished data) but peroxisome number was not determined. In contrast, a study in mussels found cadmium had no effect on catalase activity and decreased peroxisome volume density [102]. Moreover, cadmium (10–40 μM $Cd(NO_3)_2$, 24 h) weakly interacts and inhibits catalase activity in zebrafish [103]. These conflicting data of cadmium on catalase activity and peroxisomes could lie in the diverse model systems used.

4.7.4 Specialized Organelles

The effect of cadmium on other more specialized organelles, such as melanosomes, phagosomes, and secretory lysosomes has not been considerably documented.

4.8 Summary and Conclusions

Compartmentalization by lipid membranes to create organelles with specialized functions is essential to normal cell physiology. Cadmium is a promiscuous non-native metal and disrupts organelle function through a few fundamental mechanisms: (1) altered biophysical properties of membranes [4]; (2) ionic mimicry [29, 74]; and (3) direct macromolecular interactions [104]. With chronic low cadmium, organelle function could initially diminish but stress adaptive responses, which include organelle biogenesis and strengthened interorganellar communication, would strive to restore this loss of function. In acute and/or high cadmium, organelle function is compromised such that it is irreversible and detrimental to the cell. Our current knowledge clearly evidences cadmium effects on organelles as single entities in cellular processes but further understanding of how organelles interact and communicate with each other to coordinate the adaptive response under cadmium stress would be crucial to delineating and predicting heavy metal toxicity.

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Chapter 5
Cadmium and Its Impact on Genomic Stability

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Abstract The carcinogenicity of cadmium for humans and experimental animals has been long established, most evident for tumors in the lung and kidney, but with increasing evidence also for other tumor locations. While cadmium does not interact directly with DNA, elevated levels of reactive oxygen species (ROS), the interference with the cellular response to DNA damage including all major DNA repair systems as well as the inactivation of tumor suppressor functions appear to be of major importance, thereby increasing the susceptibility towards exogenous and endogenous DNA damage. Furthermore, the deregulation of cell growth, the resistance to apoptosis, as well as epigenetic alterations have been demonstrated in diverse experimental systems. Particularly sensitive targets appear to be proteins with zinc-binding structures, present in many DNA repair proteins, transcription factors and in the tumor suppressor protein p53. The interaction with critical thiol groups and/or the enhanced generation of ROS may also provoke an interference with cellular redox regulation of critical signaling pathways. Especially the combination of these multiple mechanisms may give rise to a high degree of genomic instability in cadmium-adapted cells, relevant not only for tumor initiation but also for later steps in tumor development.

5.1 Introduction: Epidemiology and Animal Carcinogenicity

Cadmium is a natural element of the earth's crust, but its distribution in the environment and thus human exposure is greatly influenced by industrial use and agriculture. Significant exposure occurs via inhalation at the workplace. With respect to the general population, food and tobacco smoking are the main exposure sources. Mainly

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based on sufficient evidence for an increased relative risk of lung cancer in workers occupationally exposed to cadmium, in 1993 and 2009, cadmium and its inorganic compounds were classified by the International Agency for Research on Cancer (IARC) as carcinogenic to humans (IARC Group 1) [2–4] and 2004 as carcinogens group 1 (carcinogenic to humans) by the German MAK Commission [5]. Cohorts in United Kingdom, Sweden and United States have been investigated, including extensive follow-up studies [6–9], revealing elevated risks of lung cancer in most cases. Some major constraints are, however, the small number of long-term, highly exposed workers and the lack of historical data on exposure to cadmium in some studies. Furthermore, confounding factors are cigarette smoke and simultaneous exposure to nickel and arsenic [4, 5]. Supportive evidence from environmental inhalative exposure provided a study in Belgium with subjects living near three smelters when compared to subjects not exposed to elevated levels of cadmium, investigated from 1985 until 2004. Based on urinary cadmium excretion and cadmium in garden soil as exposure indicators, elevated lung cancer risks were observed in the high-exposure group [10]. With respect to other cancer sites, especially the kidney may be of elevated risk due to high and persistent cadmium accumulation, going along with nephrotoxicity close to environmental exposure levels. No elevated risks for renal cancer due to cadmium exposure were observed in a Swedish or in a British cohort study [7, 9]. Nevertheless, case control studies elucidating the relative risk of kidney cancer due to occupational cadmium exposure, which have been conducted in the United States, in Finland, in Germany and in Canada and which estimated cadmium exposure via job-exposure-matrices (JEM), observed higher incidences of renal cancer upon cadmium exposure at the workplace [11–14]. Altogether, the German MAK Commission concluded that an increased relative risk of renal cancer has to be assumed [5] and also IARC stated a positive association with respect to renal and prostate cancer [4]. Finally, human cadmium exposure may also be associated with female breast and endometrial cancer, even though these tumor locations are not definitively established [15, 16]. Regarding data from experimental animals, cadmium did not induce lung tumors in the hamster [17] but at remarkably low concentrations in rats. In the latter species in long-term inhalation studies, several cadmium compounds ($CdCl_2$, $CdSO_4$, CdS, and CdO) caused lung cancer (mainly adenocarcinomas) [18, 19]. The lowest concentration inducing primary lung carcinoma in rats (15 versus 0% in controls) was 12.5 μg Cd/m^3, even though under an unusual exposure regimen (23 h/day, 7 days per week for 18 months exposure to $CdCl_2$ aerosols) [18]. In a later study, no lung tumors were induced when the rats were exposed continuously for 18 months to CdO fumes at a concentration of 10 μg Cd/m^3, whereas 21% of the animals developed tumors when exposed to 30 μg Cd/m^3 [19]. Considering oral exposure, adequately conducted studies revealed increased incidences of large granular lymphocytes, leukemia, prostate, and testis tumors in Wistar rats (summarized in [2, 5, 20]).

5.2 Direct and Indirect Genotoxicity

5.2.1 DNA Damage, Mutagenicity, and Clastogenicity

Cadmium does not cause DNA damage in cell extracts or in isolated DNA [21]. Furthermore, cadmium is not mutagenic in classical short-term test systems. Thus, in most bacterial assays, water-soluble cadmium compounds were not mutagenic, and in standard mammalian mutagenicity tests, the induction of point mutations was usually weak and/or restricted to comparatively high concentrations. In contrast, pronounced co-mutagenic effects in combination with DNA alkylating agents and with UVC radiation were observed both in bacteria and in mammalian cells, pointing towards an interaction with the cellular response to DNA damage (see below). In contrast to the missing mutagenicity, in mammalian cells cadmium compounds provoked clastogenic effects such as chromosomal aberrations and micronuclei [2, 5, 22–24]. This was also demonstrated by the pronounced positive effects of cadmium chloride in a modified mammalian test system capable of detecting large multi-locus deletions [25]. The clastogenicity is moreover evident in vivo in exposed rodents, while evidence for chromosomal damage in cadmium-exposed humans via environmental or workplace exposure is equivocal, partly due to simultaneous exposure to other metal compounds [2, 5, 26]. Recently, a reanalysis of the relationship between cadmium exposure and micronuclei in lymphocytes of exposed workers was performed, based on five studies published so far. While two studies with high exposure showed significant increases in micronuclei, three studies gave negative results, which may be due to lower exposure levels [27].

5.2.2 Oxidative Stress

Reactive oxygen species (ROS) such as superoxide anions (O_2^-) are continuously generated not only as by-products of mitochondrial respiration, due to incomplete reduction of oxygen to H_2O but also formed in large quantities during the immune defense against invading pathogens in phagocytes via NADPH oxidase. Also, hydrogen peroxide (H_2O_2) is produced in specific cellular compartments and acts as a "second messenger" to regulate many important biological processes (for review see [28]). To enable the use of oxygen for energy production and yet to minimize oxygen-derived toxicity, a complex antioxidant network has evolved, including the scavenging of reactive species by glutathione and vitamins, the enzymatic conversion of highly reactive oxygen species to less harmful ones by superoxide dismutase, catalase, and glutathione peroxidase, and finally the repair or elimination of damaged macromolecules. However, even under normal cellular conditions, protection is not complete and for example, a measurable amount of oxidatively induced DNA damage exists in mammalian cells. Oxidative stress occurs if the equilibrium between the generation of ROS and the efficiency of their detoxification is disrupted [29]. Elevated

levels of ROS have been implicated in the initiation as well as in later steps of carcinogenicity. Especially transition metal ions play an important role in the induction of oxidatively induced DNA damage. While neither superoxide anions nor hydrogen peroxide are able to react with DNA directly, in the presence of transition metals like iron, copper, cobalt, or nickel, they are converted into highly reactive hydroxyl radicals by Fenton-type reactions. DNA damage induced by ROS includes a range of lesions like DNA base modifications, sugar lesions, DNA single- and double-strand breaks, DNA–protein crosslinks, and abasic sites (for review see [30]). Among these, several oxidatively induced DNA base modifications such as 8-oxoG have miscoding and thus premutagenic properties and therefore may act as initiators in carcinogenesis [31]. In contrast, cadmium ions are not able to participate in redox reactions under physiological conditions, yet, oxidative stress and the interference with cellular redox regulation may be of high relevance in cadmium-induced carcinogenicity. Increased levels of ROS due to cadmium exposure have been observed both in vitro and in vivo [32]. Thus, different cadmium compounds have been shown to induce DNA strand breaks and oxidatively induced DNA base modifications in mammalian cells, but effects were usually small and/or restricted to comparatively high concentrations (e.g., [33, 34]). Similarly, the induction of DNA strand breaks and chromosomal aberrations by cadmium in mammalian cells was suppressed by antioxidants and antioxidant enzymes, indicating the involvement of ROS [35–37]. A pronounced oxidative stress response was also observed on the transcriptional level after exposure to BEAS-2B and A549 cells [38]. Even though cadmium ions themselves are not redox-active, several indirect effects may account for these observations, namely the release of Fenton-reactive metal ions from metallothioneins [39], the disturbance of the mitochondrial respiratory chain [40] and the inhibition of antioxidant enzymes, such as catalase, superoxide dismutase, glutathione reductase, and glutathione peroxidase [29]. Regarding oxidative DNA damage and resulting clastogenicity in cells and in vivo, an impaired DNA repair appears to be of major relevance (see below). Thus, ROS may be involved in cadmium-induced genotoxicity, but—perhaps more important—also in later steps of cadmium-induced carcinogenicity. With respect to the latter, moderately elevated levels of ROS have been implicated in later steps of tumor formation, such as cell proliferation due to mitotic stimuli and the activation of redox-sensitive transcription factors (see below). Furthermore, due to its reactivity towards thiol groups, cadmium may interfere directly with redox-controlled signaling pathways [37, 41, 42].

5.3 Interactions with DNA Repair and Tumor Suppressor Functions

Maintenance of genetic information is essential for basically all cellular processes and for the prevention of tumor development. However, many environmental agents as well as food mutagens have been identified which compromise genetic stability by

inducing different types of DNA lesions. They include ionizing radiation, UV radiation, alkylating agents, polycyclic aromatic hydrocarbons as well as heterocyclic aromatic amines. Furthermore, the DNA is also damaged by endogenous processes, such as ROS generation due to leakage of the electron transport chain in cellular respiration [37, 43]. DNA damage interferes with DNA transcription and replication; potential consequences are cell cycle arrest, programmed cell death, mutagenesis, genomic instability, and cancer. To maintain the integrity of the genome, a complex DNA damage response network has evolved, consisting of DNA repair systems, cell cycle control, and apoptosis in case of heavily damaged DNA [44–46]. Cadmium has been shown to impair almost all major DNA repair pathways. Convincing evidence is available for its interference with nucleotide excision repair (NER), base excision repair (BER), and mismatch repair (MMR), with some information also on its impact on DNA double-strand break repair; frequently, effects were observed at comparatively low, noncytotoxic concentrations (reviewed in [47–50]). Since DNA repair systems are not only required for the repair of DNA damage induced by environmental, workplace, and food mutagens but also for the elimination of DNA lesions due to endogenous processes and to keep replication errors low, the disturbance of DNA repair processes may explain co-mutagenic effects in combination with UVC radiation, benzo[*a*]pyrene and alkylating agents on one side but may also lead to genomic instability and thus contribute to cadmium-induced carcinogenicity on the other side (for reviews see [22, 23, 49]).

5.3.1 Nucleotide Excision Repair (NER)

NER is the most versatile repair system involved in the removal of structurally unrelated bulky base adducts which cause significant helical distortions. It can be subdivided into global genome repair (GG-NER) and, as a sub-pathway, transcription-coupled nucleotide excision repair (TC-NER), which removes preferentially transcription-blocking bulky DNA lesions. At least 30 different proteins and enzymes are required in mammalian cells, including those which are defective in patients suffering from the DNA repair disorder Xeroderma Pigmentosum (XP) complementation groups A through G. The most crucial step is the damage recognition, followed by the incision at both sides of the lesion and the repair polymerization leading to the displacement of the damaged oligonucleotide; repair is completed by the ligation of the repair patch (for reviews see [44–46, 51]). Cadmium has been shown to inhibit GG-NER in several studies and with respect to different DNA damaging agents. Thus, it interfered with the removal of benzo[*a*]pyrene- and UVC-induced DNA lesions in cultured mammalian cells [34, 52, 53]. As one underlying mechanism, an interaction with zinc-binding proteins has been identified. They comprise a family of proteins where zinc is complexed to four cysteine and/or histidine residues, folding a protein domain mostly involved in DNA-protein- or protein–protein interactions [54]. First discovered in transcription factors, similar structures have been identified in DNA repair proteins and tumor suppressor proteins like p53.

Examples for DNA repair proteins with zinc-binding structures include the bacterial formamidopyrimidine-DNA glycosylase (Fpg) involved in the removal of oxidative DNA base modifications and the mammalian Xeroderma Pigmentosum group A protein (XPA) essential for the formation of the DNA damage recognition complex during NER (reviewed in [55, 56]). In subcellular test systems, cadmium diminished DNA binding of XPA to an UVC-irradiated oligonucleotide [57, 58]. One molecular mechanism related to the inactivation of zinc-binding proteins appears to involve the displacement of zinc by cadmium, as evident from the reversal of cadmium-induced protein inactivation by excess of zinc as well as from structural investigations of XPA or a peptide resembling the zinc-binding domain of XPA [57–60]. Detailed studies in cadmium-treated A549 cells revealed an impaired assembly/disassembly of the DNA damage recognition proteins XPC and XPA at the repair complex after UVC irradiation [34].

5.3.2 Base Excision Repair (BER)

In contrast to the damage recognition complex in NER that detects a rather broad spectrum of DNA lesions, BER is initiated by glycosylases, which act specifically on one or few substrates. BER is mainly responsible for the removal of different types of endogenous DNA damage, including oxidative DNA base modifications like 8-oxoguanine (8-oxoG). This process generates abasic (AP) sites, which are further processed in a multistep process with slight differences depending on the type of damage [43–45, 51]. Regarding the impact of cadmium on this repair pathway, low concentrations of cadmium inhibited the activity of the bacterial Fpg [61] and disturbed the repair of oxidatively induced DNA base damage induced by visible light as well as DNA alkylation damage in mammalian cells [33, 62]. When compared with the induction of DNA base modifications such as 8-oxoG, inhibitory effects on the repair of this lesion were observed at much lower cadmium concentrations. This has been observed by direct comparison in HeLa cells: While the induction of DNA strand breaks by cadmium was restricted to 10 μM and higher, the removal of oxidatively induced DNA base modifications by visible light and recognized by the bacterial Fpg was inhibited starting at 0.5 μM cadmium, yielding complete inhibition at 5 μM, a noncytotoxic concentration in this test system [33]. With respect to isolated DNA repair enzymes, an inhibition of the murine 8-oxoguanine DNA glycosylase 1 (mOgg1), an enzyme responsible for recognition and excision of the premutagenic 8-oxoG during BER, as well as of 8-oxodG 5'triphosphate pyrophosphohydrolase (8-oxo-dGTPase), required for the removal of 8-oxo-dG from the deoxynucleotide pool, by cadmium have been described [63, 64]. Also, cadmium has been shown to inhibit the activity of the human 8-oxoguanine glycosylase (hOGG1) in mammalian cells [65–67]. Different mechanisms may be responsible, based on different experimental results, including the inactivation of the enzyme as such [66] or the diminished DNA binding of the zinc finger containing transcription factor SP1 to the OGG1 promotor [68], presumably due to the displacement of zinc by cadmium [69].

Inhibition of the repair of oxidatively induced DNA damage is also evident in vivo: When investigating, for example, the impact of cadmium on rat testis, a target organ for cadmium carcinogenesis, a gradual decrease in testicular 8-oxo-dGTPase activity was observed, accompanied with progressive increase of 8-oxo-dG levels in testicular DNA [70]. Therefore, increases in oxidatively induced DNA damage in vivo may at least in part be due to the repair inhibition and accumulation of endogenously induced DNA lesions. One other enzyme involved in DNA damage signaling, apoptosis and BER is poly(ADP-ribose) polymerase 1 (PARP-1). It contains three zinc fingers in its DNA binding domain involved in the recognition of DNA breaks and the subsequent synthesis of poly(ADP-ribose) [71, 72]. In HeLa cells, H_2O_2-induced PARP activity was decreased by cadmium chloride [73].

5.3.3 Mismatch Repair (MMR)

One other DNA repair system of particular relevance for maintaining genomic stability is MMR. This evolutionary conserved pathway is responsible for the repair of mismatched normal bases after DNA replication, contributing significantly to the extraordinary fidelity of DNA replication. Cells deficient in MMR exert a "mutator phenotype", in which the rate of spontaneous mutations is greatly elevated. Also, microsatellite instability (MSI) is a hallmark of MMR deficiency. Defects in MMR are associated with an increased risk of different types of cancer, including hereditary human colorectal cancer. The MMR system also plays a key role in cell killing in response to alkylating agents, and MMR deficient cells are about 100 times more resistant to the cytotoxicity of alkylating agents [74–77]. Finally, MMR also participates in the DNA damage response (DDR) system by activating ATM and ATR, which regulate cell cycle control and apoptosis upon elevated levels of DNA damage [78]. With respect to cadmium, exposure towards low concentrations resulted in pronounced hypermutability in yeast. Furthermore, in extracts of human cells, cadmium inhibited at least one step leading to mismatch repair [79]. Since then, different studies demonstrated the interference by cadmium with proteins involved in the initial step of MMR, i.e., damage recognition by MSH2-MSH6 and MSH2-MSH3. Also, the induction of MSI was demonstrated in mice testis [80]. As underlying mechanisms, cadmium affected ATP binding and hydrolysis of MMR enzymes, reducing their DNA binding activity and their ability to discriminate between mismatched and matched DNA base pairing in isolated systems and in mammalian cells in culture [48, 81, 82]. Furthermore, the induction of MSI concomitantly with elevated levels of oxidatively induced DNA damage has recently been shown in human colorectal cancer cells and in zebrafish at sublethal cadmium concentrations; interestingly, in both systems, N-acetyl-L-cysteine (NAC) suppressed cadmium-induced MSI, thus linking MMR inhibition to oxidative stress [83].

5.3.4 DNA Double-Strand Break Repair (DSB)

Only little is known about the impact of cadmium on DNA double-strand break repair. Current evidence suggests that cadmium leads to the formation of DSB and inhibits their repair via nonhomologous end-joining (NHEJ) via interaction with the DNA-PK protein, leading to an over-activation of the MRE11-dependent repair pathway which in turn may favor more misrepair [84]. The delay of NHEJ upon γ-irradiation was confirmed in another study; here, the authors demonstrated an altered phosphorylation of DNA-PKcs as well as reduced expression of XRCC4 and Ligase IV [85]. Also, a significant downregulation of genes coding for ATM and BRCA1 associated with DSB repair was observed via high-throughput RT-qPCR [38]. Finally, when assessing the impact of several metal compounds on DNA double-strand break repair outcomes, exposure towards cadmium was shown to increase mutagenic, nonallelic recombination [86].

5.3.5 P53 Tumor Suppressor Functions

Besides DNA repair systems, further DNA damage responses are activated upon genotoxic stress in mammalian cells. They include cell cycle control mechanisms, increasing the time for DNA repair, as well as apoptosis eliminating heavily damaged cells. The DNA damage response is strictly coordinated, for example by the tumor suppressor protein p53. P53 regulates cell cycle control and apoptosis by several coordinated pathways and thus exerts pronounced impact on the processing of DNA damage and on genomic stability [87]. Cadmium has been shown to interfere with structure and function of p53, but opposite effects have been reported. In some studies, a stabilization of p53 through phosphorylation followed by the induction of the p53-mediated stress response was observed [88–90]; others demonstrated an inactivation of p53 via structural changes [34, 91]. P53 contains a zinc-binding structure in its DNA binding domain, essential for its tumor suppressor functions and rendering the protein redox-sensitive. Exposure of either the isolated p53 protein or human breast cancer MCF7 cells to cadmium resulted in the disruption of the zinc-binding structure, yielding a so-called “mutant” conformation; consequences were the inhibition of DNA binding and the inhibition of the activation of p53 target genes including p21. Furthermore, suppression of the p53-mediated cell cycle arrest in response to DNA damage induced by γ-irradiation was observed [91]. Similar effects were demonstrated in A549 human lung tumor cells, where CdO and $CdCl_2$ induced structural alterations of the zinc-binding domain of p53, followed by diminished induction of the p53 regulated nucleotide excision repair gene XPC and diminished removal of UVC and benzo[*a*]pyrene induced DNA damage [34]. Thus, it appears that cadmium disrupts the zinc-binding structure of p53; whether or not this is due to the displacement of zinc is currently not clear.

5.4 Impact on Gene Expression Related to Genomic Instability and Deregulation of Cell Proliferation

Cadmium interacts with the expression of a large number of genes, including stress response genes and immediate early response genes. Major stress response genes induced by cadmium are those involved in the synthesis of metallothionein (MT), those encoding heat shock proteins, glutathione (GSH) synthesis and homeostasis and those mediating the oxidative stress response [23, 24, 42, 92–94]. Recently, the impact of cadmium was investigated via a high-throughput RT-qPCR test system specifically designed for the parallel and quantitative analysis of 95 selected genes crucial for genomic stability, including stress response as well as DNA repair, cell cycle control, apoptosis, and mitotic signaling. Gene expression analyses in cadmium-treated adenocarcinoma A549 and epithelial bronchial BEAS-2B cells revealed distinct dose- and time-dependent and also cell-type specific gene expression patterns, including the induction of genes coding for metallothioneins, the oxidative stress response, cell cycle control, mitotic signaling, and apoptosis. Interestingly, while genes coding for the DNA damage response were induced, distinct DNA repair genes were downregulated at the transcriptional level. Thus, this approach provided a comprehensive overview on the interaction by cadmium with distinct signaling pathways, also reflecting molecular modes of action in cadmium-induced carcinogenicity on the functional level, such as inhibition of DNA repair and tumor suppressor reactions [38]. On the molecular level, specific interactions with transcriptions factors, an interference with cellular redox regulation [42] as well as epigenetic alterations appear to be most relevant. With respect to transcription factors, cadmium exposure may lead to their activation or inactivation, depending on the actual transcription factor under investigation. Thus, c-fos and c-jun are overexpressed in cadmium-transformed cells; they constitute the AP-1 transcription factor, activating several genes involved in cell growth and division. Other transcription factors activated by cadmium are NF-κB and Nrf2. On the other hand, a suppression of the transcription factor SP1 has been observed in cadmium-treated cells (reviewed in [93, 94]). In support of a role for a redox-mediated mechanism in cadmium-induced activation of the MAPK-pathway, cadmium was shown to inhibit serine/threonine phosphatases 2A (PP2A) and 5 (PP5), leading to the activation of Erk1/2 and JNK. This effect was inhibited by N-acetyl-L-cysteine (NAC), and resembled the effects induced by hydrogen peroxide; therefore, the authors interpreted this effect being due to the enhanced generation of ROS [95, 96]. Nevertheless, since these phosphatases contain critical cysteine residues [97], the impact of cadmium may also be explained by a direct reaction with these cysteines. One other example is the cadmium-induced activation of Nrf2 [98, 99]. The Kelch-like ECH-associated protein (Keap1) serves as negative regulator of Nrf2. It contains critical cysteine residues as redox sensors; their oxidation results in the release of Nrf2 from the Keap1/cullin-3 E3-ubiquitin ligase (cul3) complex, preventing Nrf2 degradation and allowing for Nrf2 nuclear translocation. Additionally, Nrf2 contains a conserved cysteine located in the DNA binding domain (Cys-514) redox-regulated by Ref-1. After treatment of mouse embryo fibroblasts

(MEF) with cadmium, Nrf2-Keap1 were stabilized in the cytoplasm and translocated to the nucleus, where the components dissociated. This was followed by the induction of the ARE-dependent expression of HO-1 in MEF cells and in a respective reporter gene assay [98].

In addition to directly stimulating mitogenic signals, cadmium also inhibits negative controls of cell proliferation, for example, by inactivation of p53 (see above) [91]. Furthermore, long-term treatment of prostate epithelial cells resulted in cadmium-induced malignant transformation; transformed cells exerted an acquired resistance to apoptosis, which appeared to be linked to an increase in the antiapoptotic action of Bcl-2 that perturbs the JNK signal transduction pathway [100]. One other aspect related to cadmium-induced alterations in gene expression consists in epigenetic changes. Three levels of interaction appear to be relevant, namely interference with DNA methylation patterns, histone modifications and miRNAs, all of which may be affected by cadmium in a time- and cell-type specific manner (for review see [101]). The mechanism in cadmium-induced epigenetic alterations has been further investigated in a rat liver epithelial cell line as a model for cadmium-induced malignant transformation. Cadmium provoked the suppression of ApoE, a key factor of cell invasion during malignant transformation via 5-aza-2'-deoxycytidine-sensitive hypermethylation of the regulatory region of ApoE, together with the suppression of liver X receptor α (LXRα), a transcriptional regulator for ApoE [102].

5.5 Mechanistic Considerations

Cadmium appears to be involved in tumor initiation as well as in later steps of tumor development. Regarding genotoxicity, direct interactions of cadmium ions with DNA are of little importance, as evident also by the lack of relevant mutagenicity in classical bacterial and mammalian test systems. However, indirect genotoxicity leads to clastogenicity and elevated levels of oxidative DNA damage; in this context, interactions with proteins are of high significance. Especially the DNA repair inhibitions but also altered cell proliferation and/or diminished cell cycle control have frequently been observed at low, noncytotoxic concentrations of cadmium, pointing towards particular sensitive targets of cadmium ions. Relevant mechanisms include elevated levels of ROS, interactions with homeostasis and cellular functions of essential metal ions like zinc, calcium, and iron and the interference with cellular redox regulation.

Current evidence suggests that Cd^{2+} is the ultimate damaging species since water-soluble and particulate water-insoluble cadmium compounds exert similar effects in experimental cell culture systems and in experimental animals. Thus, both cadmium chloride and largely water-insoluble cadmium oxide induced oxidative DNA lesions and inhibited the removal of benzo[*a*]pyren-induced DNA lesions. Furthermore, cadmium-induced conformational changes of p53 were comparable when applying cadmium chloride or cadmium oxide. Repair inhibitory effects were strongly correlated with cadmium levels in the nuclei, indicating the bioavailability of both compounds [34]. While water-soluble cadmium compounds are taken up via ion

channels [103], particulate cadmium compounds may be taken up by phagocytosis and, due to the low pH, may dissolve gradually in lysosomes, yielding high concentrations of cadmium ions in the cytoplasm and in the nucleus, as described in detail for nickel compounds [104, 105]. This assumption is also supported by inhalation studies where water soluble cadmium sulfate, poorly water-soluble cadmium oxide and cadmium sulfide pigment with intermediate water solubility induced lung tumors in rats [106].

Since cadmium ions exert high affinity towards SH groups, potential targets are zinc-binding structures frequently found in transcription factors as well as in DNA repair and tumor suppressor proteins [55, 56]. As indicated above, one molecular mechanism related to the inactivation of zinc-binding proteins appears to involve the competition between zinc and cadmium. Compared to Zn^{2+}, the radius of Cd^{2+} ion is larger, but still, cadmium ions can substitute for zinc ions in many enzymes and transcription factors [24, 107]. Considering the example of the nucleotide excision repair protein XPA or a peptide resembling the zinc-binding domain of XPA, binding constants for cadmium were about 1000-fold higher as compared to zinc. Replacement of zinc by cadmium yielded only minor structural alterations [59, 60], but provoked a pronounced disturbance of XPA within the assembly and disassembly of the nucleotide excision repair complex [34]. In addition to direct interactions with DNA repair proteins, cadmium may disturb DNA repair processes via interaction with zinc-containing transcription factors. Thus, human OGG1 (hOGG1), a glycosylase responsible for recognition and excision of the premutagenic 8-oxoG during BER in mammalian cells, was inhibited by cadmium [108]. Even though hOGG1 contains no zinc-binding motif itself, its inhibition was shown to be due to diminished DNA binding of the zinc finger containing transcription factor SP1 to the *OGG1* promotor [68], presumably due to the displacement of zinc by cadmium [69]. Also, a downregulation of DNA repair genes like *XPC* has been observed recently in cultured cells [34] and in vivo in mouse testes [109], which may be due to a disturbed transcriptional activity of p53. However, whether or not the inactivation of the respective zinc-binding repair proteins are mediated via displacement of zinc by cadmium or whether interactions with other protein structures, such as critical thiols outside the zinc-binding structure, are relevant for the observed inhibitions has to be further elucidated. Furthermore, systematic investigations on the relevance of these mechanisms for in vivo situations are still missing. Multiple mechanisms appear to be involved in cadmium-induced alterations of gene expression. With respect to the induction of metallothionein, cadmium ions bind directly to the transcription factor MTF1 [110]. In some other cases, specific interactions have been identified. With respect to epigenetic effects, cadmium inhibited DNA-(cytosine-5) methyltransferase and lead to diminished DNA methylation during cadmium-induced cellular transformation, provoking augmented expression of cellular proto-oncogenes [111]. One example for a direct competition with calcium concerns the cadherin-mediated cell–cell adhesion system; here, cadmium specifically displaced calcium from the protein E-cadherin and impaired the cell–cell adhesion in kidney epithelial cells [112, 113]. A fast transient increase in levels of second messengers like Ca^{2+} and inositol-1,4,5-trisphosphate by low concentrations of cadmium may be due to its binding to G-protein coupled receptors in

the plasma membrane; however, cadmium affects also intracellular signaling mediated by mitogen-activated protein kinases (MAPK) as well as cAMP-dependent and calmodulin-dependent pathways (see Chap. 3). Even though zinc-binding structures are involved in many of these pathways, at present, it is unclear whether a direct replacement of zinc is the underlying mechanism [24, 94, 114]. One hypothesis integrating many mechanistic observations consists in the interference by cadmium with the cellular redox regulation [42] (see also Chap. 3). Thus, diverse signaling pathways have been identified to be redox-regulated via reversible oxidation and reduction of thiol groups [115–117]. Cadmium has been shown to induce several redox-regulated signal transduction pathways, such as NF-κB and Nrf2, but also mitotic signaling, which may be due to the increased formation of ROS or to direct interaction with redox-sensitive cysteines in signal transduction proteins. In most cases, the molecular interactions have not been fully explored experimentally, but are subject of current research activities.

5.6 Concluding Remarks and Perspectives

In summary, cadmium-induced carcinogenicity is likely based on multiple distinct mechanisms. As opposed to direct DNA damage, interactions with proteins appear to be more relevant for carcinogenicity, and several targets have been identified such as antioxidative defense systems, DNA repair processes as well as tumor suppressor and signal transduction proteins. All these features taken alone could contribute to carcinogenicity, but most likely their combination seems to be of particular importance. Thus, long-term exposure to low concentrations of cadmium leads to adapted cells exerting increased cadmium accumulation, increased proliferation, diminished DNA repair, and cell cycle control as well as resistance to apoptosis. The outcome is a severe decrease in genomic stability, which may play an important role in cadmium-induced tumor initiation and progression (summarized in Fig. 5.1).

One important question concerns specific mechanisms explaining the organ-specificity of cadmium-induced carcinogenicity. After inhalative exposure, the lung is a plausible target organ, but other organs like kidney, prostate, breast, and endometrium may be affected as well. Since tumors in prostate, breast, and endometrium are frequently hormone-dependent one aspect addressed by several groups concerns a potential impact of cadmium on steroid hormone-dependent signaling [118]. Nevertheless, respective experimental evidence is contradictory and needs to be further explored [49]. One other key issue in cadmium-induced carcinogenicity appears to be adaptation and the role of MT. Cadmium induces several genes for cadmium and ROS tolerance such as those coding for MT, GSH synthesis and function, catalase and superoxide dismutase. Hence, a condition for prolonged cell survival in the presence of cadmium is established, which may be beneficial in terms of protection from acute cadmium toxicity, also evident from comparative studies with MT-transgenic and MT-null mice [119]. However, adaptation may be a double-edged sword, since increased MT contents lead not only to cadmium accumulation

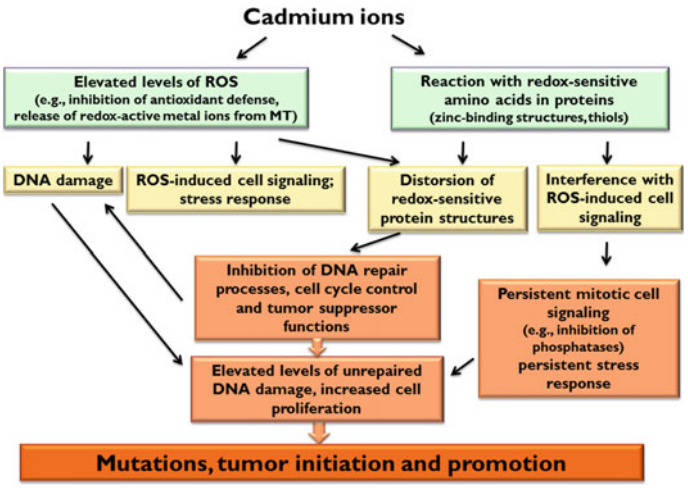

Fig. 5.1 Integrating concept of cadmium-induced carcinogenicity

and long half-lives but also to reduced DNA repair activities as well as suppressed apoptosis [92, 120].

Considering recent reports on cadmium-related carcinogenicity in different target organs under low exposure conditions, future research will have to focus on the relevance of the respective mechanisms in experimental animals and in exposed humans. Nevertheless, even though Cd blood levels are in the nanomolar concentration range and thus considerably lower as compared to low micromolar concentrations applied in most in vitro systems described above, it needs to be considered that far higher cadmium concentrations are observed for example in cadmium-exposed workers, reaching up to millimolar concentrations in the kidney cortex and high micromolar concentrations in the liver [121]. Also, indications for repair inhibition upon occupational cadmium exposure have been reported [122].

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