A REGULATORY MECHANISM OF ZINC HOMEOSTASIS INVOLVING THE MEDIATOR SUBUNIT MDT-15 AND THE TRANSCRIPTION FACTOR HIZR-1

by

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Abstract

Zinc is a metal that is essential for cell function as it plays important catalytic and structural roles in many proteins; however, excess zinc causes cell stress. Cadmium has similar chemical properties as zinc but is toxic and not required in biological systems. To maintain homeostasis, the levels of zinc detoxification genes are modulated through transcriptional regulation, which allows organisms to adapt to environmental changes. The key players in transcriptional regulation are Transcription Factors (TF), regulatory DNA elements, and coregulators such as the Mediator complex. Mediator subunit MDT-15 is required for the regulation of stress response genes in Caenorhabditis elegans, including zinc responsive genes. However, MDT-15’s physiological role and its regulatory partners in zinc homeostasis and cadmium stress response remain unknown. In this study, I investigated which TFs collaborate with MDT-15 to regulate zinc homeostasis and cadmium stress response genes, and I also examined its physiological role in zinc homeostasis. I used a fusion of the promoter of the zinc and cadmium responsive gene cdr-1 to Green Fluorescent Protein (GFP) and real-time PCR analysis as sensitive readouts to study metal response mechanisms. I found that cdr-1 induction by zinc and cadmium depends on Mediator subunits mdt-15 and cdk-8, and the TFs high zinc activated nuclear receptor-1 (hizr-1) and elt-2. Using genetic interaction studies, I found that HIZR-1 and MDT-15 function is codependent, and showed, using the yeast-two-hybrid system, that the two proteins interact physically. Interestingly, this physical association was enhanced by micromolar zinc and cadmium. To assess zinc storage, I studied the gut granules of C. elegans, which store and replenish zinc to maintain homeostasis, and found storage defects in mdt-15 and hizr-1 mutants. Lastly, I explored the regulatory conservation of this regulatory mechanism. The Insulin Secretory Granules in pancreatic β-cells require appropriate amounts of zinc to crystallize...
insulin. Using mice lacking the \textit{mdt-15} ortholog \textit{Med15} in the \(\beta\)-cells, I found that \textit{Med15} is required to express \textit{Slc30a8}, the ortholog of the \textit{mdt-15}-regulated zinc transporter \textit{cdf}-2. Collectively, my data show that \textit{mdt-15} and \textit{hizr-1} cooperate to regulate metal detoxification genes and zinc storage, through a mechanism that possibly is conserved.
Lay Summary

Zinc is a crucial metal for life, but it is important to regulate proper zinc amounts in the cell, because zinc excess or deficiency can cause disease. To regulate zinc levels, the expression of certain genes is switched ‘on' and ‘off' as needed, a process that involves various proteins. My research focuses on the proteins Mediator MDT-15, and the transcription factor HIZR-1; both play a role in detoxifying zinc. In my studies, I used C. elegans, a worm widely used for genetic studies of environmental stress responses. I found that MDT-15 and HIZR-1 physically interact, and that both are important for zinc storage in the intestine. I also examined if this regulatory mechanism is conserved in mammals and found similarity between the factors in C. elegans and the β-cells of the mouse pancreas.
Preface

I designed, conducted and/or supervised all experiments, and analyzed and interpreted all data generated, except the following:

Section 2.1, RNA samples of worms grown on cadmium (N2 and cdk-8(tm1238) worms) were provided by Jennifer Grants.

Sections 2.2 and 2.3, Jennifer Grants constructed the wild-type and mutGATA1 and mutGATA2 cdr-1::GFP reporters and conducted the experiments using these reporters in control(RNAi), mdt-15(RNAi), elt-2(RNAi), skn-1(RNAi), hsf-1(RNAi), and cdk-8(tm1238) worms grown on cadmium. Parts of these sections were included in Jennifer Grants’ thesis “Roles of mediator subunit CDK-8 in developmental and physiological responses in Caenorhabditis elegans”, 2016.

Section 2.4, Forum Bhanshali conducted one biological repeat of qPCR analysis of wild-type and mdt-15(yh8) worms grown on control and hizr-1 RNAi.

Section 2.8, Alex Kadhim and I collaboratively produced the images using a confocal microscope.

Section 2.11, Alex Kadhim prepared and provided the pancreas slides; together, we performed the confocal microscopy imaging.

A manuscript including the majority of the data in this thesis is in preparation for submission to a peer reviewed journal: Shomer N.#, Kadhim A.Z.#, Grants J.M., Xu E.E., Poon A., Lee M.Y.Y., Muhuri A., Lee D., Lee S.J.V., Lynn F.C., and Taubert S., “Toxic metal stress response and essential trace metal homeostasis require Mediator and HNF4-like nuclear receptors in worms and mouse pancreatic β-cells”. As co-first other with Alex Kadhim, I
analyzed and interpreted the data for all experiments on the *C. elegans* model, generated the figures, and will write the majority of the paper.
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<tr>
<td>AD</td>
<td>Activation domain</td>
</tr>
<tr>
<td>Adh1</td>
<td>Alcohol dehydrogenase 1</td>
</tr>
<tr>
<td>AE</td>
<td>Acrodermatitis enteropathica</td>
</tr>
<tr>
<td>ALS</td>
<td>Amyotrophic lateral sclerosis</td>
</tr>
<tr>
<td>CDF</td>
<td>Cation diffusion facilitator</td>
</tr>
<tr>
<td>CDG</td>
<td>Congenital disorder of glycosylation</td>
</tr>
<tr>
<td>CDK-8</td>
<td>Cyclin dependent kinase 8</td>
</tr>
<tr>
<td>cdr-1</td>
<td>Cadmium responsive gene 1</td>
</tr>
<tr>
<td>DBD</td>
<td>DNA binding domain</td>
</tr>
<tr>
<td>DBE</td>
<td>DAF-16 binding element</td>
</tr>
<tr>
<td>DCT-1</td>
<td>Divalent cation transporter 1</td>
</tr>
<tr>
<td>E2F</td>
<td>E2 factor</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
</tr>
<tr>
<td>EV</td>
<td>Empty vector</td>
</tr>
<tr>
<td>Foi</td>
<td>Fear of intimacy</td>
</tr>
<tr>
<td>gf</td>
<td>Gain-of-function</td>
</tr>
<tr>
<td>GFP</td>
<td>Green fluorescent protein</td>
</tr>
<tr>
<td>GWAS</td>
<td>Genome-wide association study</td>
</tr>
<tr>
<td>hizr-1</td>
<td>High zinc activated nuclear receptor 1</td>
</tr>
<tr>
<td>HNF4</td>
<td>Hepatocyte nuclear factor 4</td>
</tr>
<tr>
<td>HZA</td>
<td>High zinc activated</td>
</tr>
<tr>
<td>ISG</td>
<td>Insulin Secreting Granules</td>
</tr>
<tr>
<td>Keap1</td>
<td>Kelch-like erythroid cell-derived protein with CNC homology [ECH]-associated Protein 1</td>
</tr>
<tr>
<td>KIX</td>
<td>kinase-inducible domain (KID) interacting domain</td>
</tr>
<tr>
<td>KO</td>
<td>Knockout</td>
</tr>
<tr>
<td>LBD</td>
<td>Ligand-binding domain</td>
</tr>
<tr>
<td>lf</td>
<td>Loss-of-function</td>
</tr>
<tr>
<td>LIM</td>
<td>Lin11, Isl-1 and Mec-3</td>
</tr>
<tr>
<td>Loz1</td>
<td>Loss of zinc sensing 1</td>
</tr>
<tr>
<td>Lm</td>
<td>Lethal milk</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>LZA</td>
<td>Low zinc activation</td>
</tr>
<tr>
<td>MDT</td>
<td>Mediator</td>
</tr>
<tr>
<td>MODY</td>
<td>Maturity onset diabetes of the young</td>
</tr>
<tr>
<td>MRE</td>
<td>Metal regulatory element</td>
</tr>
<tr>
<td>MT/mtl</td>
<td>Metallothionein</td>
</tr>
<tr>
<td>MTF-1</td>
<td>Metal-responsive transcription factor 1</td>
</tr>
<tr>
<td>NHR</td>
<td>Nuclear hormone receptor</td>
</tr>
<tr>
<td>Nrf2</td>
<td>Nuclear factor erythroid 2–Related Factor 2</td>
</tr>
<tr>
<td>PC</td>
<td>Phytochelatin</td>
</tr>
<tr>
<td>Term</td>
<td>Definition</td>
</tr>
<tr>
<td>--------</td>
<td>--------------------------------------------------------------</td>
</tr>
<tr>
<td>Pol2</td>
<td>RNA polymerase 2</td>
</tr>
<tr>
<td>qPCR</td>
<td>Quantitative PCR</td>
</tr>
<tr>
<td>rf</td>
<td>Reduction-of-function</td>
</tr>
<tr>
<td>RING</td>
<td>Really interesting new gene</td>
</tr>
<tr>
<td>RNAi</td>
<td>RNA interference</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>SCD-EDS</td>
<td>Spondylocheirodysplastic Ehlers-Danlos syndrome</td>
</tr>
<tr>
<td>SLC</td>
<td>Solute-linked carrier</td>
</tr>
<tr>
<td>SNP</td>
<td>Single-nucleotide polymorphism</td>
</tr>
<tr>
<td>SOD1</td>
<td>Superoxide dismutase 1</td>
</tr>
<tr>
<td>SREBP</td>
<td>Sterol regulatory element-binding protein</td>
</tr>
<tr>
<td>sur-7</td>
<td>Suppressor of Ras 7</td>
</tr>
<tr>
<td>T1D</td>
<td>Type 1 diabetes</td>
</tr>
<tr>
<td>T2D</td>
<td>Type 2 diabetes</td>
</tr>
<tr>
<td>TF</td>
<td>Transcription factor</td>
</tr>
<tr>
<td>TMD</td>
<td>Transmembrane domain</td>
</tr>
<tr>
<td>TPEN</td>
<td>N,N,N',N'-tetrakis (2-pyridinylmethyl)-1,2-ethanediamine</td>
</tr>
<tr>
<td>TNZD</td>
<td>Transient neonatal zinc deficiency</td>
</tr>
<tr>
<td>ttm-1</td>
<td>Toxin-regulated target of p38 MAPK</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular Endothelial Growth Factor</td>
</tr>
<tr>
<td>WT</td>
<td>Wild type</td>
</tr>
<tr>
<td>Y2H</td>
<td>Yeast-two-hybrid</td>
</tr>
<tr>
<td>Zap</td>
<td>Zinc responsive activator protein 1</td>
</tr>
<tr>
<td>ZIP</td>
<td>Zrt- and Irt-like proteins</td>
</tr>
<tr>
<td>ZnF</td>
<td>Zinc finger</td>
</tr>
<tr>
<td>ZnT</td>
<td>Zn transporters</td>
</tr>
</tbody>
</table>
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To my parents and brother who support me no matter what

To my loving husband who urges me to follow my dreams

And to my little Maya who gives me a reason to do everything the best way I can
Chapter 1: Introduction

1.1 Metals in biological systems

Metals play a major role in biological systems. They comprise 17 of 30 elements necessary for life (e.g. iron, cobalt, copper, zinc), and are present in more than one third of protein structures, where they play a role in ligand-binding, function as redox centers, and act as molecular sensors in signal transduction (Thiele 1992; Sun 2010; Guengerich 2012; Waldron et al. 2009). A systematic survey of 1,371 different enzymes estimated that 47% of these proteins require metals for proper structure and/or function, while 41% contain metals at their catalytic sites (typically a transition metal, such as iron, copper, and zinc) (Andreini et al. 2008). However, essential metals are a ‘double-edged sword’, as excess of these metals causes toxicity to the cell. Therefore, their levels and bioavailability need to be regulated precisely. Unlike these essential elements, some other metals have no known biological function and are highly toxic (e.g. cadmium). Lastly, there is a third group of metals that are neither essential nor toxic and are widely used in human healthcare (e.g. platinum in some forms) (Sun 2010; Thiele 1992).

1.1.1 Zinc is an essential trace metal

Due to its chemical properties, zinc is widely used in cellular processes. Under physiological conditions, zinc does not undergo reduction or oxidation, can function both as an acid and a base, and has a variable coordination sphere that contributes to its biochemical versatility (Vallee and Falchuk 1993). Accordingly, zinc is necessary for the function of ~10% of proteins in the human proteome and ~8% of proteins in the Caenorhabditis elegans proteome (Andreini et al. 2006).
Zinc has structural, catalytic, or coactivator roles in different types of proteins, including metabolic enzymes (e.g. oxidoreductases, hydrolases, transferases, lyases, ligases, isomerases) and growth factors. It maintains the structural integrity of many TFs by binding to various zinc-binding motifs, including Lin11, Isl-1 and Mec-3 (LIM) domains, the Zinc finger (ZnF) domains, and the Really Interesting New Gene (RING) finger domains (Vallee and Falchuk 1993; Murakami and Hirano 2008). In addition, zinc is also a signaling molecule, a neuromodulator in synaptic transmission, and an intracellular signal transducer (Fukada et al. 2011).

In line with zinc’s requirement in cellular functions, zinc deficiency causes a broad range of disorders and abnormalities in humans. Zinc deficiency results in impaired growth, hair loss, skin lesions, testicular atrophy, immune dysfunction, thymic atrophy, and cognitive impairment (Plum et al. 2010; Murakami and Hirano 2008). Vice versa, exposure to high doses of zinc is also detrimental and toxic to the cell, and can influence physiological programs such as immune responses, systemic growth, endocrine functions, and neuro-sensation (Fukada et al. 2011).

Collectively, this demonstrates that the control of cellular zinc levels is crucial for the maintenance of biological functions and the health of an organism.

1.1.2 Cadmium is a nonessential toxic metal

Cadmium is a metal encountered by biological organisms as an environmental contaminant. However, cadmium has no known function in biological systems, and instead is toxic. Cadmium exposure can induce intracellular damage along with the production of reactive oxygen species (ROS), which can cause protein oxidation, DNA strand breaks, and lipid peroxidation (Thevenod and Lee 2013). Cadmium accumulates in cells (in humans, mainly in the kidney and the liver) and has a biologic half-life of approximately 20 years (Cikrt et al. 1990).
In animals, short-term exposure to low levels of cadmium triggers a cellular stress response through the induction of cadmium responsive genes (e.g. detoxification genes), allowing cells to survive, adapt, and return to normal function (Thevenod and Lee 2013). However, long periods of sublethal cadmium exposure can cause deregulation of adaptive and survival mechanisms that will create a variety of adverse effects in the organism. Chronic exposure to cadmium results in its accumulation in the placenta, and will cause placental abnormalities, decrease in birth weight, and fetal malformations (Wier et al. 1990; Frery et al. 1993). In addition, cadmium exposure also leads to kidney damage by tubular dysfunction, bone lesions, respiratory disease, neurological disorders, and various types of cancers (Brzoska and Moniuszko-Jakoniuk 2001; Waalkes et al. 1992; Dong et al. 2005).

Importantly, zinc and cadmium have similar chemical characteristics. Both metals belong to group II B of the periodic table, and thus have a similar electron configuration and are close in elemental size (Waalkes et al. 1992; Maret 2016; Essig et al. 2016). Because of these similarities, the two metals bind many of the same proteins. However, in most cases, cadmium will bind with greater affinity than zinc, resulting in zinc displacement and molecular and biological dysfunction (Jacobson and Turner 1980; Jones and Cherian 1990; Gachot and Poujeol 1992; Gachot et al. 1994; Brzoska and Moniuszko-Jakoniuk 2001). Another important consequence of the similarity of these two metals is that they are eliminated via shared detoxification mechanisms; that is, many proteins involved in detoxification of zinc are also involved in the detoxification of cadmium (Fukada et al. 2011; Brzoska and Moniuszko-Jakoniuk 2001; Dong et al. 2005; Vallee and Falchuk 1993).
1.1.3 Proteins regulating zinc homeostasis and cadmium detoxification

In a eukaryotic cell, zinc is typically distributed into four main pools: (1) bound tightly to metalloenzymes (as a cofactor) and metalloproteins (as a structural component); (2) bound with low affinity to metallothioneins (MTs); about 5%–15% of total cellular zinc; (3) stored into vesicles and intracellular organelles, usually as a supply to maintain zinc homeostasis; and (4) as cytosolic free zinc (Coyle et al. 2002; Kimura and Kambe 2016). It is important to point out that in biological systems, free zinc is present at very low concentrations (pM–low nM levels) due to efficient homeostatic mechanisms that regulate entry, storage, distribution, and excretion of zinc (Vinkenborg et al. 2009; Vallee and Falchuk 1993).

Zinc homeostasis mechanism include sequestration by zinc binding proteins such as MTs, and import/export across cellular and subcellular membranes via two zinc transporter families: the zinc exporter proteins zinc transporters (ZnT)/cation diffusion facilitators (CDF)/solute-linked carrier 30 (SLC30) that export zinc from the cytoplasm; and the zinc importer proteins Zrt- and Irt-like proteins (ZIP)/SLC39 that import zinc into the cytoplasm (Kimura and Kambe 2016). Below, I review the function and regulation of each of these protein classes in detail.

1.1.3.1 Metallothioneins in mammals

MTs are low molecular weight, sulphydryl-rich, metal-binding proteins that were first identified as cadmium binding proteins that protect against heavy metal toxicity and are evolutionary conserved in eukaryotes and bacteria (Himeno et al. 2009; Capdevila and Atrian 2011). MTs play an important protective role in cadmium stress by forming metal–MT combinations (Goering and Klaassen 1984; Elinder et al. 1987; Klaassen et al. 1999). Both zinc and cadmium induce MT transcription, but cadmium is eight times more potent in increasing MT
concentration than zinc in the liver (Eaton et al. 1980). The cadmium-protective role of MT is crucial in mammals. Masters et al. showed that mice with homozygous mutations in both MT1 and MT2 genes are extremely sensitive to hepatic poisoning by cadmium (Masters et al. 1994).

Molecularly, MTs bind zinc and copper as part of a homeostatic maintenance mechanism, regulating zinc and copper reserves (Whanger and Ridlington 1982; Petering et al. 1984). MTs are composed of two domains: the α-domain that can bind four metal atoms (usually zinc), and the β-domain that can bind three metal atoms (usually copper) (Funk et al. 1987; Chang and Huang 1996; Brzoska and Moniuszko-Jakoniuk 2001).

In mammals, MTs arise from four genes, MT1 to MT4, which evolved through a series of duplication event. In humans, the MT1 gene expanded further, and 13 paralogs are tandemly iterated within a 66.6 kb region, where eight of them are active genes (MT1A, MT1B, MT1E to MT1H, MT1M and MT1X) and five are pseudogenes (MT1C, MT1D, MT1I, MT1J and MT1L) (Moleirinho et al. 2011; Kimura and Kambe 2016). MT1 and MT2 are broadly expressed in various cell types, including most cultured cells (Figure 1.2), while MT3 and MT4 have cell type-specific expression; MT3 is mainly expressed in the brain, and MT4 is mainly expressed in epithelial tissues. In addition, both MT1 and MT2 are inducible in response to zinc, cadmium, and other stress conditions; unlike MT3 and MT4, which are not inducible (Moleirinho et al. 2011; Vasak and Meloni 2011; Kimura and Kambe 2016).

1.1.3.2 Metallothioneins in C. elegans

C. elegans has two MT genes, mtl-1 and mtl-2. Both genes contain a single intron and a short 3’ untranslated region, and although their promoter regions are non-homologous, they are both induced by cadmium and zinc (Freedman et al. 1993). MTL-2 is expressed in the intestinal
cells, and is abundantly induced in different stresses, including heavy metal stress and heat shock. MTL-1 is constitutively expressed in the pharynx and induced by heavy metals and heat shock in the intestinal cells (Figure 1.2) (Hall et al. 2012; Freedman et al. 1993).

In contrast to mammals, *C. elegans* are not hypersensitive to cadmium when MTs are mutated or knocked down with RNA interference (RNAi) (Masters et al. 1994; Hall et al. 2012). Thus, although *mtl-1* and *mtl-2* are induced transcriptionally by cadmium, other proteins likely play a more critical functional role in the cadmium stress response.

### 1.1.3.3 Zinc transporters in mammals

To maintain cellular and subcellular zinc homeostasis in the face of changing metal availability there is a need to transport zinc across biological membranes (Roh et al. 2013). Although zinc crosses membranes through general cation transporters such as Divalent Cation Transporter 1 (DCT-1), the more specific ZIP and ZnT transporters play crucial roles in zinc homeostasis (Rolfs and Hediger 1999; Huang and Tepaamorndech 2013; Jeong and Eide 2013). These zinc transporters differ in tissue specificity, cellular and subcellular localization, and expression in response to zinc and across developmental stages (Kimura and Kambe 2016).

In mammals, there are ten ZnTs (ZnT1-10) that mediate zinc efflux from the cytosol to the outer cellular matrix, or into cellular organelles (Liuzzi and Cousins 2004). Human ZnT proteins display considerable sequence homology; the majority are predicted to have six transmembrane domains (TMD; except ZnT5, which has 12 TMDs) and a long histidine-rich intracellular loop that engages zinc for transport (Seve et al. 2004; Cousins et al. 2006). Most ZnTs are localized to the cytoplasmic membrane or to intracellular compartments, e.g., ZnT5A, ZnT6, and ZnT7 are localized to the Golgi complex, whereas ZnT2, ZnT4, and ZnT8 are
localized to endosomal or lysosomal vesicles (Figure 1.2) (Wang and Zhou 2010; Emdin et al. 1980; Huang et al. 2005; Chimienti et al. 2004). ZnT1 and possibly ZnT10 are localized to the cytoplasmic membrane, and act as regulators of zinc efflux in most tissues; for example, ZnT1 is responsible for zinc absorption in the intestine by promoting zinc efflux from the enterocytes (Cousins et al. 2006; McMahon and Cousins 1998b; Bosomworth et al. 2012). Importantly, ZnT1 is one of the ZnTs that are induced by high zinc; this regulation by zinc responsive mechanisms is further discussed in section 1.1.5 (Liuzzi et al. 2001).

ZnT2 is mainly present on the surface of intracellular vesicles in the intestine, kidney, and placenta, and is strongly induced by zinc as well. This transporter has a main role in protecting cells against excess zinc toxicity by moving zinc into vesicles (McMahon and Cousins 1998a; Liuzzi et al. 2001). ZnT3 is also located on cellular vesicles but is primarily expressed in the testes and brain (Wenzel et al. 1997). ZnT4 is mainly expressed in intestine and mammary gland and is not zinc responsive (McMahon and Cousins 1998a; Andreini et al. 2006; Palmiter and Findley 1995).

The subcellular localization of a zinc transporter can differ in different tissue types. For example, ZnT5 is localized to the membrane of secretory vesicles in the pancreatic β-cells and to the apical membrane in enterocytes (Cousins et al. 2006). Similarly, ZnT1, although usually on the cytoplasmic membrane, is localized to the vesicles in the pancreatic acinar cells, and ZnT9 is localized to the nucleus membrane, but only during mitosis (Sim and Chow 1999; Liuzzi and Cousins 2004; Cousins et al. 2006).

In addition to the ZnTs, 14 ZIP transporters (ZIP1-14) transport zinc from the extracellular matrix or intracellular organelles into the cytosol (Kimura and Kambe 2016). The ZIP transporters are predicted to have eight TMDs (except ZIP6, which has six) with
extracellular amino and carboxy-termini and a long intracellular histidine-rich loop (Rogers et al. 2000; Taylor and Nicholson 2003; Liuzzi and Cousins 2004). Most ZIP proteins are found on the cytoplasmic membrane, excluding ZIP7, 9, and 13, which are located on the Endoplasmic Reticulum (ER) and the Golgi apparatus (Bin et al. 2017; Huang et al. 2005; Matsuura et al. 2009); ZIP11, which is located on the Golgi (Kelleher et al. 2012); and ZIP8, which is located on the cytoplasmic membrane and the early endosomes (Wang et al. 2012).

Zinc availability or physiologic conditions can change the localization and expression of some ZIP transporters (Cousins et al. 2006). For example, in the mouse hepatocytes, ZIP14 is mobilized to the sinusoidal membrane in acute inflammation, and plays a role in zinc uptake in the acute phase response (Cousins et al. 2006). Moreover, some ZIP transporters display a transcriptional induction in response to zinc deficiency. Among these transporters are ZIP2, which is essential for the differentiation of keratinocytes, ZIP4, which increase zinc influx in tissues involved in nutrient uptake (e.g. in the intestine and the embryonic visceral yolk sac), and ZIP10, which is involved in trafficking zinc across the erythrocyte cytoplasmic membrane. This indicates that these transporters play an important role in zinc homeostasis in low-zinc conditions (Inoue et al. 2014; Dufner-Beattie et al. 2003; Andrews 2008; Ryu et al. 2008). Different variants and mutations in these zinc transporters can lead to abnormalities and human diseases. Examples of such disorders are summarized in Table 1.1.

Cadmium uptake is presumed to be mediated by zinc transporters as well. Historically, most cadmium resistant cells are resistant because of high MT expression, but Taylor et al. found MT-null cadmium-resistant cells, in which cadmium resistance is likely due to a ZIP8 mutation that prevents cadmium import (Taylor 1976; Dalton et al. 2005). Indeed, an additional study showed that, in MT-null cadmium-resistant cells, cadmium accumulation was suppressed when
expressions of ZIP8 and ZIP14 was down-regulated, implying that both ZIP8 and ZIP14 play a role in cadmium uptake (Fujishiro et al. 2009).

### 1.1.3.4 Zinc transporters in *C. elegans*

The *C. elegans* genome contains many evolutionarily conserved genes involved in zinc metabolism. These include clear orthologs of MTs and mammalian ZnT and ZIP type zinc transporters (Figure 1.1) (Kambe et al. 2006; Freedman et al. 1993; Cousins et al. 2006).

*C. elegans* encodes 14 predicted CDF zinc transporters (Roh et al. 2013). CDF-1 is predicted to be an ortholog of mammalian ZnT1 and ZnT10 (Figure 1.1) (Roh et al. 2013). Both ZnT1 and CDF-1 reduce cytosolic zinc concentrations, and both are involved in the regulation of Ras signaling (Bruinsma et al. 2002). In *C. elegans*, CDF-1 is located on the basolateral cytoplasmic membrane of the intestinal cells, where it transports zinc from the cell cytoplasm to the pseudocoelomic cavity (Figure 1.2) (Roh et al. 2013). *cdf-1(lf)* mutants are hypersensitive to high zinc. They display decreased levels of zinc in the pseudocoelom but increased total zinc content in the intestine. Interestingly, transgenically expressing ZnT1 in *cdf-1(lf)* mutants rescues their phenotypes, which strengthens the prediction that these proteins are homologous (Bruinsma et al. 2002). However, unlike ZnT1, *cdf-1* is not induced transcriptionally by excess zinc, and its regulation mechanism is still unknown (Dietrich et al. 2016).

Like CDF-1, CDF-2 is also expressed in the intestinal cells of *C. elegans*. CDF-2 is similar in sequence and in function to mammalian ZnT2, 3, 4, and 8, and it is also located on the membrane of lysosome-related organelle called gut granules (Figure 1.1-1.2) (Roh et al. 2013). CDF-2 detoxifies surplus cytoplasmic zinc by transporting it into the gut granules. Accordingly, *cdf-2* expression levels are induced by excess zinc, and *cdf-2(lf)* mutants are hypersensitive to...
high zinc, and display a decreased level of zinc content (Roh et al. 2012). Thus, a wild-type worm can combat high zinc conditions by inducing cdf-2 expression, which results in zinc distribution to the gut granules.

The ZnT/CDF protein Toxin-regulated target of p38 MAPK (ttm-1) was identified at first as a downstream target of p38 MAP kinase (Huffman et al. 2004). Like CDF-2, TTM-1 is an ortholog of mammalian ZnT2, 3, 4, and 8 (Figure 1.1) (Roh et al. 2013). ttm-1(lf) mutants display elevated zinc in the pseudocoelom and are sensitive to high zinc in a cdf-2 mutant background (Roh et al. 2013). The ttm-1 locus expresses two mRNAs, ttm-1a and ttm-1b. TTM-1A is expressed in the intestine and hypodermis, whereas TTM-1B is expressed in the intestine, as well as in multiple other tissues such as the hypodermis, seam cells, and vulva. In the intestine, TTM-1A is localized to vesicles, whereas TTM-1B is localized to the apical cytoplasmic membrane, where it excretes zinc to the lumen (Figure 1.2). Notably, TTM-1B, but not TTM-1A is transcriptionally upregulated by high zinc (Davis et al. 2009; Roh et al. 2013).

In sum, CDFs function to coordinate zinc homeostasis, with TTM-1B and CDF-2 promoting high zinc detoxification by returning zinc to the intestinal lumen or storing it in gut granules, whereas CDF-1 distributes zinc from the intestine to other body tissues.

Besides the CDF/ZnT type transporters, 14 ZIP proteins are present in the C. elegans genome, but their roles remain generally unexplored. Dietrich et al. examined the regulation of three ZIP genes that are induced by zinc deficiency: zipt-2.1, zipt-2.3 (homologous to ZIP1, 2, and 3), and zipt-7.1 (homologous to ZIP7 and 13; Figure 1.1), but their role in zinc homeostasis is unclear (Dietrich et al. 2017).
1.1.3.5 Other proteins involved in zinc and cadmium resistance and detoxification

As mentioned, although \textit{mtl-1} and \textit{mtl-2} are induced transcriptionally by cadmium, other proteins likely play a more critical role in the cadmium stress response in \textit{C. elegans}.

Phytochelatins (PC), a group of heavy metal chelators found in some plants, fungi, and nematodes, and their biosynthesizing enzymes, are essential for resistance to cadmium toxicity in worms (Vatamaniuk \textit{et al.} 2001).

Liao \textit{et al.} described a new cadmium responsive gene in \textit{C. elegans}, \textit{cdr-1}, that is induced by cadmium and encodes a lysosomal membrane protein (Liao \textit{et al.} 2002) (Figure 1.2). In normal conditions, \textit{cdr-1}(RNAi) worms accumulate fluid droplets in their pseudocoelom, but under hypotonic stress, the phenotype was exacerbated. Therefore, CDR-1 may play a role in osmoregulation (Dong \textit{et al.} 2005).
Figure 1.1 Phylogenetic tree of zinc transporters of *C. elegans* and *Homo sapiens*

Phylogenetic tree including human and worm ZnTs, and ZIPs. Orthologs of ZnTs, mentioned in section 1.1.3.4, are in blue; orthologs of ZIPs, mentioned in section 1.1.3.4, are in green.
Figure 1.2 Zinc homeostasis and cadmium stress response proteins and their cellular localization

Different proteins, including metallothioneins and zinc transporters (ZnTs and ZIPs), are located in different parts of the cell and play a role in zinc homeostasis and cadmium stress response. Mammalian and C. elegans proteins are annotated as follows: *- mammalian, †- C. elegans. Not all C. elegans orthologs are listed; see figure 1.1 for zinc transporter orthologs.

1.1.4 Zinc homeostasis and related diseases

In biological systems, many proteins are zinc dependent. Thus, changes in zinc concentration or distribution can lead to abnormalities and, sometimes, human disease (Fukada et al. 2011). These changes can also be caused by mutations and single nucleotide polymorphisms (SNPs) of zinc regulating genes (i.e. MTs, ZnTs, and ZIPs), resulting in human genetic disorders (Kambe et al. 2008). Table 1.1. summarizes examples of zinc-related disorders, as well as phenotypes observed in animal models bearing mutations in genes involved in zinc metabolism/distribution genes. As specified in Table 1.1, unbalanced zinc homeostasis is also
related to complex diseases such as cancer, diabetes, neurodegenerative diseases, and liver
diseases. Zinc homeostasis relations to diabetes are discussed in detail in the section below.

Table 1.1 Genes involved in zinc homeostasis and related diseases and animal models

<table>
<thead>
<tr>
<th>Gene</th>
<th>Disease</th>
<th>Animal model</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>ZnT1</td>
<td></td>
<td>Mouse: ZnT1 Knockout (KO): Embryonic lethal</td>
<td>(Andrews et al. 2004; Bruinsma et al. 2002)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>*C. elegans: cdf-1(null): Embryonic lethal</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>*cdf-1(lf) mutation- Abnormal vulva formation</td>
<td></td>
</tr>
<tr>
<td>ZnT2</td>
<td>Transient neonatal zinc deficiency (TNZD); Produce zinc-deficient milk in some women</td>
<td>Mouse: ZnT3 KO: Prone to seizures brought by kainic acid treatment. Similar phenotype to the synaptic and memory deficits of Alzheimer’s disease. This transporter is required for presynaptic Erk activation and hippocampus dependent memory</td>
<td>(Chowanadisai et al. 2006; Miletta et al. 2013)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Mouse: ZnT2 KO: Designated for transient neonatal zinc deficiency (TNZD)</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>Mouse: ZnT2 KO: Designated for transient neonatal zinc deficiency (TNZD)</td>
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<tr>
<td></td>
<td></td>
<td>Mouse: ZnT2 KO: Designated for transient neonatal zinc deficiency (TNZD)</td>
<td></td>
</tr>
<tr>
<td>ZnT3</td>
<td>Increased risk of febrile seizures</td>
<td>Mouse: ZnT3 KO: Prone to seizures brought by kainic acid treatment. Similar phenotype to the synaptic and memory deficits of Alzheimer’s disease. This transporter is required for presynaptic Erk activation and hippocampus dependent memory</td>
<td>(Hildebrand et al. 2015; Cole et al. 2000; Adlard et al. 2010; Sindreu et al. 2011)</td>
</tr>
<tr>
<td>ZnT4</td>
<td></td>
<td>Mouse: lethal milk (Im) mutant- Zinc deficient milk</td>
<td>(Huang and Gitschier 1997)</td>
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<tr>
<td>ZnT5</td>
<td></td>
<td>Mouse: ZnT5 KO: Poor growth, low body fat, osteopenia, muscle weakness, impaired mast cell functions, and male-specific cardiac death</td>
<td>(Inoue et al. 2002; Nishida et al. 2009)</td>
</tr>
<tr>
<td>ZnT6</td>
<td></td>
<td>Mouse: ZnT6 KO: Growth retardation and reduced accumulation of fat</td>
<td>(Huang et al. 2007)</td>
</tr>
<tr>
<td>ZnT7</td>
<td></td>
<td>Mouse: ZnT7 KO: Growth retardation and reduced accumulation of fat</td>
<td>(Huang et al. 2007)</td>
</tr>
<tr>
<td>ZnT8</td>
<td>Type I and Type II diabetes mellitus</td>
<td>Mouse: ZnT8 KO: Impairment of insulin-crystal formation and insulin secretion</td>
<td>(Sladek et al. 2007; Nicolson et al. 2009; Wenzlau et al. 2008)</td>
</tr>
<tr>
<td>ZnT10</td>
<td>Hypermanganesemia, hepatic cirrhosis syndrome, dystonia and polycythemia</td>
<td>Mouse: ZnT10 Knockout (KO): Abnormal embryo morphogenesis in zinc limiting condition</td>
<td>(Quadri et al. 2012; Leyva-Illades et al. 2014)</td>
</tr>
<tr>
<td>ZIP1</td>
<td></td>
<td>Mouse: ZIP1or ZIP2 KO: Abnormal embryo morphogenesis in zinc limiting condition</td>
<td>(Dufner-Beattie et al. 2006)</td>
</tr>
<tr>
<td>ZIP2</td>
<td></td>
<td>*ZIP1or ZIP2 KO: Abnormal embryo morphogenesis in zinc limiting condition</td>
<td>(Peters et al. 2007)</td>
</tr>
<tr>
<td>ZIP3</td>
<td></td>
<td>Mouse: ZIP3 KO: Abnormal embryo morphogenesis and depletion of thymic pre-T cells in zinc deficient condition</td>
<td>(Dufner-Beattie et al. 2005)</td>
</tr>
<tr>
<td>ZIP4</td>
<td>Acrodermatitis Enteropathica (AE) Severe zinc deficiency</td>
<td>Mouse: ZIP4 KO: Embryonic lethal</td>
<td>(Kury et al. 2002; Wang et al. 2002; Dufner-Beattie et al. 2007)</td>
</tr>
<tr>
<td>ZIP5</td>
<td>Non-symptomatic high myopia</td>
<td>Mouse: ZIP5 KO: Abnormal embryo morphogenesis in zinc limiting condition</td>
<td></td>
</tr>
<tr>
<td>ZIP6/10</td>
<td></td>
<td>Drosophila: Fear Of Intimacy (Foi) mutant: (Foi is an ortholog of mammalian Zip6 and Zip10): Abnormal gonad formation and glial cell migration</td>
<td>(Mathews et al. 2006) (Pielage et al. 2004; Van Doren et al. 2003)</td>
</tr>
</tbody>
</table>

14
<table>
<thead>
<tr>
<th>Gene</th>
<th>Disease</th>
<th>Animal model</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>ZIP8</td>
<td>Cerebellar Atrophy Syndrome- type II Congenital Disorder of Glycosylation (CDG)</td>
<td>In Cdm mouse: ZIP8 mutants: Resistance to cadmium toxicity</td>
<td>(Park et al. 2015; Boycott et al. 2015; Dalton et al. 2005)</td>
</tr>
<tr>
<td>ZIP13</td>
<td>Spondylocheiro dysplastic Ehlers-Danlos syndrome (SCD-EDS)</td>
<td>Mouse: ZIP13 KO: Growth retardation, abnormal connective tissue development, and impaired BMP/TGF-β signaling</td>
<td>(Giunta et al. 2008; Fukada et al. 2008)</td>
</tr>
<tr>
<td>MT1 A,B,E,F, G,H,M,X</td>
<td></td>
<td>Mouse: MT-1 MT-2 KO: High sensitivity to heavy metal toxicity and lipopolysaccharide (LPS)/D-galactosamine-induced lethality, macrophage dysfunction, defects in phagocytosis and antigen presentation, reduced survival in Cu/Zn-Superoxide Dismutase (SOD1) mutants, a familial mouse model of Amyotrophic Lateral Sclerosis (ALS)</td>
<td>(Sugiura et al. 2004; Masters et al. 1994; Michalska and Choo 1993; Puttaparthi et al. 2002)</td>
</tr>
<tr>
<td>MT2A</td>
<td>SNPs are associated with an increased cancer risk</td>
<td></td>
<td></td>
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</table>

1.1.4.1 **Zinc homeostasis and diabetes**

The pancreas contains particularly high levels of zinc, with most zinc stored in the Insulin Secretory Granules (ISG) of the pancreatic β-cells (Hutton et al. 1983; Foster et al. 1993). Zinc plays an important role in the function of β-cells, affecting processes such as insulin crystallization, glucose-stimulated insulin secretion, and cell viability (Liu et al. 2015). Levels of zinc in the serum are greatly decreased in Type 1 diabetes (T1D) and Type 2 diabetes (T2D) patients (Garg et al. 1994; Basaki et al. 2012), and zinc supplementation improves T2D symptoms, both in mouse models and in human diabetic patients (Jayawardena et al. 2012). In mammals, the zinc transporter ZnT8 is located on the membrane of ISGs in β-cells and transports zinc from the cytosol into the ISGs, where it is used for insulin crystallization and storage (Chimienti et al. 2004). This transporter is almost completely restricted to the pancreas, where it has the highest expression among all zinc transporters (Lemaire et al. 2012; Chimienti et al. 2004; Chimienti et al. 2006; Chabosseau and Rutter 2016).
Genome-wide association studies (GWAS) for T2D showed an association between T2D risk and the SLC30A8 non-synonymous coding variant (rs13266634) that replaces an arginine with a tryptophan (R325W) in the C-terminus of the protein (Sladek et al. 2007). The risk variant (R325) is associated with a 17% increase in T2D risk per allele, an increased proinsulin:insulin ratio, and impaired insulin secretion in the intravenous glucose tolerance test (Shan et al. 2014; Kirchhoff et al. 2008). In addition, functional assays showed that by overexpressing each isoform, zinc accumulation in insulin granules was significantly higher for the low-risk variant (W325) versus the high-risk variant (R325) (Kim et al. 2011). This association is also supported by experiments in animal models, as many ZnT8 KO mice models have impaired glucose tolerance (Nicolson et al. 2009; Wijesekara et al. 2010; Pound et al. 2012; Tamaki et al. 2013; Rutter et al. 2016). These studies support a model whereby reduced zinc transport, caused by SLC30A8 loss-of-function (lf) mutations, increases T2D risk. However, another study identified 12 rare truncating variants in SLC30A8 that were associated with a 65% decreased risk in T2D, with the two most common rare variants causing the expression of an unstable protein (Flannick et al. 2014). This is surprising as, in contrast to previous studies, SLC30A8 haploinsufficiency associates with a protective role against T2D.

In sum, although there is evidence, through GWAS and functional studies, that proper function of ZnT8 is protective for T2D, the role of this protein remains unclear, and ZnT8 inhibition might be protective as well.

1.1.5 Metal regulated transcription

As concentrations of metals change in an organism’s environment, sensing and responding to environmental metal changes is crucial for survival. An important adaptive
response to environmental changes is transcriptional reprogramming, where the transcription of
genes is activated and repressed, as needed. The transcriptional regulation of metal regulated
genes has been studied in many different species (Thiele 1992).

1.1.5.1 Zinc regulated transcription

To maintain zinc homeostasis, transcriptional regulation occurs in various species. In
Saccharomyces cerevisiae, there are two zinc uptake systems that regulate zinc homeostasis. The
high affinity system involves the transcriptional regulation of the ZRT1 transporter gene,
whereas the lower affinity system involves the transcriptional regulation of the ZRT2 transporter
gene. Both systems are regulated by zinc concentration through the TF Zinc responsive Activator
Protein 1 (Zap1) (Zhao and Eide 1997). Another yeast, Schizosaccharomyces pombe, regulates
zinc homeostasis through the metalloregulatory factor Loss Of Zinc sensing 1 (Loz1). Loz1 is
required to repress many zinc responsive genes in low zinc conditions, such as the zinc-
dependent enzyme Alcohol Dehydrogenase 1 (Adh1) (Corkins et al. 2013).

In vertebrates, the Metal-responsive Transcription Factor 1 (MTF-1) is a key zinc
responsive transcription factor; MTF-1 is a zinc finger protein that is regulated by zinc
availability and binds to the Metal Regulatory Element (MRE) (Culotta and Hamer 1989; Laity
and Andrews 2007; Bittel et al. 1998; Chen et al. 1999; Fukada et al. 2011). In response to
excess zinc, MTF-1 activates the transcription of certain genes (e.g. ZnT1, MT1), and represses
the transcription of other genes (e.g. Zip10) in mice (Langmade et al. 2000; Andrews et al. 2001;
Lichten et al. 2011). Additionally, zinc availability also regulates zinc transporters and MTs
through MTF-1 independent pathways (Fukada et al. 2011). For example, human MT1G is
induced by Vascular Endothelial Growth Factor (VEGF) through the TF E2 factor (E2F) (Joshi
et al. 2005), and bovine MT1A and MT1E are induced by the Nuclear factor erythroid 2–Related Factor 2 (Nrf2)–Kelch-like Erythroid cell-derived protein with CNC homology [ECH]-Associated Protein 1 (Keap1) system (Fujie et al. 2016). In sum, although MTF-1 is clearly an important regulator of many metal and zinc responsive mechanisms in vertebrates, other TFs also play a role in zinc homeostasis.

1.1.5.2 Zinc regulated transcription in *C. elegans*

MTF-1 and MREs play essential roles in controlling zinc dependent gene transcription in vertebrates, while Zap1 and Loz1 TFs are necessary to regulate zinc homeostasis in yeast (Culotta and Hamer 1989; Zhao and Eide 1997; Corkins et al. 2013). Analysis of the *C. elegans* genome revealed no clear homologs of MTF-1, ZAP1, or Loz1 TFs (Dietrich et al. 2016). Moreover, although the MRE element is present in some zinc and cadmium responsive genes such as *cdr-1* and *mtl-2*, it is apparently not functional in *C. elegans*, i.e., it does not confer metal inducibility (Freedman et al. 1993; Liao et al. 2002; Dietrich et al. 2016). Nevertheless, many *C. elegans* zinc homeostasis genes are regulated by zinc; for example, the CDF genes *cdf-2* and *ttm-1b* and the MTs *mtl-1* and *mtl-2* are upregulated by high zinc (Davis et al. 2009; Roh et al. 2013).

Recently, the molecular mechanisms for this regulation were described. Roh et al. used bioinformatics to identify a regulatory element in these zinc responsive genes that is required for the response to high zinc, the High Zinc Activated (HZA) element. Following this, the TF that binds this element was identified as Nuclear Hormone Receptor 33 (NHR-33)/HIZR-1 (Roh et al. 2015; Warnhoff et al. 2017). The Ligand-Binding Domain (LBD) of HIZR-1 binds zinc, and this promotes nuclear accumulation of HIZR-1 in the gut nuclei (Warnhoff et al. 2017). This
suggests a model whereby increased zinc levels cause HIZR-1 nuclear translocation, where HIZR-1 in turn activates genes with promoters featuring HZAs, such as *ttm-1b* and *cdr-1*.

Most zinc and cadmium responsive genes are expressed exclusively in the gut (Freedman *et al.* 1993). The TF ELT-2, a master regulator of intestinal gene expression, binds the GATA DNA element (Fukushige *et al.* 1998). Accordingly, the GATA element was found adjacent to the HZA element in many promoters of zinc and cadmium responsive genes. This indicates that the HZA element might mediate the metal response, while the GATA element might mediate the specificity to intestinal cells (Roh *et al.* 2015).

In addition to the HZA element, a Low Zinc Activation (LZA) element was recently identified in the promoters of genes that are upregulated by zinc deficiency. This element is present in the promoters of the *C. elegans* ZIP genes *zipt-2.1*, *zipt-2.3*, and *zipt-7.1*, and it is sufficient for the induction of a GFP reporter in zinc deficient conditions (Dietrich *et al.* 2017).

### 1.2 The Mediator complex is a key player in transcriptional regulation

The Mediator complex (‘Mediator’) is a multi-subunit protein complex that is recruited by TFs to regulate RNA polymerase 2 (Pol2) activity, and to modulate the structure of chromatin (Soutourina 2017; Poss *et al.* 2013; Jeronimo and Robert 2017). Importantly, specific TF-Mediator subunit partnerships regulate specific biological gene sets and processes (Malik and Roeder 2010; Allen and Taatjes 2015; Grants *et al.* 2015).

#### 1.2.1 The structure and function of the Mediator complex

The Mediator complex contains 25-30 protein subunits, dependent on species, and is conserved from yeast to mammals (Bourbon 2008; Tsai *et al.* 2014; Grants *et al.* 2015). The
subunits of the Mediator are organized into four structural modules: head, middle, tail, and kinase modules. Whereas the head and middle modules interact with Pol2 to modulate transcription, the tail module predominantly binds TFs, and the kinase module associates reversibly with the rest of the Mediator (‘core’ Mediator), primarily through the middle module (Tsai et al. 2014) (Figure 1.3). The kinase module has a role in regulating the activity of Mediator and of Mediator-binding TF by phosphorylation or steric hindrance (Tsai et al. 2014; Guglielmi et al. 2004; Grants et al. 2015) (Fig. 1.3).

### 1.2.2 Mediator subunits involved in the regulation of stress response and metal homeostasis

Specific TF-Mediator subunit partnerships are involved in regulating specific stress response gene sets. In *Drosophila*, the induction of the MT genes, MtnA and MtnB, are regulated by MTF-1. However, MtnA is induced by copper, whereas MtnB is induced by cadmium, and MTF-1 seems to partner with different Mediator components to regulate each gene (Gunther et al. 2012). Specifically, MED26 is required for full activation of MtnA, whereas MED13 is required for the induction of both MtnB and MtnD (Marr et al. 2006).

In *C. elegans*, specific Mediator subunits have been identified as regulators of certain metabolic, stress, and innate immune responses (Figure 1.3). Two additional subunits, the tail module subunit MDT-15, and the cyclin dependent kinase 8 (CDK-8) kinase subunit play a regulatory role in stress responses, including metal stress and homeostasis. This will be discussed below.
1.2.2.1 The Mediator tail module subunit MDT-15

*C. elegans* MDT-15 is a tail module Mediator subunit that is required for the regulation of many metabolic and stress responses genes, including lipid metabolism, oxidative stress, xenobiotic detoxification, and innate immune response genes (Taubert *et al.* 2006; Yang *et al.* 2006; Lee *et al.* 2015; Pukkila-Worley *et al.* 2014; Goh *et al.* 2014). To regulate metabolic genes, MDT-15 interacts with the evolutionarily conserved TFs SBP-1/Sterol regulatory element-binding protein (SREBP), SKN-1/Nrf2, and Hepatocyte Nuclear Factor 4 (HNF4)-related NHRs such as NHR-49 and NHR-64 (Taubert *et al.* 2006; Yang *et al.* 2006; Goh *et al.* 2014; Arda *et al.* 2010). MDT-15 interacts with NHRs and SBP-1 through its N-terminal kinase-inducible domain interacting (KIX) domain, whereas SKN-1 binds another region in MDT-15 (Yang *et al.* 2006; Arda *et al.* 2010; Taubert *et al.* 2006; Goh *et al.* 2014). *mdt-15* is required for the induction of zinc and cadmium responsive genes, but less is known about how MDT-15 regulatory partners for metal homeostasis and stress responses (Taubert *et al.* 2008). It is important to note that MDT-15 regulates specific stresses and is not a universal coregulator to stress or adaptive responses. For example, heat stress and ER stress response genes do not require *mdt-15* for stress-induced or basal transcription (Hou *et al.* 2014; Taubert *et al.* 2008).

1.2.2.2 The Mediator kinase module subunit CDK-8

The Mediator kinase subunits CDK8 acts as corepressor or coactivator and is involved in metabolic and stress response gene regulation (Borggrefe and Yue 2011; Galbraith *et al.* 2013). CDK-8 is one of four subunits in the Mediator kinase module and opposes MDT-15/MED15 through common TF regulation or regulatory interactions between these two Mediator subunits. One example for this is that CDK-8/CDK8 negatively regulates lipogenic gene expression by
phosphorylating SBP-1/SREBP, which targets it for ubiquitination and subsequent protein degradation; in contrast, MDT-15/MED15 positively regulates lipogenic gene by serving as a coactivator for SBP-1/SREBP (Zhao et al. 2012; Xie et al. 2015; Yang et al. 2006). This is also supported by a study showing that some gene sets are inversely regulated by Cdk module and tail module subunits, including Med15 (van de Peppel et al. 2005). In addition, CDK-8 and MDT-15 also work in cooperation to regulate stress response gene sets. For example, our lab showed that CDK-8 positively regulates cadmium stress response genes, i.e. a role similar to that of MDT-15 (Grants 2016).

Figure 1.3 The Mediator complex, with emphasis on MDT-15 and CDK-8 and their regulatory roles
Model of the Mediator complex, adapted from Grants et al. (Grants et al. 2015). The Mediator head module interacts with Pol2, whereas the Mediator tail module interacts with TFs. Mediator modules are color coded, and the locations and biological activities of the Mediator subunits CDK-8 and MDT-15 are presented.
1.3 Importance, hypothesis, and objectives

The understanding of zinc homeostasis and cadmium stress responses regulation has scientific and medical importance. The nematode worm *C. elegans* is an excellent model organism to study zinc and cadmium responses, as its genome encodes evolutionarily conserved proteins and regulatory factors involved in zinc homeostasis (e.g. CDFs, ZIPs, MTs, TFs, and coregulators; see sections 1.1.3, 1.2.2.1), indicating relevance to mammalian regulation of zinc metabolism. Therefore, studying the players involved in the regulation of these metal response genes (Mediator subunits, TFs, and DNA elements) and the interactions between them in *C. elegans* can reveal much about the underlying regulatory mechanisms.

The Mediator subunit MDT-15 is required for the induction of zinc and cadmium responsive genes (Taubert *et al.* 2008), and CDK-8 is partly required for the induction of cadmium responsive genes (Grants 2016). However, what TFs and DNA elements cooperate with Mediator subunits MDT-15 and/or CDK-8 in this regulation, and whether or not CDK-8 is required for the regulation of zinc homeostasis, like MDT-15, is unclear.

The overall hypothesis of my thesis is that MDT-15 and CDK-8 work with various TFs, including HIZR-1, to drive gene expression changes and to regulate zinc homeostasis and the cadmium stress response.

My specific objectives are:

1. Define the mechanistic role of MDT-15 and CDK-8 in regulating zinc excess and deficiency response genes, and cadmium toxic response.
2. Study the physiological role of Mediator subunits and their interaction with other regulatory factors in zinc homeostasis.
Chapter 2: Results

2.1 cdk-8 was partly required for maximal zinc-induced gene expression

*C. elegans mdt-15* is required for the transcriptional induction of zinc and cadmium responsive genes, and *cdk-8* is partly required for the induction of cadmium responsive genes (Taubert *et al.* 2008; Grants 2016).

To validate these requirements and generate a comprehensive dataset for *mdt-15* and *cdk-8* mutants in both high zinc and cadmium, I used real-time quantitative PCR (qPCR) to measure the mRNA levels of metallothioneins (*mtl-1, mtl-2*), *cdr-1*, and the zinc transporters *cdf-2* and *ttm-1b*, all genes with an important role in zinc detoxification in worms. I compared the mRNA expression of these genes in N2 wild-type worms, *mdt-15(tm2182)* reduction-of-function (rf) mutants, and *cdk-8(tm1238)* null mutants without zinc supplementation (0μM Zn) and with 200μM zinc supplementation (200 μM Zn). 200μM zinc supplementation was chosen due to the examined genes being substantially induced in this concentration in previous studies (Roh *et al.* 2015). As published (Taubert *et al.* 2008), the induction of *mtl-1, mtl-2*, and *cdr-1* was significantly impaired in *mdt-15* mutants; *cdf-2* and *ttm-1* showed a similar trend (Figure 2.1A). In *cdk-8(tm1238)* null mutants, I observed a significant decrease of *cdr-1* mRNA levels upon exposure to cadmium and of *mtl-2* mRNA levels upon exposure to zinc; other zinc and cadmium responsive genes show a similar trend but did not change significantly (Figure 2.1.B-C). Thus, *cdk-8* is required for maximal mRNA expression of some genes in response to zinc or cadmium, but this requirement is less prevalent and substantial than the one observed for *mdt-15*. For most of my follow-up studies, I therefore focused on MDT-15.
Figure 2.1. *mdt-15* is required for zinc transcriptional induction, and *cdk-8* is partly required for zinc and cadmium transcriptional induction

qPCR analysis of zinc and cadmium-responsive genes. Bars represent fold induction, normalized to the average of unsupplemented N2 mRNA expression levels. Error bars: SEM, n = 3 independent trials. Statistical analysis: * p < 0.05, ** p < 0.01, *** p < 0.001 unpaired t-test comparing normalized mRNA expression of N2 supplemented worm to mutant supplemented worms. (A) Comparison of N2 worms grown on 200μM zinc to *mdt-15* mutants grown on 200μM zinc for 16 hours. (B) Comparison of N2 worms grown on 100μM cadmium to *cdk-8* mutants grown on 100μM cadmium for 4 hours. (C) Comparison of N2 worms grown on 200μM zinc to *cdk-8* mutants grown on 200μM zinc for 16 hours.

2.2 *mdt-15* and *cdk-8* were required to induce the *cdr-1* promoter under zinc and cadmium supplementation

To delineate the mechanism of MDT-15 and/or CDK-8 driven cadmium and zinc responsive transcription, I used a transcriptional *cdr-1::GFP* reporter, which encompasses 2.8 kb of the putative *cdr-1* promoter (Figure 2.2A). The *cdr-1* promoter was chosen as a model because *cdr-1* is highly cadmium and zinc responsive and requires *mdt-15* and *cdk-8* for activation (Figure 2.1), suggesting that it might be a good tool to identify DNA regulatory elements and cognate TFs that cooperate with Mediator to regulate this gene.

As expected, I observed weak basal expression of the *cdr-1::GFP* reporter, but substantial induction of fluorescence by 200μM zinc and 100μM cadmium supplementation; expression was exclusively localized to the intestine (Figure 2.2B). Knockdown of *mdt-15* by RNAi in *cdr-1::GFP* worms completely abrogated the fluorescence induction of *cdr-1P::GFP* reporter by 100μM cadmium or 200μM zinc (Figure 2.2C-D). Similarly, *cdk-8(tm1238); cdr-
1P::GFP worms also significantly reduced fluorescence induction by 100μM cadmium or 200μM zinc (Figure 2.2E-F).

This confirms that Mediator subunits mdt-15 and cdk-8 are required for induction for zinc and cadmium responsive genes, and validates the cdr-1::GFP reporter as an effective research tool for identifying factors necessary for zinc homeostasis and cadmium stress responses.
Figure 2.2 *mdt-15* and *cdk-8* are required for the induction of the *cdr-1::GFP* reporter

(A) Illustration of the *cdr-1P::GFP* reporter and putative regulatory DNA elements in the *cdr-1* promoter; note, the diagram is not to scale (adapted from (Grants 2016)). (B) Representative images of worms bearing the *cdr-1P::GFP* transcriptional reporter, both before and after exposure to 100μM cadmium or 200μM zinc. Green fluorescence represents signal from the *cdr-1P::GFP* transcriptional reporter; red fluorescence represents signal from the co-injection marker *myo-2P::mCherry*. (C-D) The bar graphs show average fluorescence intensity (arbitrary units, A.U.) of worms bearing the *cdr-1P::GFP* transcriptional reporter, exposed for four hours to 0μM or 100μM cadmium (C) or 0μM or 200μM zinc (D), and fed either empty vector (EV) or *mdt-15* RNAi, as indicated. Error bars: SEM, n > 19 worms/group. Statistical analysis: **** p < 0.0001 Two-way ANOVA, Multiple comparisons; Tukey correction. (E-F) The bar graphs show average fluorescence intensity (arbitrary units, A.U.) of worms bearing the *cdr-1P::GFP* transcriptional reporter, exposed for four hours to 0μM or 100μM cadmium (E) or 0μM or 200μM zinc (F) of N2 worms vs. *cdk-8(tm1238)* mutants. Error bars: SEM, n > 24 worms. Statistical analysis: **** p < 0.0001 Two-way ANOVA, Multiple comparisons; Tukey correction.

2.3 The TFs *hizr-1* and *elt-2* and their DNA elements HZA and GATA were required to induce the *cdr-1* promoter

To identify TFs that cooperate with *cdk-8* and *mdt-15* to regulate cadmium and zinc responsive transcription, DNA regulatory elements were identified in the *cdr-1* promoter. We identified candidate elements for SKN-1/Nrf2 (antioxidant response element, ARE), HSF-1 (heat shock response element, HSE), DAF-16/FOXO (DAF-16 binding element, DBE), ELT-2 (GATA element), and HIZR-1 (HZA element), as well as four metal response elements for which no *C. elegans* binding factor has been identified to date (MRE) (Figure 2.3A). RNAi analysis revealed that *skn-1*, *hsf-1*, and *daf-16* are not required for *cdr-1* induction by cadmium, whereas knockdown of *elt-2* or *hizr-1* abrogated fluorescence induction by cadmium (Figure 2.3B). Next, to examine whether the requirements for *elt-2* and *hizr-1* are due to direct
interaction with the *cdr-1::GFP* reporter, site-directed mutagenesis by substitution mutations was performed in the HZA (*mutHZA*) and GATA sites (*mutGATA1* and *mutGATA2*). Mutation of all these sites caused a substantial and significant decrease in basal and cadmium-induced promoter activity compared to the wild-type *cdr-1P::GFP* reporter (Figure 2.3C). Collectively, these data show that Mediator subunits MDT-15 and CDK-8 and the transcription factors ELT-2 and HIZR-1 are required to control expression from the cadmium/zinc-inducible *cdr-1* promoter.

**Figure 2.3** The TFs *elt-2* and *hizr-1* and the elements GATA and HZA are required to induce the *cdr-1* promoter

(A) Illustration of the *cdr-1P::GFP* reporter with its DNA elements and their cognate TFs; note, the diagram is not to scale. (B) The bar graphs show average fluorescence intensity (arbitrary units, A.U.) of worms bearing the *cdr-1P::GFP* transcriptional reporter before and after a 0μM or 100μM cadmium 4 hour exposure, and fed either empty vector (control RNAi) or TF RNAi, as indicated. Error bars: SEM, *n* > 19 worms. Statistical analysis: **** *p* <
Two-way ANOVA, Multiple comparisons; Tukey correction. (C) The bar graphs show average fluorescence intensity (arbitrary units, A.U.) of worms bearing variants of the *cdr-1P::GFP* transcriptional reporter: wild-type promoter, cdr-1P; with mutated HZA element, cdr-1PΔHZA; and with mutated GATA sites, cdr-1PΔGATA 1 or 2; all promoter variants were assessed with 0μM or 100μM cadmium in a 4 hour exposure. Error bars: SEM, *n* > 19 worms. Statistical analysis: **** *p* < 0.0001 Two-way ANOVA, Multiple comparisons; Tukey correction.

2.4 *mdt-15* and *hizr-1* function was co-dependent

Based on the above data, I hypothesized that MDT-15 and/or CDK-8 might interact functionally and physically with HIZR-1 and/or ELT-2 to activate metal-induced transcription. To examine a putative functional relationship between MDT-15 and HIZR-1, I studied the *hizr-1(am285)* gain-of-function (gf) mutant that induces zinc responsive genes even in the absence of zinc (Warnhoff et al. 2017). In line with published data (Warnhoff et al. 2017), qPCR analysis revealed that *mtl-1, mtl-2, cdr-1* and *cdf-2* mRNA levels are strongly induced in *hizr-1(am285)* mutants grown on control RNAi; importantly, *mdt-15* RNAi significantly reduced or abrogated these induction (Figure 2.4A). Next, I studied the *mdt-15(yh8)* gf mutant that induces MDT-15 regulated lipid metabolism genes (Svensk et al. 2013); I observed that, like *hizr-1(am285)*, the *mdt-15* gf mutation was sufficient to induce, *mtl-1, mtl-2*, and *cdr-1* expression. RNAi knockdown revealed that *mtl-2* induction required *hizr-1*, while *mtl-1* and *cdr-1* showed similar trends (Figure 2.4B). In contrast, these inductions were independent of *nhr-49*, which acts with MDT-15 to regulate lipid metabolism genes; in fact, *nhr-49* depletion by RNAi caused an induction of *mtl-1* and *mtl-2* in the *mdt-15(yh8)* gf background. Therefore, we concluded that MDT-15 and HIZR-1 interact functionally to induce zinc and cadmium responsive genes.
Figure 2.4 *mdt-15* and *hizr-1* are co-dependent for metal responsive gene induction

qPCR analysis of zinc and cadmium-responsive genes. The bars represent fold-inductions normalized to the average of unsupplemented N2 mRNA expression levels on control RNAi. Error bars: SEM. Statistical analysis: *p < 0.05, **p < 0.01, unpaired t-test comparing normalized mRNA expression of N2 supplemented worm to mutant supplemented worms. (A) Comparison of wild-type and *hizr-1*(am285) worms grown on control RNAi or *mdt-15* RNAi. n= 5 independent trials. (B) Comparison of wild-type and *mdt-15*(yh8) worms grown on control RNAi, *hizr-1* RNAi, or *nhr-49* RNAi. n= 3 independent trials.
2.5 MDT-15 physically interacted with HIZR-1 in zinc-enhanced fashion

The above data suggest that MDT-15 might interact physically with HIZR-1, as shown for other C. elegans NHRs (Taubert et al. 2006; Arda et al. 2010). To test whether HIZR-1 binds MDT-15, I used the yeast-two-hybrid (Y2H) system. Because full-length MDT-15 autoactivates transcription when used as bait in Y2H assays (Goh et al. 2014), I used a MDT-15 protein without a C-terminal domain (MDT-15-ΔCT; see Figure 2.6). I first examined the interaction between MDT-15-ΔCT (bait) and full length HIZR-1 (prey). MDT-15 and HIZR-1 showed a statistically significant interaction that was similar in strength to the interaction of MDT-15-ΔCT with a SKN-1c prey (positive control; Figure 2.5A).

With its LBD, HIZR-1 binds zinc in the micromolar range (Warnhoff et al. 2017), suggesting that zinc acts as a bona fide NHR ligand. I predicted that zinc binding might enhance the interaction of HIZR-1 with MDT-15, as shown for other ligand-stimulated NHR-coregulator interactions (Giguere 1999). Indeed, addition of zinc at various concentrations enhanced the interaction between MDT-15 and HIZR-1, with significant effects at concentrations of 5μM zinc and higher, and gradually rising until reaching a plateau at around 25μM (Fig 2.5B). As mdt-15 and hizr-1 are also required for cadmium-induced gene expression, I tested whether cadmium also enhanced MDT-15 binding to HIZR-1 and found that this was the case (Fig 2.5C). In contrast, manganese did not enhance this interaction (Fig 2.5C).

NHRs contain a zinc-finger DNA Binding Domain (DBD), suggesting that zinc-stimulated binding to a coregulator such as MDT-15 might be a common feature of NHRs. To examine the specificity of zinc- and cadmium-stimulated MDT-15–NHR interaction, I examined whether these metals affect the association of MDT-15 with NHR-64 and NHR-49. I found that NHR-64 interacts with MDT-15 as strongly as HIZR-1 does in the presence of zinc or cadmium,
while NHR-49 binding to MDT-15 has a similar interaction strength as HIZR-1 and MDT-15 in the absence of zinc; importantly, neither interaction was altered by metal supplementation (Fig 2.5C). Thus, metal-stimulation of MDT-15 interaction is not a general feature of NHRs, but specific to HIZR-1. I also ascertained that metal supplementation did not change the expression of the Y2H prey fusion proteins in all experiments, which was the case. Thus, the effects I observed likely reflect direct and specific changes in HIZR-1–MDT-15 binding upon metal addition.
Figure 2.5 MDT-15 and HIZR-1 physically interact in zinc-enhanced fashion

Protein-protein interaction analysis of MDT-15 with different TFs using the Y2H system. Expression of proteins fused to the Gal4 Activation Domain (AD) was confirmed by immunoblot, with GAPDH serving as loading control. (A) The bars represent average interaction strength between MDT-15-HIZR-1, with Empty Vector (EV)-HIZR-1 and MDT-15-EV as negative controls, and MDT-15–SKN-1c as a positive control. Average interaction strength (arbitrary units, A.U.). Error bars: SEM. n=3 independent trials. Statistical analysis: *** p < 0.001, **** p < 0.0001, One-way ANOVA, Multiple comparisons; Dunnett correction. All columns compared to “EV-HIZR-1”. (B) The bars represent average interaction strength between MDT-15–HIZR-1 with no zinc treatment or 1, 5, 10, 25, and 50μM zinc supplementation. Average interaction strength (arbitrary units, A.U.). Error bars: SEM. n=3 independent trials. Statistical analysis: *** p < 0.001, **** p < 0.0001, One-way ANOVA, Multiple comparisons; Dunnett correction. All columns compared to “no treatment”. (C) The bars represent average interaction strength between MDT-15–HIZR-1, MDT-15–NHR-64, and MDT-15–NHR-49, with no treatment, or treated with 200μM zinc, 5 μM cadmium, or 200 μM manganese. Average interaction strength (arbitrary units, A.U.). Error bars: SEM. n=3 independent trials. Statistical analysis: **** p < 0.0001, Two-way ANOVA, Multiple comparisons; Dunnett correction. Each interaction is compared to the pertinent “no treatment” control.

2.6 HIZR-1 bound MDT-15 via the KIX-domain

MDT-15 contains an N-terminal KIX-domain that binds several NHRs and the lipogenic transcription factor SBP-1 (Yang et al. 2006; Arda et al. 2010; Taubert et al. 2006). As this is the principal known transcription-factor-binding domain in MDT-15 and related proteins (Thakur et al. 2014), I predicted that HIZR-1 would physically associate with MDT-15 through the KIX-domain. To test this hypothesis, I assayed binding of HIZR-1 to an MDT-15-KIX-domain (aa 1-124), as well as a MDT-15ΔCT variant lacking the KIX-domain (MDT-15ΔKIXΔCT; aa 125-600). The binding of HIZR-1 to the MDT-15-KIX-domain was similar in strength as the binding to MDT-15ΔCT (Figure 2.6). Moreover, MDT-15ΔKIXΔCT was unable to bind HIZR-1. This
indicates that the KIX-domain is necessary and sufficient for the HIZR-1–MDT-15 interaction (Figure 2.6).

**Figure 2.6 The KIX domain of MDT-15 is necessary and sufficient to bind HIZR-1**

Protein-protein interaction analysis between HIZR-1 and MDT-15 deletion mutants MDT-15-ΔCT, MDT-15-KIX, and MDT-15-ΔKIX-ΔCT using the Y2H system; the diagram shows the deletions in the MDT-15 proteins that were analyzed. The bars represent average interaction strength (arbitrary units, A.U.). Error bars: SEM. n=3 independent trials. Statistical analysis: * p < 0.05, **** p < 0.0001, One-way ANOVA, Multiple comparisons; Dunnett correction, comparing individual values to “MDT-15-ΔCT–HIZR-1”. Expression of proteins fused to Gal4AD was confirmed by immunoblot, with GAPDH as loading control.
2.7 A HIZR-1 gain-of-function mutation strengthened binding to MDT-15

The \textit{hizr-1}(am285) \textit{gf} mutation is an aspartate 270 to asparagine (D270N) substitution in the LBD, at a residue that is conserved in other nematode species (Figure 2.7A). This mutation results in increased nuclear localization and constitutive activation of zinc responsive genes in the absence of zinc (Warnhoff \textit{et al.} 2017). To examine whether this mutation might affect the physical binding of HIZR-1 to MDT-15, I performed Y2H assays with a HIZR-1-D270N prey generated by site-directed mutagenesis. HIZR-1-D270N interacted more strongly with MDT-15 than did wild-type HIZR-1, resembling in strength the HIZR-1–MDT-15 interaction in the presence of zinc (Figure 2.7B). Nevertheless, supplemental zinc further enhanced this interaction (Figure 2.7B).
Figure 2.7 HIZR-1-D270N interaction with MDT-15 is stronger than wild type and increases with zinc treatment

(A) Alignments of HIZR-1 homologues in different Caenorhabditis species. The black arrow indicates the D270 amino acid affected by the hizr-1(am285) gf mutation. (B) Protein-protein interaction analysis between HIZR-1 and MDT-15 with and without zinc treatment, and HIZR-1-D270N and MDT-15 with and without zinc treatment. Average interaction strength (arbitrary units, A.U.). Error bars: SEM. n = 4 independent trials. Statistical analysis: ** p < 0.01, **** p < 0.0001, One-way ANOVA, Multiple comparisons; Dunnett correction, comparing individual values to “MDT-15-∆CT-HIZR-1”. Expression of proteins fused to Gal4 AD was confirmed by immunoblot, with GAPDH serving as loading control.

2.8 MDT-15 was physiologically and functionally important for zinc homeostasis

Gut granules protect against zinc toxicity by storing excess zinc; vice versa, they replenish zinc in situations of zinc deficiency (Roh et al. 2012). To study zinc storage in gut granules in wild-type and mutant worms, I used the zinc-specific fluorescent dye FluoZin-3 (Roh et al. 2012). Gut granule morphology was grossly similar in unsupplemented wild-type, mdt-15(tm2182), and cdk-8(tm1238) worms (data not shown). 200μM zinc supplementation resulted in bigger granules with a stronger fluorescent signal in wild-type worms (Figure 2.8A). Strikingly, mdt-15(tm2182) mutants displayed significantly less gut granules in high zinc conditions than wild-type worms and cdk-8(tm1238) mutants (Figure 2.8A-B). As HIZR-1 interacts with MDT-15 in zinc-stimulated fashion, I also studied hizr-1(am286) null mutants and found that these mutants also have less granules than wild type in excess zinc conditions, albeit not as few as mdt-15(tm2182) mutants (Figure 2.8A,C).
Figure 2.8 *mdt-15(tm2182)* mutants have a zinc storage defect

(A) Representative fluorescence images of worms stained with FluoZin-3, showing zinc accumulation in gut granules in wild-type, *mdt-15(tm2182)*, *cdk-8(tm1238)*, and *hizr-1(am286)* worms grown with 200µM zinc supplementation. (B) Quantification of the number of gut granules in wild-type, *mdt-15(tm2182)*, and *cdk-8(tm1238)* worms; every dot represents an individual worm. Error bars: SEM. n > 10 worms per genotype. Statistical analysis: **** p < 0.0001, One-way ANOVA, Multiple comparisons, Dunnett correction, compared to “N2”. (C) Quantification of the number of gut granules in wild-type and *hizr-1(am286)* worms; every dot represents an individual worm. Error bars: SEM. n > 13 worms per genotype. Statistical analysis: *** p < 0.001, One-way ANOVA, Multiple comparisons, Dunnett correction, compared to “N2”.  

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To examine whether *mdt-15* is functionally required in zinc homeostasis, I assessed the effect of excess zinc on an organismal phenotype, egg-laying. I found that 100μM zinc decreased the number of eggs laid by approximately 30% in wild type. In contrast, the same concentration of zinc almost completely abolished egg-laying in *mdt-15(tm2182)* mutants, indicating that this mutant is hyper-sensitive to zinc (Figure 2.9).

**Figure 2.9 mdt-15(tm2182) egg-laying is almost completely abolished in 100μM zinc**

The bars represent the average number of eggs laid over a 24 hours period by wild-type (N2) worms and *mdt-15(tm2182)* mutants grown on 0μM and 100μM zinc (*n > 51* worms per condition). Error bars: SEM; statistical analysis: **** *p < 0.0001* Two-way ANOVA, Multiple comparisons; Tukey correction.
2.9 MDT-15 was partly required for adaptation to low zinc

*mdt-15* plays an important role protecting against excessive zinc levels, but whether *mdt-15* is also involved in adaptation to reduced zinc availability has not been explored. To test whether *mdt-15* is involved in this homeostatic pathway, I compared MDT-15 regulated genes to N,N,N',N'-tetrakis (2-pyridinylmethyl)-1,2-ethanediamine (TPEN)-responsive genes (Zhang et al. 2017). TPEN is a zinc chelator that causes zinc deficiency when added to the growth media (Dietrich et al. 2017). I found a substantial and significant overlap between TPEN-responsive genes and *mdt-15* dependent genes (Figure 2.10A). To validate these results and to test whether *mdt-15* is required for low-zinc activated transcription, I grew wild-type and *mdt-15(tm2182)* worms in the presence of TPEN. I then analyzed by qPCR the expression of two ZIP genes (*zipt-7.1* and *zipt-2.3*) and several others that are induced by TPEN (*comt-5, cyp-25A3, cyp-13B1*, and *oac-14*). I also analyzed the expression of *mtl-1* as a control and observed that *mtl-1* levels were reduced in the presence of TPEN. All other genes were induced by TPEN, as expected. Notably, *comt-5, cyp-25A3, cyp-13B1*, and *oac-14* mRNA levels were substantially abrogated in *mdt-15* mutants, whereas *zipt-7.1* and *zipt-2.3* mRNA levels were not affected (Figure 2.10B).
Figure 2.10 MDT-15 is required for TPEN-induced gene expression

(A) Venn diagrams show statistically significant overlaps between TPEN-responsive genes and MDT-15 activated and repressed genes (Fisher’s exact test p-values are indicated). (B) qPCR analysis of TPEN-responsive genes in wild-type (N2) worms and mdt-15(tm2182) mutants grown on 0μM or 100μM TPEN. Bars represent fold induction, normalized to the average of un-supplemented N2 mRNA expression levels. Error bars: SEM, n = 4 independent trials. Statistical analysis: Unpaired t-test comparing normalized mRNA expression of N2 supplemented worm to mutant supplemented worms.
To assess *mdt-15*’s functional requirement in low zinc, I tested whether TPEN affected the progeny production of wild-type and *mdt-15(tm2182)* worms. Exposure to TPEN reduced progeny production by approximately 60% in wild-type worms and by approximately 92% in *mdt-15(tm2182)* (Figure 2.11).

![Figure 2.11 *mdt-15(tm2182)* egg-laying is reduced in 50μM TPEN](image)

The bars represent the average number of eggs laid over a 24 hours period by wild-type (N2) worms and *mdt-15(tm2182)* mutants grown on 0μM or 50μM TPEN. Error bars: SEM, *n* > 44 worms. Statistical analysis: **** *p* < 0.0001 Two-way ANOVA, Multiple comparisons; Tukey correction.
2.10 cdf-1 was induced by TPEN

As mentioned, CDF-1 is localized to the basolateral cytoplasmic membrane in *C. elegans* intestinal cells and transports zinc from the intestinal cell cytoplasm to the pseudocoelomic cavity. From the pseudocoelom, zinc can be transported to other *C. elegans* tissues (Roh *et al.* 2013). In previous studies, *cdf-1* mRNA levels were not induced by excess zinc (Dietrich *et al.* 2016). To examine the *cdf-1* regulatory mechanism, I verified previous studies by examining *cdf-1* mRNA levels with 200μM zinc supplementation, and observed that *cdf-1* mRNA levels was not induced, opposite to the strong induction of *mtl-1* (Figure 2.12A). To examine whether *cdf-1* is instead induced by zinc deficiency, I studied *cdf-1* levels in worms grown on 100μM TPEN, and found that there is a significant induction, while *mtl-1* levels decreased (*p* < 0.07) (Figure 2.12B). In *mdt-15* mutants *cdf-1* transcripts levels were similar to wild type (Figure 2.12B), indicating that this mechanism is *mdt-15* independent.
Figure 2.12 cdf-1 is induced by zinc deficiency

qPCR analysis of cdf-1 and mtl-1 in zinc excess and deficiency in wild-type (N2) worms and mdt-15(tm2182) mutants. Bars represent fold induction, normalized to the average of un-supplemented N2 mRNA expression levels. (A) Comparison of N2 and mdt-15(tm2182) worms grown on 0μM zinc to N2 grown on 200μM zinc. Error bars: SEM, n = 3 independent trials. Statistical analysis: **** p < 0.0001 unpaired t-test comparing normalized mRNA expression of N2 un-supplemented worm to N2 supplemented worms. (B) Comparison of N2 and mdt-15(tm2182) worms grown on 0μM zinc to N2 grown on 100μM TPEN. Error bars: SEM, n = 4 independent trials. Statistical analysis: * p < 0.05 unpaired t-test comparing normalized mRNA expression of N2 un-supplemented worm to N2 supplemented worms.
2.11 Mouse MED15 regulated the expression of the *cdf-2* ortholog SLC30A8/ZnT8 in the pancreas

In mammals, the insulin-secreting β-cells of the pancreas require appropriate amounts of zinc to store and release insulin from the ISG. Interestingly, ZnT8/SLC30A8, the mouse ortholog of the MDT-15 and HIZR-1 regulated zinc transporter CDF-2 (Figure 2.1A), is expressed exclusively in the α- and β-cells of the pancreas (Chabosseau and Rutter 2016). In β-cells, ZnT8 transports zinc from the cytosol to the ISG, where it triggers the crystallization of insulin (Chabosseau and Rutter 2016). To test whether the transcriptional regulatory mechanism of worm *cdf-2* and of mouse *Slc30a8* is conserved, *Slc30a8* transcript levels were quantified in RNA-seq experiments done on β-cells from wild-type and β-cell specific Med15 KO mice (the mouse ortholog of *mdt-15*; unpublished data by A. Kadhim, Lynn & Taubert labs). Resembling the requirement of *mdt-15* in *cdf-2* expression, Med15 ablation substantially reduced *Slc30a8* mRNA levels (A. Kadhim, personal communication). To validate this data, I performed immunofluorescence on pancreas sections of wild-type and Med15 KO mice, and found that Med15 loss results in substantially lower levels of ZnT8 protein (Figure 2.13). Thus, regulation of ZnT8-type proteins by MDT-15/MED15 appears to be evolutionarily conserved.
Figure 2.13 *Med15 KO* mice have lower levels of ZnT8 protein

Representative fluorescence images of ZnT8 protein (green) in wild-type (WT) and *Med15 KO* mouse islets. Pancreatic and Duodenal Homeobox 1 (Pdx1) is presented as control (red). Nuclei are visible with TO-PRO-3 iodine staining (blue).
Chapter 3: Discussion and Conclusions

In the nematode, *C. elegans*, exposure to high concentrations of the trace metal zinc or of the toxic metal cadmium causes strong transcriptional induction of zinc and cadmium detoxification genes. Previous studies identified the players that are involved in the transcriptional regulation of many of these genes. The Mediator subunit *mdt-15* is required for induction of zinc and cadmium responsive genes, while the TFs *elt-2* and *hizr-1*, and their DNA elements are required for induction of zinc responsive genes, specifically at the *mtl-1* promoter (Taubert et al. 2008; Roh et al. 2015; Warnhoff et al. 2017). In previous collaborative work in our lab, another graduate student and I showed that these factors regulate the *cdr-1* promoter under cadmium stress, and that the Mediator subunit *cdk-8* also plays a minor role in the induction of some metal responsive genes (Grants 2016). However, to date, these studies did not provide the mechanism of how these factors cooperate in the regulation of metal stress response, and did not address whether *cdk-8* is also involved in zinc homeostasis.

In this thesis, I address the hypothesis that the Mediator subunits MDT-15 and/or CDK-8 work through TFs to regulate zinc homeostasis and cadmium stress response. Specifically, I demonstrate that *mdt-15* and *hizr-1* are functionally co-dependent and physically interact with each other to regulate zinc and cadmium responsive genes (sections 2.4-2.5). In addition, I show that the Mediator subunit *cdk-8* is partly required for zinc inducible transcription as well as cadmium (Figure 2.1B-C). Moreover, I show a physiological role for *mdt-15* in zinc homeostasis, as loss of this Mediator subunit causes defects in zinc storage in the worm’s gut granules (section 2.8). Lastly, in collaboration with Alex Kadhim (a PhD student in our lab), I have found a potentially conserved mechanism of zinc homeostasis regulation in *Med15* β-cell specific KO diabetic mice. Thus, my study adds to our understanding of the roles of the players
involved in zinc homeostasis and cadmium stress response, as well as the physiological consequences of impaired faction of these factors and potential application in mammals (Figure 3.1).

3.1 Regulatory factors involved in zinc and cadmium responsive gene expression

To examine the factors involved in transcriptional regulation of zinc and cadmium responsive gene expression, I used two approaches. The first approach was observing RNA levels, through qPCR analysis, of the two *C. elegans* MTs, *mtl-1* and *mtl-2*, the *cdr-1* gene, and two zinc transporters, *cdf-2* and *ttm-1*. All of these genes are zinc and cadmium responsive, and some play a protective role in zinc excess or cadmium exposure (Hall *et al.* 2012; Roh *et al.* 2013). The second approach was to use a *cdr-1* promoter, fused to a GFP reporter, to examine the factors that are required for fluorescence induction under zinc excess or cadmium exposure conditions.

Both approaches have demonstrated the requirements of the two Mediator subunits *mdt-15* and *cdk-8* in zinc and cadmium induction, although *cdk-8* is only partly required (sections 2.1-2.2). These findings agree with previous studies, where *mdt-15* and *cdk-8* are required to regulate cadmium induction, and *mdt-15* is required to regulate zinc and cadmium induction (Taubert *et al.* 2008; Grants 2016). The finding that *cdk-8* is also required for zinc induction is novel, although it is not surprising, taking in consideration that zinc and cadmium bind many of the same proteins and share detoxification mechanisms (Fukada *et al.* 2011; Brzoska and Moniuszko-Jakoniuk 2001; Dong *et al.* 2005; Vallee and Falchuk 1993).

By studying the *cdr-1* promoter GFP reporter, I have shown that the TFs ELT-2 and HIZR-1 are required for fluorescence induction under cadmium exposure (collaboration with
Jennifer Grants). This effect is likely direct, as mutagenesis of their DNA regulatory elements on the promoter (the GATA and HZA elements, respectively) also abrogates the fluorescence induction of the reporter (section 2.3). GATA and HZA regulatory elements have also been investigated in the mtl-1 promoter GFP reporter, where they were required for zinc-inducible transcription (Roh et al. 2015). In addition, these regulatory elements, derived from the mtl-1 promoter, were isolated and fused to GFP as a 3X HZA&GATA::pes-10::NLS-GFP reporter that is induced by supplementary zinc or cadmium (Roh et al. 2015). This demonstrates that the GATA and HZA elements are not only required, but also sufficient to increase fluorescence signaling in the presence of cadmium and zinc. It would be interesting to examine this reporter in cdk-8 and mdt-15 mutants as another way to observe whether these coregulators work directly through these elements.

3.2 MDT-15 and HIZR-1 have a co-dependent function through physical interaction

As mentioned in section 1.2.2.1, mdt-15 is part of the tail module of Mediator, which serves as a docking site for many TFs (Tsai et al. 2014; Grants et al. 2015). In particular, mdt-15 specifically regulates metabolic processes and stress responses thorough interaction with NHRs (Taubert et al. 2006; Arda et al. 2010). Similarly, cdk-8 is required for NHR-driven transcription in Drosophila and human cells (Xie et al. 2015; Belakavadi and Fondell 2010). Thus, both proteins a priori were candidates to interact with the zinc regulatory NHR HIZR-1 in the regulation of metal response genes. Indeed, both mdt-15 and cdk-8 are required for induction of zinc and cadmium responsive genes; however, this requirement is much stronger for mdt-15 (section 2.1). Taking this into consideration, as well as the fact that ELT-2 regulates virtually all intestinal genes in worms and is not specific to metal regulated transcription (Fukushige et al. 2015).
I hypothesized that MDT-15 and HIZR-1 physically interacts as part of this stress response mechanism. To test this hypothesis, I performed Y2H assay and showed that there is a physical interaction between MDT-15 and HIZR-1. The strength of interaction was similar to the interaction of MDT-15 with SKN-1c TF, a factor known to interact functionally and physically with MDT-15 (Pang et al. 2014; Goh et al. 2014) (Figure 2.5A). Interestingly, I found that zinc supplementation increased the intensity of MDT-15 and HIZR-1 binding (Figure 2.5B). This suggests that zinc’s effect on HIZR-1 is not limited to nuclear translocation (Warnhoff et al. 2017), but also to modulating protein-protein interactions, a feature typical for *bona fide* nuclear receptor ligands (Giguere 1999).

Since cadmium and zinc share detoxification mechanisms, I predicted that cadmium supplementation might also strengthen MDT-15-HIZR-1 interaction and found that this is the case. In contrast, supplemental zinc or cadmium did not significantly strengthen the interaction of MDT-15 with NHR-49 or NHR-64, which have no apparent role in heavy metal detoxification. My data further suggests that the comparably high interaction between NHR-64 and MDT-15 may result from a constitutively present ligand (such as a fatty acid), while the relatively weak binding of MDT-15 to NHR-49 hints at a missing ligand. A NHR-64 ligand might be present in the media or in the cells in standard conditions, similar to HNF4α, which was crystallized with a fatty acid bound in its ligand binding pockets (Dhe-Paganon et al. 2002; Ashrafi et al. 2003; Liang et al. 2010; Goh et al. 2014).

As HIZR-1 is an NHR, and MDT-15 contains a KIX-domain that is known to bind NHRs along with other TFs (Arda et al. 2010; Taubert et al. 2006; Thakur et al. 2014), I examined whether HIZR-1 binds MDT-15 at the KIX-domain. As expected, I found that that is the case.
To strengthen the hypothesis that *mdt-15* and *hizr-1* function is co-dependent, I used *mdt-15*(gf) and *hizr-1*(gf) mutants, which both induce zinc responsive genes in the absence of excess zinc. *hizr-1* RNAi prevented the induction of zinc responsive genes in *mdt-15*(gf) mutants; *vice versa*, *mdt-15* RNAi prevented the induction of zinc responsive genes in *hizr-1*(gf) mutants (Figure 2.4).

Further work is needed to establish whether other physical interactions are involved in this zinc homeostasis mechanism, such as interactions of HIZR-1 with CDK-8, and/or ELT-2 with MDT-15 or CDK-8. In addition, many NHRs tend to dimerize; hence, it would be interesting to examine whether HIZR-1 homodimerizes or heterodimerizes, and whether this potential interaction may be influenced by zinc supplementation, similar to NHR-49, which regulates multiple gene sets in the fatty acid β-oxidation by partnering with different NHRs (e.g., NHR-66 to regulate sphingolipid and lipid remodeling genes, NHR-80 to regulate fatty acid desaturation related genes) (Pathare *et al.* 2012).

### 3.2.1 HIZR-1 gain-of-function mutant increases the physical interaction with MDT-15

*hizr-1*(am285) is a semidominant gf mutation that causes an induction of zinc responsive genes in the absence of zinc. This missense mutation has an asparagine, a polar residue, instead of an aspartic acid, an acidic residue, at conserved site (D270N) (Figure 2.7A). This mutation promotes HIZR-1 accumulation in the nucleus, as does the presence of zinc (Warnhoff *et al.* 2017).

As this gf mutation has a similar phenotype as zinc supplementation, I predicted that the HIZR-1-D270N protein might strengthen the interaction with MDT-15, similar to the interaction of wild-type HIZR-1 and MDT-15 with zinc supplementation. I examined HIZR-1-D270N
binding to MDT-15 with and without zinc supplementation (Figure 2.7) and found that HIZR-1-D270N without zinc supplementation increased the binding, as predicted. However, when zinc was added, the interaction of the HIZR-1-D270N-MED-15 still increased significantly. This suggests that the D270N mutation might mimic the LBD conformation when zinc is bound; however, additional zinc still increases MDT-15-HIZR-1 binding. This may suggest that the mutation causes a confirmation change in the LBD that exposes the binding site to MDT-15, independent from the ligand binding areas, and when zinc binds to the LBD, it will cause an additional change in the conformation of the protein, and further exposure of the binding site to MDT-15. A limitation of this assay is that it does not provide structural information. To understand if HIZR-1-D270N LBD conformation mimics zinc binding, structural analysis such as x-ray crystallography of HIZR-1-D270N and wild-type HIZR-1 with zinc supplementation is needed.

3.3 *mdt-15* plays a physiological role in zinc excess conditions

In the intestine of *C. elegans*, zinc is stored in lysosome-related organelles called gut granules, which function as an important part of the zinc homeostasis mechanism. In excess zinc conditions, the gut granules promote detoxification of zinc by mobilizing surplus zinc into the granules via the CDF-2 transporter, while in zinc deficiency, stored zinc is replenished to the cytoplasm (Davis *et al.* 2009; Roh *et al.* 2012).

MDT-15 is involved in transcriptional regulation of the CDF-2 transporter, and so I propose that *mdt-15* plays a physiological role in zinc detoxification by promoting zinc storage in the gut granules through CDF-2 upregulation. I examined zinc storage in the gut granules in *mdt-15, cdk-8, and hizr-1* mutants, and found that *mdt-15* and *hizr-1* mutants have significantly less
gut granules than in wild types. Though \textit{mdt-15} and \textit{hizr-1} mutants both have reduced amounts of gut granules, \textit{mdt-15} mutants have even fewer granules than \textit{hizr-1} mutants do. This may be due to MDT-15 regulating additional genes that may respond to excess of zinc and are not \textit{hizr-1} regulated.

To further examine the physiological role of \textit{mdt-15} in zinc detoxification, I performed an egg laying assay, and compared the progeny production of \textit{mdt-15} mutants to that of wild type worms. I found that the ability of \textit{mdt-15} mutants to lay eggs in high zinc conditions was almost totally abrogated (Figure 2.9). This supports my previous findings and shows that MDT-15 has an important functional role in zinc detoxification.

A limitation of this assay is that I have not examined the progeny production of \textit{hizr-1} mutants, to test if they phenocopy the \textit{mdt-15} mutant results, which could indicate that this zinc sensitivity is due to a problem in transcriptional regulation involving the \textit{mdt-15} and \textit{hizr-1} partnership.

### 3.4 Requirement of \textit{mdt-15} in zinc deficient conditions

To observe if \textit{mdt-15} is also involved in regulation of zinc deficient mechanisms, I compared MDT-15 activated and repressed genes with TPEN activated genes (Zhang \textit{et al.} 2017), and found that these gene sets share significant overlaps (Figure 2.10A). It is important to note that the microarray analysis of \textit{mdt-15} mutants was conducted under standard conditions; thus, the overlaps only include TPEN-responsive genes whose basal levels are changed in \textit{mdt-15} mutants.

I examined TPEN responsive genes in wild-type worms and in \textit{mdt-15} mutants, and found that a set of TPEN induced genes were \textit{mdt-15} dependent, although not significant.
Notably, *mdt-15* depletion had a small effect on one of the ZIP genes (*zipt*-2.3) but a more substantial effect on *comt-5*, *cyp-25A3*, *cyp-13B1*, and *oac-14* (Figure 2.10B). Thus, these data suggest that *mdt-15* may play a role in the regulation of zinc deficiency responsive genes, though the genes involved may affect TPEN detoxification rather than zinc deficiency.

To test whether *mdt-15* has a functional role in protection from zinc deficiency, I performed an egg laying assay, and found that *mdt-15* mutants are sensitive to TPEN, although this phenotype was not as striking as the one displayed by *mdt-15* mutants in zinc excess. Importantly, *mdt-15* regulates many putative xenobiotic detoxification genes (Taubert *et al.* 2008; Pukkila-Worley *et al.* 2014), and hence it is possible that the overlap between *mdt-15* regulated genes and TPEN-responsive genes reflects a detoxification response caused by TPEN, an organic molecule, rather than only zinc deficiency. To examine this further, one could compare worms grown on TPEN to worms grown on a balanced amount of TPEN and zinc. This would allow identifying genes that are induced due to zinc deficiency and not TPEN toxicity.

An additional limitation of this study is that these assays were done with *mdt-15* mutants that are hypomorphs and cause a reduction of function in the MDT-15 protein but are not *mdt-15* null mutants. In the future, performing *mdt-15* knock down with RNAi can validate the phenotype that was observed. It is important to note that RNAi knockdown has limitations as well, such as causing target mRNA to be reduced and not abolished, in addition to having off-target effects and not being effective in different tissue (i.e. neurons) (Fraser *et al.* 2000; Jackson and Linsley 2010). Combining studies with mutants and RNAi can be a comprehensive approach to conduct such a study.
3.4.1 *cdf-1* regulation in zinc deficiency

As discussed in section 2.10, *CDF-1* is a *C. elegans* zinc transporter localized to the cytoplasmic membrane of many types of cells. In intestinal cells, *CDF-1* localizes to the basolateral cytoplasmic membrane, where it transports zinc into the pseudoceolom, from where it is distributed to the rest of the body. *cdf-1* transcript levels are not regulated by supplementary zinc (Roh et al. 2013; Dietrich et al. 2016). I propose that, as this transporter localizes to the basolateral cytoplasmic membrane in the intestine, *CDF-1* protects against zinc deficiency. Accordingly, when zinc availability is low, *cdf-1* transcript levels are induced to enable zinc transport from the intestine to the pseudoceolom and to tissues that are presumably more sensitive to zinc deficiency (i.e. the neurons) (Frederickson et al. 2000). Therefore, *cdf-1* transcript levels might be induced by exposure to TPEN, similar to ZIP transporters. This is supported by RNA-seq analysis done by Zhang et al., who found that *cdf-1* was induced almost two-fold by TPEN (Zhang et al. 2017). By qPCR analysis, I found that, as expected, *cdf-1* transcript levels barely changed when exposed to supplementary zinc but are induced significantly by TPEN (Figure 2.12). This result adds to our understanding of transporter regulation in the *C. elegans* gut, and specifically regulation of CDF-1 transporter.

3.5 Conservation and potential applications of the research

*C. elegans* *CDF-2* is homologous to the mammalian zinc transporter ZnT8, and while CDF-2 transports zinc into the gut granules for storage, ZnT8 transports zinc into the insulin granules in the β-cells of the pancreas for insulin crystallization (Davis et al. 2009; Roh et al. 2013; Chimienti et al. 2004). *cdf-2* transcripts are upregulated by dietary zinc in *mdt-15* dependent fashion (Figure 2.1A) (Taubert et al. 2008). Interestingly, RNA-seq data from the
Taubert and Lynn labs has revealed that loss of mouse Med15 (mdt-15 ortholog) reduces basal mRNA levels of Slc30a8. In addition, Alex Kadhim and I have performed immunofluorescence assay, and found that ZnT8 protein is reduced in Med15 KO mice (Figure 2.13).

These results suggest an evolutionarily conserved regulatory mechanism that regulates nematode cdf-2 and mammalian Slc30a8, which might include the hizr-1 ortholog HNF4 as a partner for MED15 in regulating Slc30a8 in pancreas (Figure 3.1). As mentioned in section 1.4.1, ZnT8 plays a role in T2D, and by studying zinc homeostasis in C. elegans and establishing the players of the transcriptional regulatory mechanism, we can broaden our understanding of zinc regulatory mechanisms in mammals and its relation to diabetes.

Further work is needed to establish the zinc regulation mechanism in the pancreatic β-cells, and this can be done by examining the DNA regulatory elements requirement in the Slc30a8 promoter in the widely used mouse insulinoma cell line MIN6. This experiment can point towards a TF that might bind MED15 to regulate this response. In addition, performing coimmunoprecipitation experiments for MED15 and HIZR-1 orthologs (e.g. HNF4) may also reveal the TF(s) that regulates zinc response. Interestingly, mutations in HNF4α are associated with diabetes among other diseases, specifically maturity onset diabetes of the young (MODY) (Ellard and Colclough 2006).

Understanding of zinc regulation response can contribute knowledge pertinent to different diseases, including diabetes, and might help develop better treatments for diabetes in the future.
3.6 Conclusion

The Mediator subunits MDT-15 and the NHR HIZR-1, among other factors, regulate zinc homeostasis and cadmium stress response in C. elegans (Taubert et al. 2008; Roh et al. 2015). I have defined the interaction between MDT-15 and HIZR-1 as part of this regulatory mechanism and found that zinc and cadmium strengthen this interaction. I have also examined the physiological role of mdt-15 in zinc detoxification response through gut granule storage and examined a potentially evolutionarily conserved zinc regulatory mechanism between the C. elegans gut and the β-cells in the pancreas (Figure 3.1). As zinc homeostasis plays a role in many human diseases, including diabetes, it can be beneficial to test if the regulatory interactions I have defined in C. elegans are evolutionally conserved and what TF might interact with MED15 in this regulatory mechanism.
Figure 3.3.1 A model for a mechanism regulating zinc homeostasis that may be conserved between *C. elegans* gut cells and mouse β-cells

The model shows the genes and proteins that are *mdt-15* dependent in the *C. elegans* intestine and *Med15* dependent in mouse β-cells and that regulate zinc homeostasis in excess zinc conditions. HNF4 is an ortholog of HIZR-1 (Warnhoff et al. 2017); therefore, it can potentially be the MED15 partner for zinc homeostasis regulation.
Chapter 4: Materials and methods

4.1 *C. elegans* strains and culture

*C. elegans* strains were cultured at 20°C on nematode growth medium (NGM)-lite (0.2% NaCl, 0.4% tryptone, 0.3% KH2PO4, 0.05% K2HPO4) agar plates and supplemented with 5 μg/mL cholesterol as described (Brenner 1974). Plates were seeded with *Escherichia coli* OP50 as food source, except for RNAi, for which we used HT115. Cadmium was supplemented as cadmium chloride (CdCl₂) in NGM-lite at indicated concentrations. For zinc (ZnSO₄) and N,N,N′,N′-tetrakis(2-pyridinylmethyl)-1,2-ethanediameine (TPEN) supplementation, in qPCR and microscopy assays, we used plates containing noble agar minimal media (NAMM) (Bruinsma et al. 2008). For the egg laying assay, we used Agar A. To obtain developmentally synchronized populations for qPCR and phenotype analysis, gravid adult worms were treated with sodium hypochlorite to isolate eggs, which were allowed to hatch overnight on unseeded plates. Synchronized L1 worms were placed on seeded plates until being harvested at the desired developmental stage, as indicated.

Wild-type strain was N2 Bristol; all other strains are listed in Table 4.1.

Table 4.1 *C. elegans* strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>STE21</td>
<td><em>mdt-15(tm2182)</em> III</td>
</tr>
<tr>
<td>XA7703</td>
<td><em>cdk-8(tm1238)</em> I</td>
</tr>
<tr>
<td>STE101</td>
<td>*steEx49[cdr-1P::<em>GFP]</em></td>
</tr>
<tr>
<td>STE102</td>
<td><em>cdk-8(tm1238)</em> I; *steEx49[cdr-1P::<em>GFP]</em></td>
</tr>
<tr>
<td>STE103</td>
<td>*steEx50[cdr-1P(mutHZA):<em>GFP]</em></td>
</tr>
<tr>
<td>STE104</td>
<td>*steEx51[cdr-1P(mutGATA1):<em>GFP]</em></td>
</tr>
</tbody>
</table>
4.2 RNA isolation and quantitative qPCR

RNA was extracted from developmentally synchronized worms and prepared for gene expression analysis by qPCR analysis as described (Grants et al. 2016). I used t-tests (two-tailed, equal variance) to calculate the significance of expression changes between conditions, as indicated. qPCR primers were designed with Primer3web (bioinfo.ut.ee/primer3/) and tested on serial cDNA dilutions to analyze primer efficiency. Primer sequences are listed in Table 4.2.

Table 4.2 qPCR primers

<table>
<thead>
<tr>
<th></th>
<th>Forward primer sequences</th>
<th>Reverse primer sequences</th>
</tr>
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<tbody>
<tr>
<td>mtl-1</td>
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<tr>
<td>mtl-2</td>
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<td>GCTTTCAGAAAAAACCTCGA</td>
</tr>
<tr>
<td>cdr-1</td>
<td>TCTTCTCTCAATGGCAACTG</td>
<td>TTTGGGTAACCTTTGACGCA</td>
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<td>cdf-2</td>
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<td>act-1</td>
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<td>ubc-2</td>
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<td>CGGATTTGACAGAGGACGC</td>
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</table>

4.3 **Fluorescent reporter analysis**

Sequences of regulatory elements involved in the zinc, cadmium, or other detoxification/stress responses were identified from the literature, including ARE, MRE, HSE, DBE, HZA, GATA, HRE, and TATA box elements (Stuart *et al.* 1984; Sorger 1991; Blackwell *et al.* 1994; Chen *et al.* 1999; Furuyama *et al.* 2000; Smale and Kadonaga 2003; Adlard *et al.*; Shen *et al.* 2005; McGhee *et al.* 2007; Chiang *et al.* 2012; Roh *et al.* 2015). If available, the equivalent *C. elegans* consensus sequence for each regulatory element was identified in the literature, otherwise the eukaryotic consensus sequence was used. The *cdr-1* promoter (-1500 nucleotides upstream from transcriptional starting site) was then searched manually for presence of these candidate elements using SerialCloner 2.5.

The *cdr-1P::GFP* reporter was generated by PCR amplification of the genomic region from 2853 base pairs upstream to 11 base pairs downstream of the *cdr-1* start codon (a G>C mutation at the +3 nucleotide was introduced in the reverse primer to mutate the *cdr-1* start codon), and cloning into the pPD95.77 GFP vector from the Fire lab vector kit (Addgene plasmid #1495). Mutant transgenes were generated by site-directed mutagenesis of the pPD95.77-*cdr-1P* plasmid using the Q5 Site-Directed Mutagenesis kit (NEB E0554S). All transgenes were verified by Sanger sequencing. Transgenic strains were generated by injecting a mixture of 50 ng/μl GFP reporter plasmid, 5 ng/μl pCFJ90[myo-2p::mCherry], and 95 ng/μl
pPD95.77 empty vector into wild-type worms, and then selecting transgenic mCherry-positive progeny.

Synchronous day 1 adult worms were subjected to a 4-hour exposure of 100μM CdCl₂ or a 4-hour exposure of 200μM ZnSO₄, or 4 hours no stress control. All chemicals were dissolved in NGM-lite plates that were seeded with 5x concentrated OP50.

4.4 Gene knockdown by feeding RNAi

Knockdown by feeding RNAi was carried out on NGM-lite plates with 25 μg/mL carbenicillin, 1 mM IPTG, and 12.5 μg/mL tetracycline, and seeded twice with the appropriate HT115 RNAi bacteria clone. The mdt-15, elt-2, hizr-1, and nhr-49 clones were taken from the Ahringer library, 96-well format; mdt-15: plate 74, well C09; skin-1: plate 99, well G09; hsf-1: plate 21, well B05; daf-16: plate 18, well G12; hizr-1: plate 193, well H07; elt-2: plate 196, well E06. RNAi clones were sequenced to confirm insert identity; RNAi negative control was empty vector L4440. Synchronous L1 worms were grown on RNAi plates until they reached the required developmental stage.

4.5 FluoZin-3 Staining

FluoZin-3 acetoxymethyl ester (Molecular Probes F24195) was dissolved in dimethylsulfoxide (DMSO) to a 1 mM stock solution. FluoZin-3 and LysoTracker Red DND-99 (1 mM, Life Technologies L7528) were diluted in M9 to generate a concentration of 30μM and 20μM, respectively, and dispensed on NAMM plates, as described (Roh et al. 2012). Synchronized wild-type and mutant L4 stage worms were transferred from NGM-lite plates to these plates and cultured for 16 hrs. Worms were then transferred to NGM-lite plates without
FluoZin-3 for 30 min to reduce excess FluoZin-3 signal from the intestinal lumen before imaging.

### 4.6 Fluorescence microscopy

Worms were transferred onto 2% (w/v) agarose pads containing 15μM sodium azide (NaN₃) for microscopy. For the analysis of *cdr-1P::GFP* reporter, more than 19 worms were used per condition. The worms were imaged using DIC optics and fluorescence microscopy through a HQ camera (Photometrics, Tucson, AZ, USA) on a Zeiss Axioplan 2 microscope (Carl Zeiss Microscopy, Thornwood, NY, USA). Analysis of fluorescence intensity was performed using ImageJ software, normalizing for area and background fluorescence. For analysis of worms stained with FluoZin-3, more than 10 worms were used per condition. We used a Leica SP8 confocal microscope with Leica LAS X software and analyzed number of granules through ImageJ software 3D count; images of worms with different genotypes were taken at the same exposure time.

### 4.7 Yeast two Hybrid assay analysis and Western blot

Pairs of plasmids were transformed into strain Y187 (Clontech, Mountain View, CA, USA) and liquid β-galactosidase assays were performed as described using an OMEGASTAR plate reader (BMG Labtech, Ortenberg, Germany) to measure β-galactosidase activity and yeast growth (optical density OD550) for normalization (Goh et al. 2014). Each assay included at least three technical replicates and was repeated three or more times. Standard yeast lysis, SDS-PAGE, and Western blots were performed to detect protein expression in yeast, as described (Goh et al. 2014). Antibodies used were GAL4 AD Mouse Monoclonal Antibody (Takara Bio
USA, Inc. #630402), GAL4 DNA-BD Mouse Monoclonal Antibody (Takara Bio USA, Inc. #630403), and GAPDH Mouse Monoclonal Antibody (CB1001-500UG 6C5) for normalization.

Plasmids encoding the HIZR-1(am285) gf mutation (D270N) were generated using the NEB Q5 sited-directed mutagenesis kit and the HIZR-1 wild-type plasmid as template. The plasmid was sequenced to verify the presence of the point mutations and the absence of other mutations. Plasmids are listed in Table 4.3.

Table 4.3 Plasmids used for Y2H.

<table>
<thead>
<tr>
<th>Plasmid number</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>SPD152</td>
<td>pGADnewMCS NHR-64-LBD</td>
</tr>
<tr>
<td>SPD167</td>
<td>pGADnewMCS NHR-49-LBD</td>
</tr>
<tr>
<td>SPD226</td>
<td>pGBKnewLeu2 MDT-15-KIX</td>
</tr>
<tr>
<td>SPD228</td>
<td>pGBKnewLeu2 MDT-15-∆CT</td>
</tr>
<tr>
<td>SPD247</td>
<td>pGBKnew-MDT-15-∆CT</td>
</tr>
<tr>
<td>SPD276</td>
<td>pGBK7 newMCS</td>
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<tr>
<td>SPD278</td>
<td>pGBKnewMCSLeu2</td>
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<tr>
<td>SPD280</td>
<td>pGADT7newMCS</td>
</tr>
<tr>
<td>SPD 737</td>
<td>pGBKnewLeu2 MDT-15-∆KIX-∆CT</td>
</tr>
<tr>
<td>SPD722</td>
<td>pDEST-SKN-1c</td>
</tr>
<tr>
<td>SPD885</td>
<td>pGADnewMCS-HIZR-1</td>
</tr>
<tr>
<td>SPD917</td>
<td>pPC86-HIZR-1</td>
</tr>
<tr>
<td>SPD918</td>
<td>pGADnewMCS-HIZR-1-am285</td>
</tr>
</tbody>
</table>
4.8 Phylogenetic tree

Sequence of *Caenorhabditis* species genes were downloaded from WormBase version WS248 (http://www.wormbase.org); sequences of *Caenorhabditis* species genes were downloaded from NCBI (https://www.ncbi.nlm.nih.gov). I constructed the phylogenetic tree with MEGA 7 software using ClustalW alignment, and evolutionary history was inferred using the Maximum Likelihood method.

4.9 Sequence alignments

Alignment of *hizr-1* sequences and its homologues was done in Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo/). The sequence of *hizr-1* and the homologous sequences of other *Caenorhabditis* species were downloaded from WormBase version WS248 (http://www.wormbase.org).

4.10 Egg laying assay

60 N2 and *mdt-15(tm2182)* worms were grown from late L4 stage for 24-26 hours on agar A plates supplemented with 100μM zinc or 50μM TPEN. Eggs and L1 progeny were counted after that time and compared to worms grown on agar A plates with no supplement.

4.11 Microarray and RNA-seq data analysis and generation of Venn diagram

Lists of genes differentially expression in *mdt-15(tm2182)* mutants (S. Taubert, unpublished) were compared to lists of genes differentially expressed in TPEN (Zhang et al. 2017), and the overlap was identified as described in (Grants 2016). To calculate the *p*-values of
the overlap, Fisher’s exact test was performed with R Studio; Venn diagrams were also generated with R Studio.

4.12 Immunofluorescence

Slices of wild-type and KO mice were prepared by Alex Kadhim and Eric Xu. Immunostaining was performed on 5μm paraffin sections, as described (Xu et al. 2015). Primary antibodies were guinea pig anti-insulin (Dako Cytomation A056401-2; used at 1:500), and rabbit Znt8 antiserum (a gift from Howard Davidson; used at 1:1000). Secondary antibodies were from Jackson ImmunoResearch Laboratories (Cy™5 AffiniPure Donkey Anti-Guinea Pig IgG #706-096-148 and Alexa Fluor® 594 AffiniPure F(ab’)2 Fragment Donkey Anti-Mouse IgG #715-586-151).


———. 2006. 'Mouse ZIP1 and ZIP3 genes together are essential for adaptation to dietary zinc deficiency during pregnancy', *Genesis*, 44: 239-51.


Ellard, S., and K. Colclough. 2006. 'Mutations in the genes encoding the transcription factors hepatocyte nuclear factor 1 alpha (HNF1A) and 4 alpha (HNF4A) in maturity-onset diabetes of the young', *Hum Mutat*, 27: 854-69.


Grants, J.M. 2016. 'Roles of Mediator subunit CDK-8 in developmental and physiological responses in Caenorhabditis elegans'.


Ryu, M. S., L. A. Lichten, J. P. Liuzzi, and R. J. Cousins. 2008. 'Zinc transporters ZnT1 (Slc30a1), Zip8 (Slc39a8), and Zip10 (Slc39a10) in mouse red blood cells are differentially regulated during erythroid development and by dietary zinc deficiency', *J Nutr.*, 138: 2076-83.


