Lehrstuhl für Ernährungsphysiologie

Effects of zinc on the transcriptome and proteome of human colonic cancer cells (HT-29 cells)

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Probleme lassen sich nicht mit den Denkweisen lösen,
die zu ihnen geführt haben

Einstein

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1 Introduction

Zinc is an essential element for living organisms (1). The first evidence for this was given by Raulin in 1869, who showed, that zinc is required for the growth of the common bread mold *Aspergillus niger*. Since then, it has been demonstrated that zinc is essential for growth, development and differentiation of all types of life, including microorganisms, plants and animals. After iron, zinc is the second most abundant transition metal in the human body with a total content of 1.5-2.5 g (2). The best dietary sources for zinc are seafoods (especially oysters), meats (especially red and organ meat) and whole grain products (3). The RDA (Recommended Dietary Allowance) for zinc, which means the average daily dietary intake level that is sufficient to meet the nutrient requirements of nearly all (97-98%) healthy individuals is 8 mg per day for women and 11 mg per day for men. The bioavailability of zinc is strongly dependent on the phytate content in the diet, especially if calcium is present due to the stabilization of phytate by calcium. Once zinc is absorbed, albumin acts as the major transport protein in the plasma. An α_2-macroglobulin and amino acids also participate in this process, but to a much lower extent. (3,4). Less than 1% of the total amount of zinc in the body is circulating in blood (5). The majority of zinc is stored in bones (30 %) and muscles (60 %). Ninety percent of zinc excretion is through faeces via intestinal and pancreatic secretions and/or desquamation of intestinal cells (2,3).

1.1 Physical and chemical properties of zinc

Zinc is a first row transition metal with an electron configuration of $3d^{10}4s^2$. Due to the filled d orbital, zinc exclusively exists as a divalent cation (Zn^{2+}) in organic compounds. Zinc's lack of redox activity, in contrast to iron and copper, allows it to be utilized in biological systems without the risk of oxidant damage (6). Zinc is a strong Lewis acid which forms inert complexes, for example with amino, carboxylate and thiol groups of amino acids (namely histidine, cysteine, glutamic and aspartic acid) in proteins. The binding constants of these complexes are quite high, wherefore the concentration of intracellular free zinc ions is extremely low and is expected to be in the picomolar or nanomolar range (7,8). Due to the similarity of its physical and chemical properties with mercury and cadmium, zinc is easily replaced by these heavy metals in biological systems.

1.2 Biological functions of zinc

Zinc is an essential nutrient to all organisms, because it acts as a cofactor in a large number of proteins of intermediary metabolism, hormone secretion pathways and immune defense mechanisms (9). In order to accomplish this, zinc possesses three modes of action: it has catalytic and structural roles in hundreds of zinc-dependent enzymes and by organizing protein sub-domains (so-called zinc fingers) for the interaction with either DNA or other proteins zinc also has regulatory functions (10,11).

It is well known, that zinc plays a central role in the immune system. For example, thymulin, a nonapeptic thymic hormone that promotes T lymphocyte maturation and IL-2 production, requires zinc in an equimolar ratio for biological activity. Zinc deficiency adversely affects the size of the thymus, proliferation of lymphocytes, activity of natural killer cells and the percentage of precursors of cytotoxic T cells (12). Zinc deficiency also results in decreased cytokine production of TH1 cells, leading to an imbalance between TH1 cell and TH2 cell function (13). Moreover, the development of immunologic memory cells is impaired in zinc-deprived mice (14). This finding could be of special importance for the maintenance of vaccine efficacy in humans, but requires further exploration. There have only been a few studies conducted on the effects of high-dose zinc intake on the human immune system and the results of these are contradictionary. While some investigators reported immunodepression with zinc supplementation, others found no or even a beneficial effect. Despite several randomized clinical trials, the therapeutic and prophylactic effectiveness of zinc for treating the common cold remains uncertain (15-17).

Zinc has antioxidant properties, but as it is redox-inert itself, its effects are indirect (18): by maintaining the cellular metallothionein concentration (see 1.3) it protects sulfhydryl groups from oxidation, thereby preventing disulfide formation. Moreover, zinc contributes to the structure of superoxide dismutase (SOD), which scavenges reactive oxygen species (ROS). Lack of ROS prevents lipid peroxidation and makes zinc important for the structure and function of biomembranes. Zinc is also an effector of tubulin polymerization and acts on actin filament formation and stabilisation (19).

Furthermore, zinc plays a major role in the synthesis, storage and secretion of insulin, which is produced by the beta cells of the pancreas. Cleavage of the primarily synthesized proinsulin results in the insulin monomer, with two peptide chains (alpha and beta) cross-linked by two inter-chain disulfide bonds. In the presence of zinc, two monomers assemble *in vivo* to a dimeric insulin crystal for storage and secretion (20). The pharmacologically administered form for insulin is a hexameric zinc crystal, which is formed *in vitro* in the presence of zinc and is commonly used due to its relative high stability. The relationship between diabetes, insulin and zinc is complex and still not fully elucidated. A decrease in available zinc on one hand affects the ability of pancreatic islet cells to produce and secrete insulin, whereas on the other hand diabetes perturbs zinc homeostasis, leading to increased urinary loss and a decreased total body zinc (20).

Amongst all organs the brain has the highest zinc content per weight unit with particularly large amounts found in the lumen of glutamate-containing synaptic vesicles. It is therefore assumed, that zinc plays an important neuromodulatory role in synaptic neurotransmission of glutaminergic neurons (21). Zinc homeostasis in neurons is mainly mediated through metallothionein-3 (see 1.3), but is thought to be also maintained by the prion protein (PrP). The native isoform of this protein (PrP^{C}) might be involved in the transport of zinc into the cell or may act as a zinc sensor, whereas the infectious, disease-causing isoform (PrP^{Sc}), is known as the causative agent of the neurodegenerative transmissible spongiform encephalopathies. The role of zinc in the pathobiology of prion disease and other neurodegenerative diseases is reviewed in (22).

There is strong evidence that zinc plays a role in the regulation of apoptosis. It was initially thought that zinc affects only late stages in the apoptotic pathway, through inhibition of a DNA endonuclease (23). It is now known that caspase-6, which cleaves and activates the proenzyme form of caspase-3 (and is also responsible for the cleavage of lamins) is also a major target of zinc in this process (11). Generally, zinc supplementation increases the resistance of cells to apoptosis, whereas zinc deficiency induces the so-called programmed cell death. However, it has also been shown that zinc is capable of inducing apoptosis if high amounts are administered (24).

The prime role of zinc in enzymes, DNA replication and transcription control is described in more detail in the following sections.

1.2.1 Zinc-dependent enzymes

The first zinc-dependent enzyme, carbonic anhydrase II, was discovered by Keilin and Mann in 1940. Since then, it has been proven that zinc is an essential cofactor for more than 300 enzymes, covering all six classes of enzymes (oxidoreductases, transferases, hydrolases, lyases, isomerases and ligases) (9). As mentioned above, zinc possesses either a catalytic (e.g. carbonic anhydrase, carboxypeptidase) or a structural (e.g. superoxide dismutase) role in metalloenzymes. In enzymes containing multiple zinc atoms, it was found that different zinc atoms can have different functions within the same molecule (e.g. alkaline phosphatase).

Three types of catalytic mechanisms of zinc have been proposed (25). In the zinc-carbonyl mechanism, the substrate binds directly to zinc and displaces a metal-bound H_2O molecule. Zinc then acts as a Lewis acid to polarize the bound substrate facilitating a nucleophilic attack. In this case, zinc activates the electrophile. In the other mode, the zinc-hydroxide mechanism, the substrate does not bind directly to the metal. Zinc lowers the pK_a of the metal-bound H_2O molecule and the resulting hydroxide ion then attacks the substrate. In contrast to the first mechanism, the role of zinc here is to activate the nucleophile. The third mechanism, proposed for carbonic anhydrase, is a combination of the other two, where the substrate binds directly to the metal but does not displace the metal-bound H_2O. In this context, the function of zinc is to polarize the substrate and to activate the H_2O molecule.

Among the most important zinc enzymes are alcohol dehydrogenase in liver, carbonic anhydrase in erythrocytes, carboxypeptidase A and B in pancreatic juice and alkaline phosphatase e.g. in blood. The latter is of particular interest, since it consists of two subunits, each containing one zinc atom with catalytic and one with structural function.

1.2.2 Role of zinc in DNA replication and transcription

Zinc is of high importance for nucleic acid metabolism as it accounts for maintaining genetic stability by influencing DNA replication, DNA repair processes and apoptosis (26). As an essential cofactor for RNA polymerases and more than 2000 transcription factors, zinc is also involved in the control of gene expression.

Zinc fingers, together with leucin zippers and helix-loop-helix structures are the most common structural motifs shared by transcription factors. TFIIIA (factor A) isolated from *Xenopus* oocytes was the first example of a zinc protein controlling the transcription of a specific gene (27). Sequence analysis uncovered nine copies of an imperfect repeated motif of 30 amino acids. Two cysteine and two histidine residues of each 30-amino acid unit form a complex with a zinc atom resulting in a loop-like peptide domain, named “zinc finger”, which interacts with DNA. Although originally introduced for the multiple zinc domains of TFIIIA, this term has soon become widely used. Nowadays it describes virtually any relative short sequence, which contains four or more cysteine and/or histidine residues and which is believed to function as a nucleic-acid binding domain (28). As emphasized by Vallee et al. (28), scientists should be more precise in distinguishing “classical” zinc fingers from “nonclassical” zinc fingers such as zinc twists and zinc clusters. The following figure shows the three-dimensional structure of a “classical” zinc finger (also often referred to as Cys2-His2 type of zinc fingers) (Fig. 1A) and a schematic illustration of how the interactions between the zinc finger and the DNA occur (Fig. 1B).

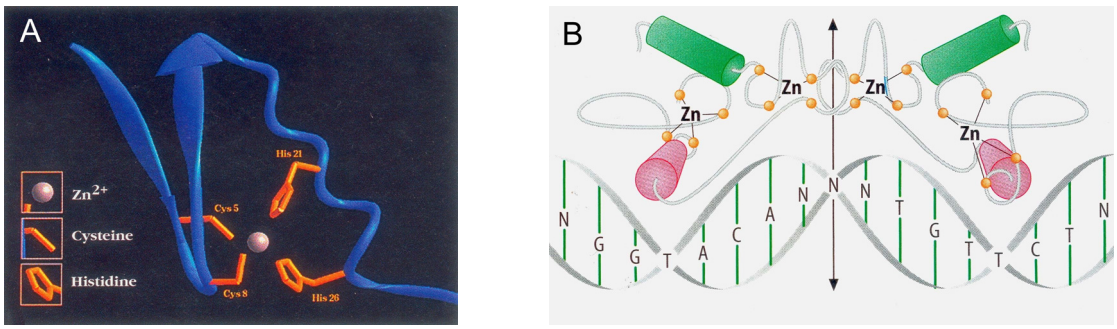

Fig. 1: Structure of a zinc finger protein and its interaction with DNA. A three-dimensional structure of a Cys2-His2 zinc finger protein (A) and a schematic view of the interaction between DNA and zinc finger (B). From (29), page 260f.

As more and more sequence information is available it has become common practice to define zinc-proteins on the basis of primary sequences, thus assuming the presence of zinc rather than determining it directly. By using sequence analysis prediction, it is estimated that more than 3 % of the human genome encode for proteins with zinc finger domains (30). Zinc fingers are the most notably recurring motifs in transcription factors, which start the transcription process through binding to DNA. However, participation of zinc in protein-protein interactions has also been demonstrated. Similar motifs were found in nuclear hormonal receptors, including estrogen, testosterone and vitamin D receptors (11).

Much of our basic understanding regarding zinc-regulated gene expression relates to studies in bacteria and yeast. In *E. coli* (31,32), *B. subtilis* (33,34) and *S. cerevisiae* (35), several metalloregulatory proteins, acting as zinc sensors and transcriptional activators/repressors, have been identified as key components of zinc-dependent gene expression. These zinc finger containing transcription factors regulate not only zinc-responsive genes, but also control their own transcription through a positive autoregulatory mechanism.

In mammalian cells, MTF-1 (metal-responsive element-binding transcription factor-1, in short metal transcription factor-1) has been recognized as a zinc-sensory transcriptional activator (36). MTF-1 is ubiquitously expressed and is mainly localized in the cytoplasm, but different stress conditions mediate its rapid translocation to the nucleus (37). It contains six zinc fingers of the Cys2-His2 type, which mediate DNA-binding and sense the level of cytosolic free zinc (38). Three different transactivation domains were also found in the C-terminus of MTF-1, contributing to the full zinc responsiveness, but their precise function is less well understood (39). Much of our knowledge about MTF-1 comes from the group of Walter Schaffner in Zürich, which has provided a significant contribution to the understanding of the structure and function of MTF-1, including cloning and functional characterization of the human MTF-1 (40). Induction of transcription of target genes (e.g. metallothioneins) requires binding of MTF-1 to cis-acting DNA elements, termed metal-responsive elements (MREs), which occur in multiple copies in the promoter region of these genes. MREs contain a well-defined 7bp core consensus sequence (TGCRCNC) and to date there is no evidence that

MTF-1 can utilize other DNA motifs to enhance transcription *in vivo* (41). The biological link between zinc, MTF-1 and metallothionein is visualised in Fig. 2.

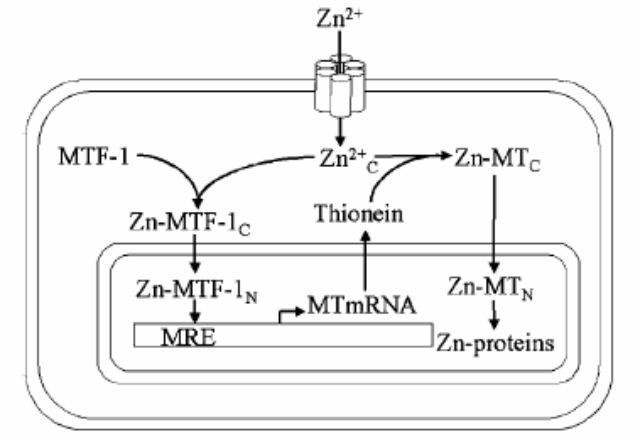

Fig. 2: Interrelations between zinc, MTF-1 and metallothionein. After complexing zinc, the metal transcription factor-1 (MTF-1) is translocated from the cytoplasm to the nucleus where it binds to metal-responsive elements (MRE) and starts transcription of thionein. Binding of zinc to this apometalloprotein leads to the formation of metallothionein. C: cytoplasmic localisation, N: nuclear localisation. From (42).

MTF-1 binding to DNA is activated by zinc, but not by other transition metals (43). From this point of view it is difficult to explain transcriptional activation of metallothioneins (MTs), the main target genes of MTF-1 by non-zinc stressors such as other transition metals and environmental stressors. Two hypothesis have been formulated to explain this paradox: firstly, increasing amounts of non-zinc heavy metals could displace zinc from intra- and/or extracellular storage proteins, therefore releasing zinc from its binding sites for a subsequent activation of MTF-1 (44). The second one is based on the finding, that specific dephosphorylation of MTF-1 contributes to its activation and that zinc as well as non-zinc stressors can alter signal transduction pathways leading to changes in the level of phosphorylation of MTF-1 (45). In addition to these two models, induction of MT transcription can also be mediated independently of MTF-1 through binding of other transcriptional activators (e.g. Nrf2) to their specific enhancer elements (e.g. AREs) in the metallothionein promotor, as discussed in section 1.3.

It is known for a long time that MTF-1 directs metallothionein expression. Interestingly, disruption of the MTF-1 gene in mice is lethal, whereas animals lacking both, MT-1 and -2, are viable (46-49). This led to the conclusion that MTF-1 controls expression of important genes other than MT-1 and -2. Some effort in

target gene search has been undertaken (41,46), however to date, only ZnT-1 was shown to be yet another *in vivo* target gene of MTF-1 (50). In addition to the regulation of gene transcription via MTF-1 other zinc-sensing metalloregulatory proteins may exist.

Despite the direct effects of zinc on gene expression as discussed above, zinc can also act indirectly by influencing superordinated signaling pathways (51). As illustrated in Fig. 3, zinc is able to increase the formation of insulin-like growth factor-1 (IGF-1) and activates both the epidermal growth factor-receptor (EGF-R) and the hepatic heavy metal ion stimulated-receptor (HHS-R). At the level of protein phosphorylation, zinc can modulate the activity of cyclic nucleotide phosphodiesterase (PDE), mitogen-activated protein kinase (MAPK), protein kinase C (PKC), protein tyrosine phosphatases (PTP), Ca^{2+}-calmodulin activated protein kinase-2 (CaMPK-2) and P70S6 kinase (P70S6K), which then influence the phosphorylation state of transcription factors (TF).

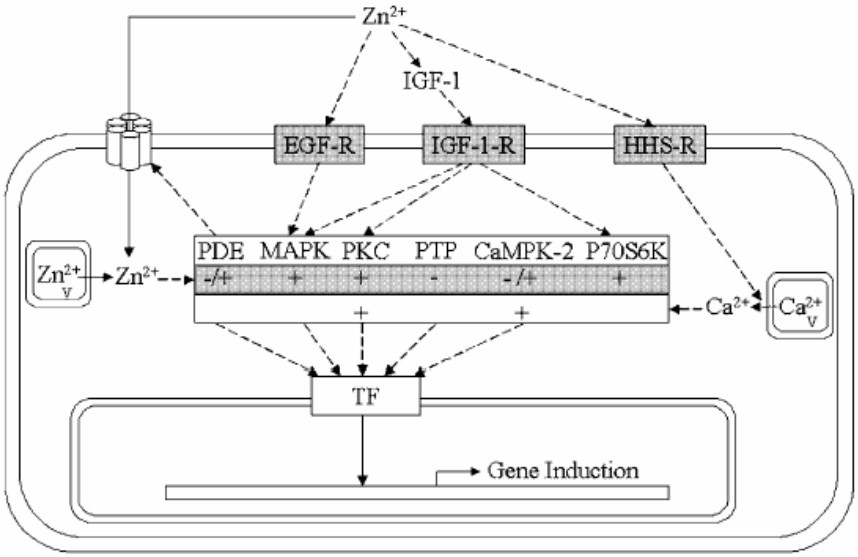

Fig. 3: Effects of zinc on selected signal transduction pathways. IGF-1 (insulin-like growth factor-1), EGF-R (epidermal growth factor-receptor), IGF-1-R (insulin-like growth factor-1-receptor), HHS-R (hepatic heavy metal ion stimulated-receptor), PDE (cyclic nucleotide phosphodiesterase), MAPK (mitogen-activated protein kinase), PCK (protein kinase C), PTP (protein tyrosine phosphatases), CaMPK-2 (Ca^{2+}-calmodulin activated protein kinase-2) and P70S6K (P70S6 kinase), TK (transcription factors). +: activation, -:inhibition, V: vesicular localisation. From (42).

1.3 Zinc homeostasis

Zinc plays a critical role for proper protein functions in a large number of zinc finger proteins and zinc-dependent enzymes, but may interfere with other metal-dependent processes or inhibit other proteins at unphysiological levels (7). Therefore eukaryotic cells must maintain an adequate intracellular zinc concentration even though extracellular or dietary zinc levels are always changing. Zinc homeostasis is maintained through influx and efflux processes, sequestration of zinc into vesicles (e.g. so called zincosomes) and buffering by zinc binding proteins (e.g. metallothioneins) (52,53). Due to the fact that zinc is a small, hydrophilic, highly charged molecule which cannot cross biological membranes by passive diffusion (54), specialised transport systems for its uptake and release are required.

In yeast, zinc homeostasis is largely mediated by the zinc transporters Zrt1, Zrt2 and Zrt3 (55). They belong to the ZIP family of metal ion transporters (Zrt-, Irt-like proteins, named after the first two members of the family identified Zrt1 and Irt1), which spans all phylogenetic levels and plays prominent roles in zinc uptake (56). Both Zrt1 and Zrt2 are highly expressed in the plasma membrane under low zinc conditions and their expression is turned off at supra-nutritional zinc levels. Zrt3 controls the export of zinc from the vacuole into the cytosol. Regulation of these genes in response to zinc is mediated by the transcriptional activator Zap1, which binds to zinc-responsive elements (ZREs) in the promoters of its target genes (55).

In mammalian cells, the mechanisms of zinc homeostasis are scarcely dissolved, but also here zinc transporters and intracellular metal-sensing proteins play key roles in controlling these processes. Among transport proteins, two families, the ZIP (Zrt-, Irt-like Protein) and CDF (Cation Diffusion Facilitator) family are of particular importance for zinc transport. As mentioned above, the ZIP family transports zinc (besides other divalent cations) into cells (53). From the 12 known ZIP members in the human genome, four are proposed to participate in zinc uptake (hZip1-4). Due to its ubiquitous expression, hZip1 is considered to be the major zinc influx transporter in human tissues (10). The latter member of this family, hZip4, was identified in 2002 by Wang et al. (57). It enables uptake of dietary zinc into intestinal enterocytes. Mutations in this gene have been identified in patients with Acrodermatitis enteropathica (AE), a rare, autosomal recessively

inherited human disease (58). Moreover, zinc uptake is also mediated by DCT1, a member of the Nramp family of transporters, which is unrelated to either ZIP or DCF proteins. DCT1 is an iron transporter that also has affinity for Zn^{2+} and other divalent cations (56).

Members of the mammalian CDF family are involved in zinc efflux or the transport of zinc into intracellular vesicles and thereby control free zinc and protect cells from zinc toxicity. To date, seven members of this family (ZnT1-7) have been identified. Whereas the first four members of this family (ZnT1-4) are quite well functionally characterised, the latter three (ZnT5-7) haven't been as thoroughly studied. The location and tissue distribution of the mammalian zinc transporters discussed in this section is summarised in Fig. 4.

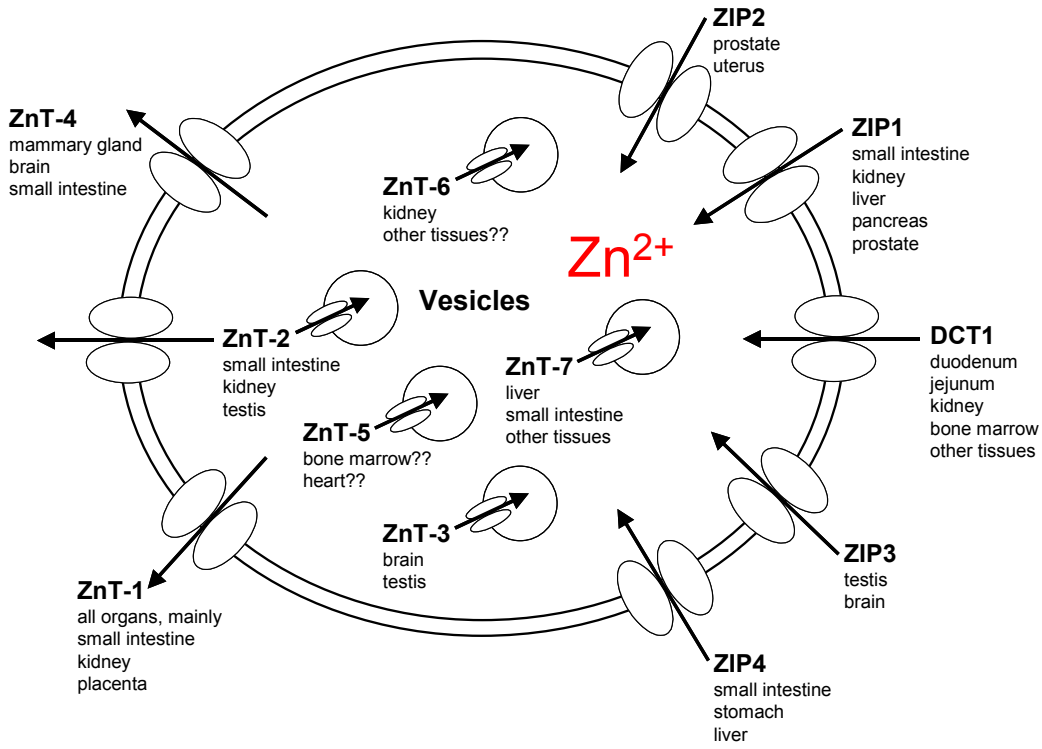

Fig. 4: Location and tissue specificity of the mammalian zinc transporters. Arrows indicate the direction of the zinc transport mediated by the corresponding transport protein. The term vesicles was used in this figure as a collective term for various intracellular compartments (endosomes, secretory granules, synaptic vesicles, Golgi apparatus, or trans-Golgi network). Modified from (54).

Besides transport processes, zinc homeostasis can also be maintained through binding or releasing zinc from zinc-binding proteins such as metallothioneins or cysteine-rich intestinal protein.

Metallothioneins (MTs) are highly conserved, low molecular weight (6-7 kDa), cysteine-rich proteins. Containing 20 cysteine residues per molecule, each thionein (the metal-free form of metallothionein) can bind up to 7 zinc or cadmium ions or up to 12 copper ions. Under physiological conditions MTs are primarily associated with zinc and secondarily with copper (44). Metal binding increases the half-life of thionein which otherwise is highly susceptible to proteolysis (59,60). Within the cell, MTs are mainly located in the cytoplasm and to a lower extent in the nucleus and lysosomes (61,62). Proposed biological functions for MTs include the detoxification of heavy metals, regulation of the metabolically active cellular zinc pool and protection against various forms of oxidative damage. Fig. 5 illustrates, how the metallothionein/thionein couple controls the concentration of readily available zinc within the cell. An increase in intracellular zinc concentration induces synthesis of thionein which then safeguards zinc via formation of MT. At low zinc levels, when zinc is needed for incorporation into zinc-dependent apometalloproteins, zinc is sequestered resulting in back-formation of thionein which has a short half-life and is removed by proteolysis (7).

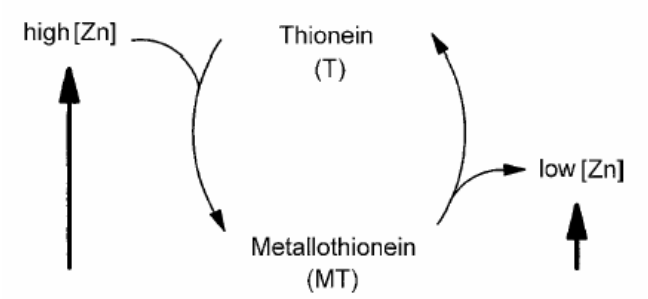

Fig. 5: Function of the MT/T couple in homeostasis of intracellular free zinc. High zinc levels induce synthesis of thionein and lead to the formation of metallothionein. Zinc is released from metallothionein when the amount of available zinc is low. From (7).

MT is a redox protein in which the redox properties do not originate from the metal atom, but from its coordination environment (7). Binding to sulfur donor atoms of cysteine ligands confers redox activity to the otherwise redox-inert zinc atom. A change in cellular redox potential to more oxidizing conditions releases zinc, whereas reductants/antioxidants restore the full potential of the sulfur ligands to bind zinc (63). Therefore, the redox properties of MT link zinc distribution to the redox state of the cell.

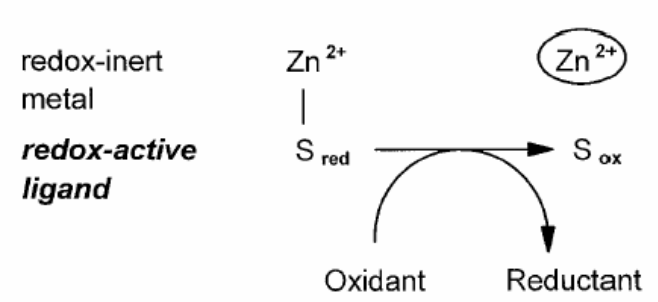

Fig. 6: Function of metallothionein as a redox protein. Release of zinc is mediated by the oxidation of the cysteine sulfur ligand. This mechanism is the basis for the link between cellular zinc and redox state. From (7).

There are four known isoforms of MT in mammals (MT-1 to MT-4). MT-1 and MT-2 are ubiquitously expressed, with the highest levels found in the liver and are strongly inducible by a variety of stimuli including metals, hormones, cytokines, oxidants, stress and irradiation (44). The other two, MT-3 and MT-4, are constitutively expressed in certain tissues and cells, with MT-3 showing almost exclusive expression in the brain (64), whereas expression of MT-4 is limited to stratified squamous epithelium of skin and tongue (65).

Gene expression in eukaryotes in general is primarily regulated at the level of initiation of transcription via binding of inducible DNA-binding proteins (so-called transactivating factors) to enhancer sequences (so-called cis-acting DNA elements), which are mostly located several hundred base pairs upstream from the promotor region. Binding of such an inducible transcription factor to its specific response element in most cases enhances or seldomly represses the formation of the transcription initiation complex (66). Basal transcription of MTs is mediated through binding of TFIID to classical TATA boxes in the core promotors of these genes. Binding of MTF-1 (see 1.2.2) to metal responsive elements (MREs) is classically required for induction by heavy-metals and oxidative stress, but also contributes to basal gene transcription (44). Synergistically with MREs, interaction between antioxidant response elements (AREs) and the transcription factor Nrf2 (NF-E2-related factor 2) is also involved in MT expression in response to reactive oxygen species (67). Independent from other regulatory sequences, glucocorticoid induction of MT-1 and MT-2 transcription requires binding of glucocorticoid hormone receptor homodimers to glucocorticoid response elements (GREs) (68).

Other hormones and cytokines (e.g. IL-6) induce expression indirectly through intracellular signaling mechanisms.

The cysteine-rich intestinal protein (CRIP) is also a zinc binding protein that binds zinc during transmucosal transport and thereby functions as an intracellular carrier for zinc (69). The rate of zinc absorption is inversely related to intestinal metallothionein levels (70). High dietary zinc levels lead to an up-regulation of intestinal metallothionein level, which then inhibits zinc absorption by binding zinc in competition with CRIP (71).

1.4 Pathologies associated with an altered zinc status

Human zinc deficiency was first reported in 1961 by Prasad in a case report on a 21 year old male Irani suffering from malnutrition and geophagia (clay eating), which led to retardation in growth and sexual development (72). Clinical symptoms of an inadequate zinc supply include loss of appetite, growth failure, immune-system dysfunction, alopecia, severe dermatitis, diarrhea and mental disorders. Zinc deficiency in humans can either be acquired or originate from an inherited disease. For example, Acrodermatitis enteropathica (AE) is a congenital genetic disorder in humans, that causes severe zinc deficiency, due to an autosomal recessive mutation of the intestinal zinc transporter Zip4 (see 1.3). Patients with this defect in zinc uptake have to be treated with a routinely adminstered oral zinc supplementation otherwise they die in late infancy (73). An acquired zinc deficiency could be due to malnutrition (e.g. parenteral nutrition, anorexia, diets), malabsorption (e.g. chronic inflammatory bowel disease, cystic fibrosis) or increased zinc metabolism or excretion (e.g. alcoholism, stress). Severe dietary zinc deficiency is still very prevalent in developing countries, affecting nearly two billion people (74). Unexpectedly, in western countries, mild forms of zinc deficiency are also quite common. Precise information about the number of people affected is not available, but findings from the “Third National Health and Nutrition Examination Survey” approved, that only 55.6 % of the surveyed population had an adequate zinc intake, with young children, adolescent females, pregnant women and elderly people at the greatest risk of inadequate zinc supply (75,76).

Despite decades of research on zinc deficiency, the effects of an excess zinc intake hasn’t been as thoroughly studied. In comparison to other trace elements

the acute toxicity of zinc is low, resulting mainly in gastrointestinal irritation and vomiting after ingestion of large amounts of zinc (1-2 g zinc sulfate) (77). Ingestions of toxic levels in the past were mainly due to eating acidic food from zinc-containing plates and dishes or inhalation of zinc fumes by enamellers or other industrial workers. In contrast to its low acute toxicity, long-term exposure to as little as 100 to 300 mg zinc per day leads to toxicity including an induced copper deficiency (78), impaired immune function and reduction of HDL cholesterol level (79). Copper deficiency secondary to zinc excess is related to an induction of metallothionein expression in absorptive intestinal cells. Metallothionein is associated primarily with zinc under normal physiological conditions, but has a much higher affinity for copper. The protein turnover of the copper-bounded form is strongly diminished, so that dietary copper bound to metallothionein is retained and becomes sequestered when enterocytes are desquamated into the intestinal lumen (80). The UL (Upper Level), which means the highest intake associated with no adverse health effects for zinc, was set to 40 mg per day for adult males and females. As zinc supplementation is common, one has to take care not to exceed these recommendations for dietary zinc intake.

1.5 Assessment of the zinc status in humans

Despite intensive research activities there is no solid biomarker for the assessment of the zinc status in humans (81). Zinc content of hair, finger nails or saliva did not prove to be valid markers for the zinc status, nor did urinary zinc excretion, rapidly exchanging zinc pools (EZP) in plasma and liver, activity of zinc-dependent enzymes and cellular components of blood (e.g. erythrocytes, thrombocytes, leukocytes). To date, plasma zinc is the most commonly used marker for determing the zinc status in humans. Although easily accessible and convenient, it has to be taken into account that plasma zinc levels are homeostatically regulated and are affected by other factors beside zinc (e.g. stress, infection, cardiac infarction). Therefore plasma zinc levels are not considered to be a sensitive and reliable biomarker for assessing a marginal (both insufficient or excessive) zinc status (82). Nevertheless, it has to be noted that plasma zinc levels have the capability to detect severe zinc deficiency states or potentially toxic intakes of zinc.

Since zinc plays a crucial role in control of gene expression, e.g. as a cofactor of numerous transcription factors (see 1.2.2), gene-based approaches such as alterations in mRNA levels of zinc-regulated genes may be used for the determination of the zinc status *in vivo*. For example, metallothionein mRNA levels (e.g. in monocytes) respond sensitively to experimental dietary zinc levels (83,84) but are also not specific since expression of MTs is induced by a variety of agents and conditions including other metals (e.g. Cd, Hg, Cu, Ni), hormones, cytokines, oxidants and stress. Therefore other zinc-regulated genes need to be identified. It is unlikely that this search will lead to the definition of one ultimate biomarker but maybe to a set of zinc-regulated genes, which could be used together in terms of a "gene expression pattern" for the determination of the zinc status in humans.

2 The aim of this work

The experimental work underlying this thesis had three different objectives. The first aim was to identify molecular targets of zinc to get a deeper insight into the molecular effects of this micronutrient in mammalian cells. As discussed previously, zinc has cofactor functions in a large number of proteins of intermediary and nucleic acid metabolism, hormone secretion pathways and immune defense mechanisms (9,85). On the basis of its multiple biological functions, zinc deficiency as well as excessive zinc supplementation causes a wide variety of symptoms. Despite decades of research, the molecular targets leading to the pleiotropic effects of zinc have only partially been identified. In the last few years high throughput screening methods for transcriptome and proteome analysis led to a shift from hypothesis-driven research to what has become known as discovery science. This means, the identification of components of a system without prior formulation of hypotheses on how these components function (86). Discovery science is a critical part of a new approach to biology, termed “systems biology”, which involves the comprehensive characterisation of the components of a biological system (e.g. DNA, RNA, proteins), ideally as a whole, leading to insights on how a system responds to a given pertubation (87). In order to follow this route and to identify zinc-dependent* genes and proteins in mammalian cells, DNA array and proteomic technologies were applied to zinc-depleted as well as zinc-supplemented HT-29 cells. This human colonic epithelial cell line was choosen as it serves as a model for the intestinal epithelium, a primary target of dietary zinc intakes *in vivo*.

Secondly, the need for development of specific and reliable biomarkers for the assessment of the zinc status *in vivo* was another driving force for this project. As argued in the previous section, it is unlikely that this function can be fulfilled by one certain gene, but by a series of markers, which react sensitivly to the dietary intake of this nutrient and therefore could allow to define a pattern of changes in gene expression that reflect a given zinc status. As a contribution to this, those genes were selected from the transcriptomic screening, which respond over a wide range of medium zinc concentrations** (<0.01 to 10 ppm) with changes in expression

levels (named zinc-sensitive* genes) and their zinc responsiveness was studied in more detail.

The third part of this work was hypothesis-driven. It aimed to discover whether identified zinc-sensitive genes could be potential new target genes of the human metal transcription factor MTF-1. This transcription factor is ubiquitously expressed and seems to have an important physiological function, as MTF-1 knockout mice die *in utero* around day 14 of gestation (46). In spite of some investigational effort made in target gene search for MTF-1, to date clear evidence exists only for metallothionein and the zinc transporter ZnT-1. This work led to the identification of a number of so-called zinc-sensitive genes. Expression levels of these genes respond to changes in medium zinc concentrations over several orders of magnitude and therefore their expression seems to be dependent primarily on zinc. The fact that MTF-1 exclusively binds zinc to enhance gene transcription led to the assumption that these genes are potential new candidates for MTF-1 target genes. This hypothesis was proven by establishing a double-stable cell line that inducibly overexpresses MTF-1.

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* In the context of this work the term “zinc-dependent” or “zinc-sensitive” denotes those genes, which respond to changes in medium zinc concentration with altered steady state mRNA and/or protein levels. Changes in mRNA levels are mainly due to an increase or decrease in the transcription rate of the gene and/or modifications in transcript stability. Accordingly, altered protein levels can be based on differences in transcription rate, translation rate or protein stability.

** The term “medium” used throughout this work stands for growth medium, therefore “medium zinc concentration” does not mean average zinc concentration, but the zinc concentration of the growth medium.

3 Materials

3.1 Equipment

Besides common laboratory equipment the following specific tools were used: Bio photometer (Eppendorf, Hamburg, Germany), Confocal laser-scanning microscope TCS SP2 including an inverted fluorescence microscope (Leica, Wetzlar, Germany), PCR Sprinter, PCR Express and Hybridization oven Shake ´n´ Stack (all from Hybaid, Middlesex, UK), Fluoroskan Ascent fluorescence multiwell-plate reader and Multiskan Ascent multiwell-plate photometer (both from Labsystems, Bornheim-Hersel, Germany), ALFexpressII™ DNA sequencer, Ettan Dalt II system for SDS-PAGE and Ettan IPGphor IEF system (all from Amersham Biosciences Europe, Freiburg, Germany), PhosphorImager Cyclone (Packard, Mariden, CT, USA), Affymetrix 428^{TM} array scanner (Affymetrix, Santa Clara, CA, USA), Atomic absorption spectrophotometer model 5100 (PerkinElmer, Überlingen, Germany), Lightcycler (Roche, Mannheim, Germany), Maldi-TOF Autoflex mass spectrometer (Bruker Daltonics, Leipzig, Germany), Speed vacuum centrifuge RC 10.10 (Jouan, Saint-Herblain, France), Steri-Cult 200 incubator (Thermo Life Sciences, Egelsbach, Germany), Laminar flow Bio48 (Faster, Ferrara, Italy).

3.2 Biochemicals and consumables

Unless otherwise specified all chemicals used (pro analysis quality) were from Sigma (Taufkirchen, Germany), Merck (Darmstadt, Germany) and Roth (Karlsruhe, Germany). Plastic ware for cell culture was obtained from Renner (Darmstadt, Germany). Media and supplements as well as other reagents for cell culture were obtained from Invitrogen (Karlsruhe, Germany). Restriction endonucleases were from Fermentas (St. Leon-Rot, Germany) and New England Biolabs (Beverly, MA, USA).

4 Methods

4.1 Cell culture methods

4.1.1 Cell culture of HT-29 cells

Human HT-29 cells were provided by American Type Culture Collection (ATCC, Rockville, MD, USA) and were used between passage 150 and 200. Cells were cultured in a humidified atmosphere of 95% air and 5% CO_2 at 37°C in RPMI 1640 medium containing 10% v/v FCS, 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin. In general, medium was replaced after 48 h. Cells were passaged at subconfluent densities using a solution containing 0.5 g/l trypsin and 0.2 g/l EDTA.

4.1.2 Experimental media

The zinc concentration in the normal culture medium after addition of the serum was approx. 3.7 µM (~0.24 ppm) as determined by atomic absorption spectrometry. To reduce the intracellular zinc concentration, adequate amounts of the membrane-permeable Zn^{2+}-chelator TPEN [N,N,N´,N´-tetrakis(2-pyridylmethyl)ethylenediamine] (20 mM in DMSO, Sigma, Taufkirchen, Germany) were added to the culture medium whereas in control experiments zinc (as $ZnCl_2$, 1000 mg/l in H_2O) was re-added to TPEN treated medium for adjusting Zn concentration to normal growth conditions. To increase zinc concentration, adequate amounts of zinc (as $ZnCl_2$, 1000 mg/l in H_2O) were added to the medium. Changes in osmolarity caused by addition of the zinc stock solution were equalized with sterile H_2O. Unless otherwise noted, zinc-depleted cells were incubated 16 h, zinc-supplemented cells 72 h (medium exchange after 48 h) with the experimental media.

4.1.3 Transfections

Transient and stable transfections were performed using the lipid-based transfection reagent FuGENE 6 (Roche Diagnostics, Mannheim, Germany) according to the recommendations of the supplier. The FuGENE 6 Reagent:DNA ratio was 6:2 (μl and μg, respectively) for all experiments.

4.1.4 Generation of a double-stable Tet-On/MTF-1 HT-29 cell line

For generation of a Tet-On cell line, HT-29 cells were grown on 6-well plates to a confluency of around 70%. 2 µg of the pTet-On vector (BD Clontech, Heidelberg, Germany) were transfected using the FuGENE 6 transfection reagent (Roche Diagnostics, Mannheim, Germany) following the instructions of the manufacturer. After transfection, culture was continued for two days before initiating selection with G418 (Geneticin G-418 sulphate, Invitrogen, Karlsruhe, Germany) at a concentration of 300 µg/ml. 44 resistant clones were picked two weeks later and further screened for high activity of the transactivator system by transient transfection with pTRE2hyg2-6xHN-Luc control vector (BD Clontech), containing the luciferase gene under control of a tetracycline-responsive element. The clone showing the highest induction (named clone 32) was selected for the second transfection. The double-stable Tet-On/MTF-1 cell line, which can grow well in the presence of both G418 and hygromycin, was established by plasmid transfection with 2 µg pMTF1/EGFP-TRE (see 4.5.2 for plasmid construction) carrying the hygromycin resistance gene. Hygromycin B (50 mg/ml in PBS, BD Clontech) at a concentration of 1.4 mg/ml was added to the culture after 48 h and resistant cells were selected, cloned and screened for doxycycline-inducible MTF-1 expression by confocal laser-scanning microscopy and real-time RT-PCR. To induce the expression of MTF-1, cells were cultured in the presence of 1 µg/ml of doxycycline (Dox, BD Clontech) for 48 h.

4.2 Fluorescence-optical measurements

4.2.1 Proliferation and cell integrity

Methods to assess proliferation and cell integrity have been described before (88). Briefly, HT-29 cells were grown under various experimental conditions. Cell counting for determination of proliferation rate and cell integrity assessment was based on SYTOX-Green (Bioprobes, Leiden, Netherlands), which becomes fluorescent after DNA binding. Therefore cells were incubated with SYTOX-Green (1:2500 dilution of a 5 mM stock solution in isotonic NaCl) to determine the number of cells with impaired integrity based on a calibration curve. Afterwards cells were lysed by 6% v/v Triton X-100 in isotonic NaCl and total cell numbers were determined. Fluorescence was measured at 538 nm after excitation at 485

nm in a fluorescence multiwell-plate reader. The percentage of cells with impaired integrity (based on permeability for SYTOX-Green) in a cell population was determined in relation to the fluorescence measured after the solubilisation of cells that assessed the total cell count.

4.2.2 Free intracellular zinc concentration

Intracellular free zinc concentrations were measured using the zinc-sensitive dye NewportGreen™ diacetate (Bioprobes, Leiden, Netherlands) according to the method described by Sensi et al. (89) with slight modifications. After washing twice with Hepes-buffered medium (HSS, composition in mM: 120 NaCl, 5.4 KCl, 0.8 $MgCl_2$, 20 Hepes, 15 glucose, 1.8 $CaCl_2$, 10 NaOH, pH 7.4), cells were loaded in the dark with 5 µM Newport green diacetate in HSS for 30 min at 37°C. Cell were then washed twice with HSS and kept in the dark for an additional 30 min at 37°C. Fluorescence emission intensity due to the binding of intracellular free zinc was measured at 530 nm after excitation at 485 nm using a fluorescence multiwell-plate reader.

4.2.3 Determination of lactate levels

Lactate levels in the cytosolic extract of HT-29 cells were measured with an UV-test (Boehringer, Mannheim, Germany) according to the manufacturer's instructions, using lactate dehydrogenase to produce pyruvate and NADH. The increase in NADH, which is stoichiometric to the amount of lactate was measured in a multiwell-plate photometer at 340 nm. For preparation of cytosolic extracts cells were trypsinised and centrifuged at 2500 g for 10 min. 750 µl of a buffer containing 2 mM EDTA, 0.1% w/v CHAPS, 5 mM DTT, 1 mM PMSF, 10 µg/ml pepstatin A, 20 µg/ml leupeptin, 10 µg/ml aprotinin and 10 mM Hepes/KOH, pH 7.4 were added to each pellet and homogenized by 10 strokes. The homogenate was centrifuged at 14000 g at 4^{o}C for 30 min and the supernatant was used for lactate analysis.

4.2.4 Detection of apoptosis

Membrane permeability as an early apoptosis marker and nuclear fragmentation as a late apoptosis marker were assessed with the DNA-binding fluorescence dyes Hoechst 33342 (early apoptosis) and Hoechst 33258 (late apoptosis, both

from Sigma, Taufkirchen, Germany). The usability of these dyes for the detection and discrimination of apoptotic subpopulations was validated by Elstein et al. (90). HT-29 cells were incubated with experimental media and pictures were taken using an inverted fluorescence microscope after staining with the dyes as described by Wenzel et al. (91).

4.3 Transcriptome analysis

4.3.1 Isolation of total RNA

RNAwiz™ (Ambion, Huntingdon, UK) was generally used for isolation of total RNA according to the manufactorer's instructions. For cDNA arrays total RNA was extracted with a slightly modified protocol as described in the Atlas™ Pure Total RNA Labeling System User Manual (Clontech, Palo Alto, CA, USA). Total RNA from Tet-On/MTF-1 clones was isolated using the RNeasy® Mini Kit (Qiagen, Hilden, Germany). RNA was stored in RNase free water at –80°C. The yield was calculated based on the absorbance at 260 nm (absorption of 1.0 is equivalent to 40 μg/ml RNA). RNA integrity was verified by an absorption ratio (A_{260}/A_{280}) >1.8 and by denaturing agarose gel electrophoresis. Intact total RNA preparations should appear as two bright bands with a ratio of intensities of the 28S and 18S rRNA of approx. 2:1.

4.3.2 DNA arrays

4.3.2.1 Atlas™ cDNA array

cDNA array analysis was performed using the Atlas™ Human 1.2 Array III (Clontech, Palo Alto, CA, USA) containing 1176 unique cDNAs spotted on a nylon membrane. Total RNA isolations, probe syntheses and array hybridisations were done according to manufacturer's protocol. Cells were grown for 72h under normal (~0.24 ppm) or high zinc (10 ppm) conditions. Total RNA from either normal or zinc-supplemented cells was reverse transcribed in the presence of [α^{33}P]-dATP (ICN Biomedicals, Eschwege, Germany) using a pooled set of primers complementary to the genes represented on the array. After hybridisation the membranes were washed and the intensity of the signals was recorded for 96h (PhosphorImager Cyclone). For quantitative analysis, the AIDA Array Evaluation software (Raytest, Straubenhardt, Germany) was used. Three independent

hybridisations were carried out. Genes were identified as significantly modulated in expression, when the average ratio in signal intensities of supplementation to control experiment was >1.5.

4.3.2.2 MWG Pan Human 10 k array

Oligonucleotide arrays on glass slides containing 9850 gene-specific oligonucleotide probes (50mer) were obtained from MWG Biotech AG (Ebersberg, Germany). RNA preparation, reverse transcription, labelling and hybridisation was performed according to the recommendations of the manufacturer. Total RNA from either control or zinc-deprived cells from three independent experiments was pooled and reverse transcription in the presence of either Cy3- or Cy5-labeled dCTP (Amersham Bioscience Europe, Freiburg, Germany) was performed to produce fluorescence labelled first-strand cDNAs. Arrays were scanned under dried conditions (Affymetrix 428^{TM} Array Scanner). The obtained data were normalised and analysed using ImaGene^{TM} 4.2 software (BioDiscovery, Los Angeles, CA, USA). Three independent hybridisations were carried out. Genes were considered as up- or down-regulated if the change was 2.0-fold or greater in at least two hybridisations.

4.3.3 Northern blot analysis

Northern blot analysis was carried out according to an established capillary blotting method (92). Total RNA (5 µg/lane) was size-fractionated on a denaturating formaldehyde gel and transferred onto a Hybond-N$^+$ nylon membrane (Amersham Biosciences Europe, Freiburg, Germany). DNA fragments, representing unique open reading frames of genes of interest were prepared as described in 4.5.1 and were used as a template to prepare randomly labelled probes by incorporating [α^{32}P]-dATP (ICN Biomedicals, Eschwege, Germany) during random primer extension reactions (MegaPrime Labelling Kit, Amersham Biosciences Europe). Resulting radiolabelled DNA probes were subsequently used for hybridisation, which was carried out at 68°C for 1 h in commercial hybridisation solution (Express Hyb, BD Clontech, Heidelberg, Germany). Membranes were first washed with 2XSSC, 0.1% w/v SDS. For the second wash 0.1XSSC, 0.1% w/v SDS was used. Hybridisation signals were visualised by exposure to a phosphor screen. Phosphorimages were scanned at different time

points depending on signal intensity. Blots were stripped by immersing the membranes in a boiling solution of 0.5% w/v SDS and reprobed for GAPDH (glyceraldehyde-3-phosphate dehydrogenase) under the same conditions.

4.3.4 Real-time RT-PCR

Quantitative RT-PCR (or real-time RT-PCR) was performed with the LightCycler instrument.

4.3.4.1 Primer design

Primer design was done with regard to primer dimer formation, self-priming formation and primer melting temperature using the HUSAR® (Heidelberg Unix Sequence Analysis Resources) software at DKFZ (German Cancer Research Center, http://genome.dkfz-heidelberg.de) or the LightCycler Probe Design software (Roche Diagnostics, Mannheim, Germany). Blast searches were done in the published sequence database GenBank (http://www.ncbi.nlm.nih.gov/BLAST/) to reveal that primers are gene-specific and if possible, those primers which span at least one intron were chosen. Primer sequences used for real-time RT-PCR are listed in 9.2.

4.3.4.2 One-step RT-PCR

One-step RT-PCR was performed using the QuantiTect™ SYBR® Green RT-PCR kit (Qiagen, Hilden, Germany) according to the recommendations of the manufacturer with a total volume of 10 µl. 100 ng total RNA was added as template to each reaction. Authenticity of the amplified products was checked by dissociation analysis and agarose gel electrophoresis.

4.3.4.3 Two-step RT-PCR

Procedures of two-step RT-PCR followed the instructions of the LightCycler - FastStart DNA Master SYBR Green I kit (Roche Diagnostics, Mannheim, Germany). To generate cDNA pools for subsequent PCR reactions, 1 µg of total RNA was reverse transcribed with 200 U SuperScript™ II RNase H$^-$ Reverse Transcriptase (Invitrogen, Karlsruhe, Germany) using 100 pmol random hexamer primers (Promega, Mannheim, Germany) according to the manufacturer's protocol. 25 ng and 3.3 ng, respectively, cDNA were added to each PCR reaction depending on the transcript level of the gene of interest. Authenticity of the

amplified products was checked by dissociation analysis and agarose gel electrophoresis.

4.3.4.4 Relative quantification

The individual level of initial target mRNA was expressed as the difference in C_T (i.e. the calculated fractional cycle number at which the fluorescence rises appreciably above background fluorescence) between control and sample (ΔC_T). The relative amount of target mRNA normalised to a reference gene (housekeeping gene) was calculated according to the following formula (93):

$$\text{RF (regulation factor)} = 2^{\Delta C_T \text{ target (control–sample)}} / 2^{\Delta C_T \text{ reference (control–sample)}}.$$

Expression levels of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and 18S ribosomal RNA (18S rRNA) were used for normalisation.

4.4 Proteome analysis

4.4.1 Western blot analysis

After incubation with experimental media, cells were washed two times with PBS and were lysed with ice-cold RIPA buffer [1XPBS, 1% v/v Nonidet P-40, 0.5% w/v Natriumdeoxycholat, 0.1% w/v SDS, with freshly added inhibitors: 10 mg/ml PMSF (10 µl/ml RIPA), Aprotinin (30 µl/ml RIPA), 100 mM Natriumorthovanadat (10 µl/ml RIPA)] (200 µl/75 cm^2 flask). Homogenisation of the cells was achieved by ultra sonification (10 strokes, low amplitude) on ice. Lysed cells were centrifuged at 10000 g for 10 min at 4°C and the supernatant containing the total cell lysate was stored at –80°C. Protein concentration was determined by optical density (OD) at 600 nm using Bio-Rad protein assay (Bio-Rad, München, Germany). Immunoblotting was carried out according to an established standard blotting method (92). Equal volumes of 2XLämmli buffer (100 mM Tris-HCl (pH 6.8), 200 mM DTT, 4% w/v SDS, 0.2% w/v Bromphenol Blue, 20% v/v Glycerol) were added to protein extracts and denatured at 95°C for 5 minutes. Proteins (60 µg protein/lane) were separated by 15% SDS-PAGE according to the method described by Schagger and Jagow (94) and transferred to a PVDF membrane (Roth, Karlsruhe, Germany). As protein molecular weight standard the prestained Precision Plus Protein Standard (Bio-Rad) was used. After blocking in TBS-T

(137 mM NaCl, 2.7 mM KCl, 24.8 mM Tris, 0.05% v/v Tween-20) containing 3% w/v nonfat dry milk, the blot was immunostained with anti-KLF4 antibody (1:100 dilution; Santa Cruz Biotechnology, Santa Cruz, CA, USA), a horseradish peroxidase conjugated anti-goat IgG antibody (1:1000 dilution; Santa Cruz Biotechnology) and 3-Amino-9-ethylcarbazole (Sigma, Taufkirchen, Germany). Staining with an anti-Actin antibody (1:1000 dilution, Santa Cruz Biotechnology) on the same blot was used for normalisation. Equal protein loading and transfer to the membrane were assessed by Ponceau S-staining (0.2% w/v Ponceau S and 3% w/v Sulfosalicylic acid in 0.1% v/v Acetic acid) of proteins prior to further blot processing. Intensities of the resulting protein bands were assessed with ImageMaster 2D software (Amersham Biosciences Europe, Freiburg, Germany).

4.4.2 2D-PAGE/MALDI-TOF-MS

4.4.2.1 Two-dimensional Polyacrylamide-gel electrophoresis (2D-PAGE)

Sample preparation and 2D-PAGE were carried out as described by Herzog et al. (95). HT-29 cells at 50-60% confluency were incubated for 16 h with either control (~0.24 ppm), zinc-depleted (<0.01 ppm) or zinc-supplemented (10 ppm) medium. Protein extracts were subsequently collected and stored at –80°C. Protein concentrations were determined by optical density (OD) at 600 nm using Bio-Rad protein assay (Bio-Rad, München, Germany). Three independent experiments were carried out, each was run in duplicate (600 μg protein/gel). Gels were scanned and analysed using the ProteomWeaver software (Definiens Imaging GmbH, München, Germany), which combined the spot detection with automatic background substraction and normalisation of the spot volumes. Changes in steady state protein levels were calculated based on the averaged volume of a given spot in all treatment gels (zinc depletion or zinc supplementation) in comparison to control gels. Spots which differed at least 2-fold were picked for MALDI-TOF-MS analysis.

4.4.2.2 Enzymatic digestion of protein spots and MALDI-TOF-MS

Digestion of proteins was performed as described elsewhere (95). Briefly, protein spots of interest were picked, destained and in-gel digestion was performed using sequencing-grade modified trypsin (Promega, Mannheim, Germany). The resulting peptide fragments were extracted and measured by MALDI-TOF-MS (matrix-

assisted laser desorption/ionization-time of flight-mass spectrometry) using the Autoflex mass spectrometer of Bruker Daltonics. Proteins were identified with Mascot Server 1.9 (Bruker Daltonics, Leipzig, Germany) based on mass searches within human sequences only. The search parameters allowed for carboxyamidomethylation of cystein and one missing cleavage. The criteria for positive identification of proteins were as follows: (i) a minimum score of 62; (ii) a mass accurancy of ± 100 ppm; (iii) at least a twofold analysis from two independent gels, and (iv) that the protein exhibits a significant difference in the number of matched peptides to the next potential hit.

4.5 Molecular biology methods

4.5.1 Preparation of DNA fragments for probe synthesis for Northern blot

cDNA fragments representing unique open reading frames of genes of interest were generated from HT-29 total RNA as template using a commercial available kit for reverse transcription (RETROscript™, Ambion, Huntingdon, UK) according to a standard protocol described therein. Primer sequences for subsequent PCR reactions are summarised in Tab. 8 and Tab. 10. Resulting DNA fragments were ligated into the pCRII-TOPO vector (Invitrogen, Karlsruhe, Germany) and transformed into chemically competent *E. coli* (One shot TOP10F´, Invitrogen) using the TOPO TA Cloning Kit from Invitrogen according to the manufacturer's protocol. After incubation overnight at 37°C, clones were picked and screened for correct size by restriction enzyme digestion and agarose gel electrophoresis. DNA from positive clones was isolated using E.Z.N.A. Plasmid Miniprep Kit II (Peqlab, Erlangen, Germany) and purified from agarose gels with QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany). Each DNA fragment was again checked for correct size by agarose gel electrophoresis and was fully sequenced.

4.5.2 Construction of a MTF1/EGFP expression plasmid

A plasmid (named pMTF1), comprising the CDS of human MTF-1, cloned into the expression vector pcDNA I/Amp (Invitrogen, La Jolla, CA, USA) was generously provided by W. Schaffner (for primary sequence see 9.4.1). Two restriction sites (XhoI and NsiI) were generated by site-directed mutagenesis. The resulting plasmid was digested with XhoI and NsiI to isolate the MTF-1 fragment containing

the entire open reading frame. The DNA fragment was ligated with the plasmid vector pEGFP-N2 (BD Clontech, Heidelberg, Germany) pre-linearised with XhoI and PstI at the multiple cloning site to obtain a plasmid, coding for a MTF-1/EGFP fusion protein (named pMTF1/EGFP-N2). The insert was checked for correct size and orientation by restriction enzyme digestion and agarose gel electrophoresis and was further sequenced. The second step was to clone the MTF-1/EGFP fusion protein into the pTRE2hyg2-6xHN vector (BD Clontech). Therefore a ClaI restriction site was generated in the pMTF1/EGFP-N2 plasmid by site-directed mutagenesis. Digestion with ClaI and NotI yielded the MTF-1/EGFP fusion protein, which was cloned into the plasmid vector pTRE2hyg2-6xHN (BD Clontech) pre-linearised with ClaI and NotI at the multiple cloning site. The resulting plasmid (named pMTF1/EGFP-TRE, for primary sequence see 9.4.2) was again checked for correct size and orientation by restriction enzyme digestion and agarose gel electrophoresis and the insert was fully sequenced. Primers used for sequencing are highlighted in the primary sequence in 9.4.2.

4.5.3 DNA sequencing

The dideoxy-DNA chain termination sequencing procedure of Sanger et al. (96) was used for DNA sequencing with the ALFExpressII™. Procedures were performed as recommended in the Thermo Sequenase Cy5 Dye Terminator Cycle Sequencing kit (Amersham Biosciences Europe, Freiburg, Germany). DNA reaction products were analysed using the ALFwin™ sequence analyser software (Amersham Biosciences Europe).

4.6 Other methods applied

4.6.1 Atomic absorption spectrometry (AAS)

Media zinc concentrations and total cellular zinc contents were determined using an atomic absorption spectrophotometer. Cell culture media were measured directly or after dilution with distilled water depending on the expected zinc concentration. For total cellular zinc contents, cells were harvested after incubation with experimental media and 20 µl were set aside for the determination of total cell numbers using a Neubauer chamber. Cells were washed once with PBS and two times with 10 mM EDTA (in PBS) to remove loosely bound zinc. Cells were lysed

by 6% v/v Triton X-100 in isotonic NaCl and cellular zinc content was measured directly in the supernatant by air acetylene flame atomic absorption spectrometry.

4.6.2 Confocal laser-scanning microscopy

Confocal laser-scanning microscopy was performed using TCS SP2 Leica Microsystems. EGFP was excited with a 488 nm laser line and was imaged between 500 and 550 nm.

4.6.3 Computational search for metal-responsive elements (MREs)

The bioinformatic search described in this section was kindly performed by Dr. Jan Budczies (GSF National Research Center for Environment and Health, Institute for Bioinformatics, Neuherberg, Germany). The upstream sequences of 18406 human RefSeq genes were retrieved from GoldenPath (UCSC genome project). The sequence collection includes only genes with transcription start (TS) annotated separately from the coding region start. To first define the appropriate search region, all 18406 sequences were scanned 5000 bps upstream from TS for a match with the IUPAC string TGCRCNC that models the functional core of a metal-response element (MRE). Employing an in-house position weight matrix algorithm, it was checked for exact-match alignments. The search was performed on the coding and on the non-coding strand. Based on the findings of this preliminary screening, further analysis were restricted to the first 1000 bps upstream from the TS.

4.7 Statistical analysis

Calculations were done using the software Prism 3.0 (GraphPad Software, Los Angeles, CA, USA). Results were analysed using unpaired Student's t test or One-way ANOVA and were considered statistically significant at a P value < 0.05. Data are given as means $\pm$ SEM.

5 Results

5.1 Transcriptome analysis

5.1.1 Zinc supplementation

5.1.1.1 Determination of experimental conditions for zinc supplementation studies

To establish culture conditions that significantly increase the cellular zinc status in HT-29 cells without altering cell viability and proliferation, different concentrations of zinc in culture medium were tested (10 to 100 ppm). These were about 40- to 400-fold higher than those in normal culture medium (~0.24 ppm). When cells were cultured in the different media for 72 h, only a zinc concentration of 10 ppm was found not to significantly affect both cell viability and proliferation (Fig. 7 A/B). Therefore the zinc concentration in the growth medium should not exceed 10 ppm in zinc supplementation studies.

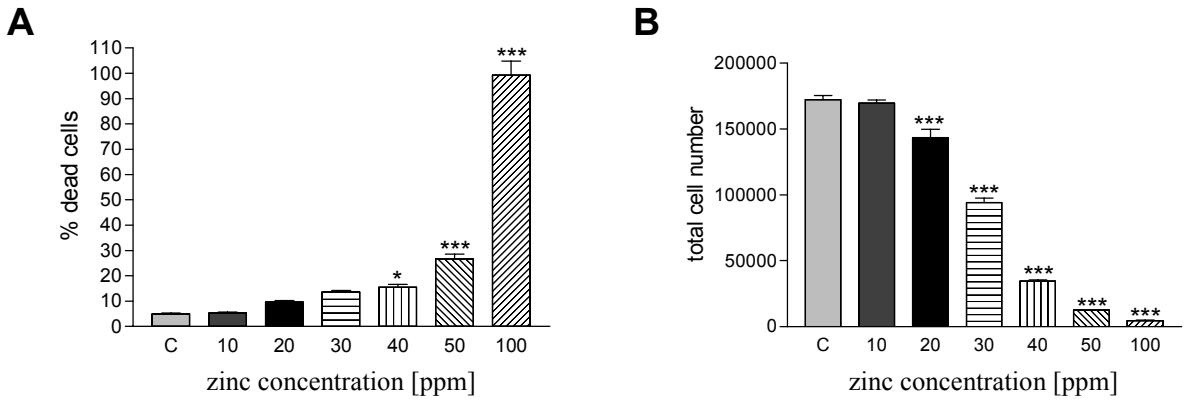


Fig. 7: Effects of high medium zinc concentrations on cell viability and proliferation. HT-29 cells were cultured for 72 h in media with normal (~0.24 ppm, C) or increased zinc concentrations (10, 20, 30, 40, 50 and 100 ppm). **A** Cell integrity was assessed by SYTOX-fluorescence. The percentage of dead cells was determined by SYTOX-fluorescence prior to cell lysis in relation to the fluorescence measured after solubilisation of the cells. **B** Proliferation was determined by cell counting using SYTOX-Green nucleic acid stain. Values are means ± SEM, n = 4, *: P<0.05, ***: P<0.001.

In addition, the expression of the metallothionein gene I (MT-1) was determined in HT-29 cells, grown for 72 h in media with a normal zinc level (~0.24 ppm) and up to 10 ppm zinc (Fig. 8).

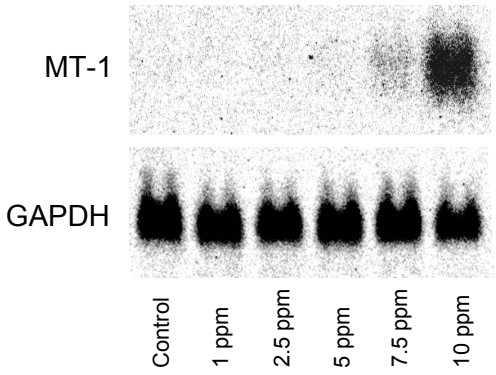


Fig. 8: Metallothionein-1 mRNA levels in response to increased medium zinc levels. HT-29 cells were cultured for 72 h in media with normal (~0.24 ppm, control) or increased zinc concentration (1, 2.5, 5, 7.5, 10 ppm) and total RNA was extracted. Equal amounts of total RNA were transferred to a nylon membrane and the blot was probed for metallothionein-1 (MT-1). The signal of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as an internal control on the same blot.

MT-1 is frequently considered a valid indicator of intracellular zinc status (7,43) and in accordance to previous results from other autors, MT-1 mRNA levels also raised in the our cell model with increasing zinc levels in the growth medium with a tremendous up-regulation at 10 ppm zinc. The fact that the optimal zinc concentration for metallothionein induction is just below the level causing cell toxicity has been described before (97). On the basis of these results and the results from the cell viability and proliferation assay, high zinc conditions were set to a medium zinc concentration of 10 ppm and an incubation time of 72h for all further studies.

5.1.1.2 Effect of high medium zinc on intracellular zinc concentration and apoptosis

As shown in Fig. 7 A/B, neither cell viability nor proliferation in HT-29 cells was adversely affected by a zinc concentration of 10 ppm. The resulting intracellular free zinc concentration was determined using the zinc-sensitive dye Newport Green. As shown in Fig. 9, intracellular free zinc levels were significantly increased. In zinc-supplemented cells, intracellular free zinc levels rapidly raised in the first two hours and remained stable over 48h. At this time point, the growth medium was changed as routinely done after two days. The slight increase in cellular free zinc levels observed in control cells probably comes from an inward flux of zinc after a one-hour incubation in HSS, a zinc-free Hepes-buffered medium used for loading of the cells with the fluorescent dye.

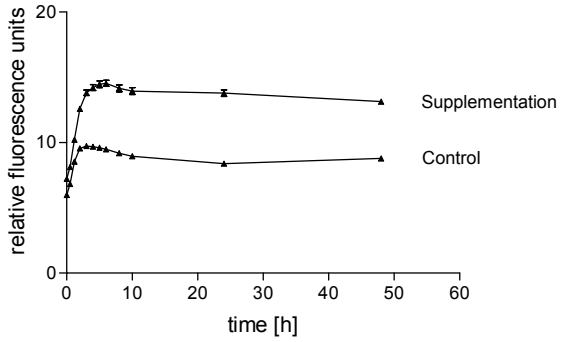


Fig. 9: Intracellular free zinc levels at high medium zinc. HT-29 cells on 24-well plates were loaded with Newport Green™ DCF Diacetat (Molecular Probes). Media with either a normal (~0.24 ppm, control) or a high zinc concentration (10 ppm, supplementation) were added and the increase in the fluorescence emission intensity due to the binding of intracellular free zinc was measured at 530 nm after excitation at 485 nm using a fluorescence multiwell-plate reader (Fluoroskan, Labsystems) for 48 h. Values are means ± SEM, n = 3.

It is interesting to note that total cellular zinc concentrations increased nearly 7-fold (as determined by atomic absorption spectrometry) when cells were exposed to 10 ppm zinc (Fig. 33), whereas intracellular free zinc levels increased only less than twofold (~1.6-fold).

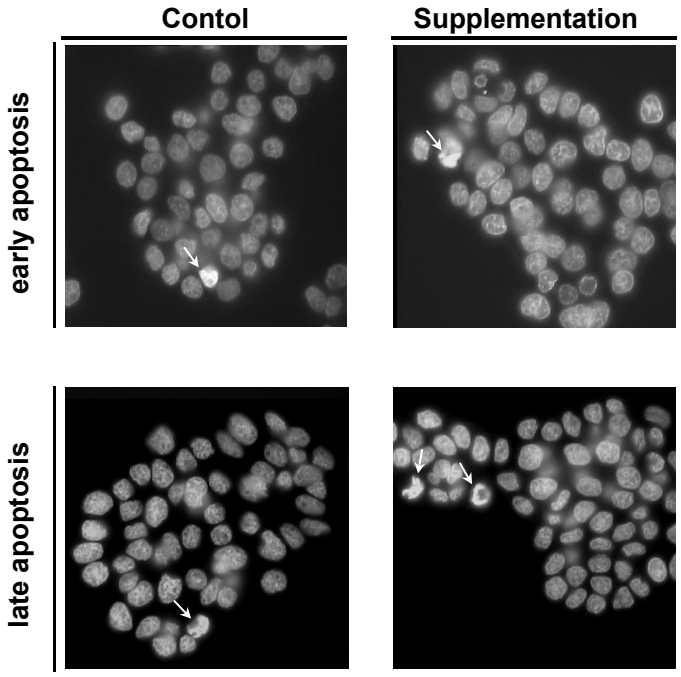


Fig. 10: Determination of early and late apoptosis events in zinc-supplemented HT-29 cells. Cells were grown for 72 h under normal (control) or high zinc (supplementation) conditions. Arrows indicate apoptotic cells due to membrane disintegration (early apoptosis) or nuclear fragmentation (late apoptosis).

Zinc protects cells against apoptosis but on the other hand causes apoptotic cell death at high concentrations (98). Therefore early as well as late apoptosis events were determined in zinc-supplemented cells. It was found that in comparison to cells in normal cell culture medium, apoptosis was not enhanced under high zinc (10 ppm) conditions (Fig. 10).

Taken together, the cell culture conditions used here simulate a high intracellular zinc concentration, but do not cause side effects such as cytotoxicity, inhibition of cell proliferation or apoptosis. These conditions should therefore allow the identification of markers of zinc action at elevated intracellular ion levels.

5.1.1.3 Identification of genes responsive to increased cellular zinc levels

In order to identify genes responsive to increased cellular zinc levels, cDNA array analysis of cells exposed to a normal or a high zinc concentration for 72 h was carried out. In a control experiment, the expression of the metallothionein gene I (MT-1) was assessed prior to hybridisation of DNA arrays, and a marked increase in mRNA levels was shown in cells cultured in medium supplemented with 10 ppm zinc (Fig. 11).

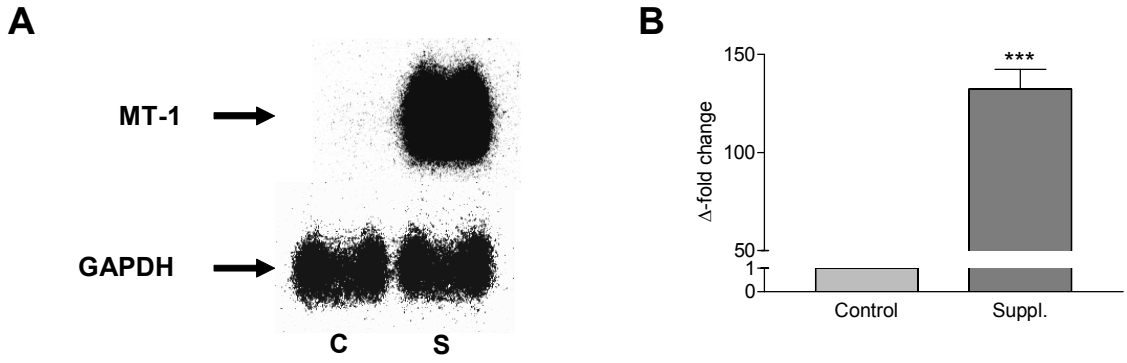
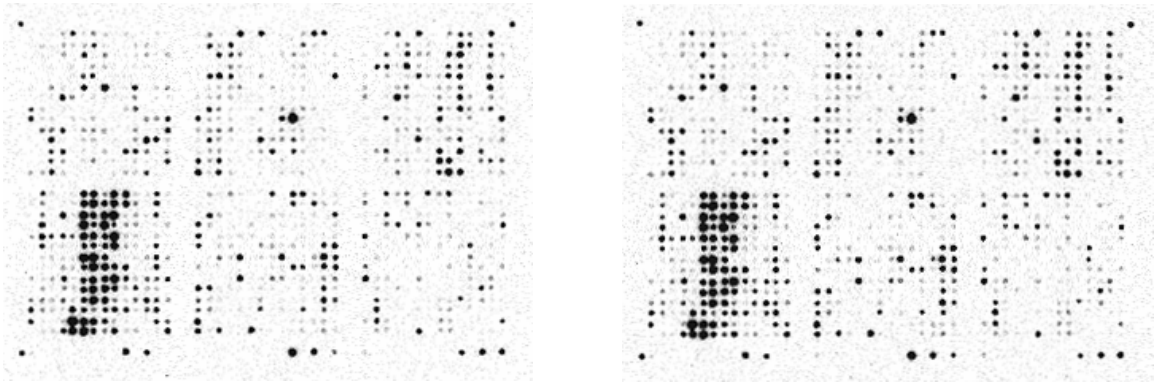


Fig. 11: mRNA levels of metallothionein-1 under high zinc conditions. HT-29 cells were cultured for 72h under normal (~0.24 ppm, control) or high zinc conditions (10 ppm, supplementation). **A** Northern blot analysis, signal for GAPDH served as an internal control, a typical blot is shown. **B** Real-time RT-PCR, values are means ± SEM, n = 3, ***: P<0.001.

Radiolabelled cDNAs synthesised from extracted mRNAs were hybridised to Atlas™ Human 1.2 Array III (Clontech), imaged with phosphorimager plates and scanned at different time points. Two representative scans are shown in Fig. 12.



Control Supplementation

Fig. 12: Representative phosphorimager scans of cDNA arrays. HT-29 cells were cultured for 72h in control (~0.24 ppm) or zinc-supplemented (10 ppm) media. Same amounts of extracted total RNA were reverse transcribed in the presence of [α^{33}P]-dATP (ICN Biomedicals) and hybridised to cDNA arrays (Atlas™ Human 1.2 Array III, Clontech). Arrays were exposed to phosphorimager plates and scanned after 96h.

Prior to data analysis, it was proven whether signal intensities of spots on the arrays were within the linear range of phosphorimager plates. Otherwise signal intensities of highly expressed genes would be underestimated due to saturation effects. To check this, acquired signal intensities of GAPDH (a widely used housekeeping gene) and 60S ribosomal protein L37 (the spot with the highest signal intensity on the array) were plotted over time (Fig. 13). A nearly perfect correlation was found, indicating that signals after 96 h are all in the linear range of phosphorimager plates and these were used for subsequent data analysis.

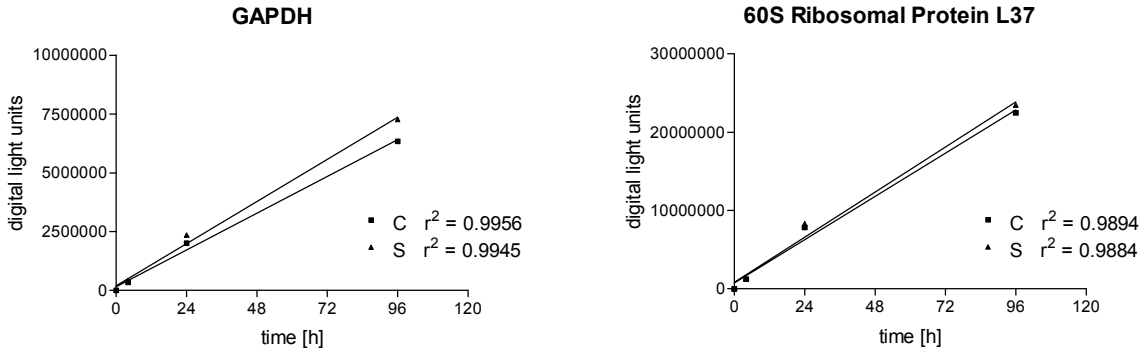


Fig. 13: Linearity of acquired signal intensities from cDNA arrays. cDNA arrays (Clontech) were hybridised with radiolabelled probes from control and zinc-supplemented cells and exposed to phosphorimager plates. Signal intensities were acquired after 4h, 24h and 96h and signals for GAPDH and 60S ribosomal protein L37 were checked for linearity, C = control, S = supplementation, r^2 = correlation coefficient.

Cross analysis of the acquired data set showed that 57 % (n = 671) of the 1176 genes on the arrays yielded signal intensities significantly above background levels. Among these, only 17 (2.5 %) genes showed differences in mRNA steady state levels at a threshold ratio of ±1.5 (average of all hybridisations). For all transcripts observed changes in gene expression were independently confirmed by quantitative RT-PCR (Tab. 1) and/or Northern blot analysis (Fig. 14).

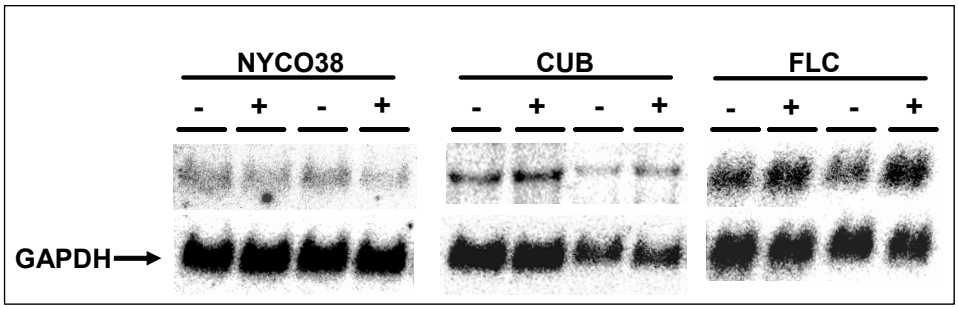


Fig. 14: Confirmation of gene regulation in response to high medium zinc concentration by Northern blot analysis. HT-29 cells were cultured for 72 h under normal (-) or high (+) zinc conditions. Blots were probed for NYCO38 (antigen NY-CO-38), CUB (hypothetical 40 kD protein) and FLC (ferritin light chain). Each blot was reprobed for GAPDH serving as internal control, n=3.

Tab. 1: Changes in steady state mRNA expression levels in response to zinc supplementation in human HT-29 cells[1]

Gene[2]	Encoded protein[3]	Function[4]	Δ-fold change at high zinc[5]		
			Array	Northern Blot	RT-PCR
X72875	Complement factor B	Immune system	+ 1.6	n. d.	+ 2.0
D29810	Hypothetical 40 kD protein	Unknown	+ 1.6	+ 1.5	+ 1.5
M11147	Ferritin light chain	Iron metabolism	+ 1.6	+ 1.5	+ 1.5
AF022080	RAS-related protein R-RAS3	Signal transduction	+ 1.5	n. d.	+ 2.2
AF048700	Gastrointestinal peptide	Unknown	- 2.3	n. d.	- 6.7
J04469	Creatine kinase, ubiquitous mitochondrial	Energy metabolism	- 1.7	n. d.	- 1.4
AF039699	Antigen NY-CO-38	Stress-induced	- 1.6	- 2.1	- 1.5
D37931	Ribonuclease 4	Stress-induced	- 1.5	n. d.	- 1.5
AF043724	Hepatitis A virus cellular receptor 1	Unknown	- 1.5	- 1.7	- 2.6
AF000985	DEAD box protein 3, Y-chromosomal	Transcription	+ 3.8	n. r.	n. d.
M97856	Nuclear autoantigenic sperm protein	Trafficking	+ 1.8	n. d.	n. r.
M85085	Cleavage stimulation factor, 64 kD subunit	Transcription	+ 1.6	n. d.	n. r.
D29956	Ubiquitin carboxyl-terminal hydrolase 11	Cell proliferation	+ 1.6	n. d.	n. r.
X68687	Zinc finger protein 33a	Transcription	+ 1.5	n. d.	n. d.
D83032	Nuclear protein, NP220	Transcription	+ 1.5	n. d.	n. r.
AF058718	Putative 13S golgi transport complex 90 kD subunit	Trafficking	+ 1.5	n. d.	u. p.
L40357	Thyroid receptor interacting protein 7	Transcription	+ 1.5	n. d.	u. p.

[1]Genes with increased (+) or decreased (-) expression levels upon zinc supplementation. Genes were originally identified by array analysis and gene regulation was independently confirmed by Northern blot and/or real-time RT-PCR. [2]GenBank accession number. [3]Name of encoded protein. [4]Proposed function of the encoded protein. [5]Magnitude of changes observed by array, Northern blot analysis or real-time RT-PCR. Values are means, n = 3, n. d., not determined, u. p., unspecific product obtained, n. r., not regulated.

Independent confirmation revealed that in the case of two genes, a specific PCR product could not be obtained and that six transcripts did not show corresponding changes in expression level. For the remaining 9 genes (52.9 %), mRNA steady state levels showed changes in the same direction as indicated on the array and those are compiled in Tab. 1. A high intracellular zinc concentration altered the expression of genes important for stress response (antigen NY-CO-38, ribonuclease 4), energy metabolism (creatine kinase) and other cellular functions (e. g. complement factor B, ferritin light chain).

DNA arrays allow to monitor the expression of thousands of genes simultaneously and are in this regard superior to conventional techniques. However, whether DNA arrays allow a quantitative assessment of gene regulation has not been fully elucidated (99). Therefore, data from DNA arrays were compared to results from Northern blot analysis and real-time PCR (Fig. 15).

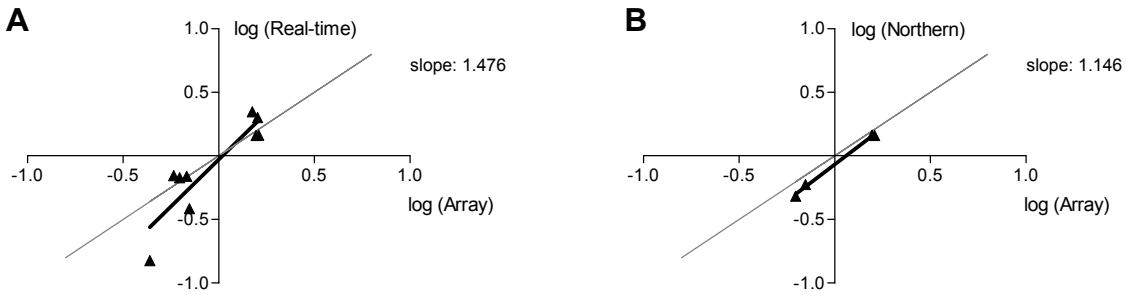


Fig. 15: Comparison of data from DNA arrays, Northern blot analysis and real-time RT-PCR of zinc-supplemented HT-29 cells. Changes in mRNA expression levels of regulated genes at high zinc determined by array analysis were plotted on a logarithmic scale against data obtained by real-time RT-PCR and Northern blot analysis, respectively, and a regression line was calculated. Grey line indicates a perfect correlation (slope 1.000) (A) DNA array vs. real-time RT-PCR, (B) DNA array vs. Northern blot.

Changes in transcript levels determined by real-time RT-PCR were often more pronounced than those found by array analysis, resulting in a regression line with a slope >1. This supports the general point of view that microarrays are a semiquantitative tool more suitable for exploring qualitative changes in gene expression (99), whereas real-time PCR is a highly sensitive, fully quantitative method. An “underestimation” of expression differences by DNA arrays has been reported previously (100,101). In contrast, changes in gene expression determined by DNA arrays and Northern blots showed a good correlation with a slope of the resulting regression line of 1.146. This is consistent with findings of Taniguchi et

al., who compared array-based results with those derived from Northern blot analysis and reasoned, that DNA microarrays provide quantitative data, which are comparable to Northern blot (102).

5.1.1.4 Time-course experiment

To study how the duration of zinc supplementation affects gene expression, HT-29 cells were cultured under high zinc conditions and changes in mRNA levels were measured for the identified zinc-dependent genes by real-time RT-PCR at various time points (Fig. 16). Genes with parallel changes in expression are shown in one graph. mRNA levels of RRAS3 (RAS-related protein R-RAS3) increased constantly and almost linearly over time. Although the magnitude of changes are extremely different for MT-1 (metallothionein-1) and CUB (hypothetical 40 kD protein), expression of both genes increased up to 24h until a plateau was reached. Expression of CFBP (complement factor B) showed an increase up to 48h and a slight decrease over the last 24h. HHAV (hepatitis A virus cellular receptor 1) showed a continuous decrease over the entire incubation period. Transcript levels of RNASE4 (ribonuclease 4) and GP (gastrointestinal peptide) were down-regulated up to 24h, then reached a steady state where they remained for the rest of the incubation. The expression of FLC (ferritin light chain), NYCO38 (antigen NY-CO-38) and MIACK (creatine kinase) was already altered by the first time point and remained more or less stable for the rest of the incubation period.

5.1.2 Zinc depletion

5.1.2.1 Determination of experimental conditions for zinc depletion studies

N,N,N´,N´-Tetrakis(2-pyridylmethyl)ethylenediamine (TPEN) is a membrane-permeable metal ion chelator with structural similarity to EDTA (103). It has a high affinity for heavy metals but only a low affinity for Ca^{2+} and Mg^{2+} (103). Despite the fact that TPEN also binds other heavy metals besides zinc, it has been successfully used in a large number of biological systems to chelate Zn^{2+} (104).

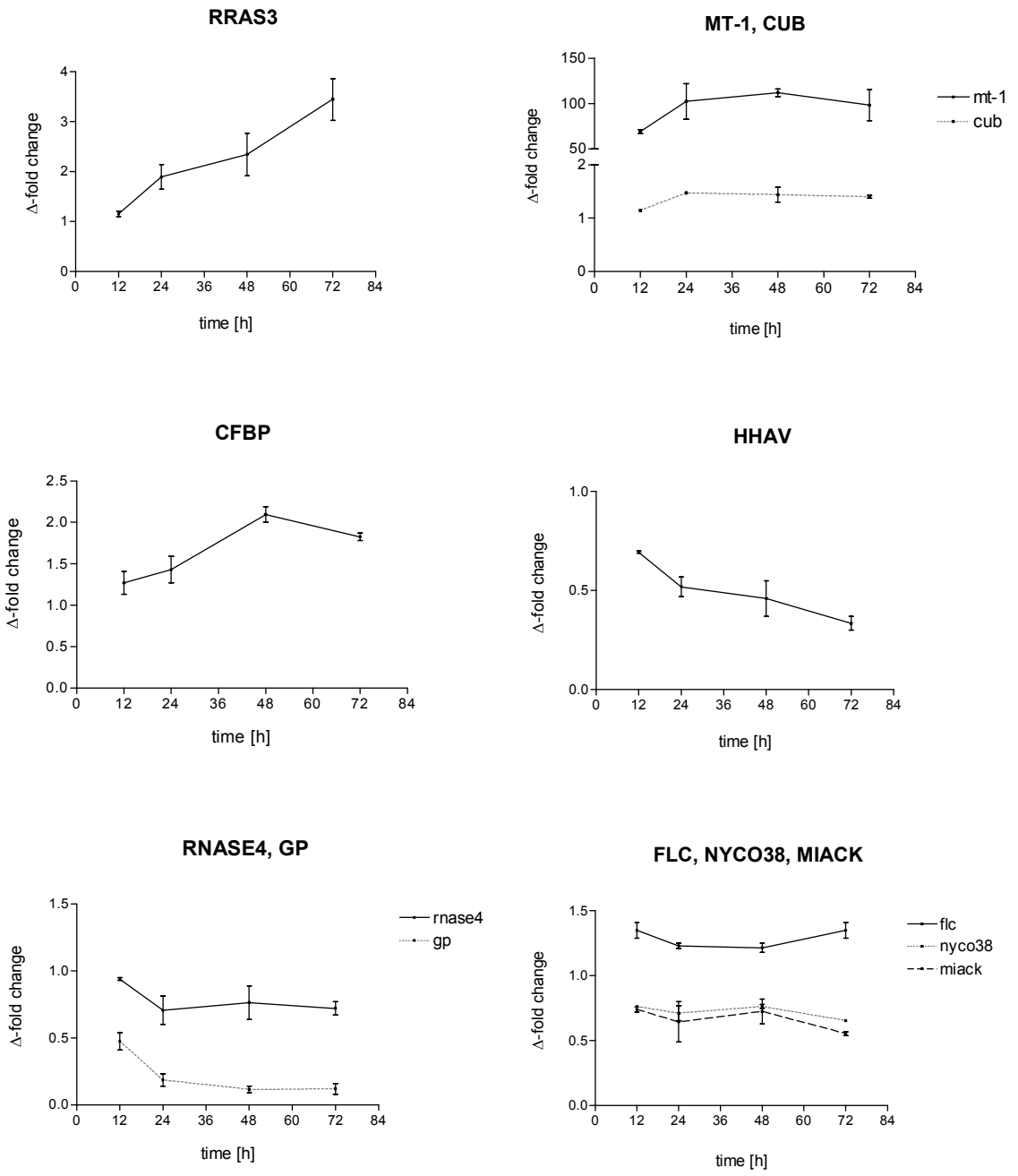
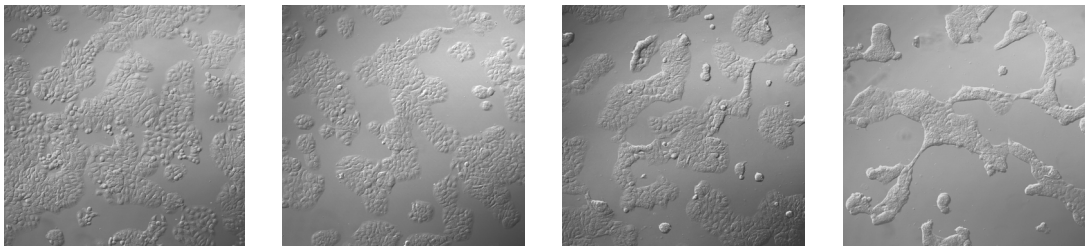


Fig. 16: Time-dependent changes in mRNA expression levels of zinc-dependent genes in response to zinc supplementation. HT-29 cells were cultured under normal (~0.24 ppm) or high (10 ppm) zinc conditions and relative changes in expression levels of the identified zinc-dependent genes were measured after 12h, 24h, 48h and 72h by real-time RT-PCR for RRAS3 (RAS-related protein R-RAS3), MT-1 (metallothionein-1), CUB (hypothetical 40 kD protein), CFBP (complement factor B), HHAV (hepatitis A virus cellular receptor 1), RNASE4 (ribonuclease 4), GP (gastrointestinal peptide), FLC (ferritin light chain), NYCO38 (antigen NY-CO-38) and MIACK (creatine kinase). Values are means ± SEM, n = 2.

To define a useful TPEN concentration for chelating intracellular zinc in HT-29 cells, increasing amounts of TPEN were added to culture medium. Cells were

incubated with these media and images were taken at time points indicated below (Fig. 17).



Control, 16h | 4 µM TPEN, 16h | 10 µM TPEN, 4h | 20 µM TPEN, 3h

Fig. 17: Effects of the zinc-chelator TPEN on cell growth. HT-29 cells were grown in normal culture medium or in media with increasing amounts of the membrane-permeable Zn^{2+}-chelator TPEN (N,N,N´,N´-Tetrakis(2-pyridylmethyl) ethylenediamine) and images were taken at indicated time points using a light-optical microscope.

When TPEN was added to the culture medium at concentrations of 10 or 20 µM, the intracellular chelation of zinc led to extensive cell death after only 4 or 3 hours, respectively, as determined by optical inspection. Zinc is important for a large number of biological processes in mammalian cells and it is therefore not surprising that severe depletion of zinc is not compatible with cell survival. In contrast, a TPEN concentration of 4 µM led to a decreased proliferation rate after 16 h but did not cause cell death. This concentration also seems reasonable, considering that the zinc concentration of the normal culture medium is ~3.7 µM (as determined by atomic absorption spectrometry) and that TPEN yields a zinc/chelate ratio of 1:1 (105).

5.1.2.2 Effects of the Zn^{2+}-chelator TPEN on cellular zinc concentration, cell viability, cell profileration, metallothionein-1 expression and apoptosis

To deplete cells of intracellular zinc, HT-29 cells were exposed to 4 µM of the membrane-permeable Zn^{2+}-chelator TPEN for 16 h. TPEN has a high affinity for zinc but can chelate other heavy metals such as iron and copper. Therefore in control experiments TPEN was added together with 4 µM zinc to study only effects originating from the deprivation of zinc. The resulting intracellular zinc concentration as determined using the zinc-sensitive dye Newport Green was significantly decreased in TPEN-treated cells (Fig. 18). Cells grown in control medium replenished with zinc had the same intracellular zinc level as cells cultured with normal culture medium.

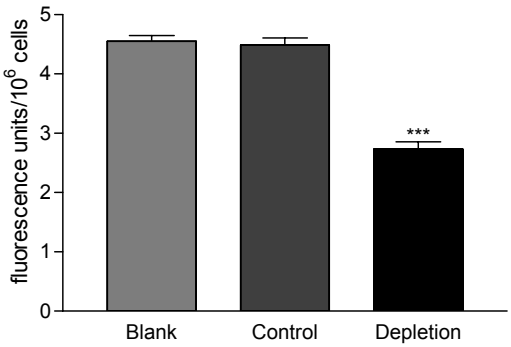


Fig. 18: Effect of TPEN on intracellular free zinc concentration. HT-29 cells were grown on 24-well plates for 16h under normal (~0.24 ppm Zn, control) or low (< 0.01 ppm Zn, depletion) zinc conditions. Cells were loaded with Newport Green™ DCF Diacetat and fluorescence emission intensity due to the binding of intracellular free zinc was measured at 530 nm after excitation at 485 nm using a fluorescence multiwell-plate reader. Blank indicates cells that were grown in medium as used for routine cell culture. Values are means ± SEM, n = 4, ***: P<0.001.

As previously observed in other cells (106), zinc depletion also significantly decreased cell proliferation in HT-29 cells (Fig. 19 B), whereas cell viability (Fig. 19 A) was not affected by low intracellular zinc concentration.

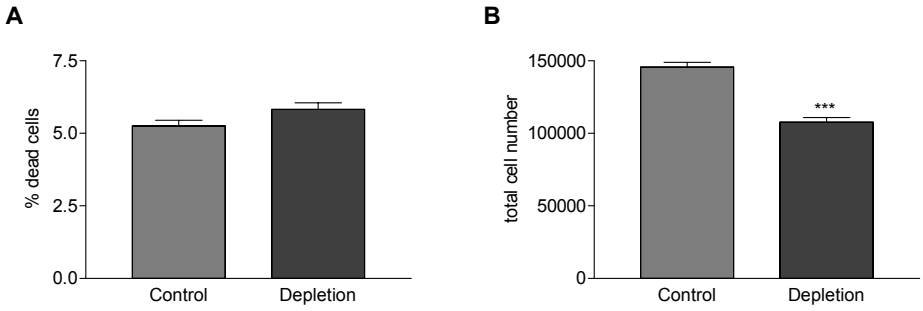


Fig. 19: Effects of intracellular zinc depletion on cell viability and cell proliferation. HT-29 cells were cultured for 16 h under normal (~0.24 ppm, control) or low zinc conditions (<0.01 ppm, depletion). **A** Cell viability was assessed by SYTOX-fluorescence. The percentage of dead cells was determined by SYTOX-fluorescence prior to cell lysis in relation to the fluorescence measured after solubilisation of the cells. **B** Proliferation was determined by cell counting using SYTOX-Green nucleic acid stain. Values are means ± SEM, n = 6, ***: P<0.001.

TPEN-induced intracellular zinc depletion is an inducer of apoptosis in a variety of cell types (107,108). The aim here was to study the effects of intracellular zinc deprivation on gene expression without activation of apoptotic cascades. To exclude this possible side effect, the occurrence of apoptosis was determined. Changes in membrane permeability is a morphological feature of early apoptosis, whereas DNA fragmentation serves as a late apoptosis marker. As shown in Fig.

20 the experimental conditions used did not lead to a higher extent of early or late apoptotic cells in comparison to control cells.

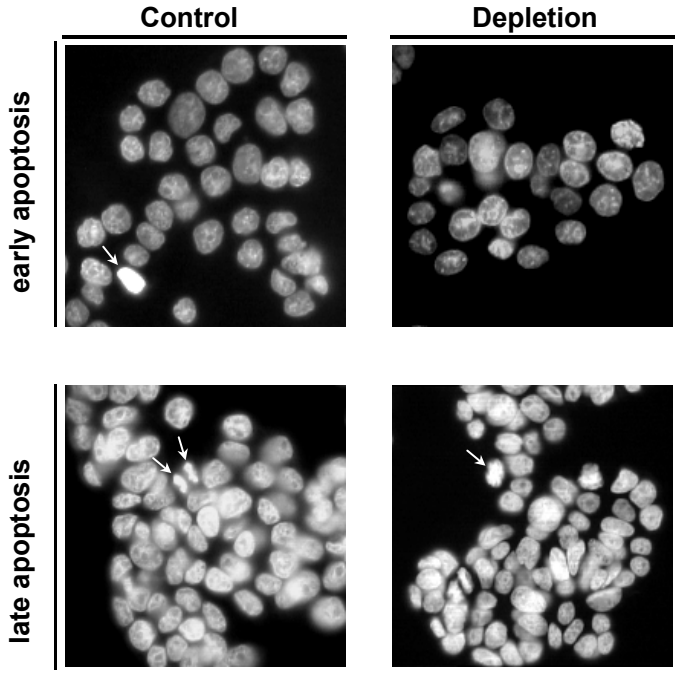


Fig. 20: Determination of early and late apoptosis events in zinc-depleted HT-29 cells. Cells were grown for 16 h under normal (control) or low zinc (depletion) conditions. Arrows indicate apoptotic cells due to membrane disintegration (early apoptosis) or nuclear fragmentation (late apoptosis).

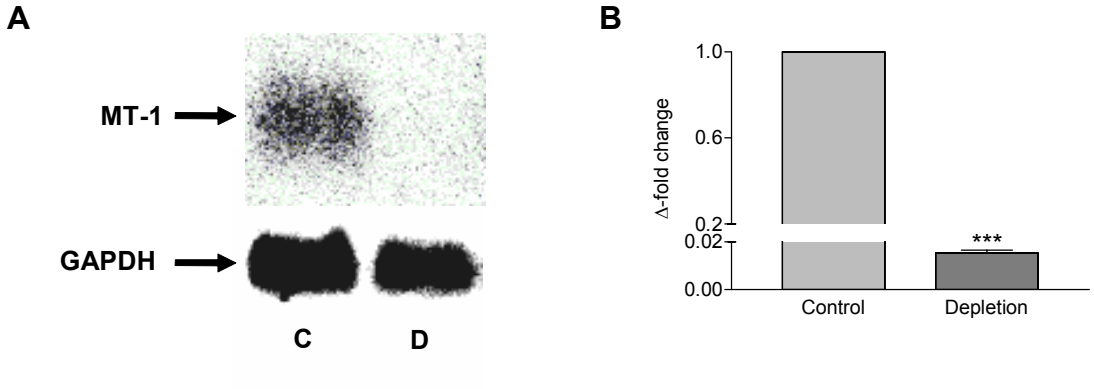


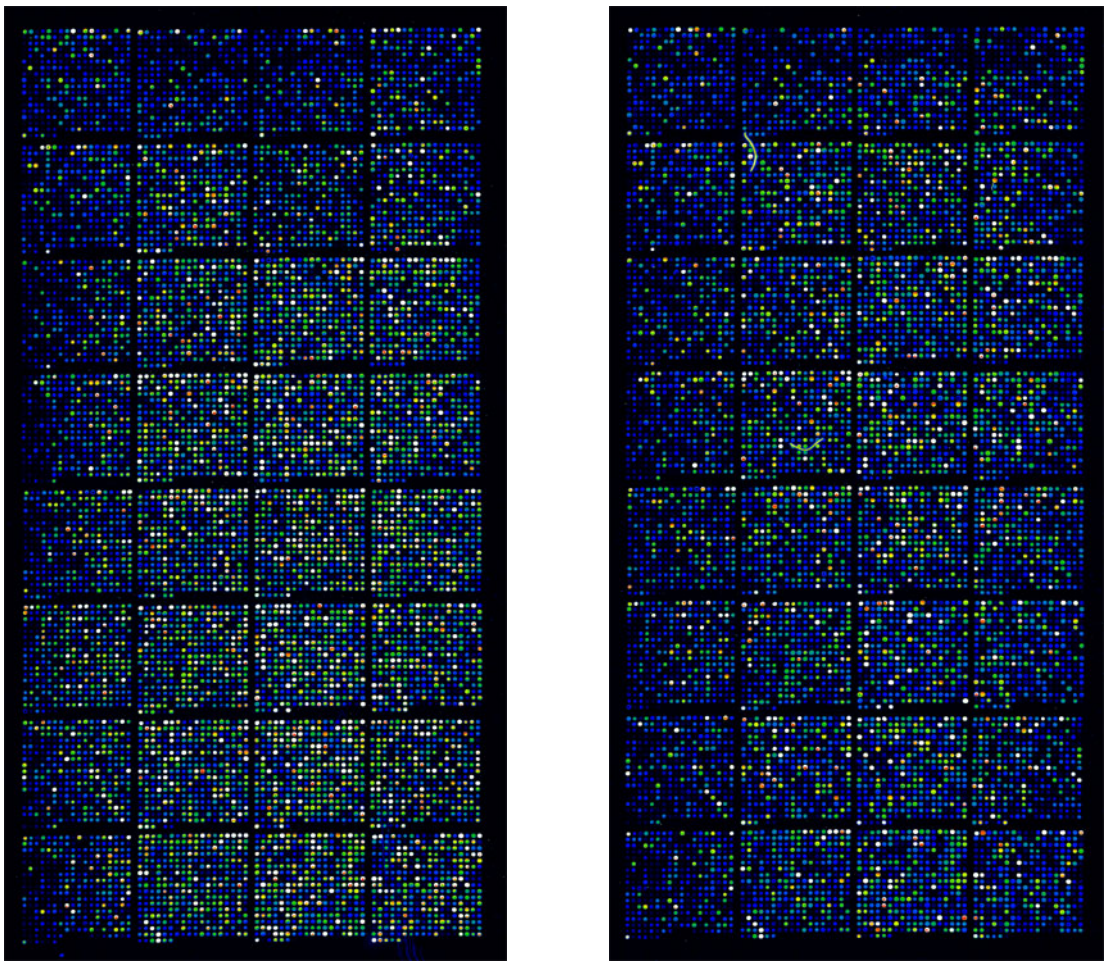
Fig. 21: mRNA levels of metallothionein-1 under low zinc conditions. HT-29 cells were cultured for 16h under normal (~0.24 ppm, control) or low zinc conditions (<0.01 ppm, depletion). **A** Northern blot analysis, signal for GAPDH served as an internal control, a typical blot is shown. **B** Real-time RT-PCR, values are means ± SEM, n = 3, ***: P<0.001.

Moreover, the expression level of the metallothionein gene I (MT-1), considered as a sensitive indicator of intracellular zinc status (7,43), was drastically reduced in zinc-depleted cells (Fig. 21). Therefore, the cell model used to simulate a zinc-

deficient state appeared suitable and did not cause impairments in cell integrity and apoptosis.

5.1.2.3 Identification of genes responsive to a low intracellular zinc concentration

For identifying genes responsive to a low intracellular zinc concentration, oligonucleotide array analysis of the transcriptome of HT-29 cells grown for 16 h under low or normal zinc conditions was carried out. Two representative images as obtained after hybridsation of DNA arrays are shown in Fig. 22.



Control Depletion

Fig. 22: Images of oligonucleotide arrays. Total RNA from control or zinc-deprived HT-29 cells was reverse transcribed in the presence of either Cy3- or C5-labeled dCTP and hybridized to oligonucleotide arrays (Pan® Human 10k Array, MWG). The two miscolored images show the resulting gene expression profiles.

Of the 9850 genes analysed, 8025 (81.5 %) were considered as expressed and changes (> 2-fold) in mRNA levels were detected for 309 (3.9 %) genes. 231 (74.8 %) genes were down-regulated whilst 78 (25.2 %) genes were up-regulated

upon intracellular zinc depletion. A complete list of all regulated genes is provided in Tab. 7.

Genes could be classified into 8 groups based on the function of the gene products or by homology with previously described proteins in public databases (Fig. 23). Identified groups encode for proteins involved in metabolism (n=58), protein metabolism (n=21), transcription (n=19), signaling (n=30), cell growth/cycle (n=15), vesicular trafficking (n=15), cell-cell interaction (n=13) and cytoskeletal homeostasis (n=10). These groups cover 59 % of the identified genes. 19 % encode for proteins with miscellaneous functions and could not be clustered (n=59). Zinc-dependent regulation was also found for a number of genes that encode for yet hypothetical proteins (n=40) or proteins with unknown function (n=29).

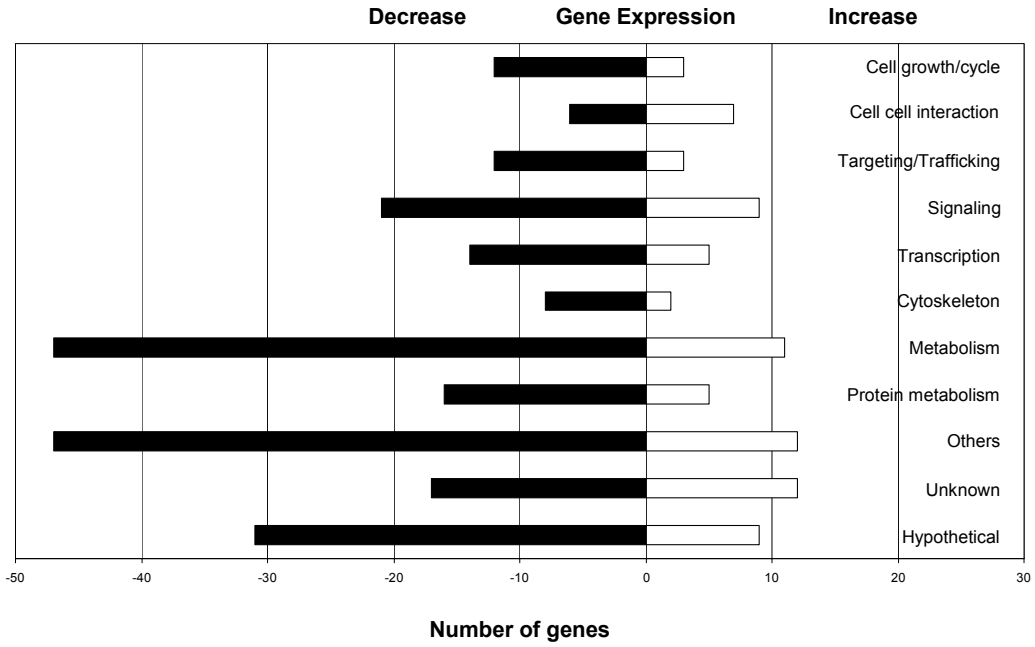


Fig. 23: Functional classification of genes with altered expression levels in response to low intracellular zinc concentration in human HT-29 cells. Open and closed bars represent the number of genes with increased or decreased mRNA levels in zinc-depleted cells as found by oligonucleotide array analysis.

5.1.2.4 Confirmation of changes in gene expression

To assess the reliability of the array results, Northern blot analysis and real-time RT-PCR for 14 selected genes from different clusters were performed. Alterations in mRNA levels were confirmed for 12 genes (85.7 %) in the same direction by quantitative RT-PCR and/or Northern blotting (Tab. 2). However, changes in

transcript levels were often 2-4 times higher than those determined on oligonucleotide arrays.

Tab. 2: Changes in steady state mRNA expression levels of selected genes in response to intracellular zinc depletion in human HT-29 cells[1]

Acc. no.[2]	Encoded protein[3]	Function[4]	Δ-fold change at low zinc concentration[5]		
			Array	Northern blot	RT-PCR
NM004235	Kruppel-like factor 4	Transcription	+ 5.3	+ 7.2	+ 9.0
NM003447	Zinc finger protein 165	Transcription	+ 3.2	+ 5.2	+ 7.3
NM006526	Zinc finger protein 217	Transcription	- 4.8	n. r.	u. p.
XM030144	H4 histone family member	DNA-binding	- 8.5	n. d.	- 4.0
NM003122	Serine protease inhibitor	Protein degradation	+3.8	n. d.	+ 3.9
NM002786	Proteasome subunit	Protein degradation	- 2.1	n. d.	- 2.5
NM004390	Cathepsin H	Protein degradation	- 2.3	n. d.	- 1.4
NM014184	Hspc163 protein	Protein folding	- 4.5	n. d.	- 19.7
NM002080	Aspartate aminotransferase 2	Amino acid metabolism	- 2.4	n. d.	- 8.2
NM000780	Cytochrome P450 subfamily VIIa	Xenobiotic metabolism	- 2.5	n. d.	u. p.
NM002495	NADH dehydrogenase Fe-S protein 4	Energy metabolism	- 3.3	- 3.2	- 7.2
NM000434	Sialidase 1	Lipid degradation	+ 5.1	n. d.	+ 7.1
NM004939	DEAD/H box polypetide 1	Apoptosis	- 5.6	n. d.	- 16.2
NM004864	Prostate differentiation factor	Cell differentiation	+ 6.9	+ 8.6	u. p.

[1]Selected zinc-dependent genes in HT-29 cells with increased (+) or decreased (-) expression levels upon zinc depletion. Genes were originally identified by array analysis and were confirmed by Northern blot analysis and/or real-time RT-PCR. [2]GenBank accession number. [3]Name of encoded protein. [4]Proposed function of the encoded protein. [5]Magnitude of changes observed by array, Northern blot analysis or real-time RT-PCR. Data indicate means for three experiments. n. d., not determined, u. p., unspecific product obtained, n. r., not regulated.

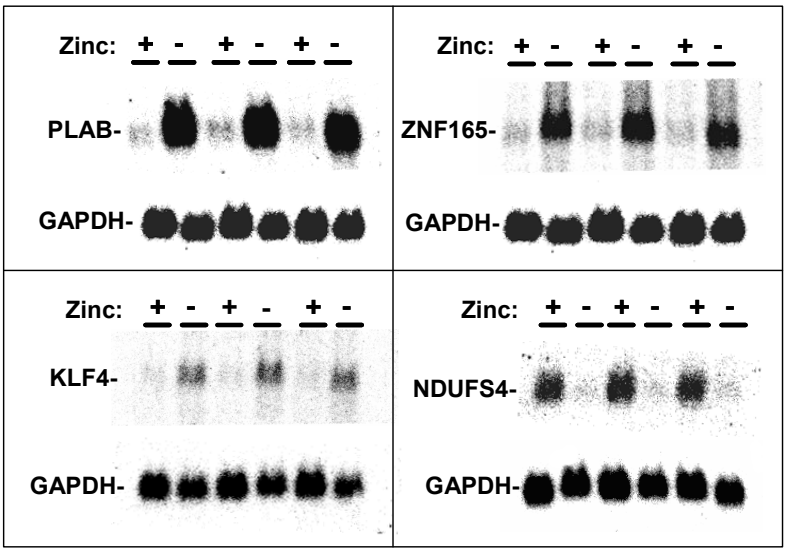


Fig. 24: Confirmation of gene regulation in response to low intracellular zinc concentration by Northern blot analysis. HT-29 cells were cultured for 16 h under normal (+) or low (-) zinc conditions. Blots were probed for PLAB (prostate differentiation factor), KLF4 (Kruppel-like factor 4), ZNF165 (zinc finger protein 165) and NDUFS4 (NADH dehydrogenase Fe-S protein 4). Each blot was reprobed for GAPDH serving as internal control, n=3.

In the case of 3 genes, the zinc finger protein 217 (ZNF217), the prostate differentiation factor (PLAB) and cytochrome P450 subfamily VIIa (CYP7A1), a specific PCR product could not be obtained. ZNF217 appeared not to be regulated by zinc depletion when the mRNA levels were determined by Northern blot analysis. This, however, is not attributable to the sensitivity of Northern blot analysis, as those performed for prostate differentiation factor (PLAB), zinc finger protein 165 (ZNF165), Kruppel-like factor 4 (KLF4) and NADH dehydrogenase Fe-S protein 4 (NDUFS4) confirmed all the results obtained by oligonucleotide array analysis and RT-PCR (Fig. 24).

In analogy to chapter 5.1.1.3, a comparison of the data from the three different techniques used was performed.

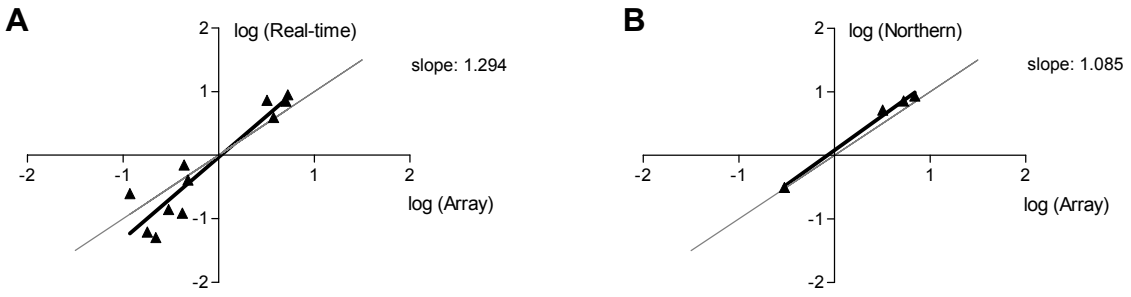


Fig. 25: Comparison of data from DNA arrays, Northern blot analysis and real-time RT-PCR of zinc-depleted HT-29 cells. Changes in mRNA expression levels of regulated genes at low zinc determined by array analysis were plotted on a logarithmic scale against data obtained by real-time RT-PCR and Northern blot analysis, respectively, and a regression line was calculated. Grey line indicates a perfect correlation (slope 1.000) (A) DNA array vs. real-time RT-PCR, (B) DNA array vs. Northern blot.

Results of the comparison between data from DNA arrays and Northern blot / real-time RT-PCR were according to those from the zinc supplementation experiment. A good correlation between data from array and Northern blot analysis was found (slope 1.085), whereas changes in transcript levels determined by real-time RT-PCR were often 2-4 times higher than those found by array analysis. Consequently microarray analysis appears to underestimate the change in expression level independent of the type of array used.

5.1.2.5 Time-course experiment

Again, as for zinc supplementation studies (see 5.1.1.4), a time-course experiment was performed, in order to elucidate how gene expression depends on incubation time. Therefore, HT-29 cells were cultured under zinc-deficient conditions and

changes in mRNA levels were measured for selected zinc-dependent genes by real-time RT-PCR at various time points (Fig. 26). Genes with parallel changes in expression are shown in one graph. mRNA levels of ZNF165 (zinc finger protein 165) and KLF4 (Kruppel-like factor 4) raised constantly and in the case of KLF4 nearly linear over time, whereas those of NEU1 (sialidase 1) and SPINK1 (serine protease inhibitor) remained almost unchanged over the first 8 hours and were only increased after this point. Expression of MT-1 (metallothionein-1) and DDX1 (DEAD/H box polypetide 1) showed an almost linear decrease within the first 8 hours which then attenuates for the rest of the incubation period. GOT2 (aspartate aminotransferase 2), HSPC163 (hspc163 protein), PSMA1 (proteasome subunit) and NDUFS4 (NADH dehydrogenase Fe-S protein 4) showed a continuous decrease between 4 and 16 hours, which was almost linear for GOT2 and HSPC163 and more weaker for the latter two. Expression of H4FG (H4 histone family member) and CTSH (cathepsin H) changed only modestly over time.

5.1.3 Zinc responsiveness of selected zinc-dependent genes

5.1.3.1 Identification of zinc-sensitive genes

Our studies investigating the effect of intracellular zinc depletion and zinc supplementation on gene expression in HT-29 cells by the use of DNA microarrays led to the identification of a considerable number of zinc-dependent genes (see paragraph 5.1.1.3 and 5.1.2.3). Based on these screenings, 20 genes were selected to further examine the zinc responsiveness of mRNA steady state expression levels by real-time RT-PCR in cells with low or high zinc status. This means that mRNA expression levels of genes regulated under high zinc conditions were determinded in zinc-depleted cells and vice versa. Whereas 11 mRNA species responded only to one condition, the mRNA levels of 9 genes changed at both low and high zinc concentrations and those were named zinc-sensitive gens. As shown in Fig. 27, the expression level of RNASE4 (ribonuclease type 4), HHAV (hepatitis A virus cellular receptor 1), GP (gastrointestinal peptide) and KLF4 (Kruppel-like factor 4) responded with up- and down-regulation to low or high zinc conditions, respectively. The mRNA's of CUB (hypothetical 40 kD protein), CFBP (complement factor B) and RRAS3 (RAS-related protein R-ras3) did display regulation in the opposite direction and two genes showed increased (FLC, ferritin

light chain) or decreased (NYCO38, antigen NY-CO-38) expression under both status conditions.

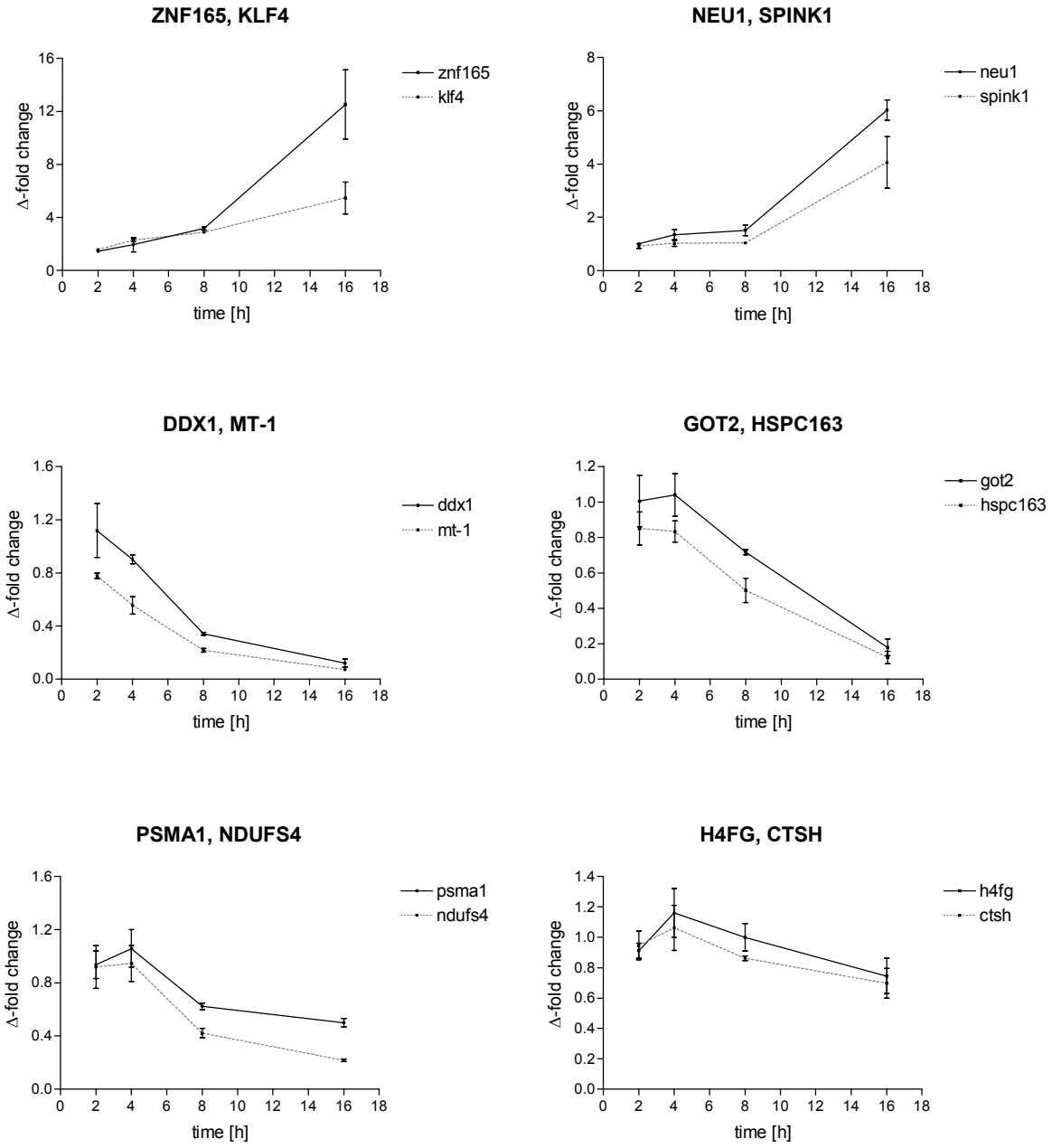


Fig. 26: Time-dependent changes in mRNA expression levels of zinc-dependent genes in response to intracellular zinc depletion. HT-29 cells were cultured under normal (~0.24 ppm) or low (<0.01 ppm) zinc conditions and relative changes in expression levels of selected zinc-dependent genes were measured after 2h, 4h, 8h and 16h by real-time RT-PCR for ZNF165 (zinc finger protein 165), KLF4 (Kruppel-like factor 4), NEU1 (sialidase 1), SPINK1 (serine protease inhibitor), MT-1 (metallothionein-1), DDX1 (DEAD/H box polypetide 1), GOT2 (aspartate aminotransferase 2), HSPC163 (hspc163 protein), PSMA1 (proteasome subunit), NDUFS4 (NADH dehydrogenase Fe-S protein 4), H4FG (H4 histone family member) and CTSH (cathepsin H). Values are means ± SEM, n = 2.

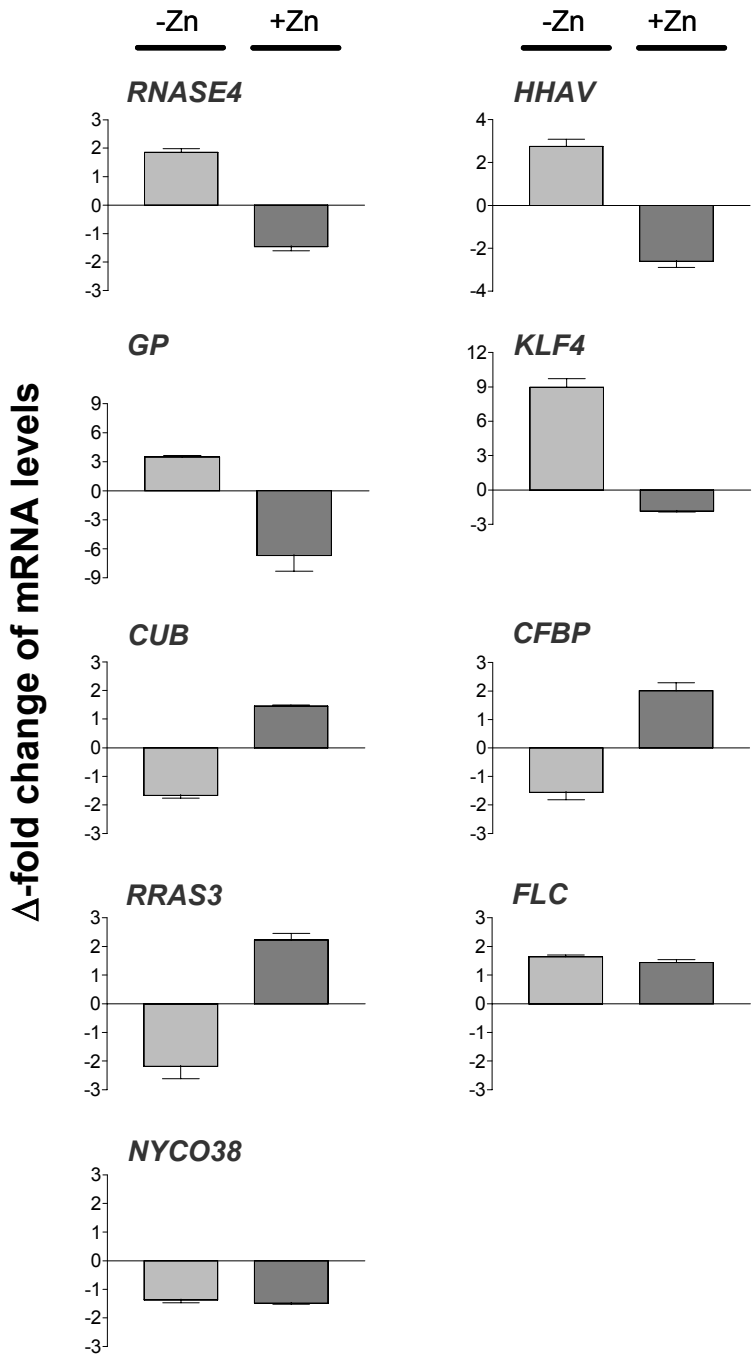


Fig. 27: Changes of mRNA levels of zinc-sensitive genes under low and high zinc conditions. Graphs show relative changes in mRNA levels of identified zinc-sensitive genes in response to zinc depletion (-Zn) and zinc supplementation (-Zn) in comparison to control cells as determined by real-time RT-PCR. RNASE4 (ribonuclease 4), GP (gastrointestinal peptide), CUB (hypothetical 40 kD protein), RRAS3 (RAS-related protein R-RAS3), NYCO38 (antigen NY-CO-38), HHAV (hepatitis A virus cellular receptor 1), KLF4 (Kruppel-like factor 4), CFBP (complement factor B) and FLC (ferritin light chain). Values are means ± SEM, n = 3.

5.1.3.2 Dependence of gene expression on medium zinc concentration

To further explore the responsiveness of the identified zinc-sensitive genes towards alterations in zinc status, the zinc concentration in the medium was systematically increased and decreased and relative changes in transcript levels were measured by real-time RT-PCR.

Expression of MT-1 (metallothionein-1) responded most sensitively amongst all genes to variations in medium zinc concentrations (Fig. 28). A 2-fold decrease of the medium zinc content already affected MT-1 mRNA levels. The most pronounced decay appeared between 0.12 ppm and 0.06 ppm zinc. A gradual increase in medium zinc lead to an increase in MT-1 transcript levels. Between 3.3 ppm and 10 ppm zinc MT-1 levels were boosted from 2.3-fold to over 110-fold. This finding is consistent with the observation made before (Fig. 8) that MT-1 levels are dramatically up-regulated at 10 ppm zinc in comparison to lower zinc concentrations.

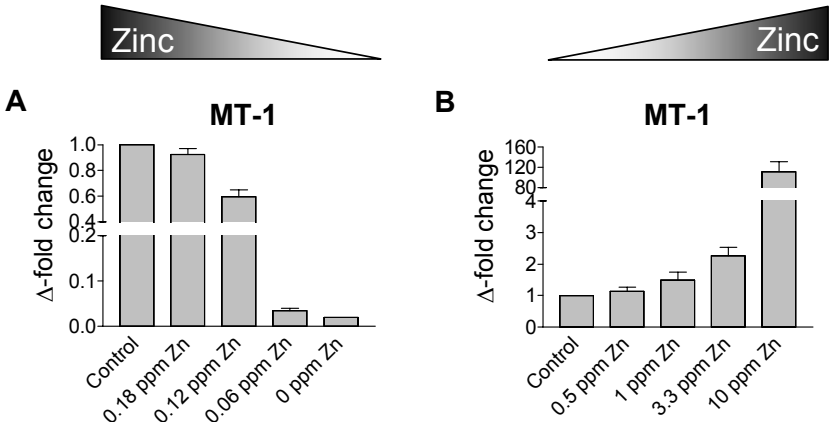


Fig. 28: Concentration-dependent changes in mRNA expression level of MT-1. HT-29 were grown in media with different zinc contents for 16h (zinc-depleted media) and 72h (zinc-supplemented media), respectively and relative changes in expression levels in comparison to control cells (~0.24 ppm zinc) were determined by real-time RT-PCR for MT-1 (Metallothionein-1). (A) zinc depletion, (B) zinc supplementation. Values are means ± SEM, n = 2.

The next figure shows three genes whose expression changed synchronously to that of MT-1, which means down-regulation under low zinc conditions and up-regulation at high zinc (Fig. 29).

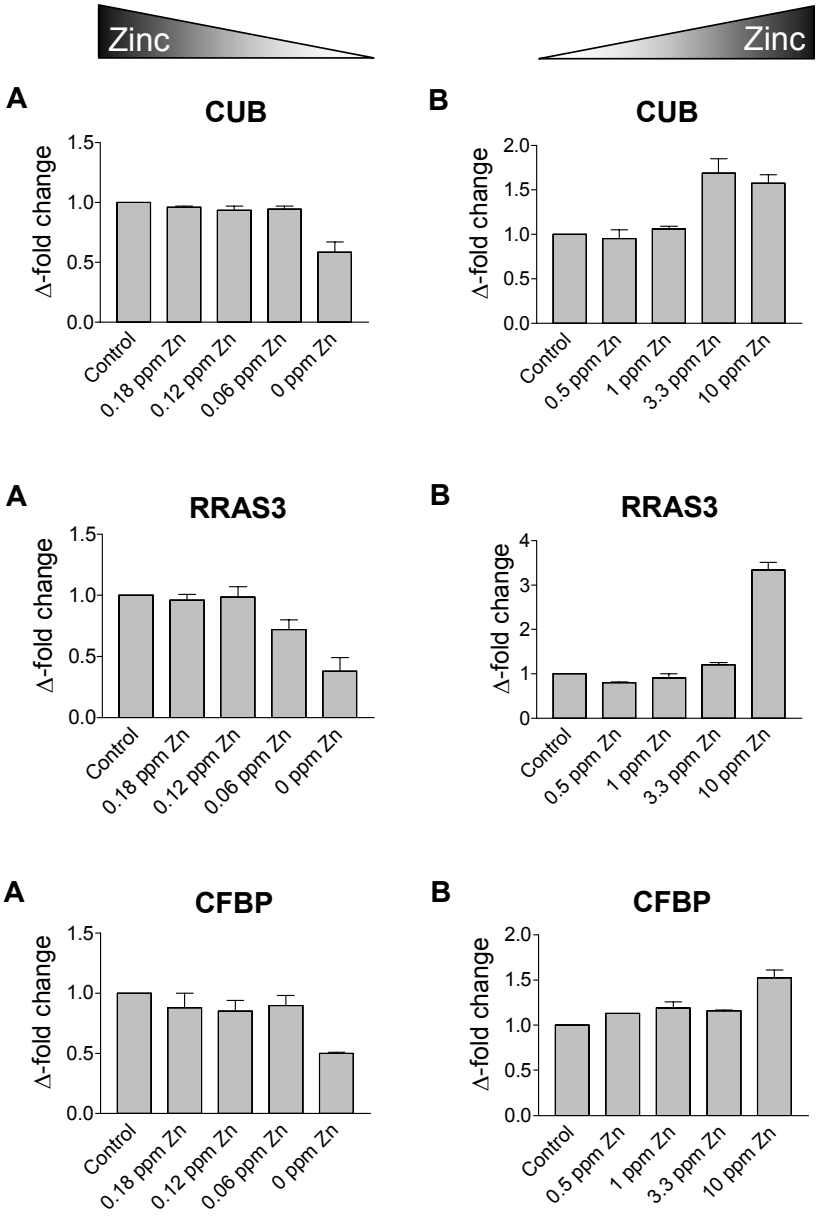


Fig. 29: Concentration-dependent changes in mRNA expression levels of CUB, RRAS3 and CFBP. HT-29 were grown in media with different zinc contents for 16h (zinc-depleted media) and 72h (zinc-supplemented media), respectively and relative changes in expression levels in comparison to control cells (~0.24 ppm zinc) were determined by real-time RT-PCR for CUB (hypothetical 40 kD protein), RRAS3 (RAS-related protein R-RAS3) and CFBP (complement factor B). (A) zinc depletion, (B) zinc supplementation. Values are means ± SEM, n = 2.

Fig. 30 summarizes genes with an opposed zinc-responsiveness compared to metallothionein-1. However, changes were observed only at the end points (0 ppm and 10 ppm zinc) of both concentration curves.

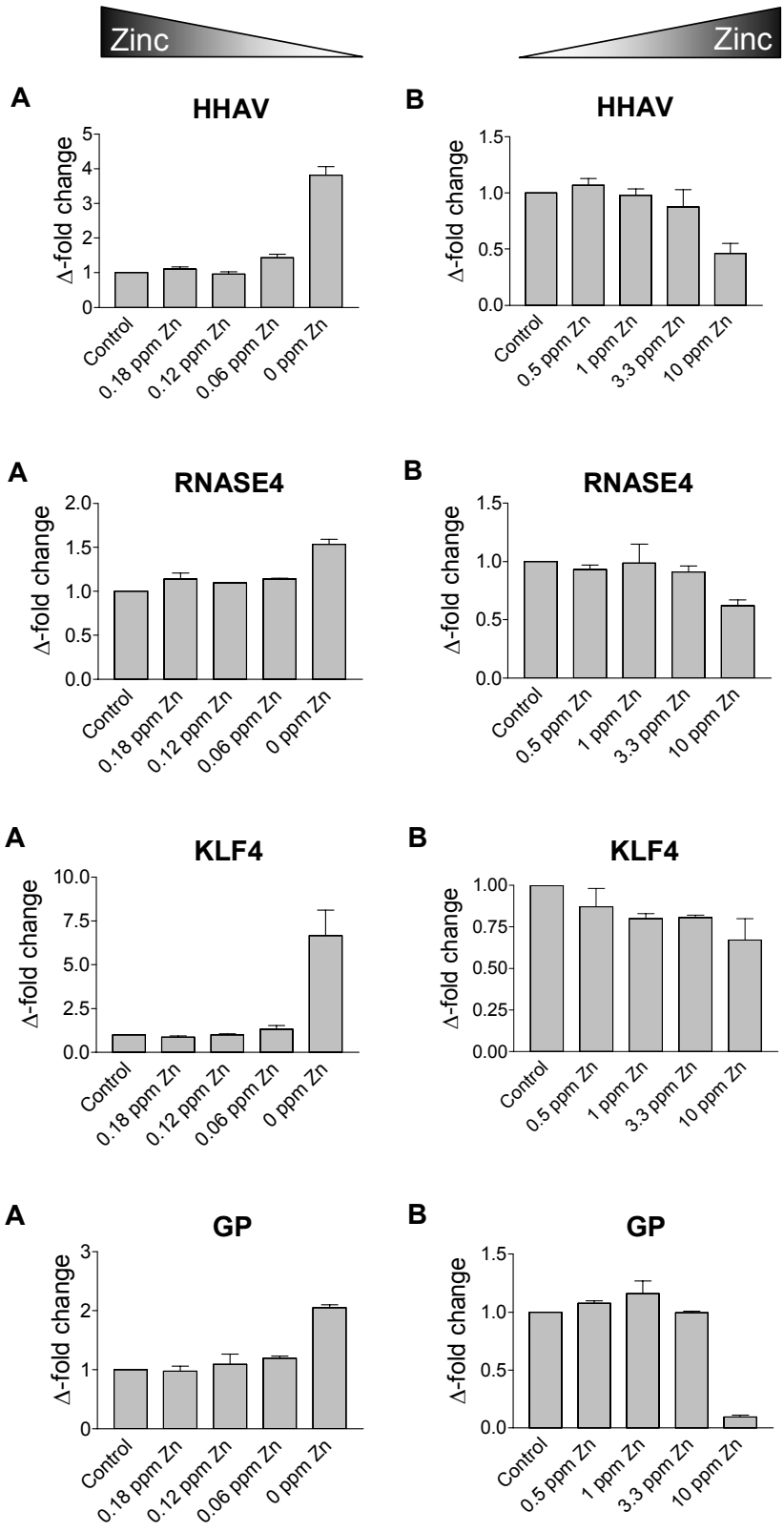


Fig. 30: Concentration-dependent changes in mRNA expression levels of HHAV, RNASE4, KLF4 and GP. HT-29 were grown in media with different zinc contents for 16h (zinc-depleted media) and 72h (zinc-supplemented media), respectively and relative changes in expression levels in comparison to control cells (~0.24 ppm zinc) were determined by real-time RT-PCR for HHAV (hepatitis A virus cellular receptor 1), RNASE4 (ribonuclease 4), KLF4 (Kruppel-like factor 4) and GP (gastrointestinal peptide). (A) zinc depletion, (B) zinc supplementation. Values are means ± SEM, n = 2.

Two genes, namely FLC (ferritin light chain) and NYCO38 (antigen NY-CO-38) were regulated under both zinc-depleted and zinc-supplemented conditions in the same direction. FLC was evenly up-regulated, whilst expression of NYCO38 was depressed.

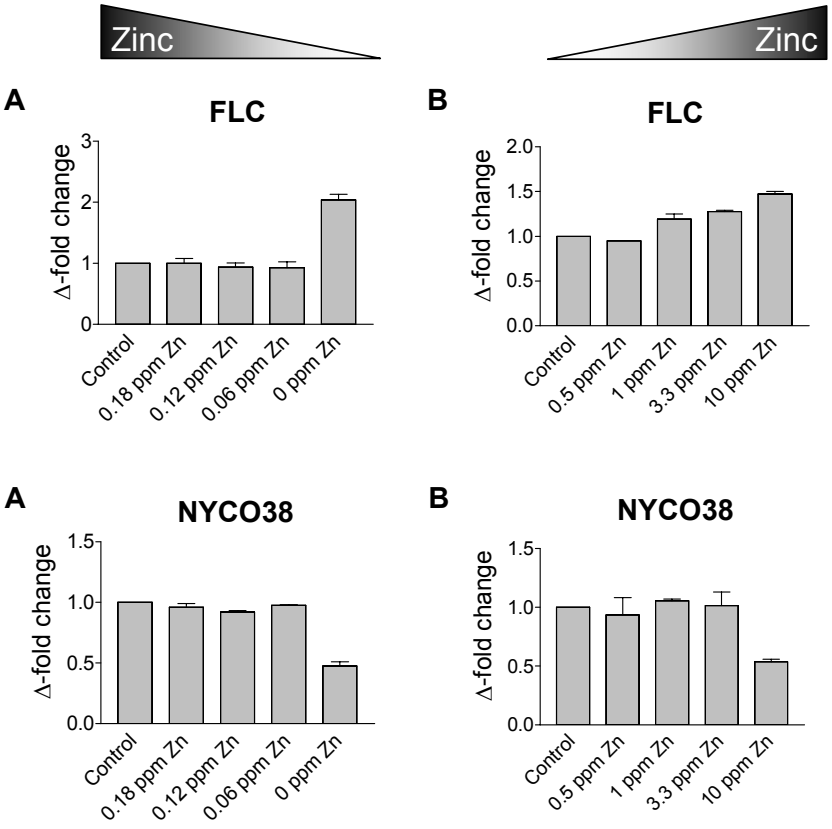


Fig. 31: Concentration-dependent changes in mRNA expression levels of FLC and NYCO38. HT-29 were grown in media with different zinc contents for 16h (zinc-depleted media) and 72h (zinc-supplemented media), respectively and relative changes in expression levels in comparison to control cells (~0.24 ppm zinc) were determined by real-time RT-PCR for FLC (ferritin light chain) and NYCO38 (antigen NY-CO-38). (A) zinc depletion, (B) zinc supplementation. Values are means ± SEM, n = 2.

In summary, changes in transcript levels for most genes were only observed in response to extreme changes in cellular zinc supply. Except MT-1, whose expression revealed a genuine responsiveness to media zinc concentrations, only CUB and RRAS3 showed a regulation at penultimate zinc concentrations (3.3 ppm zinc for CUB and 0.06 ppm zinc for RRAS3). Plotting changes in transcript levels of zinc-sensitive genes against MT-1 (Fig. 32) clearly shows that while MT-1 mRNA levels are decreasing (zinc depletion) and increasing (zinc

supplementation), respectively, expression levels of zinc-sensitive genes remain unaffected over a wide range of media zinc concentrations.

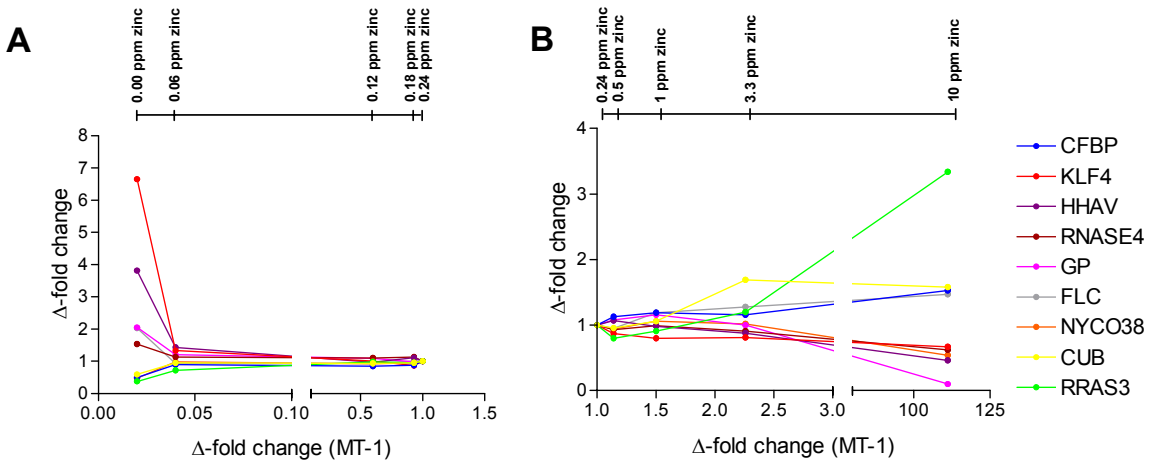


Fig. 32: Threshold level for zinc responsiveness of zinc-sensitive genes. Changes in transcript levels of zinc-sensitive genes are plotted against the expression of metallothionein-1 (MT-1). A: zinc depletion, B: zinc supplementation.

Metallothionein acts as a zinc buffer keeping the concentration of intracellular available zinc at a constant level (see Fig. 5). However, when medium zinc levels fall below 0.06 ppm or reaching 10 ppm, it seems that this safeguard effect of MT-1 is “exhausted”.

5.2 Proteome analysis

5.2.1 Identification of zinc-dependent proteins by 2D-PAGE/MALDI-TOF-MS

To identify proteins responsive to altered medium zinc levels in HT-29 cells, 2D-PAGE in combination with MALDI-TOF-MS was employed. HT-29 cells were grown for 16h in a medium with a normal, low or high zinc concentration and cellular zinc levels were measured by atomic absorption spectrometry in cells used for protein preparation (Fig. 33). Zinc-deficient conditions decreased cellular zinc levels nearly 3-fold, whereas zinc supplementation led to an increase of nearly 7-fold in comparison to control cells. After incubation, total cellular protein extracts were prepared and analysed by 2D-PAGE. 1310 proteins spots were resolved on the gels. A representative gel is shown in Fig. 34. The molecular weight and isoelectric point for each spot was calculated by the software, based on the distribution of marker proteins from a commercial wide range protein standard and

separation of proteins in the first dimension by isoelectric focussing in a linear pH range from 3-10.

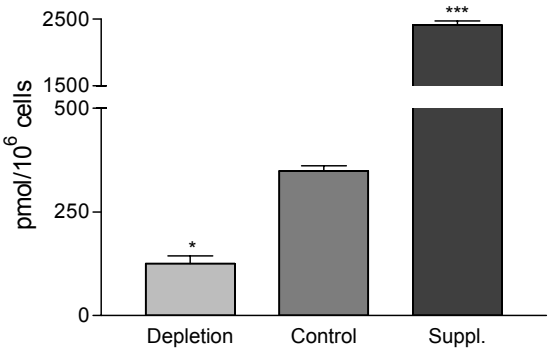


Fig. 33: Effects of zinc depletion and zinc supplementation on total cellular zinc levels. HT-29 cells were cultured for 16 h under low (<0.01 ppm, depletion), normal (~0.24 ppm, control) or high (10 ppm, supplementation) zinc conditions and cellular zinc concentrations were assayed by atomic absorption spectrophotometry. Values are means ± SEM, n = 3, *: P<0.05, ***: P<0.001.

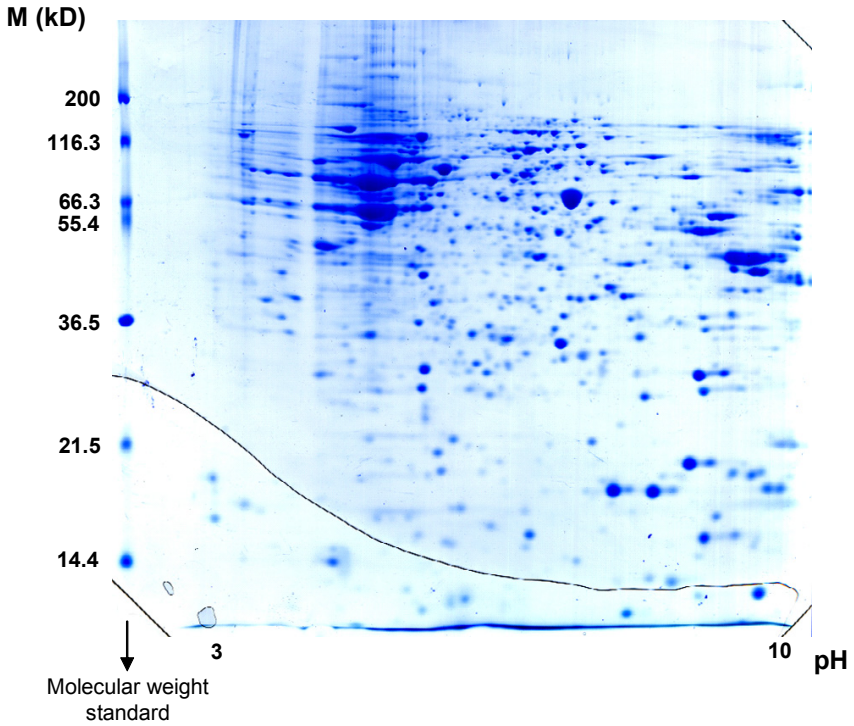


Fig. 34: Image of a representative 2D gel of proteins from HT-29 cells grown for 16h under high (10 ppm) zinc conditions. Proteins were separated on a pH 3-10 IPG strip in the first dimension and on a 12.5% SDS polyacrylamide gel in the second dimension. Proteins were visualised using Coomassie Brilliant Blue staining. Protein spots on the left side indicate marker proteins with a defined molecular weight.

Comparing protein spot densities from zinc-depleted, zinc-supplemented and control cells, 29 regulated proteins (2.2 %) were found. Proteins were considered

as up- or down-regulated if the change in steady state protein levels upon deprivation/addition of zinc was 2-fold or greater (Tab. 3). Among these, the expression of 8 proteins changed under both zinc-deficient and zinc-supplemented conditions; 2 proteins showed an opposite regulation and 6 proteins were regulated in the same direction. 14 proteins were affected only under low zinc conditions; 6 proteins showed an increase and 8 proteins a decrease in steady state expression level. 7 proteins responded only to high zinc conditions; with the expression level of one protein increased, whereas spot densities of the remaining 6 proteins were reduced.

Tab. 3: Protein spots with changed steady state levels upon zinc depletion and/or zinc supplementation [1]

Spot ID[2]	F[3]	$RF_{P(D)}$[4]	$RF_{P(S)}$[5]	Acc. no.[6]	Protein[7]	M (kD)[8]	IP[9]	Mascot Search Score[10]
$RF_{P(D)}$ and $RF_{P(S)}$ > +2								
2_66	9	+ 3.1	+ 7.5	Q8N5M4	Hypothetical protein MGC29649	29.5	9.8	68/71/84/73/55
$RF_{P(D)}$ and $RF_{P(S)}$ > -2								
1_93	13	- 3.0	- 2.2	-	-	131.4	8.1	
2_266	13	- 2.2	- 2.6	A30113	NADH2 dehydrogenase (ubiquinone) 24K chain	32.1	5.9	87/64/83
2_121	10	- 2.7	- 2.3	BAA87913	Adenylate kinase 3 alpha	35.8	9.9	74/66/71/74/76/63
1_17	9	- 2.5	- 2.4	-	-	54.1	9.1	
1_498	9	- 2.1	- 2.1	AAH15529	Ribose 5-phosphate isomerase A	35.3	7.5	90/90/81/75/105/94
$RF_{P(D)}$ > -2 and $RF_{P(S)}$ > +2								
1_469	9	n. p.	+ 3.3	-	-	106.2	9.0	
1_213	7	n. p.	+ 2.9	-	-	41.2	5.4	
$RF_{P(D)}$ > +2 and $RF_{P(S)}$ > -2								
-				-	-			
$RF_{P(D)}$ > +2								
1_525	17	+ 2.5	- 1.1	AAH11960	Proteasome 26S non-ATPase subunit 10, isoform 1	32.2	5.8	67/62/53/55
1_430	16	+ 2.7	+ 1.6	AAB66483	Serine protease	42.0	7.0	62/54/64/49
1_448	14	+ 2.0	+ 1.1	1LYWB	Cathepsin d, chain B	37.9	5.2	59/89/78/80
1_268	13	+ 2.0	+ 1.1	-	-	30.3	5.2	
1_122	12	+ 4.2	+ 1.7	-	-	95.4	9.1	
1_453	11	+ 2.4	+ 1.0	-	-	108.9	9.4	
$RF_{P(D)}$ > -2								
1_356	16	- 2.3	- 1.1	-	-	17.3	5.0	
1_243	16	- 2.3	- 1.2	-	-	37.0	9.5	
1_222	15	- 2.2	- 2.0	AAC96011	Chaperonin containing t-complex polypeptide 1, eta subunit; CCT-eta	104.8	8.0	126/166/163/105
2_674	14	- 2.4	- 1.1	VEHULA	Lamin A	148.1	6.4	142/136/162/118
1_709	13	- 2.3	- 1.9	-	-	17.6	3.6	
1_441	13	- 2.2	- 1.2	AAH07424	PRP4 pre-mRNA processing factor 4 homolog	109.5	7.7	169/60/179
1_188	13	- 2.2	- 1.1	-	-	138.1	7.9	
1_450	8	- 2.5	- 1.5	-	-	38.4	3.6	
$RF_{P(S)}$ > +2								
1_769	15	+ 1.6	+ 3.8		-	14.9	5.6	
$RF_{P(S)}$ > -2								
1_584	15	- 1.1	- 2.2	AAH14623	Serine (or cysteine) proteinase inhibitor, clade H, member 1	77.1	9.7	88/106/110
1_190	15	- 1.3	- 2.2	Q9P033	HSPC124	46.7	6.2	65/118
1_218	12	- 1.2	- 4.0	KPY1_HUMAN KPY2_HUMAN	Pyruvate kinase, muscle splice form M1 or M2	110.2	8.3	165/198/217
3_470	9	- 1.2	- 2.4	S59075	Splicing factor SRp30c	35.1	5.5	80/66
2_784	8	- 1.3	- 2.1	Q96C36	Similar to pyrroline 5-carboxylate reductase isoform (hypothetical protein)	47.7	8.2	104/66/58
2_71	7	+ 1.7	- 3.2	XNHUDM	Aspartate transaminase, mitochondrial	59.0	9.7	66/67

[1]Zinc-dependent proteins with increased (+) or decreased (-) steady state expression levels in HT-29 cells cultured for 16h under normal, low or high zinc conditions. Genes

were originally identified by 2D-PAGE and were analysed with MALDI-TOF-MS and a subsequent database search in the Mascot Search Database (MSDB). [2]Number for protein spots in 2D gels assigned by the software. [3]Frequency of a protein spot in 2D gels. [4]Regulation factor under zinc-deficient conditions. [5]Regulation factor under zinc-supplemented conditions. [6]Accession number in Mascot Search Database. [7]Name of the encoded protein. [8]Calculated molecular weight of protein spot in 2D gels. [9]Isoelectric point of protein spot in 2D gels. [10]Relative number reflecting the analogy of a given peptide mass fingerprint with that represented in the database. Values given for regulation factors are averaged values (n = 3).

Regulated protein spots were picked and analysed by MALDI-TOF-MS. 16 proteins (55 %) could be identified and these are summarized in Tab. 4. Functional classification revealed that most of the identified proteins could be linked to energy metabolism (n=5), induction of cellular stress (n=4) and protein metabolism (n=3). Furthermore it was checked whether identified proteins were represented on DNA arrays used for transcriptome analysis and if so, mean relative expression values have been included in Tab. 4. For low zinc conditions, changes in protein expression corresponded quite well with the observed changes in mRNA levels, whereas at high zinc conditions only one protein identified was present on cDNA arrays and thus protein and mRNA levels could not be compared.

Tab. 4: Functional classification of identified zinc-dependent proteins[1]

Accession no.[2]	Protein[3]	Function[4]	Δ-fold change at protein level[5]		Δ-fold change at mRNA level[6]	
			low zinc	high zinc	low zinc	high zinc
KPY1_HUMAN KPY2_HUMAN	Pyruvate kinase, muscle	Energy metabolism	- 1.2	- 4.0	+ 1.2	n. p.
XNHUDM	Aspartate transaminase, mitochondrial	Energy metabolism	+ 1.7	- 3.2	- 2.4	n. p.
A30113	NADH2 dehydrogenase 24K chain	Energy metabolism	- 2.2	- 2.6	- 1.6	n. p.
BAA87913	Adenylate kinase 3 alpha	Energy metabolism	- 2.7	- 2.3	- 1.5	n. p.
Q9P033	HSPC124, H^+-translocating pyrophosphatase	Energy metabolism	- 1.3	- 2.2	n. p.	n. p.
AAH15529	Ribose 5-phosphate isomerase A	Stress-induced	- 2.1	- 2.1	n. p.	n. p.
AAC96011	Chaperonin containing t-complex (CCT-eta)	Stress-induced	- 2.2	- 2.0	- 3.2	n. p.
S59075	Pre-mRNA splicing factor SRp30c	Stress-induced	- 1.2	- 2.4	n. p.	n. p.
AAH14623	Serine (or cysteine) proteinase inhibitor, clade H, member 1	Stress-induced	- 1.1	- 2.2	- 1.5	n. p.
AAB66483	Serine protease	Protein metabolism	+ 2.7	+ 1.6	n. p.	n. p.
AAH11960	Proteasome 26S non-ATPase subunit 10, isoform 1	Protein metabolism	+ 2.5	- 1.1	- 1.2	n. p.
1LYWB	Cathepsin d, chain B	Protein metabolism	+ 2.0	+ 1.1	n. p.	n. p.
AAH07424	PRP4 pre-mRNA processing factor 4 homolog	Splicing	- 2.2	- 1.2	+ 1.3	n. p.
VEHULA	Lamin A	Nuclear envelope protein	- 2.4	- 1.1	- 1.6	+ 1.1
Q8N5M4	Hypothetical protein MGC29649	Unknown	+ 3.1	+ 7.5	n. p.	n. p.
Q96C36	Similar to pyrroline 5-carboxylate reductase isoform	Unknown	- 1.3	- 2.1	n. p.	n. p.

[1]Identified zinc-dependent proteins in HT-29 cells with increased (+) or decreased (-) steady state expression levels upon zinc depletion and zinc supplementation, respectively. Genes were originally identified by 2D-PAGE and were analysed with MALDI-TOF-MS. [2]Accession number. [3]Name of the encoded protein. [4]Proposed function of the encoded protein. [5]Regulation factor at protein level based on 2D-PAGE results. [6]Regulation factor at mRNA level based on DNA arrays results. Values for regulation factors indicate means for three experiments. n. p., not present on array.

5.2.2 Assessment of cellular lactate levels

Among proteins which showed the most pronounced changes in steady state levels was Pyruvate kinase type M2, a key enzyme of the glycolytic pathway. Proteome analysis revealed a 4-fold down-regulation under high zinc conditions, but the enzyme appeared not affected by zinc depletion. This protein is of particular interest as tumor cells gain ATP mainly through glycolysis. Therefore cellular lactate levels were measured and those were found to be significantly reduced by about 40 % in HT-29 cells exposed to high zinc conditions in comparison to control cells (Fig. 35).

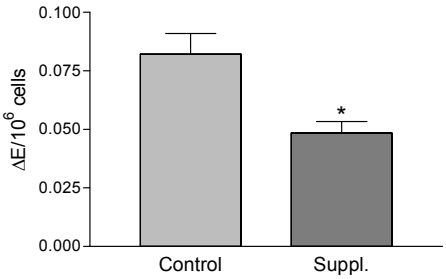


Fig. 35: Lactate levels in HT-29 cells exposed to media with normal or high medium zinc. HT-29 cells were cultured for 16 h in media containing normal (control) or high (supplementation) zinc concentrations and cellular lactate levels were determined enzymatically as described in the methods section. Values are means ± SEM, n = 5, *: P<0.05.

5.3 Comparison of results from proteome and transcriptome analysis

5.3.1 mRNA levels of zinc-dependent proteins

Most identified zinc-dependent proteins were not present on DNA arrays, especially proteins identified in cells exposed to high medium zinc, thus making a comparison between protein and mRNA levels impossible. Therefore RNA was extracted from cells treated as for proteome analysis. Primers for six of the identified proteins, namely ribose 5-phosphate isomerase A (RPIA), NADH2 dehydrogenase 24K chain (NDUFV2), chaperonin containing t-complex (CCT7), lamin A (LAMIN A), serine proteinase inhibitor (SERPINH1) and pyruvate kinase (PKM) were designed and relative expression levels of these transcripts were measured by real-time RT-PCR. The comparison of gene and protein expression levels is provided in Tab. 5.

Tab. 5: Comparison of changes in protein and mRNA expression levels of zinc-dependent proteins[1]

Gene[2]	Δ-fold change at low zinc[3]		Δ-fold change at high zinc[4]	
	protein level	mRNA level	protein level	mRNA level
CCT7	- 2.2	- 8.3	- 2.0	+ 1.2
LAMIN A	- 2.4	- 1.6	- 1.1	+ 1.4
RPIA	- 2.1	- 9.1	- 2.1	- 1.2
NDUFV2	- 2.2	- 2.2	- 2.6	+ 1.3
PKM	- 1.2	- 1.2	- 4.0	+ 1.6
SERPINH1	- 1.1	- 1.8	- 2.2	+ 1.3

[1]Selected zinc-dependent proteins in HT-29 cells with increased (+) or decreased (-) steady state expression levels upon zinc depletion (low zinc) and zinc supplementation (high zinc), respectively. Genes were originally identified by 2D-PAGE/MALDI-TOF-MS and corresponding mRNA levels were determined by real-time RT-PCR. [2]Name of the encoded protein, CCT7 (chaperonin containing t-complex), LAMIN A (lamin A), RPIA (ribose 5-phosphate isomerase A), NDUFV2 (NADH2 dehydrogenase 24K chain), PKM (pyruvate kinase) and SERPINH1 (serine proteinase inhibitor). [3]Regulation factor at low zinc. [4]Regulation factor at high zinc. Regulation factors given are the mean of three (protein level) or two (mRNA level) independent experiments.

In zinc-deprived cells, most assayed transcripts were markedly down-regulated, whereas in zinc-supplemented cells mRNA levels tended to be slightly, although in most cases not significantly, up-regulated. Levels of protein and mRNA under low zinc conditions showed a good correlation except for SERPINH1, which was found to be down-regulated at the mRNA level while unaffected at the protein level. mRNA levels of CCT7 and RPIA were drastically reduced (8.3-fold and 9.1-fold respectively), whereas changes on the protein level were only modestly (about 2-fold) altered. For zinc supplementation a correlation between protein and mRNA could not be observed. mRNA expression levels were not significantly changed, except for PKM, whereas protein levels in most cases were decreased.

5.3.2 Regulation of the Kruppel-like factor 4 on the protein level

The zinc finger-containing transcription factor KLF4 appears to be an important gene, regulated at the mRNA level by zinc depletion as well as zinc supplementation. In light of this, Western blot analysis was carried out to determine whether the effect observed at the transcript level is reflected in the abundance of KLF4-protein. The KLF4-antibody recognized a single protein band with the appropriate molecular weight in protein preparations of HT-29 cells (Fig. 36). Densitometric analysis yielded a 1.9-fold increase in KLF4 steady state protein levels under low zinc conditions. However, the increase observed on the

protein level was lower than that detected on mRNA level (1.9-fold versus 9.0-fold) and protein abundance of KLF4 was not altered under high zinc conditions.

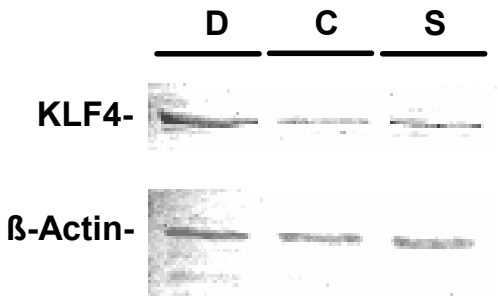


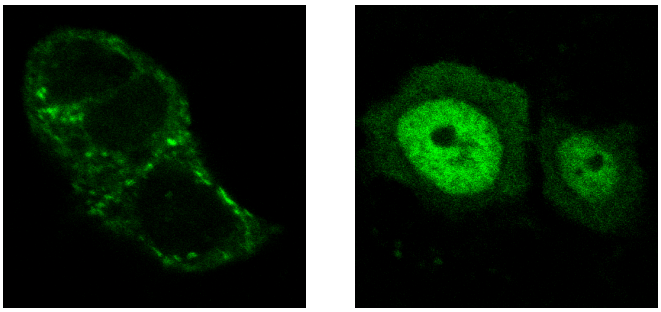
Fig. 36: Western blot analysis of Kruppel-like factor 4 in human HT-29 cells cultured under normal (C), zinc-depleted (D) or zinc-supplemented (S) conditions for 16 h. KLF4 protein levels were compared with ß-actin as an internal control on the same membrane. Relative change was normalised for ß-actin in three independent experiments. A typical blot is shown.

5.4 Target gene search for MTF-1

Our studies on the effect of zinc supplementation (see 5.1.1) and zinc depletion (see 5.1.2) on gene expression in the human adenocarcinoma cell line HT-29 led to the identification of a considerable number of genes responding with changes in steady state mRNA levels to alterations in cellular zinc status. Among 20 genes studied in more detail, mRNA steady state levels of 9 genes responded to both high and low medium zinc concentrations. As “primarily” zinc-dependent, we assessed, whether these zinc-sensitive genes are commonly controlled by the zinc-finger transcription factor MTF-1.

5.4.1 Alterations in transcript levels of zinc-sensitive genes upon overexpression of MTF-1

To assess whether the expression of the identified zinc-sensitive genes is mediated by the zinc-finger transcription factor MTF-1, a double-stable HT-29 cell line inducibly overexpressing MTF-1 was established. MTF-1 was tagged with EGFP and transient transfection of the plasmid, encoding for the MTF-1/EGFP fusion construct under control of a constitutive promotor revealed, that the fusion protein is expressed and mainly localised in the nucleus (Fig. 37).



pEGFP-N2 pMTF1/EGFP-N2

Fig. 37: Transient transfection of a plasmid encoding a MTF-1-EGFP fusion protein. HT-29 cells were transiently transfected with the vector pMTF1-EGFP-N2 containing the MTF-1 under control of the immediate early promotor of CMV ($P_{CMV\ IE}$). Pictures were taken 48h post transfection by confocal microscopy. EGFP was excited with a 488 nm laser line and imaged between 500 and 550 nm, left panel: control transfection with the vector alone, right panel: vector containing MTF-1-EGFP fusion construct.

Establishing a Tet-On gene expression system involves two consecutive stable transfections. After the second transfection positive colonies were selected based on the assessment of GFP fluorescence with a confocal laser-scanning microscope. Positive colonies were further analysed by measuring MTF-1 mRNA levels of induced cells in comparison to non-induced cells with real-time RT-PCR and three clones (namely clone 32a, 32f and 32p) were chosen for subsequent experiments. A typical image obtained from positive clones is provided in Fig. 38A. This shows that addition of doxycycline results in the appearance of numerous green cells in the culture (+Dox), whereas no fluorescence could be detected in non-induced control cells (-Dox). As expected for a transcription factor, the MTF-1/EGFP fusion protein is mainly localized in the nucleus (Fig. 38B).

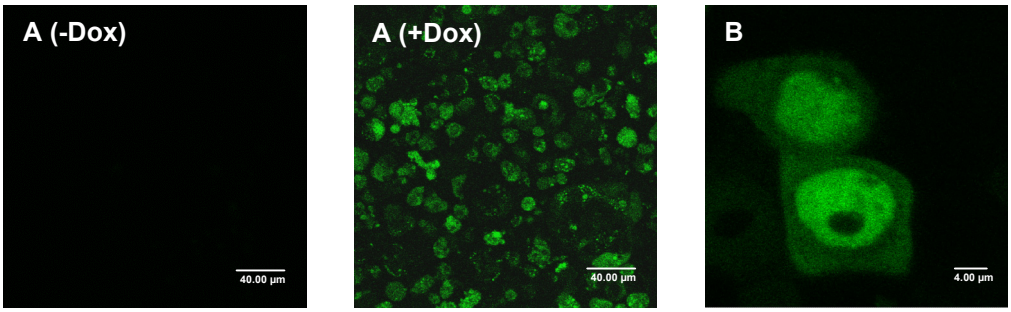


Fig. 38: Inducible expression of MTF-1 in stably transfected HT-29 cells. Cell lines stably overexpressing a MTF-1/EGFP fusion protein under control of doxycycline (Dox) were established. Pictures were taken 48h post induction with a confocal laser-scanning microscope. EGFP was excited with a 488 nm laser line and imaged between 500 and 550 nm, A: cells after treatment with (+) or without (-) 1 µg/ml Dox, B: cells after treatment with 1 µg/ml Dox (higher magnification).

Transcript levels of MTF-1 were up-regulated at least 15-fold by doxycycline in the three cell clones selected for analysis (Fig. 39). Subsequently the relative expression levels of the previously identified zinc-sensitive genes were determined in induced cells in comparison to non-induced control cells using real-time RT-PCR. Results are summarised in Tab. 6.

Tab. 6: Changes in mRNA expression levels of zinc-sensitive genes in response to doxycycline[1]

Gene[b]	MREs[c]	Δ-fold change: +Dox/-Dox[d]		
		clone 32a	clone 32f	clone 32p
Kruppel-like factor 4	**3**	**+ 3.3**	**+ 1.8**	**+ 1.7**
Hepatitis A virus cellular receptor 1	**2**	**+ 1.7**	**+ 1.4**	**+ 1.6**
Gastrointestinal peptide	1	n. a.	n. a.	- 1.1
Complement factor B	**0**	**+ 7.6**	**+ 1.2**	**+ 1.7**
Ferritin light chain	0	- 1.4	+ 1.2	- 1.1
Hypothetical 40 kD protein	0	+ 1.2	+ 1.1	± 1.0
Antigen NY-CO-38	0	- 1.4	+ 1.3	- 1.4
RAS-related protein R-RAS3	0	- 1.9	- 1.3	- 1.1
Ribonuclease 4	0	- 2.9	+ 1.6	± 1.0

[1]Increased (+) or decreased (-) expression levels of zinc-sensitive genes in cell clones overexpressing MTF-1. Genes were originally identified as zinc-sensitive by DNA array analysis in combination with real-time RT-PCR and their expression was determined in three different clones stably overexpressing MTF-1 after addition of Dox to the culture medium. Genes considered as regulated are shown in bold letters. [2]Name of encoded protein. [3]Number of MREs (metal responsive elements) [4]Magnitude of changes observed by real-time RT-PCR. Genes were considered as regulated if the change was 1.5-fold or greater in at least two clones, n. a., not available.

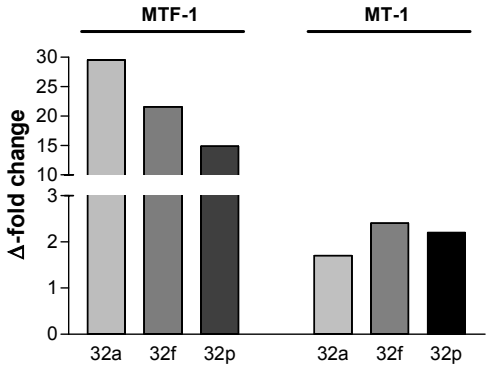


Fig. 39: mRNA levels of MTF-1 and MT-1 in induced Tet-On/MTF-1 cells. Relative changes in mRNA levels of MTF-1 (metal transcription factor-1) and MT-1 (metallothionein-1) in induced cells in comparison to non-induced cells. Transcript levels were assessed in three cell clones (named 32a, 32f and 32p), overexpressing MTF-1 48h after induction with 1μg/ml Dox by real-time RT-PCR.

Transcript levels were classified as regulated when the changes in mRNA levels were 1.5-fold or greater in at least two of the three cell clones. Three genes, namely Kruppel-like factor 4, hepatitis A virus cellular receptor 1 and complement factor B matched this criterion. Observed changes were in the same range as those found for metallothionein-1 (MT-1), which showed on average an induction of about 2-fold (Fig. 39). Metallothionein-1 is one of MTF-1's best characterized target genes and its increased expression served as an internal control for the function of the recombinant MTF-1 protein to act as a transcriptional activator.

5.4.2 Computational search for MREs in promoters of the identified zinc-sensitive genes

Since it is known that MTF-1 binds to characteristic metal-responsive elements (MREs) present in the promotors of its target genes, an in silico search for MREs in the 5´prime regions of the identified zinc-sensitive genes was employed as a complementary approach. A first screening using the algorithm described in the method section was performed in the upstream sequences of 18406 human genes, to define the appropriate search region.

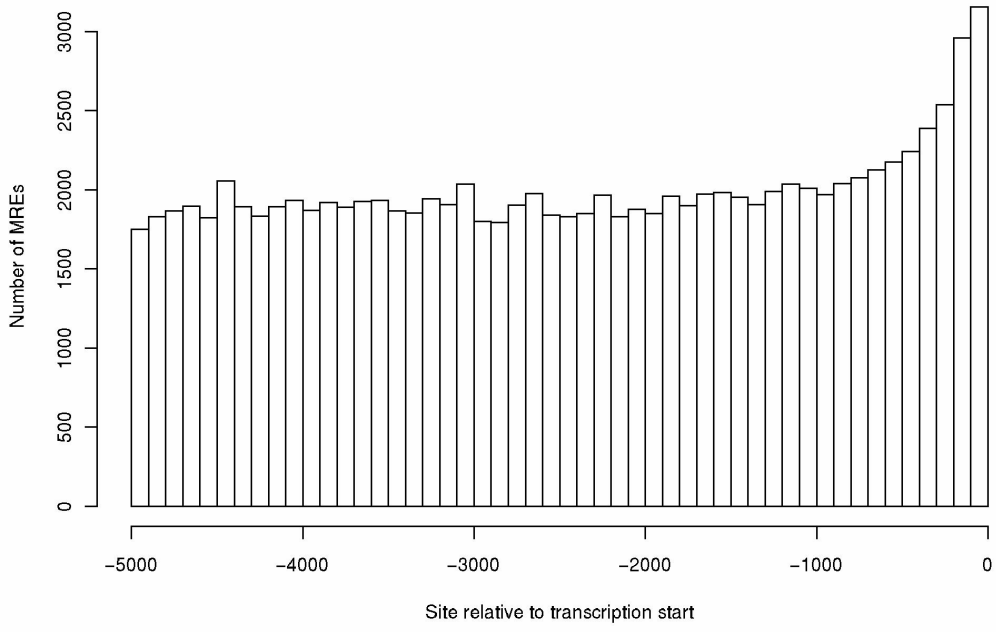


Fig. 40: Number of metal-responsive elements (MREs) found in the upstream region of 18406 human genes. MREs were identified performing a computational search in the promoters of 18406 human genes using the algorithm described in the methods section.

An uniform distribution of MREs was found over a distant region (bps -5000 to -1000 from the transcription start) with a peak in the proximity of the transcription start (Fig. 40). Remarkably, the number of MREs found in the distant region was almost equal to the number of MREs predicted using a random sequence model. As a higher probability to find functional MREs in the proximity of the transcription start than in the distant regions can be expected, further analysis concentrated on the first 1000 bps upstream from the transcription start. Based on this approach, the following number of MREs in the promotor sequences of the identified zinc-senstive genes were identifed: three MREs in KLF4, two MREs in HHAV and one MRE in GP (Tab. 6). The relative position and direction of the MREs is shown in Fig. 41. No MREs were found in the promoters of FLC, CUB, NYCO38, RRAS3, RNASE4 and CFBP (Tab. 6).

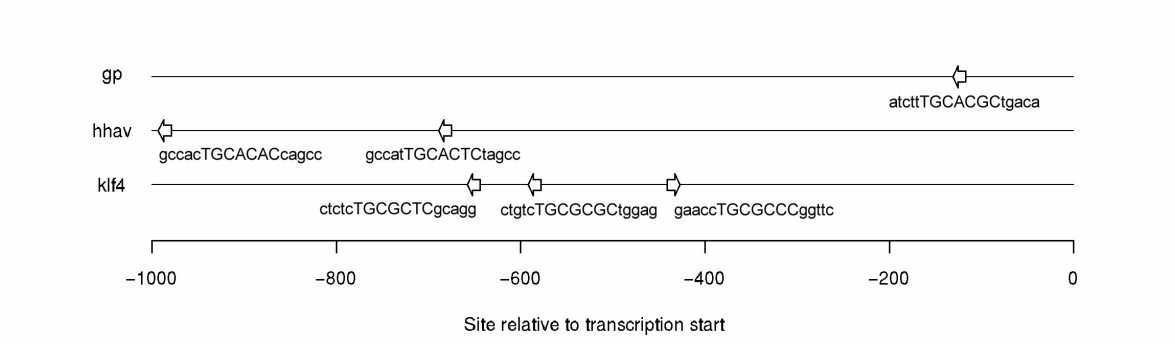


Fig. 41: Putative MREs found in the 5´pre regions of the identified zinc-sensitive genes. Arrows represent the position of the MREs. Arrows pointing to the right refer to MREs on the coding strand, arrows pointing to the left to MREs on the non-coding strand. The core consensus sequence (in capitals) with their five flanking bases on each side is shown below each corresponding arrow.

6 Discussion

Zinc is an essential nutrient in all organisms and plays a pivotal role in a wide variety of biochemical processes. On the basis of its multiple biological functions, zinc deficiency as well as zinc overload causes a wide variety of clinical symptoms. Despite being the subject of intensive research over the last decades, the underlying biochemical pathways which become dysfunctional by alterations in cellular zinc status have only partially been resolved. Moreover, assessing the zinc status *in vivo* is problematic since specific, sensitive and reliable indicators are still lacking (82,109,110). In this work the effect of cellular zinc status on gene and protein expression was studied in the human cell line HT-29, which was derived from a human colonic adenocarcinoma and grows to a monolayer of unpolarised and undifferentiated cells under standard culture conditions (111,112). Since epithelial cells are primarily affected by dietary zinc intakes *in vivo*, this cell line was used as an *in vitro* model to study the response to altered zinc status. In comparison to complex tissues obtained from animals, this *in vitro* approach offers the possibility to work with a homogeneous cell population that can be used under standardised conditions to define the molecular targets of zinc. In accordance with Griffin et al. (87), the screening was performed on the transcriptome and the proteome level as “it is only through the integration of different levels of information that a system can be described comprehensively”. The identified genes and proteins provide new insights into the molecular actions of zinc and may help to establish biomarkers for the assessment of zinc status in the future.

6.1 Transcriptome analysis

6.1.1 Zinc supplementation

Although essential for mammalian cells, zinc is capable of inducing apoptosis and can produce irreversible changes that lead to cell death at unphysiological concentrations. The underlying mechanisms of these effects are unknown, but zinc may bind to inappropriate sites in proteins or compete with other metal ions for enzymes. The side effects of a high zinc load are of growing importance in view of the upper safe limits of intake and the fact that zinc is a frequently used supplement and is widely available in form of many over-the-counter products. Numerous studies have demonstrated that the intracellular concentration of zinc is

of critical importance for cell growth, energy metabolism and survival in many cell types. In particular, epithelial tissues of the intestine are exposed to high concentrations of dietary zinc and elevated zinc levels have been shown to impair primary epithelial cell proliferation (113). As a model for the intestinal epithelium the human colonic epithelial cell line HT-29 was used to identify molecular targets of an elevated but yet non-toxic intracellular zinc concentration. A zinc concentration of 10 ppm in the growth medium was selected because it proved to be high enough to significantly increase total as well as intracellular free zinc levels and to induce transcription of MT-1, a gene highly responsive to zinc. This zinc concentration on the other hand is not too high to cause severe changes in cell proliferation and cell viability or to induce apoptosis.

Transcriptome analysis revealed 17 potential molecular targets that responded with changes in steady state expression levels to zinc supplementation. An independent confirmation of gene regulation was performed by Northern blotting and real-time RT-PCR. The latter allows rapid, highly sensitive and accurate quantification of transcript levels and therefore is now considered the “technique of choice” for validating gene expression changes identified with DNA arrays (114). Unexpectedly, for nearly half of the regulated genes identified by the arrays, changes in mRNA expression levels could not be confirmed. However, nonreproducibility of microarray results does not necessarily mean that these results are invalid. This is because RNA secondary structures, splice variants, complex polyA tails, low transcript expression, and many other factors can influence both microarray and quantitative PCR results (114). The relatively small number of differentially expressed genes identified upon zinc supplementation is surprising, as one would expect a far greater quantity of genes when medium zinc is shifted from 0.24 to 10 ppm. However, it has to be taken into account, that total cellular zinc was raised only about 7-fold and that intracellular free zinc levels only increased about 1.6-fold to medium zinc levels that were 40-fold higher than at normal growth conditions. A similar increase in intracellular zinc levels at a medium zinc concentration of 150 μM (~10 ppm) were observed by Richard et al. (115) in cultured human skin fibroblasts. These relatively small changes of intracellular free ion levels may be due to the fact that exposure of cells to high concentrations of zinc activates several protective mechanisms including the

down-regulation of zinc import proteins and the up-regulation of zinc efflux proteins (56,116) which in turn prevent a zinc overload. In addition, high zinc levels lead to an induction of zinc-binding proteins such as metallothionein (97,116), which serve as a “metal sponge”, thereby counteracting an increase in intracellular free zinc levels. Consistent with this, a drastic increase in MT-1 mRNA levels in response to zinc-supplementation was also observed in our cell culture model.

A number of studies with various cell culture models suggested that the effects of high intracellular zinc concentrations are mediated in part by the generation of reactive oxygen species (ROS) (117,118). Metallothioneins (MTs) are capable of scavenging ROS due to their high cysteine content and the increased MT expression observed could therefore represent a protective mechanism used by cells against ROS. So, when exposed to high amounts of zinc, cells activate a number of protective mechanisms that result in only a modest increase in intracellular free zinc which then exerts its effects on gene transcription. Although modest, the increase in intracellular free zinc did however alter the steady state levels of mRNAs that so far have not been described as regulated by the cellular zinc status and which appear to be particularly sensitive to elevations in free zinc in colonic cancer cells.

Of special interest is the finding that mitochondrial creatine kinase (MIACK), an enzyme involved in cellular energy production, is down-regulated under high zinc conditions, as this is consistent with results from proteome analysis, supporting the concept that cellular ATP production in response to a high zinc exposure is impaired (see 6.2). Complement factor B (CFBP) plays an important role in activating the alternative complement pathway and zinc was previously shown to play a role in the degradation of the key complement components C3b and C4b (119). However, that expression of complement factor B mRNA is sensitive to changes of cellular zinc status has not yet been described in the literature. The down-regulation of the protease inhibitor Kazal type 4 (gastrointestinal peptide, GP) in HT-29 cells in response to elevated zinc levels is most interesting with respect to its proposed functions. This 60 amino-acid peptide was originally isolated from the gastrointestinal tract but is also expressed in neurons (120,121). The primary function of GP derived from the gastrointestinal tract is thought to be the inhibition of insulin secretion (122,123). It is known that zinc plays an important

role in the synthesis, storage and secretion of insulin but again a link between this peptide and zinc has not been reported to date.

For most of the other identified targets, a link to particular cellular processes could not be established. Hepatitis A virus cellular receptor 1 (HHAV) was originally isolated from a cDNA expression library of African green monkey kidney (AGMK) cells and was shown to be a functional cellular receptor for hepatitis A virus (124,125). Recent studies showed, that HHAV blocks differentiation of proximal tubular epithelial cells and thereby suggested a potential involvement of HHAV in the development and progression of kidney carcinomas (126). Transcript levels of ribonuclease 4 (RNASE4) and antigen NY-CO-38 (NYCO38) were found to be repressed. Although the precise molecular functions of these genes are still unclear, they have been associated with stress factors such as viral infections (127) or autoimmune reactions (128). The down-regulation of these genes also substantiates the findings from proteome analysis that elevated zinc levels may adversely affect cellular stress response. The hypothetical 40 kD protein (CUB) is an endothelial and smooth muscle cell-derived neuropilin-like protein with unknown molecular function (129).

In addition to cDNA array analysis a time-course experiment was performed and the changes in expression levels of identified zinc-regulated genes were measured over time by real-time RT-PCR. The theoretical basis for these experiments was that genes sharing similar expression profiles might be functionally coupled or co-regulated and thereby provide insights into gene-gene interactions, gene function and pathway identification (130). Transcript levels of antigen NY-CO-38 (NYCO38) and mitochondrial creatine kinase (MIACK) changed synchronously over time but were only affected weakly. Metallothionein-1 (MT-1) and hypothetical 40 kD protein (CUB) as well as ribonuclease 4 (RNASE4) and gastrointestinal peptide (GP) also showed changes in the same direction but with quite different magnitudes, suggesting that these genes may not be co-regulated. Therefore, the time-course experiment performed in cells exposed to a high medium zinc concentration did not reveal any evidence for interaction or co-regulation of the genes.

In summary, DNA array analysis performed in the epithelial cell line HT-29 exposed to a zinc concentration that increased intracellular free zinc to a non-toxic level identified an unexpected small number of molecular targets. However, most of the identified genes have not been linked to cellular zinc status before and could therefore provide new avenues for further studies on the molecular functions of zinc. Moreover, the only modest alteration of intracellular free zinc levels points to the critical importance of measuring actual intracellular ion concentrations when performing studies on dose-response relationships.

6.1.2 Zinc depletion

Zinc deficiency in mammals causes a wide variety of symptoms including retarded growth, diarrhea, anorexia, impaired immunity, skin lesions and abnormal development (72). Despite decades of research, the molecular basis for these dysfunctions is still not known. Within the last few years, subtracted library hybridisation (131) and mRNA differential display (132) approaches have led to the identification of zinc-dependent genes in animal models of dietary zinc deficiency. More recently, DNA array analyses identified mammalian genes in small intestine, thymus and hepatocytes which respond with altered expression levels to changes in zinc status (100,133,134). The human colon adenocarcinoma cell line HT-29 was used here to identify zinc-dependent genes under conditions of a zinc deficiency. Transcriptome analysis based on oligonucleotide arrays showed, that the depletion of intracellular zinc caused altered mRNA expression of around 3 % of the 9850 genes represented on the array. Changes in expression levels of representative transcripts were independently confirmed by Northern blot analysis and/or quantitative RT-PCR, demonstrating the reliability of the data derived from the arrays. In most cases the alternative methods for transcript profiling revealed greater differences than those found by oligonucleotide arrays. Therefore, the defined threshold-value of a 2-fold change in mRNA-expression level appears valid and allows a conservative estimate of the numbers of genes with changed transcript levels upon zinc depletion. Similar changes in global gene expression were found by differential mRNA display and cDNA array analysis in murine thymocytes and human monocytes (135,136) with approximately 5 % of genes identified as zinc-responsive.

Identified genes that responded to an altered zinc status in HT-29 cells could be grouped into eight functional categories. Four of these gene classes (cell growth/cycle, signaling, cytoskeleton, metabolism) were also identified in a recent microarray study examining the effect of zinc deprivation and zinc excess on the expression of 22,216 genes in a human mononuclear cell line (135). Genes within those groups seem to represent a first comprehensive collection of genes which are zinc-responsive independent of the cell type. However, which genes from these collections are directly or indirectly regulated by zinc is still not known. Future studies employing bioinformatic approaches such as multiple expectation-maximization for motif elicitation (MEME) (137) or motif alignment search tool (MAST) (138) should allow the identification of similar regulatory elements in the promoters of collected genes. Similar strategies were extremely useful in defining a zinc-responsive regulon in yeast (139). The 46 genes of this regulon are controlled by the metalloregulatory protein Zap1p. In mammalian cells the zinc-finger protein MTF-1 has been identified as a zinc-sensing metalloregulatory protein (49). Zinc binds to MTF-1 and provides a positive signal for the expression of genes which contain metalloregulatory promotor elements (so-called metal-responsive elements, MREs). The number of genes regulated by MTF-1 is currently not known, but a first list of genes containing such regulatory elements has been generated based on computer searches (41). MTF-1 is usually up-regulated in cellular zinc deficiency as found also in our cell model. Recently, the MTF-1 ortholog MTF-2 was shown to be down-regulated by zinc depletion in human mononuclear cells (135) and this reciprocal regulation of MTF-1 and MTF-2 was suggested to allow the opposite regulation of gene clusters with enhanced or lowered expression levels. Of course, database searches for metalloregulatory elements and reporter assays of the candidate gene promotors are necessary to define new MTF-1 and MTF-2 target genes.

More than 20 % of the genes with significantly altered expression levels encode hypothetical proteins or proteins with unknown function. It is interesting to note, that among these seven CGI-genes were found to be regulated by zinc depletion. CGI-genes have recently been identified by the comparative gene identification approach (140) and comprise more than 150 putative full-length gene transcripts. Although the functions of these genes are not known yet, their high degree of

homology and conservation from *C. elegans* to human suggests a fundamental role in the control of cellular processes.

The largest gene cluster encompasses 58 genes encoding enzymes of the intermediate metabolism. Remarkably, 81 % of these genes were down-regulated by zinc depletion including those of proteins involved in energy metabolism such as ATP synthase (subunit f), cytochrome c, subcomplex of the NADH dehydrogenase 1 and other dehydrogenases. This observation suggests that the cellular energy metabolism in HT-29 cells is impaired by zinc depletion and such changes have indeed been demonstrated in different cell types such as hepatocytes (141) and lymphocytes (142). An impaired cellular energy charge may be explained by a reduced activity of zinc-dependent enzymes involved in energy-yielding pathways but may also be mediated at least in part through the reduced expression of genes encoding enzymes of energy metabolism. Within the cluster of genes important for metabolism, several zinc-sensitive genes encoding proteins for protein synthesis, protein degradation and amino acid metabolism were identified. Genes such as the translation initiation factor isoforms 4g and 4a (EIF4G1, EIF4A2), elongin b, proteasome subunit (PSMD3), ubiquitin specific protease 7 (USP7) and aspartate aminotransferase 2 (GOT2) and methionyl-aminopeptidase 2 (METAP2) showed 2.0- to 3.0-fold reduced expression levels in zinc-depleted cells. Consistent with the findings in HT-29, genes involved in protein degradation such as ubiquitin, ubiquitin-conjugating enzymes E2I and the ubiquitin-specific protease 24 were also identified in human mononuclear cells, grown under low zinc conditions (135,143). It may be speculated that the changes in protein metabolism cause a reduced protein turn-over as observed in different cell types in zinc-deficient animals (144) as well as in the model organism *Candida albicans* (145).

Based on experiments examining the zinc requirement for proliferation in cultured cells, Chestes and Boyne (146) hypothesised that zinc was required for proteins important for cell cycle control. Consistent with this hypothesis, 15 zinc-sensitive genes involved in regulating cell growth and cell cycle progression were found. Whereas transcript levels of genes that increase proliferation (e. g. cyclin a, cyclin-dependent kinase 8) were down-regulated 2.1- to 3.9-fold, genes that suppress cell proliferation such as the cyclin-dependent kinase inhibitor 2d or the growth-

arrest and DNA-inducible factor GADD45A were found up-regulated 2.7- to 4.0-fold in zinc-depleted cells. A variety of studies examining the role of zinc in signaling pathways (42) suggest that zinc depletion could also affect membrane signaling systems and intracellular second messengers that coordinate cell cycle and cell proliferation (147). It was also observed, that genes encoding proteins for signaling processes (e.g. protein tyrosine phosphatase, phospholipase a2-activating protein) displayed changed expression levels under low zinc conditions. The observed decrease in cell proliferation of zinc-deprived HT-29 cells appears to represent the integrated “read-out” of these alterations in gene expression of proteins that control the cell cycle. Within that context, the identified zinc-finger containing transcription factor KLF4 (Kruppel-like factor 4) is a particularly interesting gene and could provide a direct link between the cellular zinc status and growth inhibition. KLF4 was cloned as a gene whose expression accompanies growth arrest (148). KLF4 was shown to up-regulate expression of several inhibitors of the cell cycle such as cyclin-dependent kinases. It also suppresses the expression of genes that are positive regulators of the cell cycle such as the cyclins. An up-regulation of KLF4 therefore should lead to a reduced cell growth. In agreement with this hypothesis, zinc depletion in this cell model caused an increase in the steady state expression level of the KLF4-mRNA with a simultaneously increased protein level as demonstrated by Western blot analysis. An altered expression of KLF4 by alterations of the cellular zinc status has not been observed previously and may provide a new mechanism by which the function of zinc in regulating cell growth is mediated.

Additionally, a time-course experiment was performed to determine whether similar expression patterns could be identified amongst the zinc-regulated genes. Time-course, as well as dose-response profiles in general, have the power to provide insights into the interrelationship of genes. Surprisingly, it was always groups of two genes that showed signs of co-regulation as a fist hint that their transcriptional regulation could be related. If more genes had been studied, those with similar expression profiles could be clustered and bioinformatic tools would then allow the identification of over-represented motifs in the upstream regions of co-expressed genes (149).

In summary, the results of this study demonstrate that oligonucleotide-based gene expression analysis provides a powerful tool for the identification of genes that respond to a cellular depletion of zinc. The pattern of genes affected by zinc could be a basis for further studies to define the zinc regulon in mammalian cells. In addition, it was demonstrated for the first time, that expression of KLF-4 at both mRNA and protein level increases in zinc-depleted cells. This could be a new mechanism to explain in part the effect of zinc in regulation of cell growth.

6.1.3 Zinc responsiveness of selected zinc-dependent genes

Our studies in HT-29 cells led to the identification of a considerable number of genes that were sensitive to zinc deficiency and zinc supplementation. In view of this, we examined whether these identified transcripts change expression levels under both low and high zinc conditions. A set of 9 out of 20 genes responded over a wide range of medium zinc concentrations with reduced or increased expression levels. It is interesting to note that 8 of these 9 genes were initially identified as regulated by a high zinc load and shown here to respond also to a zinc deprivation, whereas amongst the genes from the zinc depletion study only one, namely Kruppel-like factor 4 (KLF4) was also affected by zinc supplementation.

By gradually increasing or decreasing medium zinc concentrations, the zinc responsiveness of the identified transcripts was determined by quantitative RT-PCR. The expression of most genes remained unaffected over a wide range of medium zinc concentrations (both high and low zinc) and responded only to the most severe conditions. However, expression of metallothionein-1 showed a very pronounced zinc responsiveness. Even a 2-fold decrease and a 4-fold increase of medium zinc concentration already affected MT-1 mRNA levels. This high sensitivity of metallothionein towards alterations in zinc status may explain the lack of effects on other genes over a large concentration range, as metallothionein by binding and release of zinc functions as the prime intracellular homeostatic system for zinc (7). An increase in intracellular free zinc is counteracted by the de-novo synthesis of the metallothionein apoform thionein, which subsequently complexes zinc, whereas zinc is released from metallothionein if the amount of available intracellular zinc is low. Therefore metallothionein acting as a zinc buffer appears

to prevent experimentally evoked changes in intracellular free zinc levels over a wide range of medium zinc concentrations. Indeed, it was demonstrated in this work, that drastic changes in medium zinc levels result in only moderate changes in intracellular free zinc. However, if a certain zinc threshold-level is crossed, the buffering effect of metallothionein is “exhausted” and the resulting change in intracellular free zinc level then exerts its effect on gene expression.

6.2 Proteome analysis

Quantitative analysis of global mRNA levels is currently the preferred method for studying the response of a biological system (cells, tissues etc.) to a given experimental condition or perturbation (150). This is because techniques for transcriptome analysis such as differential display (151), DNA arrays (152) or SAGE (serial analysis of gene expression) (153) are fast and sensitive methods, whereas proteome analysis is much more time-consuming and is still limited to proteins with a high abundance. It is therefore common practice to measure mRNA levels and to implicitly translate them into levels of activity of the corresponding protein in the cell. Although transcriptome analysis has proven to be very powerful, on its own it is not sufficient for the characterisation of a biological system as a whole (87). It was shown in several studies that in many cases the amount of mRNA is not a reliable indicator of the corresponding protein abundance (154-156). This is because changes in protein steady state levels based on posttranscriptional mechanisms are not predictable based on measurements of the mRNA level. Such mechanisms include control of protein translation, protein stability or protein modification. Therefore proteome analysis was performed as a complementary approach in addition to transcriptional profiling to elucidate how zinc influences different levels of gene expression.

It was known from transcriptome analysis that established culture conditions were suitable to alter cellular zinc levels in HT-29 cells accordingly. For proteome analysis the duration of the incubation with experimental media was set to 16 h for both zinc-deficient and zinc-supplemented conditions to have a better comparability between both experimental settings. Proteome analysis revealed 16 molecular targets that responded with changes in steady state protein levels to a decrease and/or increase in medium zinc concentration. For most of the identified

proteins lower steady state expression levels were observed. More than half of the targets could be linked to an impaired state of cellular ATP production and to cellular stress response.

Four of the molecular targets consistently down-regulated by the higher intracellular zinc level were: serine (or cysteine) proteinase inhibitor, pre-mRNA splicing factor SRp30c, ribose 5-phosphate isomerase A and chaperonin containing t-complex. Although the molecular functions of these proteins are quite different, all targets have been reported to respond to various stress factors such as heat shock (157,158), viral infections (159) or accumulation of nuclear bodies (160). Therefore, a reduced expression of these targets as caused by a high zinc concentration may increase the sensitivity of the cells to toxic effects of environmental stress stimuli. Greater sensitivity towards oxidative stress has indeed been observed in human skin fibroblasts grown in zinc-supplemented medium (115).

A decrease in the protein levels under high zinc conditions was also observed for pyruvate kinase, adenylate kinase, $NADH_2$ dehydrogenase, H^+-translocating pyrophosphatase and aspartate transaminase. These enzymes are known to have a function in cellular ATP production, and reduced cellular ATP levels in response to a high zinc exposure have indeed been demonstrated in a number of studies with different cell types as well as in mitochondrial preparations (161-163). As a result of these studies, it seems that the reduction in cellular ATP levels is mediated mainly via inhibition of the electron transport chain, a reduced ATP production by loss of mitochondrial membrane potential and an increased mitochondria permeability; for review see (118). Enzymes of the tricarboxylic acid cycle such as aconitase were also shown to be inhibited by zinc (164). Here, additional evidence for an impaired cellular ATP status under high zinc conditions is provided, with a decrease in the expression of a number of proteins, that are important for ATP generation.

Since cancer cells produce ATP mainly by glycolysis even in the presence of a high oxygen tension, the regulation of this pathway by a higher cellular zinc concentration is of particular importance. Pyruvate kinase (muscle type), a key protein of the glycolytic pathway was found to be present at reduced steady state

levels under high zinc conditions. A fall in ATP levels generally stimulates aerobic glycolysis by de-inhibition of fructose-6-P kinase. Furthermore, NADH, generated by enhanced activity of glyceraldehyde-3-P-dehydrogenase, increases the reducing power that together with increased activity of lactate dehydrogenase (LDH) leads to high cellular lactate formation. A reduced level of pyruvate kinase protein levels therefore could reduce the cells' capability to gain energy from glycolysis. When cellular lactate levels were determined in zinc-supplemented cells, levels were indeed decreased by about 40 % as compared to that of control cells. Pyruvate kinase type M2 (PKM) is generally over-expressed in tumor cells and is considered as a valid tumor marker for a variety of tumors including those of colonic tissue (165-167). The reduced steady state level of this glycolytic enzyme at increased cellular zinc concentration in HT-29 cells is therefore a very interesting observation, suggesting that even a modest increase in intracellular free zinc (1.6-fold) can cause a reduction in tumor cell glycolysis and energy production. This in turn could initiate a pleiotropic cell response and could also be the basis for the more toxic effects of zinc, observed when this metal is provided in higher concentrations. Since pyruvate kinase type M2 is frequently used as a tumor marker, the decline in protein levels following an increase in zinc concentration warrants further studies in view of the therapeutic potential of zinc to inhibit tumor cell growth.

Alterations in expression levels of energy-related and stress-induced proteins under zinc-deficient conditions were in most cases in the same direction as observed under high zinc but were often less pronounced. An exception is aspartate transaminase (GOT2), which is up-regulated at low zinc, while it was found to be down-regulated at high cellular zinc levels. GOT2 is involved in the transamination of amino acids into α-keto acids which are routed for complete metabolism through the citric acid cycle, and increased activities of this enzyme were indeed found in the liver of zinc-deficient rats (168). This enzyme also acts as a fatty acid-binding protein, mediating cellular uptake of long-chain free fatty acids (169,170).

Three identified molecular targets which play a role in protein degradation, namely serine protease, proteasome 26S non-ATPase subunit 10, and cathepsin d were consistently up-regulated under zinc-deficient conditions, indicating that protein

catabolism is enhanced when zinc becomes limiting. There are two possible explanations for this. First, zinc deficiency results in down-regulation of a number of chaperones, important for the folding of newly synthesized proteins, as found in the present study (e.g. chaperonin containing t-complex) and by other authors (143). The higher expression levels of proteins important for proteolysis may therefore be a consequence of higher amounts of improperly folded proteins, which are potentially toxic to cells and have to be eliminated through degradation. Another reason for enhanced levels of proteases could be that cells under zinc-limited conditions mobilize zinc from metalloproteins for use in essential cellular processes. However, it has to be noted that alterations in protein expression are contrary to that observed on mRNA level, where transcript levels of genes important for proteolysis were reduced (e.g. cathepsin h, proteasome 26S non-ATPase subunit 3 and subunit 11). Of particular interest is the finding that serine protease (kallikrein 6, KLK6) is regulated by zinc. This enzyme is a member of the human kallikrein gene family with currently 15 members (171). It was originally cloned from a cDNA library prepared from a human colon adenocarcinoma cell line (COLO 201), but shows highest expression in brain (172). KLK6 is frequently overexpressed in ovarian tumours and therefore was proposed as a biomarker for ovarian cancer (173). Studies also suggested a potential involvement in the development and progression of Alzheimer's disease (AD), but the precise mechanism is unclear (174,175). The zinc-dependent regulation of KLK6 is a novel finding and may help to elucidate the role of zinc in neurodegenerative diseases such as AD.

To determine a possible correlation of protein and mRNA level in our cell culture model, alterations of identified zinc-regulated proteins were checked also at the transcript level. In general, comparing results from proteome analysis with array results, a good correlation was found between both levels at low zinc. The smaller number of genes represented on the array used for the zinc supplementation study made such a comparison invaluable. Therefore, transcript levels of selected proteins were measured by real-time RT-PCR. Again, for low zinc conditions, alterations observed for protein levels were also detected at the mRNA level, but were often more pronounced. At high zinc, however, a correlation between mRNA and protein could not be found. Although the cause for this discrepancy is not

clear, it highlights the importance of posttranscriptional mechanisms controlling gene expression processes. Taken together, changes at the protein level, at least in this cell culture model, appeared in general to be more moderate than those found at the mRNA level. Our comparison of protein and mRNA abundance supports the conclusion of other authors (87,154) that information obtained from one level does not necessarily reflect the situation at the other level of gene expression control.

The following figure summarises the results from transcriptome and proteome analysis of HT-29 cells under high and low zinc conditions (Fig. 42).

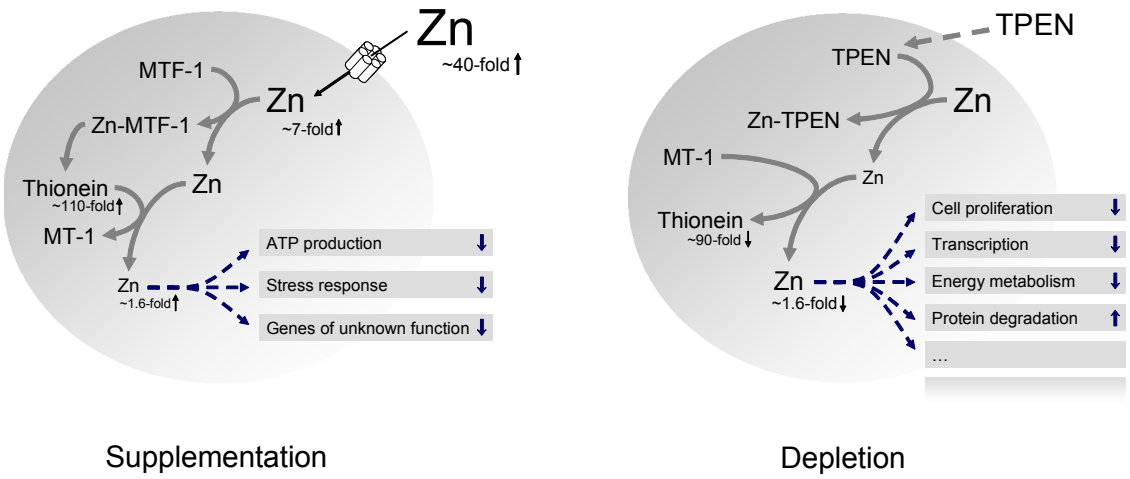


Fig. 42: Effects of zinc supplementation and zinc depletion on HT-29 cells. Zinc supplementation: A medium zinc concentration, which is about 40 times higher than in normal culture medium, increases the total cellular zinc levels by about 7-fold. The transcription factor MTF-1 is activated by zinc and induces the transcription of thionein (~110-fold), which buffers zinc via formation of MT-1. The resulting modest increase in intracellular free zinc (~1.6-fold) leads to changes in the expression of genes and proteins involved for example in ATP production and stress response. Zinc depletion: A decrease in intracellular zinc caused by the membrane-permeable zinc chelator TPEN is counteracted by the release of zinc from the intracellular zinc buffer MT-1. Thionein has a short half-life and is removed by proteolysis. Even the resulting modest decrease in intracellular free zinc levels (~1.6-fold) affects the expression of an impressive number of genes and proteins important for basic cellular metabolic functions such as cell proliferation, transcription, energy production and protein degradation.

6.3 Target gene search for MTF-1

Among the best characterized zinc-dependent genes in higher eukaryotes are the metallothioneins which are regulated by the zinc-finger containing transcription factor MTF-1 (metal-responsive element-binding transcription factor-1 or in short metal transcription factor-1). MTF-1 is a metalloregulatory protein whose DNA-

binding activity is reversibly activated in response to changes in free zinc concentrations in the cell, thus serving as an intracellular free zinc sensor (176-178). Interestingly, disruption of the MTF-1 gene in mice is lethal, whereas animals lacking both MT-1 and -2 are viable (46-49). This led to the conclusion, that MTF-1 controls expression of important genes other than MT-1 and -2 and those target genes have been searched systematically (41,46). However, to date only the mammalian zinc efflux transporter ZnT-1 was shown to be an additional *in vivo* target gene of MTF-1 (50).

This work led to the identification of a set of zinc-dependent genes, which are sensitive to high and low zinc concentrations. It was assessed whether expression control of these genes also occurred via the zinc-finger transcription factor MTF-1 by generating a HT-29 cell line that overexpresses MTF-1. The Tet-On™ gene expression system was used as it allows the rapid and high-level induction of genes of interest (179). In comparison to control cells, overexpression of MTF-1 in this cell model resulted in the up-regulation of three of the previously identified zinc-sensitive genes (Kruppel-like factor 4, hepatitis A virus cellular receptor 1 and complement factor B). The consistent up-regulation is not surprising, since MTF-1 acts as a transcriptional activator, enhancing the transcription of its target genes (180). Induction of transcription of target genes requires binding of MTF-1 to cis-acting DNA elements, termed metal-responsive elements (MREs), which occur in multiple copies in the promoter regions of these genes. MREs contain a well-defined 7 bp core consensus sequence (TGCRCNC; R is a purine and N any base) within an extended consensus sequence of 12 bp (181-183) and to date there is no evidence that MTF-1 can utilize other DNA motifs to enhance transcription *in vivo* (41). As a complementary approach an in-silico search for MREs in the promoters of the identified zinc-sensitive genes was performed. A perfect match with the MRE core consensus sequence was used as search criterion, because experiments with point mutants suggested that this core sequence plays a crucial role in heavy-metal response, while mutations in the flanking regions are less critical (183,184). It is thought that two or more MREs are required for a promotor to exhibit significant metal responsiveness (181,185), and three and two MREs, respectively, were indeed found in the promotor of Kruppel-like factor 4 and hepatitis A virus cellular receptor 1. In the case of the third gene,

complement factor B, no MRE was found in the 5´prime region. However this does not necessarily mean that this gene is not a candidate for MTF-1 control as the core consensus needs not to be preserved in its entire structure (182) as given by examples from mouse and human with mutations at position 1, 5 or 7, for example, MREa in mZnT-1 (TGCAGAC), see (185). All other genes screened did not match this criterion for a putative MTF-1 target gene as they contained no conserved MREs in their promotor regions and did not or not consistently show changes in mRNA levels by increasing protein levels of MTF-1. The role of MTF-1 for transcriptional regulation of the gastrointestinal peptide gene remains unclear, as one MRE was found in the promotor, but mRNA levels could not be measured due to very low expression levels in the cell clones.

The finding that expression of KLF4 is increased in cells overexpressing MTF-1 is particularly interesting for several reasons. KLF4 (or GKLF for Gut-enriched Kruppel-like factor) is a zinc finger-containing, epithelial-specific transcription factor, that functions as a negative regulator of cell proliferation (148) by blocking G1/S progression of the cell cycle (186). Several studies were conducted to explain the function of KLF4 as a cell cycle checkpoint protein. Stone et al. (187) proposed that the expression of KLF4 is regulated by the tumor suppressor APC (adenomatous polyposis coli). KLF4, in turn, negatively affects β-catenin levels which otherwise would activate several downstream growth-promoting genes including cyclin D1, resulting in an abnormal cell growth in colon or an increased tumorigenicity of colon cancer cells. A role of KLF4 in the p53-dependent induction of the cyclin-dependent kinase inhibitor $p21^{WAF1/Cip1}$ was also suggested (188,189). In this work it was demonstrated for the first time, that expression of KLF4-mRNA and -protein increases in zinc-depleted HT-29 cells (190). An increased expression level of KLF4 in cells overexpressing MTF-1 was also shown. Moreover, three perfect matching MREs were found in its upstream regulatory sequence. Taken together, the results presented here indicate, that MTF-1 also plays a crucial role in transcriptional regulation of KLF4, which is a new aspect in understanding how zinc affects cell growth.

Target gene search for MTF-1 so far was based on database search for metal responsive elements (MREs) or DNA array results from knockout studies (41,46,191). Both strategies generally result in a long list of putative target genes

for which a link to MTF-1 has to be proven for each candidate gene separately. The “reverse” approach used here has the advantage that the search for potential target genes is narrowed to a small number of zinc-sensitive genes, which in the second step were checked for their MTF-1 responsiveness. A good correlation between the results from the experimental approach and the subsequent database search was found, indicating that an in-silico search for MREs offers valuable clues in identification of potential new target genes for MTF-1. The data presented here imply that MTF-1 may be involved in the transcriptional regulation of Kruppel-like factor 4, hepatitis A virus cellular receptor 1 and possibly - although less convincing - for complement factor B. However, whether MTF-1 directly or indirectly regulates expression of these putative new target genes remains to be determined in further studies including transient co-transfections of MTF-1 with reporter plasmids driven by promotor constructs of genes under investigation and electrophoretic mobility shift assays (EMSAs).

7 Summary

Zinc is essential for the structural and functional integrity of cells and plays a pivotal role in the control of gene expression. Despite intensive research activities over the last decades, the molecular targets leading to the pleiotropic effects of zinc have only partially been identified. In this work, transcriptome and proteome analysis using the human colon adenocarcinoma cell line HT-29 as a model were performed to identify genes and proteins with altered steady state expression levels upon alteration of the cellular zinc status.

For transcriptome profiling two commercially available DNA array formats were used and real-time RT-PCR/Northern blot analysis were employed for the independent confirmation of array results. DNA array analysis of HT-29 cells, exposed to a high but non-toxic zinc concentration identified an unexpectedly small number of molecular targets. However, most of the identified genes have so far never been linked to alterations in cellular zinc status. Unlike zinc supplementation, a low intracellular zinc concentration caused changes in the steady state mRNA levels of 309 genes; most of them with down-regulation. Genes identified as regulated based on microarray data encode proteins important for central pathways of intermediary metabolism and basic cellular functions such as signaling, cell cycle control and growth, vesicular trafficking, cell-cell interaction, cytoskeleton and transcription control. Proteome analysis by 2D-PAGE yielded 29 proteins that are regulated by zinc depletion and/or zinc supplementation. Thereof, 16 proteins were identified by MALDI-TOF-MS analysis. Most of the targets could be linked to an impaired state of cellular ATP production and cellular stress response. Comparison of data obtained from transcriptome and proteome analysis showed a good correlation in case of zinc depletion studies, whereas only a weak correlation was found in case of cells exposed to high medium zinc.

Among the identified zinc-dependent genes, 9 were found to respond to a low as well as a high cellular zinc status with changes in expression levels. As those are obviously primarily zinc-dependent, it was investigated whether these genes are controlled by the zinc-finger transcription factor MTF-1. Using a conditional expression system and by employing an in-silico analysis for metal responsive elements (MREs) within promoters of zinc-sensitive genes, it became evident that Kruppel-like factor 4, hepatitis A virus cellular receptor 1 and complement factor B are three potential new target genes of MTF-1.

In conclusion, the methods employed in the present thesis proved suitable to identify an impressive number of new molecular targets of zinc. The pattern of genes and proteins that respond to alterations in cellular zinc status may represent a reference for further studies to define the zinc regulon in mammalian cells.

8 Zusammenfassung

Zink ist essentiell für die strukturelle und funktionelle Integrität von Zellen und spielt eine wichtige Rolle in der Genexpression. Obwohl die vielfältigen Effekte von Zink in den letzten Jahrzehnten Gegenstand intensiver Forschung waren, konnten die zugrundeliegenden molekularen Mechanismen nur teilweise aufgeklärt werden. In der vorliegenden Arbeit wurde am Model der humanen Dickdarmkrebszellinie HT-29 ein Screening auf Transkriptom- und Proteomebene durchgeführt, um Gene und Proteine zu identifizieren, die mit veränderten Expressionsspiegeln auf ein verändertes Zinkangebot (Mangel, Überschuß) reagieren.

Für das Screening auf Transkriptionsebene wurden zwei unterschiedliche, kommerziell erhältliche DNA-Chip-Formate verwendet. Zur unabhängigen Bestätigung der Resultate dienten die real-time RT-PCR- und Northern Blot-Analyse. Die Array-Analyse von HT-29 Zellen, die einer hohen aber nicht-toxischen Zinkkonzentration ausgesetzt wurden, ergab eine unerwartet kleine Anzahl von verändert exprimierten Transkripten. Gleichwohl wurden die meisten der identifizierten Gene bislang nicht mit Veränderungen im Zinkangebot in Verbindung gebracht. Hingegen wiesen unter einem experimentellen Zinkmangel 309 Gene signifikante Veränderungen der mRNA-Spiegel auf; die meisten von ihnen waren vermindert exprimiert. Sie kodieren für Proteine, die eine wichtige Rolle im Intermediärstoffwechsel sowie für grundlegenden Funktionen der Zelle spielen, wie Signalweitergabe, Zellzykluskontrolle und -wachstum, Trafficking, Zell-Zell-Interaktion, Zytoskelettdynamik und Transkription. Die Proteomanalyse mittels 2D-PAGE ergab 29 Proteine, die in ihrer Expression durch Zinkmangel und/oder -zulage signifikant verändert waren. Von diesen konnten 16 Proteine mittels MALDI-TOF-MS identifiziert werden. Die Mehrzahl der Proteine ist beteiligt an der ATP-Produktion und der zellulären Stressantwort. Ein Vergleich der Daten der Transkriptom- und Proteomanalyse ergab eine gute Übereinstimmung der beiden Expressionsspiegel für die Zinkdepletierungsversuche, wohingegen nur eine schwache Korrelation für die Zinkzulageversuche nachweisbar war.

Neun der identifizierten zink-abhängigen Gene waren sowohl unter Bedingungen eines niedrigen als auch eines hohen Zinkstatus differenziell exprimiert. Da somit die Expression dieser Gene primär zink-abhängig zu sein schien, wurde experimentell geprüft, ob sie unter der Kontrolle des Zinkfinger-Transkriptionsfaktors MTF-1 (metal transcription factor-1) stehen. Durch Etablierung eines konditionalen Expressionssystems und mittels einer bioinformatischen Suche nach Response-Elementen in den Promotorsequenzen dieser Gene wurde gezeigt, daß Kruppel-like Factor 4, Hepatitis A

virus cellular receptor 1 und Complement Factor B drei potentielle neue Targetgene von MTF-1 sind.

Zusammenfassend läßt sich feststellen, daß sich die angewendeten Screening-Methoden als geeignet erwiesen, um eine Vielzahl neuer Zielgene zu identifizieren, welche Änderungen des zellulären Zinkspiegels in Zellantworten übersetzen. Die identifizierten Marker bieten die Grundlage für weitere Studien, das Zink-Regulon in Säugetierzellen zu entschlüsseln.

9 Addendum

9.1 Complete list of genes identified as responsive to zinc depletion

Tab. 7: List of genes regulated by low zinc conditions. Complete list of genes with altered mRNA expression levels in response to intracellular zinc depletion in human HT-29 cells

Cell growth/cycle						
		-Zn/+Zn: fold change				
Acc. no.	Encoded protein	Exp. 1	Exp. 2	Exp. 3	Mean	SD
NM_079421	cyclin-dependent kinase inhibitor 2d; cdkn2d	3,57	4,84	3,54	3,99	0,74
NM_001924	growth arrest and dna-damage-inducible, alpha; gadd45a	2,81	3,47	4,10	3,46	0,64
NM_013376	cdk4-binding protein p34sei1; sei1	2,73	3,30	2,04	2,69	0,63
NM_021930	rad50-interacting protein 1; flj11785	-5,05	-1,91	-4,59	-3,85	1,70
NM_002754	mitogen-activated protein kinase 13; mapk13	-2,82	-3,17	-3,22	-3,07	0,22
XM_003560	mad2-like 1; mad2l1	-3,15	-2,73	-2,23	-2,70	0,46
NM_001237	cyclin a; ccna2	-2,74	-2,70	-2,31	-2,58	0,24
NM_001260	cyclin-dependent kinase 8; cdk8	-3,96	-2,11	-1,47	-2,51	1,30
NM_002358	mad2-like 1; mad2l1	-3,11	-2,61	-1,73	-2,48	0,70
NM_003564	transgelin 2; tagln2	-0,72	-2,52	-3,86	-2,37	1,57
NM_004642	cdk2-associated protein 1; cdk2ap1	-1,67	-2,31	-2,70	-2,23	0,52
NM_014287	pm5 protein; pm5	-1,77	-2,71	-2,15	-2,21	0,47
AJ012584	nucks protein; nucks	-2,17	-1,63	-2,73	-2,18	0,55
NM_006716	activator of s phase kinase; ask	-2,58	-1,61	-2,22	-2,13	0,49
NM_006306	smc1 (structural maintenance of chromosomes 1, yeast)-like 1; smc1l1	-2,04	-2,39	-1,95	-2,13	0,24
Cell cell interaction						
		-Zn/+Zn: fold change				
Acc. no.	Encoded protein	Exp. 1	Exp. 2	Exp. 3	Mean	SD
NM_033049	mucin 13, epithelial transmembrane; muc13	3,60	3,11	2,37	3,02	0,62
NM_000574	decay accelerating factor for complement (cd55, cromer blood group system); daf	2,15	2,18	3,62	2,65	0,84
NM_031500	protocadherin alpha 4, isoform 2 precursor; pcdha4	2,30	2,78	1,98	2,35	0,41
NM_022137	secreted modular calcium-binding protein 1; smoc1	2,69	2,66	1,63	2,33	0,60
NM_004306	annexin a13; anxa13	2,67	1,51	2,45	2,21	0,62
NM_005625	syndecan binding protein (syntenin); sdcbp	1,45	2,21	2,83	2,16	0,69
NM_021195	claudin 6; cldn6	2,40	2,09	1,90	2,13	0,25
NM_002296	lamin b receptor; lbr	-1,72	-2,19	-2,93	-2,28	0,61
NM_005573	lamin b1; lmnb1	-2,88	-2,14	-1,78	-2,27	0,56
NM_006499	lectin, galactoside-binding, soluble, 8 (galectin 8); lgals8	-2,03	-2,76	-1,88	-2,22	0,47
NM_007328	killer cell lectin-like receptor subfamily c, member 1 isoform nkg2-b; klrc1	-2,63	-2,45	-1,58	-2,22	0,56
NM_004004	gap junction protein, beta 2, 26kd (connexin 26); gjb2	-2,87	-2,33	-0,93	-2,04	1,00
NM_003813	a disintegrin and metalloproteinase domain 21 preproprotein; adam21	-1,60	-2,18	-2,11	-1,96	0,32
Targeting/Trafficking						
		-Zn/+Zn: fold change				
Acc. no.	Encoded protein	Exp. 1	Exp. 2	Exp. 3	Mean	SD
XM_011184	sec24-related protein d; sec24d	4,05	3,50	3,46	3,67	0,33
NM_001282	adaptor-related protein complex 2, beta 1 subunit; ap2b1	2,75	2,74	1,71	2,40	0,60
NM_014606	hect domain and rld 3; herc3	2,11	2,27	2,02	2,13	0,13
NM_006370	vesicle-associated soluble nsf attachment protein receptor (v-sn; vti2	-3,96	-2,67	-4,86	-3,83	1,10

NM_004069	adaptor-related protein complex 2, sigma 1 subunit, isoform ap17; ap2s1	-3,28	-2,59	-3,36	-3,08	0,43
NM_005102	zygin 2; fez2	-3,17	-2,15	-2,66	-2,66	0,51
NM_002618	peroxisome biogenesis factor 13; pex13	-4,17	-2,10	-1,50	-2,59	1,40
NM_002270	karyopherin (importin) beta 2; kpnb2	-2,66	-2,43	-2,23	-2,44	0,21
NM_014748	kiaa0064 gene product; kiaa0064	-3,15	-2,29	-1,46	-2,30	0,84
NM_005733	rab6 interacting, kinesin-like (rabkinesin6); rab6kifl	-2,18	-2,61	-1,98	-2,26	0,32
NM_007357	low density lipoprotein receptor defect c complementing; ldlc	-2,23	-2,99	-1,54	-2,26	0,73
NM_016338	ran binding protein 11; loc51194	-2,29	-2,36	-1,86	-2,17	0,27
NM_001316	cse1 chromosome segregation 1-like (yeast); cse1l	-2,17	-2,30	-1,72	-2,06	0,31
NM_012458	translocase of inner mitochondrial membrane 13 homolog b (yeast); timm13b	-2,05	-1,49	-2,30	-1,95	0,42
NM_006423	rab acceptor 1 (prenylated); rabac1	-1,37	-2,11	-2,36	-1,95	0,51
Signaling						
		-Zn/+Zn: fold change				
Acc. no.	**Encoded protein**	**Exp. 1**	**Exp. 2**	**Exp. 3**	**Mean**	**SD**
NM_014399	tetraspan net-6 protein; net-6	2,28	2,32	2,99	2,53	0,40
NM_004440	epha7	1,91	2,80	2,84	2,52	0,53
NM_004417	dual specificity phosphatase 1; dusp1	3,00	2,26	1,94	2,40	0,54
NM_006024	tax1 (human t-cell leukemia virus type i) binding protein 1; tax1bp1	1,62	2,53	2,55	2,23	0,53
NM_003567	breast cancer antiestrogen resistance 3; bcar3	2,19	2,48	1,96	2,21	0,26
NM_002923	regulator of g-protein signalling 2, 24kd; rgs2	2,07	2,10	2,44	2,20	0,21
NM_004253	phospholipase a2-activating protein; plaa	1,96	2,40	2,24	2,20	0,22
NM_002844	protein tyrosine phosphatase, receptor type, k; ptprk	2,02	2,04	2,11	2,06	0,05
NM_001233	caveolin 2; cav2	2,27	2,29	1,46	2,01	0,47
NM_018492	pdz-binding kinase; topk	-8,76	-1,05	-2,54	-4,12	4,09
NM_007240	dual specificity phosphatase 12; dusp12	-4,14	-3,39	-3,21	-3,58	0,49
NM_022344	protein kinase njmu-r1; njmu-r1	-3,74	-2,09	-3,37	-3,07	0,86
NM_002710	protein phosphatase 1, catalytic subunit, gamma isoform; ppp1cc	-2,46	-2,95	-2,99	-2,80	0,30
NM_013258	apoptosis-associated speck-like protein containing a card; asc	-2,91	-2,68	-2,70	-2,76	0,13
NM_001992	coagulation factor ii receptor precursor; f2r	-2,80	-2,79	-2,61	-2,73	0,11
NM_003467	chemokine (c-x-c motif), receptor 4 (fusin); cxcr4	-2,25	-3,27	-2,22	-2,58	0,60
NM_004981	potassium inwardly-rectifying channel, subfamily j, member 4; kcnj4	-2,65	-2,26	-2,56	-2,49	0,20
NM_018841	g-protein gamma-12 subunit; loc55970	-1,90	-2,40	-3,11	-2,47	0,61
NM_001144	autocrine motility factor receptor; amfr	-2,33	-2,93	-1,83	-2,36	0,55
NM_012116	cas-br-m (murine) ectropic retroviral transforming sequence c; cblc	-1,97	-2,13	-2,84	-2,31	0,46
NM_017934	pleckstrin homology domain interacting protein; phip	-2,74	-1,79	-2,08	-2,20	0,49
NM_000180	guanylate cyclase 2d, membrane (retina-specific); gucy2d	-2,54	-2,10	-1,95	-2,20	0,31
XM_004846	pdgfa associated protein 1; pdap1	-1,89	-2,18	-2,43	-2,17	0,27
NM_014225	protein phosphatase 2 (formerly 2a), regulatory subunit a (pr 65), alpha isoform; ppp2r1a	-1,40	-2,19	-2,81	-2,13	0,70
NM_004444	ephrin receptor ephb4 precursor; ephb4	-1,71	-2,18	-2,48	-2,12	0,39
NM_006453	transducin (beta)-like 3; tbl3	-2,09	-2,07	-2,05	-2,07	0,02
NM_006504	protein tyrosine phosphatase, receptor type, e; ptpre	-2,19	-1,70	-2,21	-2,04	0,29
NM_006252	protein kinase, amp-activated, alpha 2 catalytic subunit; prkaa2	-2,10	-1,98	-2,01	-2,03	0,06
NM_006866	leukocyte immunoglobulin-like receptor, subfamily a (with tm domain), member 2; lilra2	-2,02	-2,00	-1,99	-2,00	0,01
NM_033015	fas-activated serine/threonine kinase, isoform 2; fastk	-1,47	-2,02	-2,02	-1,84	0,32
Transcription						
		-Zn/+Zn: fold change				
Acc. no.	**Encoded protein**	**Exp. 1**	**Exp. 2**	**Exp. 3**	**Mean**	**SD**
NM_004235	kruppel-like factor 4 (gut); klf4	5,19	5,40	5,30	5,30	0,10
NM_021969	short heterodimer partner; nr0b2	5,31	4,83	3,48	4,54	0,95
NM_003447	zinc finger protein 165; znf165	2,91	3,88	2,84	3,21	0,58

NM_016061	cgi-127 protein; loc51646	2,32	2,34	1,55	2,07	0,45
NM_001450	four and a half lim domains 2; fhl2	2,04	2,07	1,87	1,99	0,11
NM_006526	zinc finger protein 217; znf217	-4,68	-5,09	-4,74	-4,84	0,22
NM_012086	general transcription factor iiic, polypeptide 3 (102kd); gtf3c3	-3,08	-3,80	-4,11	-3,66	0,53
NM_006963	zinc finger protein 22 (kox 15); znf22	-4,06	-2,89	-1,31	-2,75	1,38
NM_002467	v-myc myelocytomatosis viral oncogene homolog (avian); myc	-2,49	-2,56	-2,87	-2,64	0,20
NM_016283	adrenal gland protein ad-004; loc51578	-2,74	-3,02	-1,91	-2,56	0,57
NM_012333	c-myc binding protein; mycbp	-1,98	-2,19	-2,64	-2,27	0,34
NM_018450	uncharacterized bone marrow protein bm029; bm029	-2,42	-2,53	-1,77	-2,24	0,41
NM_002140	heterogeneous nuclear ribonucleoprotein k, isoform a; hnrpk	-1,58	-2,56	-2,40	-2,18	0,53
NM_006713	activated rna polymerase ii transcription cofactor 4; pc4	-2,55	-2,34	-1,47	-2,12	0,57
NM_005180	murine leukemia viral (bmi-1) oncogene homolog; bmi1	-2,34	-2,08	-1,81	-2,08	0,26
NM_018951	homeo box a10; hoxa10	-2,09	-2,24	-1,75	-2,03	0,25
NM_003851	cellular repressor of e1a-stimulated genes; creg	-2,25	-2,14	-1,68	-2,02	0,30
NM_005170	achaete-scute complex homolog-like 2; ascl2	-2,04	-1,82	-2,12	-2,00	0,16
NM_007331	whsc1 protein; whsc1	-2,23	-1,28	-2,17	-1,89	0,53

Cytoskeleton

		-Zn/+Zn: fold change				
Acc. no.	**Encoded protein**	**Exp. 1**	**Exp. 2**	**Exp. 3**	**Mean**	**SD**
NM_001458	gamma filamin; flnc	1,99	2,20	3,13	2,44	0,60
NM_003116	sperm associated antigen 4; spag4	2,22	2,68	1,92	2,28	0,38
NM_006097	myosin regulatory light chain 2, smooth muscle isoform; myrl2	-4,19	-3,23	-2,58	-3,33	0,81
NM_001667	adp-ribosylation factor-like 2; arl2	-3,37	-1,93	-2,69	-2,66	0,72
NM_004924	actinin, alpha 4; actn4	-1,72	-2,20	-3,21	-2,37	0,76
NM_016638	srp25 nuclear protein; loc51329	-1,81	-2,48	-2,63	-2,31	0,44
NM_007317	kinesin-like 4; knsl4	-2,59	-1,83	-2,25	-2,22	0,38
NM_004517	integrin-linked kinase; ilk	-1,65	-2,30	-2,71	-2,22	0,54
NM_018204	cytoskeleton associated protein 2; ckap2	-1,80	-2,52	-2,10	-2,14	0,36
NM_004523	kinesin-like 1; knsl1	-2,55	-1,73	-2,05	-2,11	0,41

Metabolism

		-Zn/+Zn: fold change				
Acc. no.	**Encoded protein**	**Exp. 1**	**Exp. 2**	**Exp. 3**	**Mean**	**SD**
NM_000434	neuraminidase precursor; neu1	5,46	5,74	3,94	5,05	0,97
NM_000963	prostaglandin-endoperoxide synthase 2 (prostaglandin g/h synthase and cyclooxygenase); ptgs2	5,00	1,91	2,82	3,24	1,59
NM_005794	short-chain alcohol dehydrogenase family member; hep27	3,59	2,10	3,64	3,11	0,87
NM_001673	asparagine synthetase; asns	2,72	2,57	3,47	2,92	0,48
NM_000636	superoxide dismutase 2, mitochondrial; sod2	2,76	1,85	2,55	2,38	0,48
NM_002394	antigen identified by monoclonal antibodies 4f2, tra1.10, trop4, and t43; slc3a2	2,64	2,45	2,06	2,38	0,30
NM_003889	pregnane x receptor, isoform 1l; nr1i2	2,62	2,27	2,22	2,37	0,22
NM_004569	phosphatidylinositol glycan, class h; pigh	2,77	2,28	1,63	2,23	0,57
NM_000169	galactosidase, alpha; gla	2,06	2,24	2,37	2,23	0,16
NM_004786	thioredoxin-like, 32kd; txnl	1,70	2,27	2,44	2,14	0,39
NM_003850	succinate-coa ligase, adp-forming, beta subunit; sucla2	1,85	2,01	2,04	1,97	0,10
NM_006516	solute carrier family 2 (facilitated glucose transporter), member 1; slc2a1	-2,94	-5,08	-8,55	-5,53	2,83
NM_014473	putative dimethyladenosine transferase; hsa9761	-4,88	-4,50	-3,25	-4,21	0,85
NM_004731	solute carrier family 16 (monocarboxylic acid transporters), member 7; slc16a7	-4,52	-4,13	-3,00	-3,88	0,79
NM_001428	enolase 1; eno1	-0,70	-2,86	-7,54	-3,70	3,50
NM_000310	palmitoyl-protein thioesterase 1 (ceroid-lipofuscinosis, neuronal 1, infantile); ppt1	-2,84	-3,80	-3,86	-3,50	0,57
NM_005003	nadh dehydrogenase (ubiquinone) 1, alpha/beta subcomplex, 1 (8kd, sdap); ndufab1	-3,53	-3,44	-3,51	-3,49	0,05

NM_002495	nadh dehydrogenase (ubiquinone) fe-s protein 4 (18kd) (nadh-coenzyme q reductase); ndufs4	-4,24	-2,99	-2,78	-3,34	0,79
NM_004889	atp synthase, h+ transporting, mitochondrial f0 complex, subunit f, isoform 2; atp5j2	-2,01	-3,40	-4,44	-3,28	1,22
NM_005787	not56 (d. melanogaster)-like protein; not56l	-3,18	-2,34	-3,46	-2,99	0,58
NM_020244	cholinephosphotransferase 1; loc56994	-3,04	-2,83	-2,52	-2,80	0,26
NM_015423	alpha-aminoadipic semialdehyde dehydrogenase-phosphopantetheinyl transferase; aasdhppt	-2,12	-3,06	-2,94	-2,71	0,51
NM_003849	succinate-coa ligase, gdp-forming, alpha subunit; suclg1	-2,54	-2,26	-3,12	-2,64	0,44
NM_000414	hydroxysteroid (17-beta) dehydrogenase 4; hsd17b4	-3,39	-2,54	-1,86	-2,60	0,77
NM_004549	nadh dehydrogenase (ubiquinone) 1, subcomplex unknown, 2 (14.5kd, b14.5b); ndufc2	-4,00	-1,44	-2,34	-2,59	1,30
NM_001216	carbonic anhydrase ix precursor; ca9	-1,60	-2,82	-3,20	-2,54	0,84
NM_000780	cytochrome p450, subfamily viia, polypeptide 1; cyp7a1	-2,24	-2,79	-2,55	-2,53	0,27
NM_000143	fumarate hydratase; fh	-3,07	-2,82	-1,63	-2,51	0,77
NM_002491	nadh dehydrogenase (ubiquinone) 1 beta subcomplex, 3 (12kd, b12); ndufb3	-3,20	-2,41	-1,89	-2,50	0,66
NM_004548	nadh dehydrogenase (ubiquinone) 1 beta subcomplex, 10 (22kd, pdsw); ndufb10	-2,50	-2,94	-2,02	-2,49	0,46
NM_000120	epoxide hydrolase 1, microsomal (xenobiotic); ephx1	-2,10	-2,71	-2,62	-2,48	0,33
NM_003878	gamma-glutamyl hydrolase (conjugase, folylpolygammaglutamyl hydrolase) precursor; ggh	-2,62	-2,58	-2,22	-2,47	0,22
NM_000191	3-hydroxymethyl-3-methylglutaryl-coenzyme a lyase (hydroxymethylglutaricaciduria); hmgcl	-3,58	-1,42	-2,39	-2,46	1,08
NM_033496	hexokinase 1, isoform hki-r; hk1	-2,69	-3,00	-1,64	-2,44	0,71
NM_005687	phenylalanyl-trna synthetase beta-subunit; phehb	-3,14	-2,47	-1,71	-2,44	0,72
NM_001441	fatty acid amide hydrolase; faah	-3,16	-2,40	-1,55	-2,37	0,81
NM_017512	rts beta protein; hsrtsbeta	-2,68	-2,19	-2,21	-2,36	0,28
NM_015239	kiaa1035 protein; kiaa1035	-2,50	-2,25	-2,29	-2,34	0,14
NM_005000	nadh dehydrogenase (ubiquinone) 1 alpha subcomplex, 5; ndufa5	-2,87	-1,92	-2,22	-2,34	0,49
NM_002489	nadh dehydrogenase (ubiquinone) 1 alpha subcomplex, 4 (9kd, mlrq); ndufa4	-2,92	-1,99	-2,10	-2,34	0,51
NM_020062	glut4 enhancer factor; gef	-2,98	-2,05	-1,90	-2,31	0,58
NM_002413	microsomal glutathione s-transferase 2; mgst2	-2,81	-1,78	-2,32	-2,30	0,51
NM_000709	branched chain keto acid dehydrogenase e1, alpha polypeptide (maple syrup urine disease); bckdha	-1,89	-2,01	-3,00	-2,30	0,61
NM_001608	acyl-coenzyme a dehydrogenase, long chain precursor; acadl	-2,24	-2,34	-2,29	-2,29	0,05
NM_001398	peroxisomal enoyl-coenzyme a hydratase-like protein; ech1	-2,93	-1,68	-2,22	-2,27	0,63
NM_012103	ancient ubiquitous protein 1; aup1	-2,18	-2,04	-2,52	-2,25	0,24
NM_018947	cytochrome c; hcs	-2,39	-2,38	-1,92	-2,23	0,27
NM_005049	pwp2 periodic tryptophan protein homolog (yeast); pwp2h	-2,40	-2,21	-1,81	-2,14	0,30
NM_001634	s-adenosylmethionine decarboxylase 1 precursor; amd1	-2,06	-2,24	-2,09	-2,13	0,10
NM_003129	squalene monooxygenase; sqle	-1,62	-2,67	-2,07	-2,12	0,53
NM_002940	atp-binding cassette, sub-family e, member 1; abce1	-2,14	-2,21	-1,87	-2,07	0,18
NM_016142	steroid dehydrogenase homolog; loc51144	-2,15	-2,16	-1,73	-2,02	0,25
NM_001914	cytochrome b-5; cyb5	-1,68	-2,04	-2,23	-1,98	0,28
NM_015629	pre-mrna processing factor 31 homolog (yeast); prpf31	-2,04	-1,40	-2,34	-1,93	0,48
NM_021004	peroxisomal short-chain alcohol dehydrogenase; scad-srl	-2,00	-2,04	-1,66	-1,90	0,21
NM_001071	thymidylate synthetase; tyms	-2,00	-1,60	-2,04	-1,88	0,24
NM_022109	cdw92	-1,20	-2,30	-2,13	-1,87	0,59
gapdh	glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA, complete ""cds;"" glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12)	-0,69	-2,11	-2,40	-1,73	0,91
Protein metabolism						
		-Zn/+Zn: fold change				
Acc. no.	**Encoded protein**	**Exp. 1**	**Exp. 2**	**Exp. 3**	**Mean**	**SD**
NM_003122	serine protease inhibitor, kazal type 1; spink1	3,27	3,90	4,11	3,76	0,44

NM_019843	eif4e-transporter; 4e-t	3,32	3,60	2,00	2,98	0,86
NM_014685	homocysteine-inducible, endoplasmic reticulum stress-inducible, ubiquitin-like domain member 1; herpud1	3,21	2,22	2,92	2,78	0,51
NM_001751	cysteinyl-trna synthetase; cars	2,40	2,61	2,36	2,46	0,13
NM_006513	seryl-trna synthetase; sars	2,22	2,41	2,71	2,45	0,25
NM_007108	elongin b; tceb2	-3,46	-3,05	-2,38	-2,96	0,55
NM_003418	zinc finger protein 9; znf9	-2,93	-3,07	-2,67	-2,89	0,20
NM_002809	proteasome (prosome, macropain) 26s subunit, non-atpase, 3; psmd3	-1,73	-3,02	-3,84	-2,86	1,06
NM_003470	ubiquitin specific protease 7 (herpes virus-associated); usp7	-3,39	-2,95	-2,13	-2,82	0,64
NM_021078	gcn5 general control of amino-acid synthesis 5-like 2 (yeast); gcn5l2	-1,66	-2,82	-3,59	-2,69	0,98
NM_001967	eukaryotic translation initiation factor 4a, isoform 2; eif4a2	-1,73	-3,81	-2,27	-2,60	1,08
NM_015920	40s ribosomal protein s27 isoform; loc51065	-3,13	-2,64	-1,96	-2,57	0,59
NM_004247	u5 snrnp-specific protein, 116 kd; u5-116kd	-3,20	-2,93	-1,44	-2,52	0,95
NM_002080	aspartate aminotransferase 2 precursor; got2	-2,19	-1,49	-3,49	-2,39	1,01
NM_004390	cathepsin h; ctsh	-1,45	-2,25	-3,20	-2,30	0,87
NM_012413	glutaminyl-peptide cyclotransferase precursor; qpct	-2,23	-2,86	-1,68	-2,26	0,59
NM_004953	eukaryotic translation initiation factor 4 gamma, 1; eif4g1	-1,82	-2,57	-2,27	-2,22	0,38
NM_006838	methionyl aminopeptidase 2; metap2	-2,45	-1,73	-2,23	-2,13	0,37
NM_001748	calpain 2, large subunit; capn2	-2,02	-2,37	-1,99	-2,13	0,21
NM_002786	proteasome (prosome, macropain) subunit, alpha type, 1; psma1	-2,19	-2,24	-1,84	-2,09	0,22
NM_002815	proteasome (prosome, macropain) 26s subunit, non-atpase, 11; psmd11	-2,20	-2,17	-1,57	-1,98	0,36

Others

		-Zn/+Zn: fold change				
Acc. no.	**Encoded protein**	**Exp. 1**	**Exp. 2**	**Exp. 3**	**Mean**	**SD**
NM_004864	prostate differentiation factor; plab	3,61	8,89	8,25	6,92	2,88
NM_001657	amphiregulin (schwannoma-derived growth factor); areg	3,18	2,84	5,20	3,74	1,28
NM_000510	follicle stimulating hormone, beta polypeptide; fshb	3,95	3,61	2,26	3,27	0,89
NM_004323	bcl2-associated athanogene; bag1	2,16	2,32	3,19	2,56	0,56
NM_005083	u2 small nuclear ribonucleoprotein auxiliary factor, small subunit-related protein 1; u2af1rs1	2,76	2,51	1,95	2,41	0,41
NM_022338	chromosome 11 open reading frame 24; c11orf24	2,19	2,45	2,10	2,24	0,18
NM_016090	rna binding motif protein 7; rbm7	2,07	1,93	2,49	2,17	0,29
NM_001831	clusterin (complement lysis inhibitor, sp-40,40, sulfated glycoprotein 2, testosterone-repressed prostate message 2, apolipoprotein j); clu	2,30	1,98	2,13	2,14	0,16
NM_016306	dnaj (hsp40) homolog, subfamily b, member 11; dnajb11	1,75	2,28	2,22	2,08	0,29
NM_004482	polypeptide n-acetylgalactosaminyltransferase 3; galnt3	2,21	1,89	2,06	2,05	0,16
XM_030485	similar to growth arrest and dna-damage-inducible protein gadd45 beta (negative growth-regulatory protein myd118) (myeloid differentiation primary response protein myd118) (h. sapiens); loc126563	2,67	2,15	1,09	1,97	0,80
NM_002776	kallikrein 10; klk10	2,00	2,14	1,55	1,90	0,31
XM_030144	h4 histone family, member g; h4fg	-6,80	-9,18	-9,45	-8,48	1,46
NM_004939	dead/h (asp-glu-ala-asp/his) box polypeptide 1; ddx1	-4,97	-5,71	-6,03	-5,57	0,54
NM_014184	hspc163 protein; hspc163	-5,47	-2,72	-5,41	-4,54	1,57
NM_004893	h2a histone family, member y; h2afy	-4,24	-4,43	-3,10	-3,92	0,72
NM_006595	apoptosis inhibitor 5; api5	-3,35	-2,96	-3,96	-3,42	0,51
NM_005969	nucleosome assembly protein 1-like 4; nap1l4	-1,52	-2,34	-5,97	-3,28	2,37
NM_002513	non-metastatic cells 3, protein expressed in; nme3	-4,13	-2,81	-2,87	-3,27	0,75
NM_006429	chaperonin containing tcp1, subunit 7 (eta); cct7	-2,25	-3,20	-3,99	-3,15	0,87
NM_002402	mesoderm specific transcript homolog (mouse); mest	-4,24	-1,63	-3,38	-3,08	1,33
NM_001545	immature colon carcinoma transcript 1; ict1	-3,89	-2,68	-1,90	-2,83	1,01
NM_004626	wingless-type mmtv integration site family, member 11 precursor; wnt11	-2,93	-2,71	-2,79	-2,81	0,11
NM_006430	chaperonin containing tcp1, subunit 4 (delta); cct4	-2,67	-2,67	-2,96	-2,76	0,17

NM_031243	heterogeneous nuclear ribonucleoprotein a2/b1, isoform b1; hnrpa2b1	-2,60	-2,77	-2,87	-2,75	0,14
NM_006402	hepatitis b virus x-interacting protein; xip	-1,99	-3,36	-2,86	-2,74	0,69
NM_020202	nit protein 2; nit2	-3,64	-2,58	-1,97	-2,73	0,85
NM_004356	cd81 antigen (target of antiproliferative antibody 1); cd81	-1,77	-3,05	-3,34	-2,72	0,84
NM_004765	b-cell cll/lymphoma 7c; bcl7c	-3,33	-2,82	-1,92	-2,69	0,71
NM_012403	pp32 related 1; pp32r1	-2,43	-2,87	-2,68	-2,66	0,22
NM_016097	hspc039 protein; hspc039	-2,92	-2,64	-2,40	-2,65	0,26
NM_016161	alpha-1,4-n-acetylglucosaminyltransferase; loc51146	-4,08	-1,44	-2,37	-2,63	1,34
NM_000244	multiple endocrine neoplasia i; men1	-2,47	-1,59	-3,82	-2,63	1,12
NM_020158	exosome component rrp46; rrp46	-2,71	-2,42	-2,69	-2,61	0,17
NM_014267	small acidic protein; image145052	-1,72	-2,98	-2,68	-2,46	0,66
NM_006397	ribonuclease hi, large subunit; rnasehi	-3,12	-1,54	-2,67	-2,44	0,81
NM_006764	interferon-related developmental regulator 2; ifrd2	-2,45	-2,19	-2,64	-2,42	0,23
NM_006305	putative human hla class ii associated protein i; phap1	-2,46	-2,86	-1,90	-2,41	0,48
XM_012128	ny-ren-58 antigen; loc51134	-1,50	-2,16	-3,47	-2,38	1,00
NM_000179	muts homolog 6 (e. coli); msh6	-3,42	-1,47	-2,21	-2,37	0,98
NM_000269	non-metastatic cells 1 protein; nme1	-1,59	-2,56	-2,92	-2,36	0,69
NM_004653	smcy homolog, y chromosome (mouse); smcy	-1,99	-2,55	-2,46	-2,33	0,30
NM_001196	bh3 interacting domain death agonist; bid	-3,43	-2,01	-1,43	-2,29	1,03
NM_004760	serine/threonine kinase 17a (apoptosis-inducing); stk17a	-2,20	-2,30	-2,10	-2,20	0,10
NM_002692	polymerase (dna directed), epsilon 2; pole2	-2,16	-2,19	-2,26	-2,20	0,05
NM_012321	u6 snrna-associated sm-like protein; lsm4	-1,47	-2,21	-2,84	-2,17	0,68
NM_006445	u5 snrnp-specific protein; prpf8	-1,42	-2,25	-2,75	-2,14	0,67
NM_002945	replication protein a1 (70kd); rpa1	-1,64	-2,49	-2,28	-2,13	0,44
AF151045	hspc211	-2,14	-2,38	-1,75	-2,09	0,32
NM_004818	prp28, u5 snrnp 100 kd protein; u5-100k	-2,72	-2,08	-1,42	-2,07	0,65
NM_001356	dead/h (asp-glu-ala-asp/his) box polypeptide 3; ddx3	-1,32	-2,17	-2,68	-2,05	0,68
NM_005646	tar (hiv) rna binding protein 1; tarbp1	-2,59	-1,40	-2,12	-2,03	0,60
NM_006940	sry (sex determining region y)-box 5; sox5	-2,19	-2,16	-1,75	-2,03	0,25
NM_016310	dna directed rna polymerase iii polypeptide k; polr3k	-2,18	-2,08	-1,80	-2,02	0,20
NM_017423	polypeptide n-acetylgalactosaminyltransferase 7; galnt7	-2,27	-1,51	-2,24	-2,01	0,43
NM_006938	small nuclear ribonucleoprotein d1 polypeptide (16kd); snrpd1	-2,05	-2,08	-1,72	-1,95	0,20
NM_002691	polymerase (dna directed), delta 1, catalytic subunit (125kd); pold1	-1,35	-2,41	-2,00	-1,92	0,53
NM_016059	peptidylprolyl isomerase (cyclophilin)-like 1; ppil1	-1,59	-2,08	-2,06	-1,91	0,28
NM_007375	tar dna binding protein; tardbp	-1,21	-2,16	-2,27	-1,88	0,58
Unknown						
		-Zn/+Zn: fold change				
Acc. no.	**Encoded protein**	**Exp. 1**	**Exp. 2**	**Exp. 3**	**Mean**	**SD**
NM_016140	brain specific protein; loc51673	5,85	4,51	4,54	4,97	0,77
NM_015946	cgi-17 protein; pelo	2,52	2,83	3,70	3,02	0,61
NM_007268	ig superfamily protein; z39ig	3,03	3,06	2,14	2,74	0,53
NM_014138	pro0659 protein; pro0659	2,87	1,86	2,83	2,52	0,57
NM_018491	cobw-like protein; loc55871	2,07	2,21	3,22	2,50	0,63
NM_014039	ptd012 protein; ptd012	2,57	2,63	2,28	2,49	0,19
AF223467	npd008 protein; npd008	1,95	2,82	2,59	2,45	0,45
NM_006460	hmba-inducible; his1	2,29	2,02	2,54	2,28	0,26
NM_016078	cgi-148 protein; loc51030	2,29	2,21	2,10	2,20	0,09
NM_018445	ad-015 protein; loc55829	2,17	2,68	1,50	2,11	0,59
NM_003831	sudd (suppressor of bimd6, aspergillus nidulans) homolog; sudd	2,30	2,25	1,29	1,95	0,57
NM_016027	cgi-83 protein; loc51110	1,61	2,03	2,04	1,89	0,24
BC012078	unknown (protein for mgc:19976)	-4,63	-3,96	-2,98	-3,86	0,83
BC009441	unknown (protein for mgc:15858)	-2,20	-3,42	-5,42	-3,68	1,62
NM_016028	cgi-85 protein; loc51111	-4,13	-4,03	-2,29	-3,48	1,04
NM_080390	my048 protein; my048	-3,29	-3,48	-2,25	-3,01	0,66
NM_015703	cgi-96 protein; cgi-96	-3,14	-2,72	-2,04	-2,64	0,55
BC002509	unknown (protein for mgc:2941)	-1,63	-2,45	-3,71	-2,60	1,05

NM_016044	cgi-105 protein; loc51011	-2,66	-2,64	-2,27	-2,52	0,22
NM_016271	strin protein; strin	-2,18	-2,81	-2,53	-2,51	0,32
NM_016055	cgi-118 protein; mrpl48	-2,44	-2,90	-2,13	-2,49	0,39
NM_014111	pro2086 protein; pro2086	-2,23	-2,42	-2,27	-2,31	0,10
NM_003634	nipsnap homolog 1; nipsnap1	-2,67	-2,73	-1,47	-2,29	0,71
NM_018477	uncharacterized hypothalamus protein harp11; harp11	-2,71	-1,60	-2,38	-2,23	0,57
NM_015698	t54 protein; t54	-2,60	-1,78	-2,02	-2,13	0,42
NM_014008	jm1 protein; jm1	-2,33	-1,51	-2,34	-2,06	0,48
XM_011354	serologically defined colon cancer antigen 10; sdccag10	-2,28	-2,18	-1,68	-2,05	0,32
NM_006589	chromosome 1 open reading frame 2; c1orf2	-2,14	-1,44	-2,05	-1,88	0,38
NM_018442	pc326 protein; pc326	-2,03	-2,08	-1,34	-1,82	0,42
Hypothetical						
		-Zn/+Zn: fold change				
Acc. no.	**Encoded protein**	**Exp. 1**	**Exp. 2**	**Exp. 3**	**Mean**	**SD**
NM_016410	hypothetical protein; hspc177	2,27	2,44	3,23	2,65	0,51
NM_020317	hypothetical protein dj465n24.2.1; dj465n24.2.1	2,37	3,11	2,39	2,62	0,42
XM_006601	hypothetical protein flj10659; flj10659	2,31	1,90	2,56	2,26	0,33
NM_017813	hypothetical protein flj20421; flj20421	2,25	2,32	2,08	2,22	0,12
NM_024107	hypothetical protein mgc3123; mgc3123	2,52	1,66	2,39	2,19	0,46
NM_018988	hypothetical protein; flj20330	2,41	2,22	1,79	2,14	0,32
NM_017633	hypothetical protein flj20037; flj20037	2,27	2,08	2,06	2,14	0,12
NM_024681	hypothetical protein flj12242; flj12242	1,86	2,15	2,20	2,07	0,18
NM_018095	hypothetical protein flj10450; flj10450	2,20	2,05	1,37	1,87	0,44
NM_024707	hypothetical protein flj13956; flj13956	-3,78	-3,91	-4,28	-3,99	0,26
NM_024927	hypothetical protein flj21019; flj21019	-4,63	-3,95	-3,24	-3,94	0,70
NM_018087	hypothetical protein flj10407; flj10407	-4,92	-4,21	-2,38	-3,83	1,31
XM_071438	hypothetical protein flj14069; flj14069	-2,10	-3,48	-5,26	-3,61	1,58
NM_018034	hypothetical protein flj10233; flj10233	-4,20	-1,65	-3,27	-3,04	1,29
XM_040853	hypothetical protein xp_040853; loc94573	-1,98	-3,63	-2,69	-2,77	0,83
NM_017615	hypothetical protein flj20003; flj20003	-4,95	-1,31	-2,04	-2,77	1,93
NM_019095	hypothetical protein; loc54675	-3,04	-2,64	-2,09	-2,59	0,48
NM_018010	hypothetical protein flj10147; flj10147	-3,06	-2,66	-2,04	-2,58	0,51
NM_014388	novel putative protein similar to yil091c yeast hypothetical 84 kd protein from sga1-ktr7; dj434o14.5	-3,22	-2,31	-1,90	-2,48	0,68
NM_018307	hypothetical protein flj11040; flj11040	-3,04	-2,02	-2,33	-2,46	0,52
BC000953	hypothetical protein flj11159	-2,92	-2,42	-1,74	-2,36	0,60
XM_052092	wd40 repeat domain 11 protein; wdr11	-3,12	-2,27	-1,68	-2,36	0,72
NM_030577	hypothetical protein mgc10993; mgc10993	-3,68	-2,22	-1,08	-2,33	1,30
NM_024844	hypothetical protein flj12549; flj12549	-2,74	-1,69	-2,50	-2,31	0,55
NM_018035	hypothetical protein flj10241; flj10241	-1,67	-2,13	-3,03	-2,28	0,69
NM_018122	hypothetical protein flj10514; flj10514	-2,67	-2,10	-1,81	-2,19	0,44
NM_021925	hypothetical protein flj21820; flj21820	-2,36	-2,30	-1,85	-2,17	0,28
NM_017816	hypothetical protein flj20425; flj20425	-2,06	-2,37	-2,03	-2,16	0,19
NM_016468	hypothetical protein; loc51241	-2,12	-1,99	-2,17	-2,09	0,10
XM_071638	hypothetical protein xp_071638; loc139328	-2,05	-2,12	-2,10	-2,09	0,03
NM_020153	hypothetical protein; loc56912	-2,60	-2,12	-1,54	-2,08	0,53
NM_015918	hypothetical protein; hspc004	-2,08	-2,03	-2,10	-2,07	0,04
NM_024856	hypothetical protein flj12983; flj12983	-2,07	-2,40	-1,70	-2,06	0,35
NM_018077	hypothetical protein flj10377; flj10377	-2,32	-1,62	-2,13	-2,02	0,36
NM_024524	hypothetical protein flj20986; flj20986	-1,39	-2,23	-2,37	-2,00	0,53
XM_003666	hypothetical protein flj20124	-2,28	-2,00	-1,61	-1,96	0,34
AL080140	hypothetical protein; dkfzp434l243	-2,00	-2,15	-1,58	-1,91	0,30
NM_018360	hypothetical protein flj11209; flj11209	-1,45	-2,14	-2,14	-1,91	0,40
NM_022068	hypothetical protein flj23403; flj23403	-2,11	-2,03	-1,48	-1,87	0,34
NM_017806	hypothetical protein flj20406; flj20406	-2,10	-1,37	-2,12	-1,86	0,43

9.2 Primers

Sequences of primers used for Northern blot analysis and real-time RT-PCR are listed below.

9.2.1 Zinc supplementation

Tab. 8: Oligonucleotide primers used for Northern blot analysis in zinc supplementation studies. Genes were originally identified by array analysis in response to zinc supplementation in HT-29 cells and primers were used for independent confirmation by Northern blot analysis.

Acc. no.	Encoded protein	Sequence of forward primer (FP) and backward primer (BP)	Amplicon size
NM002046	Glyceraldehyde-3-phosphate dehydrogenase	FP: 5´-GAC CAC AGT CCA TGC CAT CAC T-3´ BP: 5´-TCC ACC ACC CTG TTG CTG TAG-3´	453 bp
X64177	Metallothionein-1	FP: 5´-ATG GAC CCC AAC TGC TCC TGC-3´ BP: 5´-GGA ATG TAG CAA ATG AGT CGG-3´	279 bp
AF000985	DEAD box protein 3, Y-chromosomal	FP: 5´-TTG GCT GTA GGC AGA GTA GGC-3´ BP: 5´-ATG TCT AGT CCT CGT GCT GCC-3´	314 bp
D29810	Hypothetical 40 kD protein	FP: 5´-GGA ATC CAT GTT TCT GGA CGC-3´ BP: 5´-CAG GCT CTC TGT ACA CAG TCC-3´	687 bp
M11147	Ferritin light chain	FP: 5´-ATT ATT CCA CCG ACG TGG AGG-3´ BP: 5´-TAG TCG TGC TTG AGA GTG AGC-3´	505 bp
AF039699	Antigen NY-CO-38	FP: 5´-CTT CAT CAG CCT GGT AGG CTC-3´ BP: 5´-CAT CTC CTT CCG GTA TCT CTC-3´	445 bp
AF043724	Hepatitis A virus cellular receptor 1	FP: 5´-GCC TCA TCC TAC ATC TGG CAG-3´ BP: 5´-TGG AAT GCT CGT TGT CGT TGG-3´	551 bp

Tab. 9: Oligonucleotide primers used for real-time RT-PCR in zinc supplementation studies. Genes were originally identified by array analysis in response to zinc supplementation in HT-29 cells and primers were used for independent confirmation by real-time RT-PCR; T_m, melting point of the PCR product, u. p., unspecific PCR product obtained.

Acc. no.	Encoded protein	Sequence of forward primer (FP) and backward primer (BP)	Amplicon size	T_m (two-step)
NM002046	Glyceraldehyde-3-phosphate dehydrogenase	FP: 5´-GGC TCT CCA GAA CAT CAT CCC TGC-3´ BP: 5´-GGG TGT CGC TGT TGA AGT CAG AGG-3´	269 bp	89.3 °C
X64177	Metallothionein-1	FP: 5´-CTC CTG CAA GAA GAG CTG CT-3´ BP: 5´-TCA GGC ACA GCA GCT GCA CTT-3´	106 bp	90.1 °C
M85085	Cleavage stimulation factor, 64 kD subunit	FP: 5´-AGG CTT GTT AGG AGA TGC-3´ BP: 5´-CTG GGC TCG GGT AAT G-3´	186 bp	87.4 °C
X68687	Zinc finger protein 33a	FP: 5´-AAC CCA TAC ACA AGA AAA GCC C-3´ BP: 5´-CCC TGT GAT GTA CTG TGA GTG A-3´	170 bp	81.1 °C
D83032	Nuclear protein, NP220	FP: 5´-TCC GTC GGT CTC GAA G-3´ BP: 5´-CCA TGC CCA GAT CGT T-3´	250 bp	82.0 °C
L40357	Thyroid receptor interacting protein 7	FP: 5´-CCA CAA GAC GGT CTG C-3´ BP: 5´-CTC CCT CGT TAT CTA CAG ATT-3´	228 bp	u. p.
M97856	Nuclear autoantigenic sperm protein	FP: 5´-ATG GGG TTG ATA CCA AGG-3´ BP: 5´-AGC TCT AGG TTC CCA AT-3	241 bp	81.4 °C
AF058718	Putative 13S golgi transport complex 90 kD subunit	FP: 5´-GAC GAG AAT TGG GGC T-3´ BP: 5´-GCT CTA GTA GGC GCT T-3´	341 bp	u. p.
D29810	Hypothetical 40 kD protein	FP: 5´-GCA CGT TTA TTA GAG TGA ATC CTA CCC-3´ BP: 5´-CTA CTG CGA GGT TGT AGT GGT TGC-3´	218 bp	84.8 °C
X72875	Complement factor B	FP: 5´-ATC CGG GAC TTG CTA TAC-3´ BP: 5´-CCC ATA CAG CTC TCG TG-3´	326 bp	85.0 °C
D29956	Ubiquitin carboxyl-terminal hydrolase 11	FP: 5´-TCC TAC GAT GGC AGG T-3´ BP: 5´-AGT TAC TCG TGG TCC C-3´	300 bp	81.5 °C
M11147	Ferritin light chain	FP: 5´-ATT ATT CCA CCG ACG TGG AGG-3´ BP: 5´-TAG TCG TGC TTG AGA GTG AGC-3´	505 bp	89.7 °C
AF022080	RAS-related protein R-RAS3	FP: 5´-CTC ATC GTC TAC TCC GTC ACT G-3´ BP: 5´-ATC AAA TCG ACC TTG TTG GC-3´	128 bp	87.2 °C
J04469	Creatine kinase, ubiquitous mitochondrial	FP: 5´-AGT GTT TGA AAG ATT CTG CC-3´ BP: 5´-TTT GCC TAG TCG GTC C-3´	289 bp	86.4 °C
D37931	Ribonuclease 4	FP: 5´-ACT TGA TGA TGC AAA GAC G-3´ BP: 5´-CGT CTA GTG CTC GCT AT-3´	229 bp	85.5 °C
AF048700	Gastrointestinal peptide	FP: 5´-CAT GGT AGA GTC TCC AAC CTG TTC-3´ BP: 5´-ACT GTT CTC CAG CTG ACA CTT CAT-3´	191 bp	85.3 °C
AF039699	Antigen NY-CO-38	FP: 5´-GAC ACA TCG GCC TGA TCC-3´ BP: 5´-ACC TTC TTC TCC TTG TTT TCC C-3´	142 bp	87.9 °C
AF043724	Hepatitis A virus cellular receptor 1	FP: 5´-GGA GAT TGT GCC ACC CAA GGT CAC-3´ BP: 5´-GCT ACT GGT TCA TGG TTC TGC CTG-3´	297 bp	86.3 °C

9.2.2 Zinc depletion

Tab. 10: Oligonucleotide primers used for Northern blot analysis in zinc depletion studies. Genes were originally identified by array analysis in response to intracellular zinc depletion in HT-29 cells and primers were used for independent confirmation by Northern blot analysis.

Acc. no.	Encoded protein	Sequence of forward primer (FP) and backward primer (BP)	Amplicon size
NM002046	Glyceraldehyde-3-phosphate dehydrogenase	FP: 5´-GAC CAC AGT CCA TGC CAT CAC T-3´ BP: 5´-TCC ACC ACC CTG TTG CTG TAG-3´	453 bp
X64177	Metallothionein-1	FP: 5´-ATG GAC CCC AAC TGC TCC TGC-3´ BP: 5´-GGA ATG TAG CAA ATG AGT CGG-3´	279 bp
NM004235	Kruppel-like factor 4	FP: 5´-CAG AAT TGG ACC CGG TGT AC-3´ BP: 5´-TTG CTG ACG CTG ATG ACC-3´	139 bp
NM003447	Zink finger protein 165	FP: 5´-AAG CAG TAC TCC AGG TTC AAG C-3´ BP: 5´-ACC TTC ATC CTG GGC AGA C-3´	365 bp
NM006526	Zink finger protein 217	FP: 5´-AGA CGG ATC TGA GGA TGG G-3´ BP: 5´-TTT TCA AAT TTT TGG TTT GCG-3´	350 bp
NM002495	NADH dehydrogenase Fe-S protein 4	FP: 5´-AGA TTG GCA CAG GAC CAG AC-3´ BP: 5´-CGC TCC CTG GTA TCA AAC TC-3´	197 bp
NM004864	Prostate differentiation factor	FP: 5´-GAG AGT TGC GGA AAC GCT AC-3´ BP: 5´-AGT GCA ACT CCA GCT GGG-3´	390 bp

Tab. 11: Oligonucleotide primers used for real-time RT-PCR in zinc depletion studies. Genes were originally identified by array analysis in response to intracellular zinc depletion in HT-29 cells and primers were used for independent confirmation by real-time RT-PCR; T_m, melting point of the PCR product, u. p., unspecific PCR product obtained.

Acc. no.	Encoded protein	Sequence of forward primer (FP) and backward primer (BP)	Amplicon size	T_m (two-step)
X03205	18S ribosomal RNA	FP: 5´-CCA AAG TCT TTG GGT TCC GG -3´ BP: 5´-ACC AAC TAA GAA CGG CCA TG -3´	205 bp	86.9 °C
X64177	Metallothionein-1	FP: 5´-CTC CTG CAA GAA GAG CTG CT-3´ BP: 5´-TCA GGC ACA GCA GCT GCA CTT-3´	106 bp	90.1 °C
NM000780	Cytochrome P450 subfamily VIIa	FP: 5´-TTC CAG CCC TGG TAG CAG-3´ BP: 5´-TCT CCA GAT CAT CAA AGG TGG-3´	177 bp	u. p.
NM014184	Hspc163 protein	FP: 5´-TAC TGC TCA TGT CAT TGC ACT-3´ BP: 5´-TTC ATG TGT GAC TTC AGC TGC-3´	154 bp	82.3 °C
NM003122	Serin protease inhibitor	FP: 5´-ATC TTT CTT CTC AGT GCC TTG G-3´ BP: 5´-GGA TAG AAG TCT GGC GTT TCC-3´	199 bp	83.2 °C
NM004939	DEAD/H box polypetide 1	FP: 5´-CAA GTT AGA TTC CTG GTC CTG G-3´ BP: 5´-CAA GCC TTT CCC AGA GTC TG-3´	292 bp	82.1 °C
NM004235	Kruppel-like factor 4	FP: 5´-CAG AAT TGG ACC CGG TGT AC-3´ BP: 5´-TTG CTG ACG CTG ATG ACC-3´	139 bp	92.6 °C
NM002495	NADH dehydrogenase Fe-S protein 4	FP: 5´-AGA TTG GCA CAG GAC CAG AC-3´ BP: 5´-CGC TCC CTG GTA TCA AAC TC-3´	197 bp	81.4 °C
NM003447	Zink finger protein 165	FP: 5´-AAG CAG TAC TCC AGG TTC AAG C-3´ BP: 5´-ACC TTC ATC CTG GGC AGA C-3´	365 bp	83.3 °C
NM000434	Sialidase 1	FP: 5´-ACA CCC CGG AAT CTC TCC-3´ BP: 5´-CCA GGA GGC ACC ATG ATC-3´	174 bp	89.2 °C
XM030144	H4 histone family member	FP: 5´-TGC TAA GCG CCA TCG TAA G-3´ BP: 5´-AGC CAT ACA GAG TGC GCC-3´	257 bp	86.6 °C
NM002786	Proteasome subunit	FP: 5´-TCG ATA GAC CAC TGC CTG TG-3´ BP: 5´-AGG GCC CAT ATC ATC ATA ACC-3´	131 bp	83.0 °C
NM004390	Cathepsin H	FP: 5´-GGC AGT TGC TGG ACT TTC TC-3´ BP: 5´-GGA TAT ACT CGA AAG CCT GGC-3´	166 bp	89.2 °C
NM002080	Aspartate aminotransferase 2	FP: 5´-TTG CGG TTT TGA CTT CAC AG-3´ BP: 5´-ACC ACT GGC AAA GCC TTG-3´	199 bp	85.9 °C
NM006526	Zink finger protein 217	FP: 5´-AGA CGG ATC TGA GGA TGG G-3´ BP: 5´-TTT TCA AAT TTT TGG TTT GCG-3´	350 bp	u. p.
NM004864	Prostate differentiation factor	FP: 5´-GAG AGT TGC GGA AAC GCT AC-3´ BP: 5´-AGT GCA ACT CCA GCT GGG-3´	390 bp	u. p.

9.2.3 mRNA levels of zinc-dependent proteins

Tab. 12: Oligonucleotide primers used for real-time RT-PCR of zinc-dependent proteins. Genes were originally identified to show changes in protein levels by 2D-PAGE

in response to zinc depletion and/or zinc supplementation in HT-29 cells and primers were used for assessment of corresponding mRNA levels by real-time RT-PCR; T_m, melting point of the PCR product.

Acc. no.	Encoded protein	Sequence of forward primer (FP) and backward primer (BP)	Amplicon size	T_m (two-step)
X03205	18S ribosomal RNA	FP: 5´-CCA AAG TCT TTG GGT TCC GG -3´ BP: 5´-ACC AAC TAA GAA CGG CCA TG -3´	205 bp	86.9 °C
BC014623	Serine proteinase inhibitor	FP. 5´-AAC TAC TAC GAC GAC GAG-3´ BP: 5´-TGC CAT CTG TGT CCA AC-3´	361 bp	89.0 °C
AF026292	Chaperonin containing t-complex	FP: 5`-CAA GGA CTA TTC CAG GAA AAC-3´ BP: 5´-ACA GAC ACG ATC AGG C-3´	277 bp	88.2 °C
NM002654	Pyruvate kinase, muscle	FP: 5´-GAT TGG ACG GTG CAA C-3´ BP: 5´-GGT CGC TGG TAA TGG G-3´	284 bp	89.7 °C
BC015529	Ribose 5-phosphate isomerase	FP: 5´-GCT ATG CTA GTC GCT TCA-3´ BP: 5´-ATT TGT GTA CCC GGT CA-3´	243 bp	87.5 °C
BC033088	Lamin A	FP: 5´-TGG AAA CGG AGA ACG C-3´ BP: 5´-TGG TAT TGC ACG CTT TCA-3´	210 bp	91.8 °C
NM021074	NADH dehydrogenase flavoprotein 2	FP: 5´-ATT TAG CCC AAA GGC AG-3´ BP: 5´-TGG TGC GTT CAC ACA G-3´	302 bp	83.6 °C

9.2.4 MTF-1

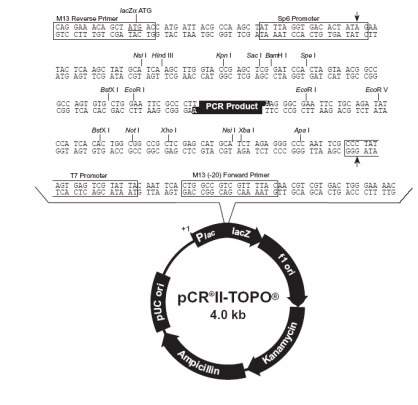
Tab. 13: Oligonucleotide primers used for real-time RT-PCR of MTF-1. Primers were used for detection and quantification of MTF-1 in the Tet-On/MTF-1 HT-29 cell line by real-time RT-PCR; T_m, melting point of the PCR product.

Acc. no.	Encoded protein	Sequence of forward primer (FP) and backward primer (BP)	Amplicon size	T_m (one-step)	T_m (two-step)
X03205	18S ribosomal RNA	FP: 5´-CCA AAG TCT TTG GGT TCC GG -3´ BP: 5´-ACC AAC TAA GAA CGG CCA TG -3´	205 bp	83.0 °C	86.9 °C
NM005955	Metal transcription factor-1	FP: 5´-GAG CCT TCT GTC CAC AGA TTC-3´ BP: 5´-GAG GCC AGG TTG CAG TAC AAG-3´	259 bp	80.2 °C	83.9 °C

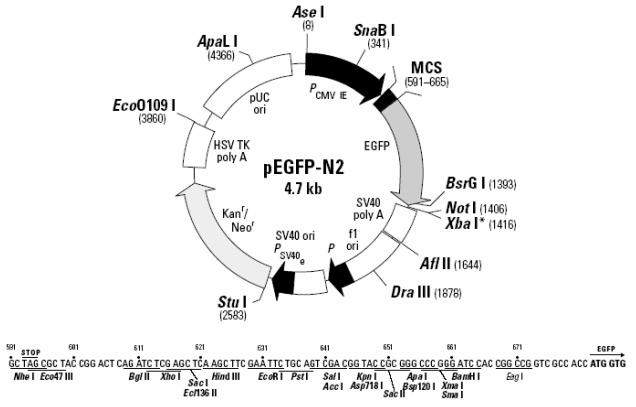
9.3 Vectors

For probe synthesis for Northern blot and construction of the MTF1/EGFP expression plasmid the following commercial available vectors were used:

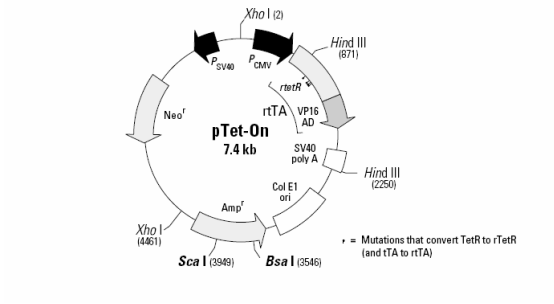
pCRII-TOPO (Invitrogen, Karlsruhe, Germany)



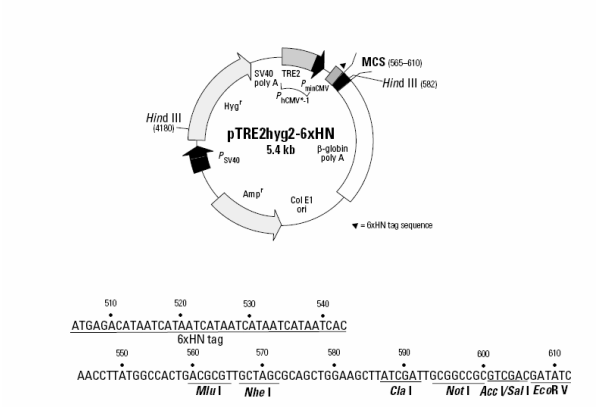
pEGFP-N2 (BD Clontech, Heidelberg, Germany)



pTet-On (BD Clontech, Heidelberg, Germany)



pTRE2hyg2-6xHN (BD Clontech, Heidelberg, Germany)



9.4 MTF-1 plasmids

9.4.1 pMTF1

The following plasmid, comprising the coding sequence (CDS) of human MTF-1 (3123-5384, shown in bold letters), cloned into the expression vector pcDNA I/Amp (Invitrogen, La Jolla, CA, USA) was generously provided by W. Schaffner.

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  1 GGCGTAATCT GCTGCTTGCA AACAAAAAAA CCACCGCTAC CAGCGGTGGT TTGTTTGCCG
 61 GATCAAGAGC TACCAACTCT TTTTCCGAAG GTAACTGGCT TCAGCAGAGC GCAGATACCA
121 AATACTGTCC TTCTAGTGTA GCCGTAGTTA GGCCACCACT TCAAGAACTC TGTAGCACCG
181 CCTACATACC TCGCTCTGCT AATCCTGTTA CCAGTGGCTG CTGCCAGTGG CGATAAGTCG
241 TGTCTTACCG GGTTGGACTC AAGACGATAG TTACCGGATA AGGCGCAGCG GTCGGGCTGA
301 ACGGGGGGTT CGTGCACACA GCCCAGCTTG GAGCGAACGA CCTACACCGA ACTGAGATAC
361 CTACAGCGTG AGCATTGAGA AAGCGCCACG CTTCCCGAAG GGAGAAAGGC GGACAGGTAT
421 CCGGTAAGCG GCAGGGTCGG AACAGGAGAG CGCACGAGGG AGCTTCCAGG GGGAAACGCC
481 TGGTATCTTT ATAGTCCTGT CGGGTTTCGC CACCTCTGAC TTGAGCGTCG ATTTTTGTGA
541 TGCTCGTCAG GGGGGCGGAG CCTATGGAAA AACGCCAGCA ACGCAAGCTA GCTTCTAGCT
601 AGAAATTGTA AACGTTAATA TTTTGTTAAA ATTCGCGTTA AATTTTTGTT AAATCAGCTC
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661 ATTTTTTAAC CAATAGGCCG AAATCGGCAA AATCCCTTAT AAATCAAAAG AATAGCCCGA
721 GATAGGGTTG AGTGTTGTTC CAGTTTGGAA CAAGAGTCCA CTATTAAAGA ACGTGGACTC
781 CAACGTCAAA GGGCGAAAAA CCGTCTATCA GGGCGATGGC CGCCCACTAC GTGAACCATC
841 ACCCAAATCA AGTTTTTTGG GGTCGAGGTG CCGTAAAGCA CTAAATCGGA ACCCTAAAGG
901 GAGCCCCCGA TTTAGAGCTT GACGGGGAAA GCCGGCGAAC GTGGCGAGAA AGGAAGGGAA
961 GAAAGCGAAA GGAGCGGGCG CTAGGGCGCT GGCAAGTGTA GCGGTCACGC TGCGCGTAAC
1021 CACCACACCC GCCGCGCTTA ATGCGCCGCT ACAGGGCGCG TACTATGGTT GCTTTGACGA
1081 GACCGTATAA CGTGCTTTCC TCGTTGGAAT CAGAGCGGGA GCTAAACAGG AGGCCGATTA
1141 AAGGGATTTT AGACAGGAAC GGTACGCCAG CTGGATTACC AAAGGGCCTC GTGATACGCC
1201 TATTTTTATA GGTTAATGTC ATGATAATAA TGGTTTCTTA GACGTCAGGT GGCACTTTTC
1261 GGGGAAATGT GCGCGGAACC CCTATTTGTT TATTTTTCTA AATACATTCA AATATGTATC
1321 CGCTCATGAG ACAATAACCC TGATAAATGC TTCAATAATA TTGAAAAAGG AAGAGTATGA
1381 GTATTCAACA TTTCCGTGTC GCCCTTATTC CCTTTTTTGC GGCATTTTGC CTTCCTGTTT
1441 TTGCTCACCC AGAAACGCTG GTGAAAGTAA AAGATGCTGA AGATCAGTTG GGTGCACGAG
1501 TGGGTTACAT CGAACTGGAT CTCAACAGCG GTAAGATCCT TGAGAGTTTT CGCCCCGAAG
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1681 AGTACTCACC AGTCACAGAA AAGCATCTTA CGGATGGCAT GACAGTAAGA GAATTATGCA
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2461 GTATGTTCCC ATAGTAACGC CAATAGGGAC TTTCCATTGA CGTCAATGGG TGGACTATTT
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2581 TGACGTCAAT GACGGTAAAT GGCCCGCCTG GCATTATGCC CAGTACATGA CCTTATGGGA
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2701 TTGGCAGTAC ATCAATGGGC GTGGATAGCG GTTTGACTCA CGGGGATTTC CAAGTCTCCA
2761 CCCCATTGAC GTCAATGGGA GTTTGTTTTG GCACCAAAAT CAACGGGACT TTCCAAAATG
2821 TCGTAACAAC TCCGCCCCAT TGACGCAAAT GGGCGGTAGG CGTGTACGGT GGGAGGTCTA
2881 TATAAGCAGA GCTCTCTGGC TAACTAGAGA ACCCACTGCT TACTGGCTTA TCGAAATTAA
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3001 AATTCGATAC TCCGGAGCCC GTCAGTATCG GCGGAATTCC GGTGCTGCCG CCGTTGCCGG
3061 GAGCCGCGGA GACAAGTCAT TACGTTTTCA TTTCTCACTA CTGGGCTGAG CACAACTGAA
3121 CC**ATGGGGGA ACACAGTCCA GACAACAACA TCATCTACTT TGAGGCAGAG GAAGATGAGC**
3181 **TGACCCCCGA TGATAAAATG CTCAGGTTTG TGGATAAAAA CGGACTGGTG CCTTCCTCAT**
3241 **CTGGAACTGT TTATGATAGG ACCACTGTTC TTATTGAGCA GGACCCTGGC ACTTTGGAGG**
3301 **ATGAAGATGA CGACGGACAG TGCGGAGAAC ACTTGCCTTT TCTAGTAGGG GGTGAAGAGG**
3361 **GCTTTCACCT GATAGATCAT GAAGCAATGT CCCAGGGTTA TGTGCAGCAC ATTATCTCAC**
3421 **CAGATCAGAT TCATTTGACA ATAAACCCTG GTTCCACACC CATGCCAAGA AATATTGAAG**
3481 **GTGCAACCCT CACTCTGCAG TCGGAATGTC CGGAAACAAA ACGTAAAGAA GTAAAGCGGT**
3541 **ACCAATGTAC CTTTGAGGGC TGTCCCCGCA CCTACAGCAC AGCAGGCAAC CTGCGAACCC**
3601 **ACCAGAAGAC TCACCGAGGA GAGTACACCT TTGTCTGTAA TCAGGAGGGC TGTGGCAAAG**
3661 **CCTTCCTTAC CTCTCACAGC CTCAGGATCC ACGTGCGAGT GCACACGAAG GAGAAGCCAT**
3721 **TTGAGTGTGA CGTGCAGGGC TGTGAGAAGG CATTCAACAC ACTGTACAGG CTGAAAGCAC**
3781 **ATCAGAGGCT TCACACAGGG AAAACGTTTA ACTGTGAATC TGAAGGCTGC AGCAAATACT**
3841 **TCACCACACT CAGTGATCTG AGGAAGCACA TTCGAACTCA TACAGGGGAA AAGCCATTTC**
3901 **GGTGCGATCA CGATGGCTGT GGAAAAGCAT TTGCAGCAAG CCACCACCTT AAAACTCACG**
3961 **TTCGTACACA TACTGGTGAA AGACCCTTCT TCTGCCCCAG TAATGGCTGT GAGAAAACAT**
4021 **TCAGCACTCA ATACAGTCTC AAAAGTCACA TGAAAGGTCA TGATAACAAA GGACACTCAT**
4081 **ACAATGCACT TCCACAACAC AATGGATCAG AGGATACAAA TCACTCACTT TGTCTAAGTG**
4141 **ACTTGAGCCT TCTGTCCACA GATTCTGAAT TGCGAGAAAA TTCCAGTACG ACCCAGGGCC**
4201 **AGGACCTCAG CACAATTTCA CCAGCAATCA TCTTTGAATC AATGTTCCAG AATTCAGATG**
4261 **ATACGGCAAT TCAGGAAGAT CCTCAACAGA CAGCTTCCTT GACTGAAAGT TTTAATGGTG**
4321 **ATGCAGAGTC AGTCAGTGAT GTTCCGCCAT CCACAGGAAA TTCAGCATCT TTATCTCTTC**
4381 **CACTTGTACT GCAACCTGGC CTCTCCGAGC CACCCCAGCC TCTACTACCT GCCTCAGCTC**
4441 **CGTCTGCTCC TCCGCCTGCT CCCTCCCTAG GACCTGGCTC CCAGCAAGCT GCATTTGGCA**
4501 **ACCCCCCTGC TCTCTTACAA CCTCCAGAAG TGCCTGTTCC CCACAGCACA CAGTTTGCTG**
4561 **CTAATCATCA AGAGTTTCTT CCGCACCCCC AGGCACCGCA GCCCATTGTA CCAGGACTTT**
4621 **CTGTTGTTGC TGGGGCTTCT GCATCAGCAG CGGCAGTGGC ATCAGCTGTG GCAGCACCAG**
4681 **CCCCACCACA AAGTACTACT GAGCCCCTGC CAGCCATGGT CCAGACTCTG CCCCTGGGTG**

4741 **CCAACTCTGT CCTAACTAAT AATCCCACAA TAACCATCAC CCCAACTCCC AACACAGCTA**
4801 **TCCTGCAGTC CAGCCTAGTC ATGGGAGAAC AGAACTTACA ATGGATATTA AATGGTGCCA**
4861 **CCAGTTCTCC ACAAAACCAA GAACAAATTC AGCAAGCATC TAAAGTTGAG AAGGTGTTTT**
4921 **TTACCACTGC AGTACCAGTA GCCAGTAGCC CAGGGAGCTC TGTCCAGCAG ATTGGCCTCA**
4981 **GTGTTCCTGT GATCATCATC AAACAAGAAG AGGCATGTCA GTGTCAGTGT GCATGCCGGG**
5041 **ACTCTGCAAA GGAGCGGGCA TCCAGCAGGA GAAAGGGCTG CTCCTCCCCA CCCCCTCCAG**
5101 **AGCCGAGCCC CCAGGCTCCT GATGGGCCCA GCCTGCAGCT CCCAGCGCAG ACTTTCTCTT**
5161 **CAGCCCCTGT TCCCGGGTCA TCATCCTCTA CCTTGCCCTC CTCCTGTGAG CAAAGCCGAC**
5221 **AAGCAGAGAC TCCTTCAGAC CCTCAGACAG AAACATTAAG TGCCATGGAT GTGTCAGAGT**
5281 **TTCTATCCCT CCAGAGCCTG GACACCCCGT CCAATCTGAT TCCCATTGAA GCACTACTGC**
5341 **AGGGGGAGGA GGAGATGGGC CTCACCAGCA GCTTCTCCAA GTGA**AGGGCC CATGTGTGCT
5401 CACCTCTGGG AAAAGCGGGT GAGCAGGAGG CATGAGGTAC AATGCCTGCC ATCATGGGTC
5461 AGAAATTTGA AGGATGAAGA AATCTACTGT TTGAAATCCT CACCTTTCAG ACGTATTTTC
5521 TTTATTCACA TCCCAGGAGC ATCCATTTTA AGGAACTATT CTTTGGAAAA AAACAAAAAA
5581 CAAAAAAAAC AACAAAAAAA GCTAAGTTAT AAGTGAACTG TTTGGCTGCA CTGTATGTCA
5641 CTTTTGCTTG TTGTCATGTG AACTTGGAAA CTAAGGTTAC TCGTGTGCAT AAAAATTCTA
5701 AATGAAAGGG TGTGGTTTCC ATCAATCTAT GCTGCCCATC GCTTGCACTG GGGTCTTTGT
5761 GGATCGGGCA GGAGTTTTCA GTGTGTTGGG TGTTGCTCCT TCCTATGTGT CTTTTGAATC
5821 TGAGGCTGAC ATTTGCTTGG AAGGCCAGAC CCTTGCTCCA TCAGAGAGGG CAGTGGCAAA
5881 GGCCAGTGAG GCAGCTGTGA GTTGGACAGG GTTCAGGTGA GATGGTGTTG TCATTTGTGC
5941 TTAGTGTTGG TGGTGCTCAG GGTGGATAAC ACGGGTCGTT CTGCAGCCCG CTTCAGCACA
6001 AATAGGCAGC TTAAGGCCTG GCTCACAGGC TGTGGGGTTG ATCTGGCTCT GCAGAGGCCC
6061 TAGGCAGCTT GTTGACTGCT GTCTGTTGAT GACGTGTGTG CAAAGCAGGC TCTAGCAACA
6121 TGATCACTGT CCTTGCCTTC CTGGTTCTTT CTCTCGGTTG GTTGCCAGGG CTTGCAGATC
6181 GCAGTGAATT TTCCTTGGGG AACATCGCTG TTTTGTCCTA GAGTGAACTT GTGGCTTATG
6241 GCCAGTGCTG TTTGGTGGTC TGCCTTCTTT TTAATGGTAT TTTCTTCCTC AGAGCAGAAG
6301 GGCTGCATTT TGCTTATCAG AAGAAGGTGC AGATTTAAGG GAATTCGATA TCAAGCTTAT
6361 CGATACCGTC GACCCGAGCA TGCATCTAGA GGATCTTTGT GAAGGAACCT TACTTCTGTG
6421 GTGTGACATA ATTGGACAAA CTACCTACAG AGATTTAAAG CTCTAAGGTA AATATAAAAT
6481 TTTTAAGTGT ATAATGTGTT AAACTACTGA TTCTAATTGT TTGTGTATTT TAGATTCCAA
6541 CCTATGGAAC TGATGAATGG GAGCAGTGGT GGAATGCCTT TAATGAGGAA AACCTGTTTT
6601 GCTCAGAAGA AATGCCATCT AGTGATGATG AGGCTACTGC TGACTCTCAA CATTCTACTC
6661 CTCCAAAAAA GAAGAGAAAG GTAGAAGACC CCAAGGACTT TCCTTCAGAA TTGCTAAGTT
6721 TTTTGAGTCA TGCTGTGTTT AGTAATAGAA CTCTTGCTTG CTTTGCTATT TACACCACAA
6781 AGGAAAAAGC TGCACTGCTA TACAAGAAAA TTATGGAAAA ATATTTGATG TATAGTGCCT
6841 TGACTAGAGA TCATAATCAG CCATACCACA TTTGTAGAGG TTTTACTTGC TTTAAAAAAC
6901 CTCCCACACC TCCCCCTGAA CCTGAAACAT AAAATGAATG CAATTGTTGT TGTTAACTTG
6961 TTTATTGCAG CTTATAATGG TTACAAATAA AGCAATAGCA TCACAAATTT CACAAATAAA
7021 GCATTTTTTT CACTGCATTC TAGTTGTGGT TTGTCCAAAC TCATCAATGT ATCTTATCAT
7081 GTCTGGATCA TCCCGCCATG GTATCAACGC CATATTTCTA TTTACAGTAG GGACCTCTTC
7141 GTTGTGTAGG TACCGCTGTA TTCCTAGGGA AATAGTAGAG GCACCTTGAA CTGTCTGCAT
7201 CAGCCATATA GCCCCCGCTG TTCGACTTAC AAACACAGGC ACAGTACTGA CAAACCCATA
7261 CACCTCCTCT GAAATACCCA TAGTTGCTAG GGCTGTCTCC GAACTCATTA CACCCTCCAA
7321 AGTCAGAGCT GTAATTTCGC CATCAAGGGC AGCGAGGGCT TCTCCAGATA AAATAGCTTC
7381 TGCCGAGAGT CCCGTAAGGG TAGACACTTC AGCTAATCCC TCGATGAGGT CTACTAGAAT
7441 AGTCAGTGCG GCTCCCATTT TGAAAATTCA CTTACTTGAT CAGCTTCAGA AGATGGCGGA
7501 GGGCCTCCAA CACAGTAATT TTCCTCCCGA CTCTTAAAAT AGAAAATGTC AAGTCAGTTA
7561 AGCAGGAAGT GGACTAACTG ACGCAGCTGG CCGTGCGACA TCCTCTTTTA ATTAGTTGCT
7621 AGGCAACGCC CTCCAGAGGG CGTGTGGTTT TGCAAGAGGA AGCAAAAGCC TCTCCACCCA
7681 GGCCTAGAAT GTTTCCACCC AATCATTACT ATGACAACAG CTGTTTTTTT TAGTATTAAG
7741 CAGAGGCCGG GGACCCCTGG GCCCGCTTAC TCTGGAGAAA AAGAAGAGAG GCATTGTAGA
7801 GGCTTCCAGA GGCAACTTGT CAAAACAGGA CTGCTTCTAT TTCTGTCACA CTGTCTGGCC
7861 CTGTCACAAG GTCCAGCACC TCCATACCCC CTTTAATAAG CAGTTTGGGA ACGGGTGCGG
7921 GTCTTACTCC GCCCATCCCG CCCCTAACTC CGCCCAGTTC CGCCCATTCT CCGCCCCATG
7981 GCTGACTAAT TTTTTTTATT TATGCAGAGG CCGAGGCCGC CTCGGCCTCT GAGCTATTCC
8041 AGAAGTAGTG AGGAGGCTTT TTTGGAGGCC TAGGCTTTTG CAAAAAGCTA ATTC

9.4.2 pMTF1/EGFP-TRE

The following plasmid encodes for a MTF-1/EGFP fusion protein under control of a tetracycline-responsive element, which was used for transfection to generate the double-stable Tet-On/MTF-1 cell line. Coding sequence of MTF-1 (595-2856) is again shown in bold letters, whereas CDS of EGFP (2902-3621) is underlined.

Forward primers used for sequencing are marked with yellow, backward primers with green rectangles.

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   1 CTCGAGTTTA CCACTCCCTA TCAGTGATAG AGAAAAGTGA AAGTCGAGTT TACCACTCCC
  61 TATCAGTGAT AGAGAAAAGT GAAAGTCGAG TTTACCACTC CCTATCAGTG ATAGAGAAAA
 121 GTGAAAGTCG AGTTTACCAC TCCCTATCAG TGATAGAGAA AAGTGAAAGT CGAGTTTACC
 181 ACTCCCTATC AGTGATAGAG AAAAGTGAAA GTCGAGTTTA CCACTCCCTA TCAGTGATAG
 241 AGAAAAGTGA AAGTCGAGTT TACCACTCCC TATCAGTGAT AGAGAAAAGT GAAAGTCGAG
 301 CTCGGTACCC GGGTCGAGGT AGGCGTGTAC GGTGGGAGGC CTATATAAGC AGAGCTCGTT
 361 TAGTGAACCG TCAGATCGCC TGGAGACGCC ATCCACGCTG TTTTGACCTC CATAGAAGAC
 421 ACCGGGACCG ATCCAGCCTC CGCGGCCCCG AATTCGAGCT CGGTACCCGG GGATCCTCTA
 481 GTCAGCTGAC GCGAGCTAGA CACCATGAGA CATAATCATA ATCATAATCA TAATCATAAT
 541 CACAACCTTA TGGCCACTGA CGCGTTGCTA GCGCAGCTGG AAGCTTATCG ATCCATGGGG
 601 GAACACAGTC CAGACAACAA CATCATCTAC TTTGAGGCAG AGGAAGATGA GCTGACCCCC
 661 GATGATAAAA TGCTCAGGTT TGTGGATAAA AACGGACTGG TGCCTTCCTC ATCTGGAACT
 721 GTTTATGATA GGACCACTGT TCTTATTGAG CAGGACCCTG GCACTTTGGA GGATGAAGAT
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 841 CTGATAGATC ATGAAGCAAT GTCCCAGGGT TATGTGCAGC ACATTATCTC ACCAGATCAG
 901 ATTCATTTGA CAATAAACCC TGGTTCCACA CCCATGCCAA GAAATATTGA AGGTGCAACC
 961 CTCACTCTGC AGTCGGAATG TCCGGAAACA AAACGTAAAG AAGTAAAGCG GTACCAATGT
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1441 CATACTGGTG AAAGACCCTT CTTCTGCCCC AGTAATGGCT GTGAGAAAAC ATTCAGCACT
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1681 AGCACAATTT CACCAGCAAT CATCTTTGAA TCAATGTTCC AGAATTCAGA TGATACGGCA
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1981 GCTCTCTTAC AACCTCCAGA AGTGCCTGTT CCCCACAGCA CACAGTTTGC TGCTAATCAT
2041 CAAGAGTTTC TTCCGCACCC CCAGGCACCG CAGCCCATTG TACCAGGACT TTCTGTTGTT
2101 GCTGGGGCTT CTGCATCAGC AGCGGCAGTG GCATCAGCTG TGGCAGCACC AGCCCCACCA
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2281 TCCAGCCTAG TCATGGGAGA ACAGAACTTA CAATGGATAT TAAATGGTGC CACCAGTTCT
2341 CCACAAAACC AAGAACAAAT TCAGCAAGCA TCTAAAGTTG AGAAGGTGTT TTTTACCACT
2401 GCAGTACCAG TAGCCAGTAG CCCAGGGAGC TCTGTCCAGC AGATTGGCCT CAGTGTTCCT
2461 GTGATCATCA TCAAACAAGA AGAGGCATGT CAGTGTCAGT GTGCATGCCG GGACTCTGCA
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3001 GGCGAGGGCG ATGCCACCTA CGGCAAGCTG ACCCTGAAGT TCATCTGCAC CACCGGCAAG
3061 CTGCCCGTGC CCTGGCCCAC CCTCGTGACC ACCCTGACCT ACGGCGTGCA GTGCTTCAGC
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3181 GTCCAGGAGC GCACCATCTT CTTCAAGGAC GACGGCAACT ACAAGACCCG CGCCGAGGTG
3241 AAGTTCGAGG GCGACACCCT GGTGAACCGC ATCGAGCTGA AGGGCATCGA CTTCAAGGAG
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3421 GACGGCAGCG TGCAGCTCGC CGACCACTAC CAGCAGAACA CCCCCATCGG CGACGGCCCC
3481 GTGCTGCTGC CCGACAACCA CTACCTGAGC ACCCAGTCCG CCCTGAGCAA AGACCCCAAC
3541 GAGAAGCGCG ATCACATGGT CCTGCTGGAG TTCGTGACCG CCGCCGGGAT CACTCTCGGC
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5941 GATACCGCGA GACCCACGCT CACCGGCTCC AGATTTATCA GCAATAAACC AGCCAGCCGG
6001 AAGGGCCGAG CGCAGAAGTG GTCCTGCAAC TTTATCCGCC TCCATCCAGT CTATTAATTG
6061 TTGCCGGGAA GCTAGAGTAA GTAGTTCGCC AGTTAATAGT TTGCGCAACG TTGTTGCCAT
6121 TGCTACAGGC ATCGTGGTGT CACGCTCGTC GTTTGGTATG GCTTCATTCA GCTCCGGTTC
6181 CCAACGATCA AGGCGAGTTA CATGATCCCC CATGTTGTGC AAAAAAGCGG TTAGCTCCTT
6241 CGGTCCTCCG ATCGTTGTCA GAAGTAAGTT GGCCGCAGTG TTATCACTCA TGGTTATGGC
6301 AGCACTGCAT AATTCTCTTA CTGTCATGCC ATCCGTAAGA TGCTTTTCTG TGACTGGTGA
6361 GTACTCAACC AAGTCATTCT GAGAATAGTG TATGCGGCGA CCGAGTTGCT CTTGCCCGGC
6421 GTCAATACGG GATAATACCG CGCCACATAG CAGAACTTTA AAAGTGCTCA TCATTGGAAA
6481 ACGTTCTTCG GGGCGAAAAC TCTCAAGGAT CTTACCGCTG TTGAGATCCA GTTCGATGTA
6541 ACCCACTCGT GCACCCAACT GATCTTCAGC ATCTTTTACT TTCACCAGCG TTTCTGGGTG
6601 AGCAAAAACA GGAAGGCAAA ATGCCGCAAA AAAGGGAATA AGGGCGACAC GGAAATGTTG
6661 AATACTCATA CTCTTCCTTT TTCAATATTA TTGAAGCATT TATCAGGGTT ATTGTCTCAT
6721 GAGCGGATAC ATATTTGAAT GTATTTAGAA AAATAAACAA ATAGGGGTTC CGCGCACATT
6781 TCCCCGAAAA GTGCCACCTG ACGTCTAAGA AACCATTATT ATCATGACAT TAACCTATAA
6841 AAATAGGCGT ATCACGAGGC CCTTTCGTCT TCACTCGAGT GTGTCAGTTA GGGTGTGGAA
6901 AGTCCCCAGG CTCCCCAGCA GGCAGAAGTA TGCAAAGCAT GCATCTCAAT TAGTCAGCAA
6961 CCAGGTGTGG AAAGTCCCCA GGCTCCCCAG CAGGCAGAAG TATGCAAAGC ATGCATCTCA
7021 ATTAGTCAGC AACCATAGTC CCGCCCCTAA CTCCGCCCAT CCCGCCCCTA ACTCCGCCCA
7081 GTTCCGCCCA TTCTCCGCCC CATGGCTGAC TAATTTTTTT TATTTATGCA GAGGCCGAGG
7141 CCGCCTCGGC CTCTGAGCTA TTCCAGAAGT AGTGAGGAGG CTTTTTTGGA GGCCTAGGCT
7201 TTTGCAAAAA GCTTCCATGA AAAAGCCTGA ACTCACCGCG ACGTCTGTCG AGAAGTTTCT
7261 GATCGAAAAG TTCGACAGCG TCTCCGACCT GATGCAGCTC TCGGAGGGCG AAGAATCTCG
7321 TGCTTTCAGC TTCGATGTAG GAGGGCGTGG ATATGTCCTG CGGGTAAATA GCTGCGCCGA
7381 TGGTTTCTAC AAAGATCGTT ATGTTTATCG GCACTTTGCA TCGGCCGCGC TCCCGATTCC
7441 GGAAGTGCTT GACATTGGGG AATTCAGCGA GAGCCTGACC TATTGCATCT CCCGCCGTGC
7501 ACAGGGTGTC ACGTTGCAAG ACCTGCCTGA AACCGAACTG CCCGCTGTTC TGCAGCCGGT
7561 CGCGGAGGCC ATGGATGCGA TCGCTGCGGC CGATCTTAGC CAGACGAGCG GGTTCGGCCC
7621 ATTCGGACCG CAAGGAATCG GTCAATACAC TACATGGCGT GATTTCATAT GCGCGATTGC
7681 TGATCCCCAT GTGTATCACT GGCAAACTGT GATGGACGAC ACCGTCAGTG CGTCCGTCGC
7741 GCAGGCTCTC GATGAGCTGA TGCTTTGGGC CGAGGACTGC CCCGAAGTCC GGCACCTCGT
7801 GCACGCGGAT TTCGGCTCCA ACAATGTCCT GACGGACAAT GGCCGCATAA CAGCGGTCAT
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7861 TGACTGGAGC GAGGCGATGT TCGGGGATTC CCAATACGAG GTCGCCAACA TCTTCTTCTG
7921 GAGGCCGTGG TTGGCTTGTA TGGAGCAGCA GACGCGCTAC TTCGAGCGGA GGCATCCGGA
7981 GCTTGCAGGA TCGCCGCGGC TCCGGGCGTA TATGCTCCGC ATTGGTCTTG ACCAACTCTA
8041 TCAGAGCTTG GTTGACGGCA ATTTCGATGA TGCAGCTTGG GCGCAGGGTC GATGCGACGC
8101 AATCGTCCGA TCCGGAGCCG GGACTGTCGG GCGTACACAA ATCGCCCGCA GAAGCGCGGC
8161 CGTCTGGACC GATGGCTGTG TAGAAGTACT CGCCGATAGT GGAAACCGAC GCCCCAGCAC
8221 TCGTCCGAGG GCAAAGGAAT AGAGTTCTAG AGGATCATAA TCAGCCATAC CACATTTGTA
8281 GAGGTTTTAC TTGCTTTAAA AAACCTCCCA CACCTCCCCC TGAACCTGAA ACATAAAATG
8341 AATGCAATTG TTGTTGTTAA CTTGTTTATT GCAGCTTATA ATGGTTACAA ATAAAGCAAT
8401 AGCATCACAA ATTTCACAAA TAAAGCATTT TTTTCACTGC
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9.5 List of abbreviations

2D	Two-dimensional
AAS	Atomic absorption spectrometry
AE	Acrodermatitis enteropathica
ATCC	American Type Culture Collection
ATP	Adenosine triphosphate
BLAST	Basic Local Alignment Search Tool
bp	Base pairs
cDNA	complementary Deoxyribonucleic acid
CHAPS	3-[(3-Cholamidopropyl)dimethyl-ammonio]-1-propanesulfonate
Cy3	Fluorescence dye, emission 570
Cy5	Fluorescence dye, emission 670
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate (dATP, dCTP, dGTP, dTTP)
DTT	Dithiothreitol
EDTA	Ethylendiamine-tetraaceticacid
EGFP	Enhanced green fluorescence protein
Fig.	Figure
FCS	Fetal calf serum
g	Acceleration of gravity
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
h	Hour(s)
IEF	Isoelectric focusing
IPG	Immobilized pH gradient
kD	Kilodalton
M	Molar (mol/l)
MALDI	Matrix assisted laser desorption ionization
MRE	Metal responsive element
mRNA	messenger RNA

MS	Mass spectrometry
MT	Metallothionein
MTF-1	Metal responsive element-binding transcription factor-1
n	Number (of samples, replicates, experiments)
NCBI	National Center for Biotechnology Information
Oligo-d(T)	Oligodeoxythymidine phosphate
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
pH	Logarithm of the reciprocal of hydrogen-ion concentration
PMSF	Phenylmethanesulfonyl fluoride
PVDF	Polyvinylidene difluoride
RNA	Ribonucleic acid
RT	Reverse transcription
RT-PCR	Reverse transcription polymerase chain reaction
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate-Polyacrylamide gel electrophoresis
SSC	Sodium citrate-Sodium chloride buffer
Tab.	Table
TBS-T	Tris buffered saline plus Tween 20
TOF	Time of flight
TRIS	Tris-(hydroxymethyl)-aminomethane
Zn	Zinc

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Parts of this work have already been published:

Original papers and submitted manuscripts

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Kindermann B, Döring F, Fuchs D, Pfaffl M, Daniel H. Effects of increased cellular zinc levels on gene and protein expression in HT-29 cells (submitted).

Kindermann B, Döring F, Budczies J, Daniel H. Zinc-sensitive genes as potential new target genes of the metal transcription factor-1 (MTF-1) (submitted).

Oral presentations

Kindermann B, Daniel H, Döring F. Identifizierung Zink-sensitiver Gengruppen mittels DNA-Arrays in humanen Darmzellen. 40th Scientific Congress of the German Nutrition Society, March 2003, Potsdam, Germany.

Kindermann B, Daniel H, Döring F. Effects of zinc on the transcriptome and proteome in the human colon adenocarcinoma cell line HT-29. Colloquium of the Molecular Nutrition Unit, November 2003, Freising, Germany.

Poster presentations

Kindermann B, Döring F, Pfaffl M, Daniel H. Einsatz der cDNA-Array-Technologie zur Identifizierung Zink-sensitiver Gene in vitro. – presented at Status Seminar Chip Technologies, January 2002, Dechema, Frankfurt, Germany and 39th Scientific Congress of the German Nutrition Society, March 2002, Jena, Germany.

Kindermann B, Döring F, Pfaffl M, Daniel H. Application of the cDNA array technology for the identification of zinc-responsive genes in mammalian cells. The first International Nutrigenomics Conference, February 2002, Noordwijk aan Zee, Netherlands.

Kindermann B, Döring F, Herzog A, Fuchs D, Wenzel U, Daniel H. Identification of genes and proteins responsive to intracellular zinc concentration in the human colon cancer cell line HT-29 by transcriptome and proteome analysis. The second International Nutrigenomics Conference, November 2003, Amsterdam, Netherlands.

Erklärung

Hiermit versichere ich, dass ich die vorliegende Arbeit

Effects of zinc on the transcriptome and proteome of human colonic cancer cells (HT-29 cells)

selbstständig verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel benutzt habe. Die den benutzten Quellen wörtlich oder inhaltlich entnommenen Stellen sind als solche kenntlich gemacht.

Diese Arbeit hat in gleicher oder ähnlicher Form noch keiner anderen Prüfungsbehörde vorgelegen.

Freising,

Birgit Kindermann

Nun ist es also geschafft. Aber auch wenn immer die Rede von „meiner“ Arbeit ist, weiß ich natürlich, dass so etwas nicht allein gelingen kann. Deshalb gilt mein Dank an dieser Stelle:

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Isabelle Frey, die mir zuliebe immer geschwitzt hat,

Lothar für eine unbeschreibliche Menge Geduld und dem Verständnis, dass die Zellen eben auch am Wochenende „Futter“ brauchen,

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Zum Schluss möchte ich noch sagen, dass an diesem Institut by far die beste Atmosphäre herrscht und dass die letzten vier Jahre (abgesehen von etlichen physischen und psychischen Tiefpunkten natürlich) tierisch viel Spaß gemacht haben.

Dankeschön !!!