BIOINFORMATIC ANALYSES OF ZINC-ACTIVATED TRANSCRIPTION FACTOR BINDING SITES

by

MICHAEL FRANCIS

(Under the Direction of Arthur Grider)

ABSTRACT

In humans, the zinc (Zn)-activated transcription factors (TFs) metal response element-binding transcription factor 1 (MTF-1) and ZNF658 regulate gene expression by binding to their associated DNA motifs, the metal regulatory element (MRE) and the zinc transcriptional regulatory element (ZTRE), respectively, upon activation by cellular Zn. Genome-wide bioinformatic analyses were performed to better understand how these motifs may affect TF binding. The effects of the number of MREs and their distance from transcriptional start sites (TSSs) were examined in a secondary analysis of gene expression. MTF-1 is also predicted to form complex regulatory networks with miRNAs via the MRE in the promoters of miRNAs and their target genes. ZNF658 may be responsible for a global Zn deficiency response by binding selectively to ZTRE permutations that occur closer to transcriptional start sites (ex: ZTRE-E) versus others (ex: ZTRE-A) under varied Zn concentrations.

INDEX WORDS: Metal response element-binding transcription factor 1 (MTF-1), ZNF658, zinc, microRNAs (miRNAs), transcriptional regulation, nutrigenomics, bioinformatics

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DEDICATION

To Dolores Inserra, who believed in me,

and

to Stephen Marotta, an American patriot,

and

to Barclay Powers.

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

Introduction

Complex regulatory networks in humans that are populated by zinc (Zn)-finger proteins (ZFPs) are a great hidden treasure trove of biological data. Many of these ZFPs are transcription factors (TFs) that control which genes are expressed and when. Certain ZFPs are directly activated by Zn, meaning the amount of Zn a person consumes can have direct genetic consequences. This, in tandem with the necessity for structural Zn by thousands of proteins, equates to a vast and interesting role of Zn at the transcriptional level.

The action of the most well-studied Zn-activated TF, metal response element-binding transcription factor 1 (MTF-1), has traditionally been understood in terms of Zn's own homeostatic processes, via the induced expression of cellular Zn transporters and metallothionine. However, recent experiments demonstrate that MTF-1 affects the expression of hundreds, or even thousands of genes, the products of whom belong to widely varied processes and pathways.

Of course there are many factors besides Zn that are essential to maintaining a finely-tuned and complex human organism, and one category that has been moving toward center stage in the past decade is microRNAs (miRNAs)—small, noncoding RNAs that can deactivate (or amplify) gene expression post-transcriptionally. These miRNAs themselves can have their expression influenced by Zn levels, with the result as multi-layered regulatory circuits. The elucidation of these and other three dimensional interaction pathways will be the topic of future research for decades and represents a giant hurdle in understanding how life really does what it does.

Although MTF-1 has been the main focus of Zn-activated transcriptional regulation research for decades, a new factor has been recently discovered—ZNF658. It is possible that ZNF658 oversees the mechanism of the response to critically low Zn in cells, and acts as a master regulator to turn on or off certain protein expression to compensate. This function is seen by the Zap1 TF in yeast and ZNF658 may have an analogous role in humans.

The main text of this thesis consists of three published manuscripts from the past two years of research. In Chapter 2, we test in a secondary analysis whether the amount or location of MTF-1 binding sites affected gene expression levels. In Chapter 3, we lay the foundation of the potential regulatory networks between MTF-1 and miRNAs, as pertains to human bone development signaling pathways (TGF-β, MAPK, and Wnt). And in Chapter 4, we perform the first genome-wide analysis of ZNF658 binding sites, and make new functional predictions based on our results.

Literature Review

Zinc (Zn) has widespread and essential roles in all living organisms, and follows iron as the second most abundant trace mineral in humans. Deficiency can cause problems in health and development, with four percent of the world population affected (Wuehler, Peerson et al. 2005), and children as the most at-risk demographic (Hambidge and Krebs 2007, Tuerk and Fazel 2009). In humans, ten percent of all proteins require Zn for their structure and function (Andreini, Banci et al. 2006). Most of these proteins are involved in gene regulation, with the largest category being Zn-finger proteins (ZFPs) (Klug 2010). Though Zn is ubiquitous in proteins involved in a wide range of physiological processes, one area that Zn has a notable role is bone development pathways. A special class of ZFPs are those whose function is in direct response to cellular Zn

concentrations, hereafter referred to as Zn-activated transcription factors (ZATFs). A deeper understanding of ZATFs is crucial to discovering the mechanisms involved in the Zn-mediated regulation of these pathways. Two notable ZATFs are the well-studied metal response element-binding transcription factor 1 (MTF-1), and the recently characterized ZNF658. ZATFs may also increase their regulatory extent by altering the transcription levels of microRNA (miRNA) gene products.

Zn and bone development: assessment

Because of the strong link between Zn status and bone growth, the Zn status of populations suspected of Zn deficiency can be assessed by functional length- or height-for-age measurements (International Zinc Nutrition Consultative, Brown et al. 2004, Hess, Lonnerdal et al. 2009). In animal models, diets lacking Zn will result in reduced bone growth (Keller, Chu et al. 2000, Chu, Mouat et al. 2003, Grider, Mouat et al. 2007). Zn status also directly relates to certain osteogenic biomarkers (Cho, Lomeda et al. 2007, Seo, Cho et al. 2010, Berger, Pollock et al. 2015).

Zn and bone development: regulatory pathways

The signaling pathways involved in the regulation of bone formation and growth are transforming growth factor β/bone morphogenic protein (TGF-β/BMP), wingless-type (Wnt), p38 mitogen-activated protein kinase (MAPK), Hedgehog, Notch, and fibroblast growth factor (FGF), with significant overlap between these (Chen, Deng et al. 2012). TGF-β/BMP, Wnt, MAPK, and Hedgehog pathways also have overlap with the IGF-1 pathway (Longobardi, O'Rear et al. 2006, Guntur and Rosen 2013, Tahimic, Wang et al. 2013). Insulin-like growth factor-1 (IGF-1) is a hormone which plays an important role in childhood growth. In children, IGF-1 levels are correlated with Zn status in some studies (Bougle, Sabatier et al. 2004, Imamoglu, Bereket et al. 2005, Cesur, Yordam et al. 2009, Hamza, Hamed et al. 2012), but not all show this association

(Park, Choi et al. 2017). It has been demonstrated that Zn increases IGF-1 synthesis in bone (Igarashi and Yamaguchi 2001) as well as IGF-1 binding to its receptor (McCusker 1998, McCusker, Kaleko et al. 1998, Sackett and McCusker 1998, Sackett and McCusker 1998).

Zn-activated TFs: overview

Zn is a required component of Cys_2His_2 ZFPs, which make up 3% of the human genome and are predicted to be the largest class of TFs. However the majority of Cys_2His_2 ZFPs have unknown functions, and those whose functions are known have widely varied functions. This indicates a potentially vast regulatory network that is largely unstudied (Najafabadi, Mnaimneh et al. 2015). A small group of these ZFPs, ZATFs, are known to bind to DNA in direct response to stimulation of their Zn sensors. In eukaryotes the known ZATFs are Zap1 (*Saccharomyces cerevisiae*), Loz1 (*Schizosaccharomyces pombe*), bZip19/bZip23 (*Arabidopsis thaliana*), and MTF-1 (*Homo sapiens*, conserved among metazoan species) (Choi and Bird 2014). The newly discovered ZATF ZNF658, which has been shown to bind to DNA and regulate gene transcription in response to cellular Zn levels (Ogo, Tyson et al. 2015), may also be added to this list.

MTF-1 binds to the MRE

The metal regulatory element (MRE) was discovered in 1984, as a 12 base conserved DNA sequence that when altered or deleted, changed the expression levels of mouse metallothionine (MT) gene product (G.W. Stuart). The following year, that same lab deduced what is now the core MRE consensus sequence, 5'-TGCRCNC-3' (R= A or G, N = any nucleotide) (Stuart, Searle et al. 1985). In 1988, the ZATF now known as MTF-1 was discovered as that protein which binds to the MRE and is responsible for its regulatory effects (Westin and Schaffner). For many years, MTF-1 was considered exclusively a positive transcriptional regulator of *MT* and *SLC30A1* gene products in response to changes in intracellular Zn concentrations, and thus was a function of Zn regulating

its own homeostasis (G.W. Stuart 1984, O'Halloran 1993, Langmade, Ravindra et al. 2000, Saydam, Adams et al. 2002).

MTF-1 has six Zn fingers; the N-terminal fingers 1-4 have a high affinity for both Zn and the MRE, while fingers 5 and 6 have a weaker affinity for the MRE and bind to the 3' end of the motif. However, all six fingers are necessary for MTF-1 to form a complex with the MRE. Fingers 5-6 may also modulate the affinity of MTF-1 for the various permutations of the MRE (Labbé, Prévost et al. 1991, Chen, Chu et al. 1999). MTF-1 binds with the highest affinity to the nucleotide sequence 5'-TGCACTC-3', which is sometimes labeled MREd (Labbé, Prévost et al. 1991).

We now know that MTF-1 responds not exclusively to Zn, but also to metal ion exposure, hypoxia, oxidative stress, or elevated temperature (Andrews 2001, Lichtlen and Schaffner 2001). Its regulatory effects have been shown as present in the cellular antioxidant response (Stoytcheva, Vladimirov et al. 2010), insulin synthesis (Huang, Yan et al. 2010, Li, Cui et al. 2014), immunity and inflammation/hypoxia (Cramer, Nagy et al. 2005, Murphy, Sato et al. 2005, Murphy, Kimura et al. 2008), iron and copper homeostasis (Selvaraj, Balamurugan et al. 2005, Balesaria, Ramesh et al. 2010, Troadec, Ward et al. 2010), and epigenetic histone modification (Okumura, Li et al. 2011). MTF-1 is gaining recognition for its unique physiological role, and as a target for drug discovery and development to treat epileptogenesis (Van Loo, Schaub et al. 2015), osteoarthritis (Kim, Jeon et al. 2014), and prion disease (Bellingham, Coleman et al. 2009). Finally, MTF-1 has also been suggested to be a master regulator of microRNA (miRNA) expression (Lee, Li et al. 2007), which is discussed later in this review and explored in depth in Chapter 3.f

<u>miRNAs: overview</u>

The most studied family of non-coding RNAs (ncRNAs) are miRNAs: 19-24 nt sequences commonly understood as post-transcriptional repressors of gene expression (Lee, Feinbaum et al.

1993), though recently they have shown to have the potential to upregulate gene expression as well (Ørom, Nielsen et al. , Vasudevan, Tong et al. 2007, Eiring, Harb et al. 2010). During their biogenesis, miRNAs are transcribed by RNA polymerase II into long double-stranded primary miRNA (pri-miRNA) which can be hundreds or thousands of base pairs in length. The defining characteristics of pri-miRNA are local stem-loop structures, and capped and polyadenylated 5' and 3' tails (Bartel , Lee, Kim et al. 2004). The RNA-binding protein DiGeorge syndrome critical region 8 (DGCR8) associates with the RNase-III-type protein Drosha, to form the large Microprocessor complex which splices pri-miRNA into 60-90 base pair long pre-miRNA, which isolates and releases the functional hairpin/stem-loop structure (Gregory, Chendrimada et al. 2006). The pre-miRNA is exported to the cytoplasm by a nuclear transport receptor, Exportin-5, then cleaved near the terminal loop by the RNase-III-type protein Dicer to generate a 19-24 base pair miRNA duplex (Kim 2004). The duplex is loaded onto an Argonaute family protein (AGO) to form the active RNA-induced-silencing-complex (RISC). The more thermodynamically stable strand of this duplex is kept as the mature (or guide) miRNA while the other (passenger or miRNA*) strand is degraded (Schwarz, Hutvagner et al. 2003, Kim, Han et al. 2009).

miRNAs downregulate gene expression at a post-transcriptional level by interacting with messenger RNA (mRNA) to cause its translational repression or cleavage. miRNA-induced repression typically occurs via sequence-specific binding of AGO or GW182 proteins to the target mRNA 3'-UTR (Chekulaeva and Filipowicz 2009). There are also mechanisms for upregulation of genes by miRNAs which include: recruiting protein complexes to AU-rich regions of mRNA, increasing translation by repressing proteins that block a target gene, enhancing ribosome biogenesis, and activating target gene translation during cell cycle arrest (Ling, Fabbri et al. 2013). miRNA can mediate both short and long-range cellular communication and can function locally

or enter the bloodstream and act on neighboring cells or at distant sites in a hormone-like fashion. (Mitchell, Parkin et al. 2008, Fabbri, Paone et al. 2012).

miRNAs and bone development

The regulatory effects of miRNAs are involved with in osteogenic differentiation processes. Runt-related transcription factor 2 (Runx2) is a "master gene" required for osteoblastic differentiation from mesenchymal precursors, as well as bone growth. Dicer-processed miRNAs control commitment and differentiation of osteoblasts, which is mechanistically dependent upon transcriptional regulation of Dicer by Runx2 binding to its promoter. Dicer and Runx2 are closely linked and their expressions increase simultaneously during osteogenic differentiation in embryos (Tu, Zhang et al. 2007, Vimalraj, Arumugam et al. 2015, Wysokinski, Pawlowska et al. 2015, Zheng, Tu et al. 2017).

The widely studied miR-34a is a known inhibitor of osteoblast differentiation and bone formation. In models of human stromal (skeletal, mesenchymal) stem cell (hMSC) differentiation, overexpression of miR-34a in hMSCs reduced heterotopic bone formation by 60%. Conversely in in miR-34a-deficient hMSC *in vivo* bone formation was increased by 200% (Chen, Holmstrom et al. 2014).

miRNAs and Zn

Our Zn-related interest in miRNAs stems from the fact that MRE motifs occur frequently in the promoter region of miRNA genes, and the two classes of regulators may form complex regulatory circuits of gene expression (Arora, Rana et al. 2013). Results from *in vivo* and *in vitro* studies indicate Zn-dependent differential miRNA expression (Ryu, Langkamp-Henken et al. 2011, Liuzzi 2014, Grider, Lewis et al. 2015). Zn has also been shown to regulate the activity of matrix-degrading metalloproteinases in osteoarthritis (OA) via MTF-1 in conjunction with

miRNAs. miR-488 is significantly decreased in OA chrondrocytes, which results in the overexpression of its usual suppression target, the ZIP8 zinc transporter, in human osteoarthritic chondrocytes (Song, Kim et al. 2013). The overexpression of ZIP8 causes increased zinc levels within chondrocytes, leading to the activation of MTF-1. MTF-1 activates the transcription of matrix-degrading enzyme genes (*MMP3*, *9*, *12*, *13*, *ADMTS5*) which increases cartilage degradation and OA severity (Kim, Jeon et al. 2014). Activation of the Zinc-ZIP8-MTF-1 axis with the hypoxia inducible factor (HIF)-2α in mice amplified pathogenesis of OA in mice (Lee, Won et al. 2016).

ZNF658 binds to the ZTRE

The ZTRE was first reported in 2012 as a palindromic DNA motif responsible for Zn-mediated gene repression (Coneyworth, Jackson et al.). They found that removal of this motif eliminated the Zn-dependent repression of *SLC30A5* (ZnT5), *SLC30A10* (ZnT10), and *CBWD* genes. The nucleotides of their proposed ZTRE sequence, written with ambiguity codes, is 5′-CMCDCCYN$_{0–100}$RGGHGKG-3′ (where M=C or A; D=A, G, or T; Y=C or T; R=A or G; H=A, C, or T; and K=T or G) (Coneyworth, Jackson et al. 2012). The 5’- and 3’- flanking sequences are required to be reverse palindromic for functionality. There are no known criteria for the middle “N” bases, which can range from zero to one hundred in length (Coneyworth, Jackson et al. 2012). The variability in TF binding to palindromic motifs is often attributable to TF dimerization (Peirano and Wegner 2000, Huang, Jankowski et al. 2015).

Three years later the same lab published a follow-up paper to their ZTRE study, and identified the specific TF responsible for binding this sequence, via mass spectrometry, called ZNF658 (Ogo, Tyson et al. 2015). ZNF658 was also shown to repress genes under conditions of high Zn. They found that introduction of ZNF658 siRNA to Caco-2 cells had significant effects

on the up- or down-regulation of 124 genes in their Illumina HT12 bead chip panel, including a large number of rRNA genes (Ogo, Tyson et al. 2015).

ZNF658 is homologous to Zap1

In order to understand the function of ZNF658, it may be useful to look at the homologous ZATF and possible evolutionary precursor in *S. cerevisiae*, called Zap1. The essential DNA-binding ZFs of these two proteins are aligned and highly similar (Ogo, Tyson et al. 2015). Zap1 is the main regulator of Zn homeostasis in *S. cerevisiae* and accomplishes this via Zn-dependent binding to an 11-mer reverse palindromic motif, the Zn-Responsive Element (ZRE). Zap1 displays low activity in Zn-adequate cells and high activity during Zn deficiency (Lyons, Gasch et al. 2000).

One of the most interesting roles of Zap1 is its ability to initiate a "Zn-sparing" response by sensing Zn deficiency and repressing levels of some Zn-binding proteins, while simultaneously increasing expression of replacement proteins that have lower Zn requirements (Merchant and Helmann 2012). For example, alcohol dehydrogenase isoenzymes in *S. cerevisiae* Adh1 and Adh3 each have structural requirements of two Zn atoms, and are repressed by Zap1 during Zn deficiency. Meanwhile the expression of Adh4, which requires one Zn atom or potentially an iron atom in its place, is induced (Merchant and Helmann 2012).

Zap1 can increase its own expression in Zn deficiency via autoregulation. This increase activates a certain set of target genes by enabling the increased concentration Zap1 to bind to lower binding affinity ZREs (Wu, Bird et al. 2008). These lower affinity ZREs are found closer to target gene TSSs, and are also sometimes sites of Zap1-mediated repression. This is thought to be a classical-type repression via Zap1 blocking transcription initiation sites (Bird, Blankman et al. 2004).

The two independent activation domains of Zap1, AD1 and AD2, which act as Zn sensors and can initiate DNA binding, are also relevant to delineating the similarities between Zap1 and ZNF658 (Bird, Zhao et al. 2000). AD1 is considered the dominant activation domain, while AD2 allows for maximum target gene activation in situations of Zn deficiency or environmental stress.

When Zn binds to AD1 or AD2, a conformation change occurs which inhibits Zap1 from transcriptional activation via coactivator recruitment (Frey and Eide 2012). The AD1 domain shares a region of similarity when the amino acid sequences of Zap1 and ZNF658 are aligned (Ogo, Tyson et al. 2015). ZNF658 also contains a KRAB (Kruppel associated box) domain near its N-terminus; these are typically associated with transcriptional repression via the recruitment of KAP1, and subsequent chromatin remodeling, to repress transposable elements (TEs) (Lupo, Cesaro et al. 2013). However, not all KRAB domains bind to KAP1, and they are also involved with transcriptional activation, plus a variety of other functions (Ecco, Imbeault et al. 2017).

References

Andreini, C., L. Banci, I. Bertini and A. Rosato (2006). "Counting the zinc-proteins encoded in the human genome." J Prot Res 5(1): 196-201.

Andrews, G. K. (2001). "Cellular zinc sensors: MTF-1 regulation of gene expression." Biometals 14(3): 223-237.

Arora, S., R. Rana, A. Chhabra, A. Jaiswal and V. Rani (2013). "miRNA-transcription factor interactions: a combinatorial regulation of gene expression." Mol Genet Genomics 288(3/4): 77-87.

Balesaria, S., B. Ramesh, H. McArdle, H. K. Bayele and S. K. S. Srai (2010). "Divalent metal-dependent regulation of hepcidin expression by MTF-1." FEBS Lett 584(4): 719-725.

Bartel, D. P. "MicroRNAs: genomics, biogenesis, mechanism, and function." Cell 116(2): 281-297.

Bellingham, S. A., L. A. Coleman, C. L. Masters, J. Camakaris and A. F. Hill (2009). "Regulation of prion gene expression by transcription factors sp1 and metal transcription factor-1." J Biol Chem 284(2): 1291-1301.

Berger, P. K., N. K. Pollock, E. M. Laing, V. Chertin, P. J. Bernard, A. Grider, S. A. Shapses, K. H. Ding, C. M. Isales and R. D. Lewis (2015). "Zinc supplementation increases procollagen type 1 amino-terminal propeptide in premenarcheal girls: a randomized controlled trial." J Nutr 145(12): 2699-2704.

Bird, A. J., E. Blankman, D. J. Stillman, D. J. Eide and D. R. Winge (2004). "The Zap1 transcriptional activator also acts as a repressor by binding downstream of the TATA box in <em>ZRT2</em&gt." Embo J 23(5): 1123.

Bird, A. J., H. Zhao, H. Luo, L. T. Jensen, C. Srinivasan, M. Evans-Galea, D. R. Winge and D. J. Eide (2000). "A dual role for zinc fingers in both DNA binding and zinc sensing by the Zap1 transcriptional activator." Embo J 19(14): 3704-3713.

Bougle, D. L., J. P. Sabatier, G. Guaydier-Souquieres, F. Guillon-Metz, D. Laroche, P. Jauzac and F. Bureau (2004). "Zinc status and bone mineralisation in adolescent girls." J Trace Elem Med Bio 18(1): 17-21.

Cesur, Y., N. Yordam and M. Dogan (2009). "Serum insulin-like growth factor-i and insulin-like growth factor binding protein-3 levels in children with zinc deficiency and the effect of zinc supplementation on these parameters." J Pediatr Endocr Met 22(12): 1137-1143.

Chekulaeva, M. and W. Filipowicz (2009). "Mechanisms of miRNA-mediated post-transcriptional regulation in animal cells." Curr Opin Cell Biol 21(3): 452-460.

Chen, G., C. Deng and Y. P. Li (2012). "TGF-beta and BMP signaling in osteoblast differentiation and bone formation." Int J Biol Sci 8(2): 272-288.

Chen, L., K. Holmstrom, W. Qiu, N. Ditzel, K. Shi, L. Hokland and M. Kassem (2014). "MicroRNA-34a inhibits osteoblast differentiation and in vivo bone formation of human stromal stem cells." Stem Cells 32(4): 902-912.

Chen, X., M. Chu and D. P. Giedroc (1999). "MRE-binding transcription factor-1: weak zinc-binding finger domains 5 and 6 modulate the structure, affinity, and specificity of the metal-response element complex." Biochemistry 38(39): 12915-12925.

Cho, Y. E., R. A. Lomeda, S. H. Ryu, H. Y. Sohn, H. I. Shin, J. H. Beattie and I. S. Kwun (2007). "Zinc deficiency negatively affects alkaline phosphatase and the concentration of Ca, Mg and P in rats." Nutr Res Pract 1(2): 113-119.

Choi, S. and A. J. Bird (2014). "Zinc'ing sensibly: controlling zinc homeostasis at the transcriptional level." Metallomics 6(7): 1198-1215.

Chu, Y., M. F. Mouat, R. B. Harris, J. A. Coffield and A. Grider (2003). "Water maze performance and changes in serum corticosterone levels in zinc-deprived and pair-fed rats." Physiol Behav 78(4-5): 569-578.

Coneyworth, L. J., K. A. Jackson, J. Tyson, H. J. Bosomworth, E. van der Hagen, G. M. Hann, O. A. Ogo, D. C. Swann, J. C. Mathers, R. A. Valentine and D. Ford (2012). "Identification of the human zinc transcriptional regulatory element (ZTRE): a palindromic protein-binding dna sequence responsible for zinc-induced transcriptional repression." J Biol Chem 287(43): 36567-36581.

Cramer, M., I. Nagy, J. Murphy Brian, M. Gassmann, O. Hottiger Michael, O. Georgiev and W. Schaffner (2005). NF-κB contributes to transcription of placenta growth factor and interacts with metal responsive transcription factor-1 in hypoxic human cells. Biol Chem. 386: 865.

Ecco, G., M. Imbeault and D. Trono (2017). "KRAB zinc finger proteins." Development 144(15): 2719.

Eiring, A. M., J. G. Harb, P. Neviani, C. Garton, J. J. Oaks, R. Spizzo, S. Liu, S. Schwind, R. Santhanam, C. J. Hickey, H. Becker, J. C. Chandler, R. Andino, J. Cortes, P. Hokland, C. S. Huettner, R. Bhatia, D. C. Roy, S. A. Liebhaber, M. A. Caligiuri, G. Marcucci, R. Garzon, C. M. Croce, G. A. Calin and D. Perrotti (2010). "miR-328 functions as an RNA decoy to modulate HNRNP E2 regulation of mRNA translation in leukemic blasts." Cell 140(5): 652-665.

Fabbri, M., A. Paone, F. Calore, R. Galli, E. Gaudio, R. Santhanam, F. Lovat, P. Fadda, C. Mao, G. J. Nuovo, N. Zanesi, M. Crawford, G. H. Ozer, D. Wernicke, H. Alder, M. A. Caligiuri, P. Nana-Sinkam, D. Perrotti and C. M. Croce (2012). "MicroRNAs bind to Toll-like receptors to induce prometastatic inflammatory response." P Natl Acad Sci USA 109(31): E2110-E2116.

Frey, A. G. and D. J. Eide (2012). "Zinc-responsive coactivator recruitment by the yeast Zap1 transcription factor." MicrobiologyOpen 1(2): 105-114.

G.W. Stuart, P. F. S., H.Y. Chen, R.L. Brinster, R.D. Palmiter (1984). "A 12-base-pair DNA motif that is repeated several times in metallothionein gene promoters confers metal regulation to a heterologous gene." Proc. Natl. Acad. Sci 81: 7318–7322.

Gregory, R. I., T. P. Chendrimada and R. Shiekhattar (2006). MicroRNA biogenesis: isolation and characterization of the microprocessor complex. Methods Mol Biol. 342: 33-47.

Grider, A., R. D. Lewis, E. M. Laing, A. A. Bakre and R. A. Tripp (2015). "Zinc affects miR-548n, SMAD4, SMAD5 expression in HepG2 hepatocyte and HEp-2 lung cell lines." Biometals 28(6): 959-966.

Grider, A., M. F. Mouat and A. G. Scrimgeour (2007). "Consumption of a moderately Zn-deficient and Zn-supplemented diet affects soluble protein expression in rat soleus muscle." J Nutr Biochem 18(11): 753-759.

Guntur, A. R. and C. J. Rosen (2013). "IGF-1 regulation of key signaling pathways in bone." Bonekey Rep 2: 437.

Hambidge, K. M. and N. F. Krebs (2007). "Zinc deficiency: A special challenge." J Nutr 137(4): 1101-1105.

Hamza, R. T., A. I. Hamed and M. T. Sallam (2012). "Effect of zinc supplementation on growth hormone insulin growth factor axis in short Egyptian children with zinc deficiency." Ital J Pediatr 38.

Hess, S. Y., B. Lonnerdal, C. Hotz, J. A. Rivera and K. H. Brown (2009). "Recent advances in knowledge of zinc nutrition and human health." Food Nutr Bull 30(1 Suppl): S5-11.

Huang, L., M. Yan and C. P. Kirschke (2010). "Over-expression of ZnT7 increases insulin synthesis and secretion in pancreatic β-cells by promoting insulin gene transcription." Exp Cell Res 316(16): 2630-2643.

Huang, Y.-H., A. Jankowski, K. S. E. Cheah, S. Prabhakar and R. Jauch (2015). "SOXE transcription factors form selective dimers on non-compact DNA motifs through

multifaceted interactions between dimerization and high-mobility group domains." Sci Rep 5: 10398.

Igarashi, A. and M. Yamaguchi (2001). "Increase in bone growth factors with healing rat fractures: The enhancing effect of zinc." Int J Mol Med 8(4): 433-438.

Imamoglu, S., A. Bereket, S. Turan and G. Haklar (2005). "Effect of zinc supplementation on growth hormone secretion, IGF-I, IGFBP-3, somatomedin generation, alkaline phosphatase, osteocalcin and growth in prepubertal children with idiopathic short stature." J Pediatr Endocr Met 18(1): 69-74.

International Zinc Nutrition Consultative, G., K. H. Brown, J. A. Rivera, Z. Bhutta, R. S. Gibson, J. C. King, B. Lonnerdal, M. T. Ruel, B. Sandtrom, E. Wasantwisut and C. Hotz (2004). "International Zinc Nutrition Consultative Group (IZiNCG) technical document #1. Assessment of the risk of zinc deficiency in populations and options for its control." Food Nutr Bull 25(1 Suppl 2): S99-203.

Keller, K. A., Y. Chu, A. Grider and J. A. Coffield (2000). "Supplementation with L-histidine during dietary zinc repletion improves short-term memory in zinc-restricted young adult male rats." J Nutr 130(6): 1633-1640.

Kim, J.-H., J. Jeon, M. Shin, Y. Won, M. Lee, J.-S. Kwak, G. Lee, J. Rhee, J.-H. Ryu, C.-H. Chun and J.-S. Chun (2014). "Regulation of the catabolic cascade in osteoarthritis by the Zinc-ZIP8-MTF1 axis." Cell 156(4): 730-743.

Kim, V. N. (2004). "MicroRNA precursors in motion: exportin-5 mediates their nuclear export." Trends Cell Biol 14(4): 156-159.

Kim, V. N., J. Han and M. C. Siomi (2009). "Biogenesis of small RNAs in animals." Nat Rev Mol Cell Biol 10(2): 126-139.

Klug, A. (2010). "The discovery of zinc fingers and their applications in gene regulation and genome manipulation." Annu Rev Biochem 79: 213-231.

Labbé, S., J. Prévost, P. Remondelli, A. Leone and C. Séguin (1991). "A nuclear factor binds to the metal regulatory elements of the mouse gene encoding metallothionein-I." Nucleic Acids Res 19(15): 4225-4231.

Langmade, S. J., R. Ravindra, P. J. Daniels and G. K. Andrews (2000). "The transcription factor MTF-1 mediates metal regulation of the mouse ZnT1 gene." J Biol Chem 275(44): 34803-34809.

Lee, J., Z. Li, R. Brower-Sinning and B. John (2007). "Regulatory circuit of human microRNA biogenesis." PLOS Comput Biol 3(4): e67.

Lee, M., Y. Won, Y. Shin, J. H. Kim and J. S. Chun (2016). "Reciprocal activation of hypoxia-inducible factor (HIF)-2alpha and the zinc-ZIP8-MTF1 axis amplifies catabolic signaling in osteoarthritis." Osteoarthr Cartilage 24(1): 134-145.

Lee, R. C., R. L. Feinbaum and V. Ambros (1993). "The C. elegans heterochronic gene lin-4 encodes small RNAs with antisense complementarity to lin-14." Cell 75(5): 843-854.

Lee, Y., M. Kim, J. Han, K. H. Yeom, S. Lee, S. H. Baek and V. N. Kim (2004). "MicroRNA genes are transcribed by RNA polymerase II." EMBO J 23(20): 4051.

Li, B., W. Cui, Y. Tan, P. Luo, Q. Chen, C. Zhang, W. Qu, L. Miao and L. Cai (2014). "Zinc is essential for the transcription function of Nrf2 in human renal tubule cells in vitro and mouse kidney in vivo under the diabetic condition." J Cell Mol Med 18(5): 895-906.

Lichtlen, P. and W. Schaffner (2001). "Putting its fingers on stressful situations: the heavy metal-regulatory transcription factor MTF-1." BioEssays 23(11): 1010-1017.

Ling, H., M. Fabbri and G. A. Calin (2013). "MicroRNAs and other non-coding RNAs as targets for anticancer drug development." Nat Rev Drug Discov 12(11): 847-865.

Liuzzi, J. P. (2014). "Up-regulation of miR-34a by zinc deficiency." Vitam Miner 3(1): 119.

Longobardi, L., L. O'Rear, S. Aakula, B. Johnstone, K. Shimer, A. Chytil, W. A. Horton, H. L. Moses and A. Spagnoli (2006). "Effect of IGF-I in the chondrogenesis of bone marrow mesenchymal stem cells in the presence or absence of TGF-beta signaling." J Bone Miner Res 21(4): 626-636.

Lupo, A., E. Cesaro, G. Montano, D. Zurlo, P. Izzo and P. Costanzo (2013). "KRAB-zinc finger proteins: a repressor family displaying multiple biological functions." Curr Genomics 14(4): 268-278.

Lyons, T. J., A. P. Gasch, L. A. Gaither, D. Botstein, P. O. Brown and D. J. Eide (2000). "Genome-wide characterization of the Zap1p zinc-responsive regulon in yeast." P Natl A Sci 97(14): 7957.

McCusker, R. H. (1998). "Controlling insulin-like growth factor activity and the modulation of insulin-like growth factor binding protein and receptor binding." J Dairy Sci 81(6): 1790-1800.

McCusker, R. H., M. Kaleko and R. L. Sackett (1998). "Multivalent cations and ligand affinity of the type 1 insulin-like growth factor receptor on P2A2-LISN muscle cells." J Cell Physiol 176(2): 392-401.

Merchant, S. S. and J. D. Helmann (2012). "Elemental economy: microbial strategies for optimizing growth in the face of nutrient limitation." Adv Microb Physiol 60: 91-210.

Mitchell, P. S., R. K. Parkin, E. M. Kroh, B. R. Fritz, S. K. Wyman, E. L. Pogosova-Agadjanyan, A. Peterson, J. Noteboom, K. C. Briant, A. Allen, D. W. Lin, N. Urban, C. W. Drescher,

B. S. Knudsen, D. L. Stirewalt, R. Gentleman, R. L. Vessella, P. S. Nelson, D. B. Martin and M. Tewari (2008). "Circulating microRNAs as stable blood-based markers for cancer detection." P Natl A Sci 105(30): 10513.

Murphy, B. J., T. Kimura, B. G. Sato, Y. Shi and G. K. Andrews (2008). "Metallothionein induction by hypoxia involves cooperative interactions between metal-responsive transcription factor-1 and hypoxia-inducible transcription factor-1α." Mol Cancer Res 6(3): 483-490.

Murphy, B. J., B. G. Sato, T. P. Dalton and K. R. Laderoute (2005). "The metal-responsive transcription factor-1 contributes to HIF-1 activation during hypoxic stress." Biochem Bioph Res Co 337(3): 860-867.

Najafabadi, H. S., S. Mnaimneh, F. W. Schmitges, M. Garton, K. N. Lam, A. Yang, M. Albu, M. T. Weirauch, E. Radovani, P. M. Kim, J. Greenblatt, B. J. Frey and T. R. Hughes (2015). "C2H2 zinc finger proteins greatly expand the human regulatory lexicon." Nat Biotechnol 33: 555.

O'Halloran, T. (1993). "Transition metals in control of gene expression." Science 261(5122): 715-725.

Ogo, O. A., J. Tyson, S. J. Cockell, A. Howard, R. A. Valentine and D. Ford (2015). "The zinc finger protein ZNF658 regulates the transcription of genes involved in zinc homeostasis and affects ribosome biogenesis through the zinc transcriptional regulatory element." Mol Cell Biol 35(6): 977-987.

Okumura, F., Y. Li, N. Itoh, T. Nakanishi, M. Isobe, G. K. Andrews and T. Kimura (2011). "The zinc-sensing transcription factor MTF-1 mediates zinc-induced epigenetic changes in

chromatin of the mouse metallothionein-I promoter." BBA-Gene Regul Mech 1809(1): 56-62.

Ørom, U. A., F. C. Nielsen and A. H. Lund "MicroRNA-10a binds the 5' UTR of ribosomal protein mrnas and enhances their translation." Mol Cell 30(4): 460-471.

Park, S. G., H. N. Choi, H. R. Yang and J. E. Yim (2017). "Effects of zinc supplementation on catch-up growth in children with failure to thrive." Nutr Res Pract 11(6): 487-491.

Peirano, R. I. and M. Wegner (2000). "The glial transcription factor Sox10 binds to DNA both as monomer and dimer with different functional consequences." Nucleic Acids Res 28(16): 3047-3055.

Ryu, M. S., B. Langkamp-Henken, S. M. Chang, M. N. Shankar and R. J. Cousins (2011). "Genomic analysis, cytokine expression, and microRNA profiling reveal biomarkers of human dietary zinc depletion and homeostasis." P Natl A Sci USA 108(52): 20970-20975.

Sackett, R. L. and R. H. McCusker (1998). "Multivalent cations depress ligand affinity of insulin-like growth factor-binding proteins-3 and -5 on human GM-10 fibroblast cell surfaces." J Cell Biochem 69(3): 364-375.

Sackett, R. L. and R. H. McCusker (1998). "Multivalent cations depress ligand binding to cell-associated insulin-like growth factor binding protein-5 on human glioblastoma cells." Endocrinology 139(4): 1943-1951.

Saydam, N., T. K. Adams, F. Steiner, W. Schaffner and J. H. Freedman (2002). "Regulation of metallothionein transcription by the metal-responsive transcription factor MTF-1 - Identification of signal transduction cascades that control metal-inducible transcription." J Biol Chem 277(23): 20438-20445.

Schwarz, D. S., G. Hutvagner, T. Du, Z. Xu, N. Aronin and P. D. Zamore (2003). "Asymmetry in the assembly of the RNAi enzyme complex." Cell 115(2): 199-208.

Selvaraj, A., K. Balamurugan, H. Yepiskoposyan, H. Zhou, D. Egli, O. Georgiev, D. J. Thiele and W. Schaffner (2005). "Metal-responsive transcription factor (MTF-1) handles both extremes, copper load and copper starvation, by activating different genes." Gene Dev 19(8): 891-896.

Seo, H. J., Y. E. Cho, T. Kim, H. I. Shin and I. S. Kwun (2010). "Zinc may increase bone formation through stimulating cell proliferation, alkaline phosphatase activity and collagen synthesis in osteoblastic MC3T3-E1 cells." Nutr Res Pract 4(5): 356-361.

Song, J., D. Kim, C. H. Lee, M. S. Lee, C.-H. Chun and E.-J. Jin (2013). "MicroRNA-488 regulates zinc transporter SLC39A8/ZIP8 during pathogenesis of osteoarthritis." J Biomed Sci 20(1): 31-31.

Stoytcheva, Z. R., V. Vladimirov, V. Douet, I. Stoychev and M. J. Berry (2010). "Metal transcription factor-1 regulation via MREs in the transcribed regions of selenoprotein H and other metal-responsive genes." Biochim Biophys Acta 1800(3): 416-424.

Stuart, G. W., P. F. Searle and R. D. Palmiter (1985). "Identification of multiple metal regulatory elements in mouse metallothionein-I promoter by assaying synthetic sequences." Nature 317(6040): 828.

Tahimic, C. G., Y. Wang and D. D. Bikle (2013). "Anabolic effects of IGF-1 signaling on the skeleton." Front Endocrinol (Lausanne) 4: 6.

Troadec, M.-B., D. M. Ward, E. Lo, J. Kaplan and I. De Domenico (2010). "Induction of FPN1 transcription by MTF-1 reveals a role for ferroportin in transition metal efflux." Blood 116(22): 4657-4664.

Tu, Q., J. Zhang, L. James, J. Dickson, J. Tang, P. Yang and J. Chen (2007). "Cbfa1/Runx2-deficiency delays bone wound healing and locally delivered Cbfa1/Runx2 promotes bone repair in animal models." Wound Repair Regen 15(3): 404-412.

Tuerk, M. J. and N. Fazel (2009). "Zinc deficiency." Curr Opin Gastroen 25(2): 136-143.

Van Loo, K. M. J., C. Schaub, J. Pitsch, R. Kulbida, T. Opitz, D. Ekstein, A. Dalal, H. Urbach, H. Beck, Y. Yaari, S. Schoch and A. J. Becker (2015). "Zinc regulates a key transcriptional pathway for epileptogenesis via metal-regulatory transcription factor 1." Nat Commun 6: 8688.

Vasudevan, S., Y. Tong and J. A. Steitz (2007). "Switching from repression to activation: microrna s can up-regulate translation." Science 318(5858): 1931.

Vimalraj, S., B. Arumugam, P. J. Miranda and N. Selvamurugan (2015). "Runx2: structure, function, and phosphorylation in osteoblast differentiation." Int J Biol Macromol 78: 202-208.

Westin, G. and W. Schaffner (1988). "A zinc-responsive factor interacts with a metal-regulated enhancer element (MRE) of the mouse metallothionein-I gene." EMBO J 7(12): 3763-3770.

Wu, C. Y., A. J. Bird, L. M. Chung, M. A. Newton, D. R. Winge and D. J. Eide (2008). "Differential control of Zap1-regulated genes in response to zinc deficiency in Saccharomyces cerevisiae." BMC Genomics 9: 370.

Wuehler, S. E., J. M. Peerson and K. H. Brown (2005). "Use of national food balance data to estimate the adequacy of zinc in national food supplies: methodology and regional estimates." Public Health Nutr 8(7): 812-819.

Wysokinski, D., E. Pawlowska and J. Blasiak (2015). "RUNX2: a master bone growth regulator that may be involved in the dna damage response." DNA Cell Biol 34(5): 305-315.

Zheng, L., Q. Tu, S. Meng, L. Zhang, L. Yu, J. Song, Y. Hu, L. Sui, J. Zhang, M. Dard, J. Cheng, D. Murray, Y. Tang, J. B. Lian, G. S. Stein and J. Chen (2017). "Runx2/DICER/miRNA pathway in regulating osteogenesis." J Cell Physiol 232(1): 182-191.

CHAPTER 2

BIOINFORMATIC ANALYSIS OF THE METAL RESPONSE ELEMENT AND ZINC-DEPENDENT GENE REGULATION VIA THE METAL RESPONSE ELEMENT-BINDING TRANSCRIPTION FACTOR 1 IN CACO-2 CELLS[1]

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Abstract

The purpose of this study was to determine the correlation between the position or number of metal regulatory elements (MREs) near gene transcriptional or translational start sites, and the strength of metal response element-binding transcription factor 1 (MTF-1) regulation. A secondary analysis was performed *in silico* on published results measuring the effects of Zn and MTF-1 on transcriptional regulation of genes (n=120) in the Caco-2 cell line. MRE sequence variations throughout the human genome were sorted using a position weight matrix. Three null hypotheses (H_0) were tested: (1) there is no correlation between the number of MREs and MTF-1 transcriptional strength, (2) there is no correlation between the distance of the MRE upstream from the transcriptional start site (TSS) and MTF-1 transcriptional strength, and (3) there is no correlation between the distance of the MRE downstream from the translational start site (TrSS) and MTF-1 transcriptional strength. Spearman correlation was used to test for significance ($p < 0.05$). From our results we rejected the first H_0; we observed a significant correlation between the total number of MRE sequences -7Kbp upstream from the TSS, within the 5' untranslated region, and +1Kbp downstream from the TrSS, versus the strength of MTF-1 regulation ($r = 0.202$; $p = 0.027$). The second and third H_0 were accepted. These results expand our understanding of the role of the MRE in Zn-dependent gene regulation. The data indicate that Zn influences the transcriptional control of gene expression beyond maintaining intracellular Zn homeostasis.

Introduction

It is estimated that 2800 human proteins or about 10% of our proteome is composed of metalloproteins that require zinc (Zn) for biological function, regulation of activities, or structural purposes (Andreini et al. 2006; Klug 2010). The large majority of Zn-binding proteins are involved in the regulation of gene expression, with the most abundant class being Zn-finger proteins (Klug 2010). Intracellular Zn homeostasis is controlled through the coordination of Zn with the metal response element-binding transcription factor 1 (MTF-1), a 72.5 kDa Zn-finger protein in the Cys_2His_2 family of transcription factors. MTF-1 has traditionally been understood in the context of positive transcriptional regulation of metallothionein (MT) and *SLC30A1* gene product in response to fluctuating intracellular Zn concentrations, and the resultant maintenance of Zn homeostasis driven by the expression of these genes (G.W. Stuart 1984; O'Halloran 1993; Langmade et al. 2000; Saydam et al. 2002). However, the physiological role of MTF-1 is more wide-ranging. MTF-1 regulates gene expression in response to metal ion exposure, hypoxia, oxidative stress, and/or elevated temperature (Andrews 2001; Lichtlen and Schaffner 2001). Its regulatory effects have been implicated in the cellular antioxidant response (Stoytcheva et al. 2010), insulin synthesis (Huang et al. 2010; Li et al. 2014), growth and development (Grider et al. 2017), immunity and inflammation/hypoxia (Cramer et al. 2005; Murphy et al. 2005; Murphy et al. 2008), iron and copper homeostasis (Selvaraj et al. 2005; Balesaria et al. 2010; Troadec et al. 2010), and epigenetic histone modification (Okumura et al. 2011). It has been suggested that MTF-1 is a master regulator of microRNA (miRNA) expression, along with c-Myb, NF-Y, Sp1, and AP-2α (Lee et al. 2007). MTF-1 is also currently a target for drug discovery and development to treat epileptogenesis (Van Loo et al. 2015), osteoarthritis (Kim et al. 2014), and prion disease (Bellingham et al. 2009).

As the name suggests, MTF-1 binds to the metal response element (MRE), a cis-regulatory element which consists of a highly conserved 5'-TGCRCNC-3' core (R= A or G, N = A, C, G, or T) (Günther et al. 2012b). This seven-nucleotide consensus sequence is fairly ubiquitous in the genome and occurs over 2 million times (Kearse et al. 2012). The 5' and 3' flanking regions of the MRE may affect transcriptional activity (Koizumi et al. 1999), and there is the potential that a longer sequence beyond the 7-mer core sequence can alter the affinity and function of MTF-1 binding to the MRE.

Like many transcription factors, MTF-1 resides in the cytoplasm and, upon activation, translocates into the nucleus (Smirnova et al. 2000; Saydam et al. 2001). A domain near the C-terminus of MTF-1 regulates its homodimerization, which is important for metal-induced transcriptional activity. Interestingly, although Zn increases the transcriptional activity of MTF-1, it does not enhance dimerization of MTF-1. Copper (Cu) activates MTF-1 binding to a lesser degree than Zn but enhances dimerization more than Zn to synergistically enhance gene regulation (Günther et al. 2012a).

MTF-1 is conserved across metazoan species and has been characterized in human (Brugnera et al. 1994), mouse (Andrews et al. 2001), capybara (Lindert et al. 2008), *Drosophila melanogaster* (Zhang et al. 2001), pufferfish (Maur et al. 1999), zebrafish (Hogstrand et al. 2008), trout (Dalton et al. 2000), and chicken (Laity and Andrews 2007). MTF-1 knockout mice die *in utero* around day 14 from liver decay and edema. It is not known whether MTF-1 has a primary role in embryonic liver development, or if this is a cascading effect resulting from impaired cellular pathways and/or increased sensitivity to heavy metal content at this stage (Günes et al. 1998; Lichtlen et al. 2001; Wang et al. 2004). It is important to note that mouse and capybara MTF-1 (mMTF-1) is less sensitive to Zn-induced mMRE binding and subsequent regulation than human

MTF-1 (Brugnera et al. 1994; Lindert et al. 2008). Interestingly, changing only three amino acids in the protein sequence of mMTF-1 to resemble the human counterpart in the nuclear export signal of the acidic activation domain will augment its metal inducibility to nearly match that of human MTF-1 (Lindert et al. 2009).

MTF-1 may also exhibit preferential binding to the "N" base in 5'-TGCRCNC-3' depending on the metal stimuli. Cadmium treatment in *Drosophila* genomic tiling arrays (ChIP-chip) conferred to MTF-1 a binding preference to a C or T in the "N" position of the MRE, and Cu treatment gave a preference to G or A in that position, with little overlap between the two. Therefore, permutations of the core MRE motif could function to regulate genes which are expressed in response to various mineral stimuli other than Zn (Sims et al. 2012).

Of the six Zn fingers in MTF-1, the N-terminal fingers 1-4 have a high affinity for both Zn and the MRE. These fingers bind to the TGCRC side of the MRE sequence. Fingers 5 and 6 have a weaker affinity for the MRE and bind to the 3' end of the sequence. However, all six fingers contribute to the ability of MTF-1 to form a complex with the MRE. It has also been suggested that fingers 5 and 6 can modulate the specificity and affinity of MTF-1 for the MRE (Labbé et al. 1991; Chen et al. 1999). MTF-1 exhibits the highest affinity binding to the sequence 5'-TGCACTC-3', labeled MREd (Labbé et al. 1991; Stoytcheva et al. 2010).

MTF-1 also exhibits transcriptional regulation when binding to an MRE downstream from the transcription start site (TSS) of a gene. Human selenoprotein H (*SELENOH*) is downregulated by the action of MTF-1 binding to an MRE in the 5' untranslated region (UTR) of the gene, 92 base pairs (bp) upstream from its translational start site (TrSS) (Stoytcheva et al. 2010). Similarly, an MRE within the first exon was shown to be responsible for downregulation of ZIP10 (*SLC39A10*) in zebrafish (Zheng et al. 2008). This upstream regulation by MTF-1 may be related

to the RNA polymerase II stalling mechanism in genes which require rapid transcriptional response in cells (Muse et al. 2007). However, the relevancy of transcription factors binding to exonic regulatory elements is not clear, and it is currently a subject of debate whether this binding is passive and nonfunctional, or if it can contribute to recruitment of the transcription machinery (Stergachis et al. 2013; Xing and He 2015; Agoglia and Fraser 2016).

The purpose of this study was to determine whether the number or location of MRE are correlated with the degree of Zn-dependent transcriptional regulation as mediated through MTF-1. Three null hypotheses (H_0) were tested: (1) there is no correlation between the number of MREs and MTF-1 transcriptional strength, (2) there is no correlation between the distance of the MRE upstream from the transcriptional start site and MTF-1 transcriptional strength, and (3) there is no correlation between the distance of the MRE downstream from the translational start site and MTF-1 transcriptional strength. The results from this study will further our understanding of the role of Zn in transcriptional regulation of gene expression, as well as in other Zn-dependent cellular, biochemical and physiological processes.

Materials and Methods

All analyses were performed using the human hg38 genome assembly (Dec. 2013, Genome Reference Consortium GRCh38). A search was performed to determine the genomic positions of the MRE consensus sequence with five bases added to the 5' and 3' ends (5'-nnnnnTGCRCNCnnnnn-3') (Geneious; v10.2.3) (Kearse et al. 2012). The resulting sequences (n = 2,214,485) were applied to a position weight matrix for analysis (R v3.4.3; seqLogo v1440) (Figure 1) (Schneider and Stephens 1990; Bembom 2017). Chi-square analysis using Excel CHISQ.DIST was used to determine significance ($p \leq 0.05$) at each position.

The genes that were analyzed in this investigation were obtained from Hardyman et al. (2016) which identified 120 protein coding genes within the Caco-2 human colonic adenocarcinoma cell line whose expression were affected by cellular Zn status and the presence/absence of MTF-1. Expression of these genes was defined as a ratio for the effect of MTF-1 on their expression: (downregulation (<1); upregulation (>1); no effect (1)) (Hardyman et al. 2016). The "absolute strength" of MTF-1 effect, controlling for direction of regulation, is defined as the absolute value of one minus the expression ratio, and was determined using the following formula:

$$\text{Strength of MTF-1 effect on gene expression (directional)} = \frac{fold\ change\ with\ control\ siRNA\ (MTF{-}1\ available)}{fold\ change\ with\ MTF{-}1\ siRNA\ (without\ MTF{-}1)}$$

$$\text{Absolute strength of MTF-1 effect on gene expression} = \left| 1 - \frac{fold\ change\ with\ control\ siRNA\ (MTF{-}1\ available)}{fold\ change\ with\ MTF{-}1\ siRNA\ (without\ MTF{-}1)} \right|$$

A recent study reported that MTF-1 transcriptional regulation also occurs when an MRE is located downstream of the gene TrSS (Stoytcheva et al. 2010). Our search range in base pairs was chosen based on data from Hardyman et. al. (2016) and Stoytcheva et. al. (2010). The Integrative Genomics Viewer (IGV; v2.3.90) was used to plot all TGCRCNC sequences that occur within -7Kbp upstream of the TSS, as well as within the 5' UTR and +1Kbp downstream from the TrSS (Robinson et al. 2011; Thorvaldsdóttir et al. 2013). For genes with multiple MRE sequences, only the closest MRE to the TSS or TrSS was considered in the distance analyses. Only MRE sequences on the same DNA strand as the associated gene were considered. Spearman correlation used to test for significance was performed using GraphPad Prism (v7.04).

Results

Within the human genome, the 'R' and 'N' bases within the MRE consensus core sequence are most frequently 'A' and 'T', respectively (Figure 1). The resultant sequence TGCACTC (MREd) is also the most commonly occurring MRE core sequence in the genome (Figure 2). The MRE consensus sequence was extended by 5 bases in the 5' and 3' directions; there are 732,932 permutations of the 17-mer MRE present in the genome. The most common 17-mer MRE sequence (10.0% of total) is GCCACTGCACTCCAGCC (Figure 1). The majority of the 17-mer MRE sequence permutations (72.8%) occur singly in the genome.

The maximum range upstream for counting the MRE sequences was determined by searching for the MRE positions upstream from the TSS of genes; certain MTF-1-dependent genes within the working gene list exhibited their first MRE -7Kbp upstream from their TSS. The lower position in the range was determined by searching for the MRE positions +1Kbp downstream from the TrSS of genes in which there were no MREs in their 5'UTR. This search strategy took into consideration genes such as *ATP6V1B1* and *HIST1H2BD* which exhibited an ~20% change in expression following treatment with the MTF-1 siRNA, yet lacked an MRE either upstream or in their 5' UTR. Therefore, the MREs downstream of their TrSS, +477 and +755 bp respectively, appear to be the most likely candidates as functional binding sites for MTF-1 (Stoytcheva et al. 2010). The position of the MRE within *KRT17* was >+1Kbp upstream from the TrSS, and it exhibited a 0.79-fold change in expression, however this gene is treated as a special case (Figure 3).

There is a significant correlation between the number of MRE motifs in the region of -7Kbp from the TSS to +1Kbp from the TrSS, versus the absolute strength of MTF-1 regulation

(n=118, r=0.205, p=0.0260; Table 2). No significant correlations were observed in null hypotheses 2 or 3 as defined above.

The possibility that a MRE sequence longer than the 7-mer consensus sequence is more predictive of MTF-1 transcriptional gene regulation was explored. A 17-mer MRE, RCCAYTGCACTCYAGCC (R= A or G, Y= C or T) was used to search -7Kbp upstream from the TSS of the 120-gene cohort. This 17-mer sequence is a combination of six of the most common permutations of the 17-base MRE sequence, and accounts for 19.5% of the total instances in the genome. Of the genes identified as actively regulated by MTF-1, a significant number (n=63, 52.5%) had one or more instances of this motif in the range from -7Kbp upstream to the TSS (χ^2 (1, N=120) = 71.492, p<0.0001).

Base composition may play a significant role in the binding affinity of MTF-1 to the MRE. To test this, a 17-base position weight matrix made from MREs associated with MTF-1 responsive genes (Table S1a, 856 MREs, 120 genes, "expected") was compared against a position weight matrix made from MREs associated with MTF-1 unresponsive genes (Table S1b, 153 MREs, 7 genes, "observed") using a chi-squared test at each base position. There are significant differences in the relative weight of bases at every variable position in 5'-*nnnnn*TGCRC*N*C*nnnnn*-3' excluding "R" (χ^2 (3, N=153) >7.815, p<0.05). However, the two most commonly occurring 17-mer MRE sequences throughout the genome (GCCACTGCACTCCAGCC, ACCACTGCACTCCAGCC) remain the first and second most common MREs in both the responsive and unresponsive sets.

Discussion

MTF-1 functions as an intracellular Zn sensor by coordinating Zn and binding to the MRE within the promoters of MT and Zn transporters resulting in their transcription (G.W. Stuart 1984;

O'Halloran 1993; Langmade et al. 2000; Saydam et al. 2002). Several studies support a role for MTF-1 regulating genes other than those directly involved with cellular Zn homeostasis, including as a master regulator of miRNA gene expression (Andrews 2001; Lichtlen and Schaffner 2001; Lee et al. 2007). The results of this study are consistent with those indicating that MTF-1 is a Zn-dependent transcriptional regulator beyond MT and intracellular Zn homeostasis. Our study investigated the target for MTF-1, the MRE, and defined its prevalence within the human genome as well as correlating its location and abundance with the degree of expression in a Caco-2 gene cohort (Hardyman et al. 2016).

It has been previously suggested that base differences in the 5' and 3' flanking regions of the MRE core sequence contribute to the binding affinity of MTF-1 to the MRE, and thus can vary the strength of gene regulation (Koizumi et al. 1999). The number of MREs in this position analysis of bases was not large enough from which to draw conclusions, but the significant differences in base permutations within MREs of genes that have varied MTF-1 expression strength and responsivity is a subject of continued interest and relevance. It was interesting to note the proximity of MREs in genes shown to have no regulatory effects from MTF-1 (Table S1b). The presence of 109 instances of the MRE in the 5' UTR of *ODZ4*, a gene unresponsive to MTF-1 regulation, raises the question of whether these MREs have any functional aspect. This discrepancy might be attributed to the base differences mentioned above. For example, in *ODZ4* the predominant base in the "N" position of its 109 MREs is A (42.1%, T=28.6%), while in the MTF-1 responsive cohort of genes it is predominately T (40.5%, A=25.0%).

For genes which have a nearby MRE but are unresponsive to MTF-1 regulation, there are other extraneous factors which could contribute to a lack of an effect. MTF-1 is hypothesized to form a transcriptional complex with p300 (or CBP) and Sp1 that is essential for gene regulation

(Ogra et al. 2001; Li et al. 2008). Absence of, or interference with, either of these factors would likely disturb MTF-1 mediated regulation. MTF-1 also forms cascading regulatory circuits with miRNAs throughout the genome. For example, in a feed forward loop MTF-1 can target a gene for upregulation, while simultaneously upregulating one or more miRNAs that can post-transcriptionally suppress the products of that same target gene; this would appear as a net zero of regulatory effects (Arora et al. 2013). Although the idea was not explored here, the 3D structure of DNA can also potentially render some MREs more or less functional in terms of their MTF-1 binding and gene regulating capacity (Woringer et al. 2014).

The mechanism of action for MTF-1 binding to the MRE, and subsequently altering transcription, has yet to be fully elucidated. However, we have shown that for a large (n=120) cohort of genes actively regulated by MTF-1, the number of MREs proximal to a gene affects its transcription. Additionally, not every gene with an MRE is regulated by MTF-1. There have not been identified extended MRE sequences that can definitively alter the function of MTF-1, but we have presented here a 17-mer sequence that appears in a significant number of MTF-1 active genes. These findings can serve to improve predictive analyses for genes, other than those directly involved with intracellular Zn homeostasis, that are regulated by MTF-1.

References

Agoglia RM, Fraser HB (2016) Disentangling sources of selection on exonic transcriptional enhancers. Mol Biol Evol 33:585-590

Andreini C, Banci L, Bertini I, Rosato A (2006) Counting the zinc-proteins encoded in the human genome. J Proteome Res 5:196-201

Andrews GK (2001) Cellular zinc sensors: MTF-1 regulation of gene expression. BioMetals 14:223-237

Andrews GK, Dae Kee L, Ravindra R, Lichtlen P, Sirito M, Sawadogo M, Schaffner W (2001) The transcription factors MTF-1 and USF1 cooperate to regulate mouse metallothionein-I expression in response to the essential metal zinc in visceral endoderm cells during early development. EMBO J 20:1114-1122

Arora S, Rana R, Chhabra A, Jaiswal A, Rani V (2013) miRNA-transcription factor interactions: a combinatorial regulation of gene expression. Mol Genet Genomics 288:77-87

Balesaria S, Ramesh B, McArdle H, Bayele HK, Srai SKS (2010) Divalent metal-dependent regulation of hepcidin expression by MTF-1. FEBS Lett 584:719-725

Bellingham SA, Coleman LA, Masters CL, Camakaris J, Hill AF (2009) Regulation of prion gene expression by transcription factors sp1 and metal transcription factor-1. J Biol Chem 284:1291-1301

Bembom O (2017) seqLogo: Sequence logos for DNA sequence alignments. R package version 1.44.0

Brugnera E, Georgiev O, Radtke F, Heuchel R, Baker E, Sutherland GR, Schaffner W (1994) Cloning, chromosomal mapping and characterization of the human metal-regulatory transcription factor MTF-1. Nucleic Acids Res 22:3167-3173

Chen X, Chu M, Giedroc DP (1999) MRE-binding transcription factor-1: weak zinc-binding finger domains 5 and 6 modulate the structure, affinity, and specificity of the metal-response element complex. Biochemistry-US 38:12915-12925

Cramer M, Nagy I, Murphy Brian J, Gassmann M, Hottiger Michael O, Georgiev O, Schaffner W (2005) NF-κB contributes to transcription of placenta growth factor and interacts with metal responsive transcription factor-1 in hypoxic human cells. Biol Chem 386:865-872

Dalton TP, Solis WA, Nebert DW, Carvan Iii MJ (2000) Characterization of the MTF-1 transcription factor from zebrafish and trout cells. Comp Biochem Phys B 126:325-335

G.W. Stuart PFS, H.Y. Chen, R.L. Brinster, R.D. Palmiter (1984) A 12-base-pair DNA motif that is repeated several times in metallothionein gene promoters confers metal regulation to a heterologous gene. Proc Natl Acad Sci USA 81:7318–7322

Grider A, Bakre AA, Laing EM, Lewis RD (2017) *In silico* analysis of microrna regulation of bone development: metal response element-binding transcription factor 1 and bone signaling pathways. Austin J Nutr Metab 4:1042

Günes C, Heuchel R, Georgiev O, Müller KH, Lichtlen P, Blüthmann H, Marino S, Aguzzi A, Schaffner W (1998) Embryonic lethality and liver degeneration in mice lacking the metal-responsive transcriptional activator MTF-1. EMBO J 17:2846-2854

Günther V, Davis AM, Georgiev O, Schaffner W (2012a) A conserved cysteine cluster, essential for transcriptional activity, mediates homodimerization of human metal-responsive transcription factor-1 (MTF-1). BBA - Mol Cell Res 1823:476-483

Günther V, Lindert U, Schaffner W (2012b) The taste of heavy metals: gene regulation by MTF-1. BBA - Mol Cell Res 1823:1416-1425

Hardyman JE, Tyson J, Jackson KA, Aldridge C, Cockell SJ, Wakeling LA, Valentine RA, Ford D (2016) Zinc sensing by metal-responsive transcription factor 1 (MTF1) controls metallothionein and ZnT1 expression to buffer the sensitivity of the transcriptome response to zinc. Metallomics 8:337-343

Hogstrand C, Zheng D, Feeney G, Cunningham P, Kille P (2008) Zinc-controlled gene expression by metal-regulatory transcription factor 1 (MTF1) in a model vertebrate, the zebrafish. Biochem Soc Trans 36:1252-1257

Huang L, Yan M, Kirschke CP (2010) Over-expression of ZnT7 increases insulin synthesis and secretion in pancreatic β-cells by promoting insulin gene transcription. Exp Cell Res 316:2630-2643

Kearse M, Moir R, Wilson A, Stones-Havas S, Cheung M, Sturrock S, Buxton S, Cooper A, Markowitz S, Duran C, Thierer T, Ashton B, Meintjes P, Drummond A (2012) Geneious Basic: An integrated and extendable desktop software platform for the organization and analysis of sequence data. Bioinformatics 28:1647-1649

Kim JH, Jeon J, Shin M, Won Y, Lee M, Kwak JS, Lee G, Rhee J, Ryu JH, Chun CH, Chun JS (2014) Regulation of the catabolic cascade in osteoarthritis by the zinc-Zip8-MTF1 axis. Cell 156:730-743

Klug A (2010) The discovery of zinc fingers and their applications in gene regulation and genome manipulation. Annu Rev Biochem 79:213-231

Koizumi S, Suzuki K, Ogra Y, Yamada H, Otsuka F (1999) Transcriptional activity and regulatory protein binding of metal-responsive elements of the human metallothionein-IIA gene. Eur J Biochem 259:635-642

Labbé S, Prévost J, Remondelli P, Leone A, Séguin C (1991) A nuclear factor binds to the metal regulatory elements of the mouse gene encoding metallothionein-I. Nucleic Acids Res 19:4225-4231

Laity JH, Andrews GK (2007) Understanding the mechanisms of zinc-sensing by metal-response element binding transcription factor-1 (MTF-1). Arch Biochem Biophys 463:201-210

Langmade SJ, Ravindra R, Daniels PJ, Andrews GK (2000) The transcription factor MTF-1 mediates metal regulation of the mouse ZnT1 gene. Journal of Biological Chemistry 275:34803-34809

Lee J, Li Z, Brower-Sinning R, John B (2007) Regulatory circuit of human microRNA biogenesis. PLOS Comput Biol 3:e67

Li B, Cui W, Tan Y, Luo P, Chen Q, Zhang C, Qu W, Miao L, Cai L (2014) Zinc is essential for the transcription function of Nrf2 in human renal tubule cells in vitro and mouse kidney in vivo under the diabetic condition. J Cell Mol Med 18:895-906

Li Y, Kimura T, Huyck RW, Laity JH, Andrews GK (2008) Zinc-induced formation of a coactivator complex containing the zinc-sensing transcription factor MTF-1, p300/CBP, and Sp1. Mol Cell Biol 28:4275-4284

Lichtlen P, Schaffner W (2001) Putting its fingers on stressful situations: the heavy metal-regulatory transcription factor MTF-1. BioEssays 23:1010-1017

Lichtlen P, Wang Y, Belser T, Georgiev O, Certa U, Sack R, Schaffner W (2001) Target gene search for the metal-responsive transcription factor MTF-1. Nucleic Acids Res 29:1514-1523

Lindert U, Cramer M, Meuli M, Georgiev O, Schaffner W (2009) Metal-responsive transcription factor 1 (mtf-1) activity is regulated by a nonconventional nuclear localization signal and a metal-responsive transactivation domain. Mol Cell Biol 29:6283-6293

Lindert U, Leuzinger L, Steiner K, Georgiev O, Schaffner W (2008) Characterization of metal-responsive transcription factor (mtf-1) from the giant rodent capybara reveals features in common with human as well as with small rodents (mouse, rat). Short communication. Chem Biodivers 5:1485-1494

Maur AAd, Belser T, Elgar G, Georgiev O, Schaffner W (1999) Characterization of the transcription factor mtf-1 from the japanese pufferfish *Fugu Rubripes* reveals evolutionary conservation of heavy metal stress response. Biol Chem 380:175-185

Murphy BJ, Kimura T, Sato BG, Shi Y, Andrews GK (2008) Metallothionein induction by hypoxia involves cooperative interactions between metal-responsive transcription factor-1 and hypoxia-inducible transcription factor-1α. Mol Cancer Res 6:483-490

Murphy BJ, Sato BG, Dalton TP, Laderoute KR (2005) The metal-responsive transcription factor-1 contributes to HIF-1 activation during hypoxic stress. Biochem Bioph Res Co 337:860-867

Muse GW, Gilchrist DA, Nechaev S, Shah R, Parker JS, Grissom SF, Zeitlinger J, Adelman K (2007) RNA polymerase is poised for activation across the genome. Nat Genet 39:1507-1511

O'Halloran T (1993) Transition metals in control of gene expression. Science 261:715-725

Ogra Y, Suzuki K, Gong P, Otsuka F, Koizumi S (2001) Negative regulatory role of sp1 in metal responsive element-mediated transcriptional activation. J Biol Chem 276:16534-16539

Okumura F, Li Y, Itoh N, Nakanishi T, Isobe M, Andrews GK, Kimura T (2011) The zinc-sensing transcription factor MTF-1 mediates zinc-induced epigenetic changes in chromatin of the mouse metallothionein-I promoter. BBA - Gene Regul Mech 1809:56-62

Robinson JT, Thorvaldsdóttir H, Winckler W, Guttman M, Lander ES, Getz G, Mesirov JP (2011) Integrative genomics viewer. Nat Biotechnol 29:24

Saydam N, Adams TK, Steiner F, Schaffner W, Freedman JH (2002) Regulation of metallothionein transcription by the metal-responsive transcription factor MTF-1 - Identification of signal transduction cascades that control metal-inducible transcription. J Biol Chem 277:20438-20445

Saydam N, Georgiev O, Nakano MY, Greber UF, Schaffner W (2001) Nucleo-cytoplasmic trafficking of metal-regulatory transcription factor 1 is regulated by diverse stress signals. J Biol Chem 276:25487-25495

Schneider TD, Stephens RM (1990) Sequence logos: a new way to display consensus sequences. Nucleic Acids Res 18:6097-6100

Selvaraj A, Balamurugan K, Yepiskoposyan H, Zhou H, Egli D, Georgiev O, Thiele DJ, Schaffner W (2005) Metal-responsive transcription factor (MTF-1) handles both extremes, copper load and copper starvation, by activating different genes. Gene Dev 19:891-896

Sims HI, Chirn G-W, Marr MT (2012) Single nucleotide in the MTF-1 binding site can determine metal-specific transcription activation. Proc Natl Acad Sci USA 109:16516-16521

Smirnova IV, Bittel DC, Ravindra R, Jiang H, Andrews GK (2000) Zinc and cadmium can promote rapid nuclear translocation of metal response element-binding transcription factor-1. J Biol Chem 275:9377-9384

Stergachis AB, Haugen E, Shafer A, Fu W, Vernot B, Reynolds A, Raubitschek A, Ziegler S, LeProust EM, Akey JM, Stamatoyannopoulos JA (2013) Exonic transcription factor binding directs codon choice and affects protein evolution. Science 342:1367-1372

Stoytcheva ZR, Vladimirov V, Douet V, Stoychev I, Berry MJ (2010) Metal transcription factor-1 regulation via MREs in the transcribed regions of selenoprotein H and other metal-responsive genes. Biochim Biophys Acta 1800:416-424

Thorvaldsdóttir H, Robinson JT, Mesirov JP (2013) Integrative Genomics Viewer (IGV): high-performance genomics data visualization and exploration. Brief Bioinform 14:178-192

Troadec M-B, Ward DM, Lo E, Kaplan J, De Domenico I (2010) Induction of FPN1 transcription by MTF-1 reveals a role for ferroportin in transition metal efflux. Blood 116:4657-4664

Van Loo KMJ, Schaub C, Pitsch J, Kulbida R, Opitz T, Ekstein D, Dalal A, Urbach H, Beck H, Yaari Y, Schoch S, Becker AJ (2015) Zinc regulates a key transcriptional pathway for epileptogenesis via metal-regulatory transcription factor 1. Nat Commun 6:8688

Wang Y, Wimmer U, Lichtlen P, Inderbitzin D, Stieger B, Meier PJ, Hunziker L, Stallmach T, Forrer R, Rülicke T, Georgiev O, Schaffner W (2004) Metal-responsive transcription factor-1 (MTF-1) is essential for embryonic liver development and heavy metal detoxification in the adult liver. FASEB J 18:1071-1079

Woringer M, Darzacq X, Izeddin I (2014) Geometry of the nucleus: a perspective on gene expression regulation. Curr Opin Chem Biol 20:112-119

Xing K, He X (2015) Reassessing the “duon” hypothesis of protein evolution. Mol Biol Evol 32:1056-1062

Zhang B, Egli D, Georgiev O, Schaffner W (2001) The drosophila homolog of mammalian zinc finger factor mtf-1 activates transcription in response to heavy metals. Mol Cell Biol 21:4505-4514

Zheng D, Feeney GP, Kille P, Hogstrand C (2008) Regulation of ZIP and ZnT zinc transporters in zebrafish gill: zinc repression of ZIP10 transcription by an intronic MRE cluster. Physiol Genomics 34:205-214

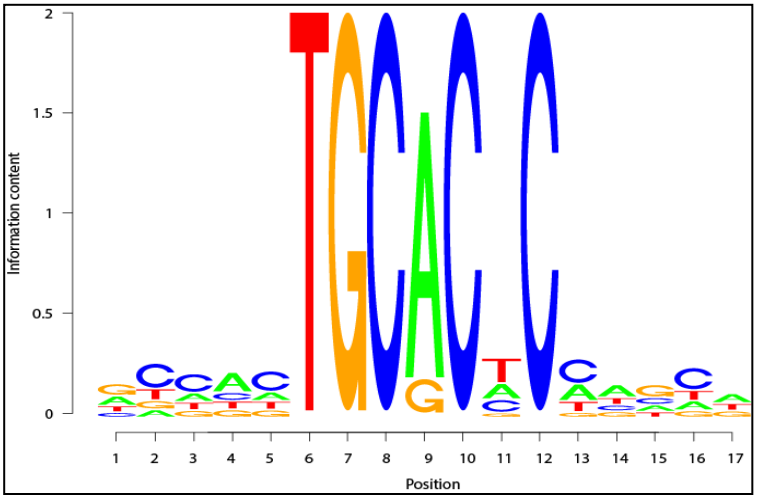

Figure 1.1. Sequence logo of the genome-wide instances of TGCRCNC with five free bases on either side (5'-nnnnnTGCRCNCnnnnn-3'). There is a strong preference for A in the "R" position and a preference for T in the "N" position. The sequence made by the predominate base at each position coincides with the most commonly occurring 7-mer MRE sequence in the genome, TGCACTC, and the most common 17-mer sequence GCCACTGCACTCCAGCA.

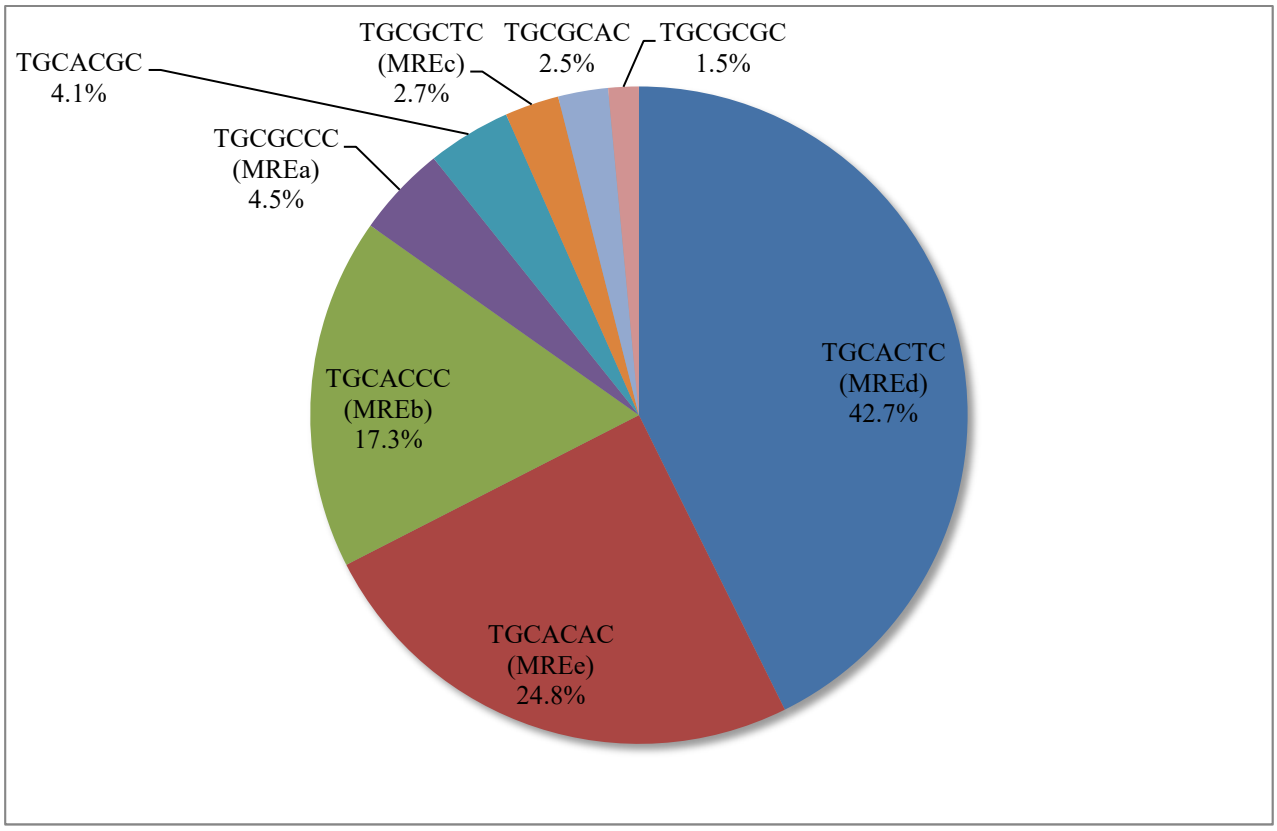

Figure 1.2. Permutations of the MRE consensus sequence 5'-TGCRCNC-3' in the human genome. Equivalent MRE names sometimes used are given in parenthesis. TGCACTC or MREd, which has been demonstrated to exhibit the highest affinity binding to MTF-1 (Stoytcheva et al. 2010), is also the most prevalent in the human genome.

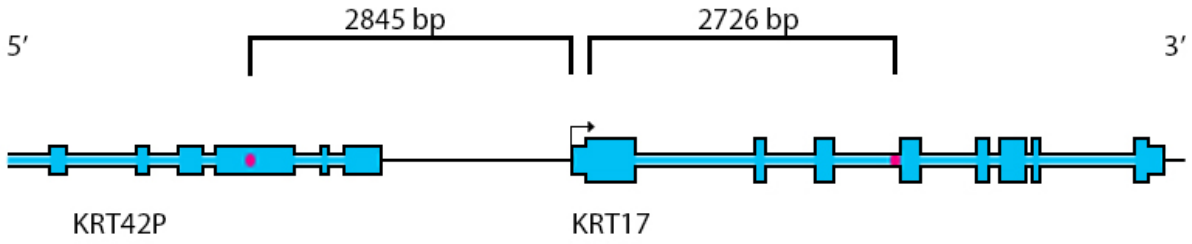

Figure 1.3. *KRT17* (chr17:41,617,440-41,626,630) exhibits a 0.79 fold change ratio in MTF-1 control versus knockdown conditions. Large bars indicate coding sequence regions, dots indicate MRE positions. There is an MRE 2726 bp downstream from the *KRT17* TrSS, and the MRE 2845 bp upstream from the TSS is in an exon of the neighboring gene, *KRT42P*. Neither of these sites conform to currently understood mechanisms for MTF-1 binding and regulation.

Table 1. Position weight matrix of all instances of nnnnnTGCRCNCnnnnn in the human genome (n=2,214,485).

	POSITION																
BASE	1	2	3	4	5	6 (T)	7 (G)	8 (C)	9 (R)	10 (C)	11 (N)	12 (C)	13	14	15	16	17
A	0.309	0.134	0.199	0.487	0.186	0	0	0	0.889	0	0.273	0	0.300	0.439	0.195	0.182	0.229
C	0.129	0.508	0.479	0.175	0.490	0	0	1	0	1	0.218	1	0.455	0.177	0.214	0.493	0.464
G	0.391	0.151	0.151	0.165	0.154	0	1	0	0.111	0	0.056	0	0.069	0.171	0.437	0.124	0.146
T	0.171	0.207	0.171	0.173	0.170	1	0	0	0	0	0.453	0	0.176	0.214	0.155	0.201	0.161

Table 1.2. Results of Spearman correlation tests comparing distance and quantity of MRE sequences vs. the strength of MTF-1 effects on the regulation of associated genes. *There is a significant correlation between the absolute strength of MTF-1 effect on gene expression, defined as $\left| 1 - \frac{\boldsymbol{fold\ change\ with\ control\ siRNA\ (MTF{-}1\ available)}}{\boldsymbol{fold\ change\ with\ MTF{-}1\ siRNA\ (without\ MTF{-}1)}} \right|$, and the total number of MREs associated with that gene.

Spearman correlation test	**Directional strength MTF-1**			**Absolute strength MTF-1**		
	n	R	p (two-tailed)	n	R	p (two-tailed)
Upstream MRE distance	111	-0.04555	0.6350	111	-0.07074	0.4606
Downstream MRE distance	50	-0.07241	0.6173	50	0.1325	0.3591
Total number of MRE instances	118	-0.06677	0.4725	118	0.205	0.0260*

CHAPTER 3

MIRNA–TARGET INTERACTIONS IN OSTEOGENIC SIGNALING PATHWAYS INVOLVING ZINC VIA THE METAL REGULATORY ELEMENT [2]

[2] Francis, M. and A. Grider. 2018. *BioMetals*. 32(1): p. 111-121.
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Abstract

Adequate zinc nutriture is necessary for normal bone growth and development, though the precise mechanisms for zinc-mediated bone growth remain poorly defined. A key transcription factor activated by zinc is metal response element-binding transcription factor 1 (MTF-1), which binds to the metal regulatory element (MRE). We hypothesize that MREs will be found upstream of miRNA genes as well as miRNA target genes in the following bone growth and development signaling pathways: TGF-β, MAPK, and Wnt. A Bioconductor-based workflow in R was designed to identify interactions between MREs, miRNAs, and target genes. MRE sequences were found upstream from 64 mature miRNAs that interact with 213 genes which have MRE sequences in their own promoter regions. *MAPK1* exhibited the most miRNA-target interactions (MTIs) in the TGF-β and MAPK signaling pathways; *CCND2* exhibited the most interactions in the Wnt signaling pathway. Hsa-miR-124-3p exhibited the most MTIs in the TGF-β and MAPK signaling pathways; hsa-miR-20b-5p exhibited the most MTIs in the Wnt signaling pathway. *MYC* and hsa-miR-34a-5p were shared between all three signaling pathways, also forming an MTI unit. JUN exhibited the most protein-protein interactions, followed by MAPK8. These *in silico* data support the hypothesis that intracellular zinc status plays a role in osteogenesis through the transcriptional regulation of miRNA genes via the zinc/MTF-1/MRE complex.

Introduction

Zinc (Zn) is an essential trace element involved in fundamental biochemical and physiological pathways. It is necessary for enzyme catalysis, protein structure, and various regulatory functions as approximately 2800 enzymes and proteins bind Zn (10% of the human genome) (Andreini et al. 2006), and approximately 3% of our genome encodes zinc finger transcription factors (Klug 2010). Approximately 4% of the worldwide population is Zn deficient (Wuehler et al. 2005), and children are the most affected (Hambidge and Krebs 2007; Tuerk and Fazel 2009). Proper Zn nutriture is necessary for normal growth and development in animals and humans. Consumption of a Zn-depleted diet resulted in reduced bone Zn and growth in rodent animal models (Chu et al. 2003; Grider et al. 2007; Keller et al. 2000). Manipulation of the Zn nutritional environment affects osteogenic biomarkers (Berger et al. 2015; Cho et al. 2007; Seo et al. 2010). Current recommendations for assessing the risk of Zn deficiency in populations includes the functional assessment of length- or height-for-age measurements (Hess et al. 2009; International Zinc Nutrition Consultative et al. 2004).

The molecular mechanisms associated with the effect of Zn on bone growth and metabolism are the focus of ongoing research. Circulating levels of insulin-like growth factor-1 (IGF-1) correlate with dietary Zn levels and Zn status in children in some studies (Bougle et al. 2004; Cesur et al. 2009; Hamza et al. 2012; Imamoglu et al. 2005) but not others (Park et al. 2017). Zn supplementation increases the synthesis of IGF-1 in bone (Igarashi and Yamaguchi 2001) and the binding of IGF-1 to its receptor (McCusker 1998; McCusker et al. 1998; Sackett and McCusker 1998a; Sackett and McCusker 1998b). Signaling pathways that are also involved with bone formation include transforming growth factor β/bone morphogenic protein (TGF-β/BMP), wingless-type (Wnt), p38 mitogen-activated protein kinase (MAPK), Hedgehog, Notch, and

fibroblast growth factor (FGF). Significant cross-talk occurs between these pathways (Chen et al. 2012). The TGF-β/BMP, Wnt, MAPK, and Hedgehog pathways also intersect with the IGF-1 pathway (Guntur and Rosen 2013; Longobardi et al. 2006; Tahimic et al. 2013).

We hypothesize that the Zn-dependent regulation of bone growth and development involves microRNAs (miRNAs) and metal regulatory elements (MREs), ultimately forming complex and precise regulatory cascades of gene expression (Arora et al. 2013). MiRNAs are small, noncoding RNAs involved in the posttranslational regulation of gene expression through binding to seed sequences in the 3' untranslated regions (UTR) of mRNA, resulting in transcriptional repression (Lee et al. 1993), or in the 5' terminal oligopyrimidine tracts of ribosomal protein mRNA, resulting in translational activation (Orom et al. 2008). MiRNAs have also been shown to form complexes with certain regulatory proteins resulting in translational activation (Eiring et al. 2010; Vasudevan et al. 2007). The MRE is a conserved 7-base motif (5'-TGCRCNC-3'; R=A or G, N = any nucleotide) that is bound by metal transcription factor-1 (MTF-1), a 72.5 kDa Zn-finger (ZF) protein in the Cys_2His_2 family of transcription factors (Stuart 1984; Langmade et al. 2000; O'Halloran 1993; Saydam et al. 2002). MTF-1 contains six ZFs and exhibits Zn-dependent binding to the MRE, with ZF1 and possibly ZF3 and ZF6 responsible for its Zn-sensing and MRE binding properties (Bittel et al. 2000; Chen et al. 1999; Chen et al. 1998; Guerrerio and Berg 2004).

Results from *in vivo* and *in vitro* studies indicate Zn-dependent differential miRNA expression (Grider et al. 2015; Liuzzi 2014; Ryu et al. 2011). MTF-1 has been proposed as a master regulator for miRNA expression (Lee et al. 2007). The results of *in silico* studies from this laboratory support the hypothesis that genes within osteogenic signaling pathways are regulated by Zn mediated by MTF-1/MRE/miRNA interactions (Grider et al. 2017). The purpose of this

investigation is to identify the locations of MREs upstream genes of miRNAs which then target genes within the TGF-β, MAPK, and Wnt signaling pathways. It must be noted, however, that previous results indicate that the presence of an MRE upstream from the transcriptional start site (TSS) of a gene does not necessitate its Zn-dependent or MTF-1 dependent regulation. The results presented here show that both miRNAs and their target genes contain MREs upstream from their TSSs; suggesting that the regulation of osteogenesis by Zn involves complex interactions between Zn, MRE/gene, and MRE/miRNA/gene associations. A model of feed-forward loops is proposed.

Materials and Methods

All analyses were performed using the human hg38 genome assembly (Dec. 2017, Genome Reference Consortium GRCh38.p12). Bioconductor packages were run using R Studio (Huber et al. 2015; Team 2016). A summary of the workflow is shown in Figure 1.

Positions of the MRE consensus sequence 5'-TGCRCNC -3' in GRCh38.p12 were recorded using Biostrings (v2.48.0) (Pagès H 2018). BiomaRt (v2.36.0) was used to find the genomic coordinates of all HUGO Gene Nomenclature Committee (HGNC)-named genes (Durinck S 2005; Durinck S 2009). Pre-miRNAs with transcriptional start sites predicted in human embryonic stem cells (hESC) by the microTSS algorithm (Georgakilas et al. 2014) were queried in the range of -7Kbp upstream and +1Kbp downstream, based on previous analysis, (Francis and Grider 2018) using GenomicRanges (v1.32.3) (Lawrence 2013). microTSS genomic coordinates were batch converted from hg19 to hg38 using liftOver (Kent et al. 2002). Genes that were interrupted between their TSSs and nearest MRE were excluded from our analysis. The pre-miRNAs with proximal MREs were converted to their mature miRNA sequences using miRBase (Griffiths-Jones 2004; Griffiths-Jones et al. 2006; Griffiths-Jones et al. 2008; Kozomara and Griffiths-Jones 2011; Kozomara and Griffiths-Jones 2014). TarBase (experimentally verified)

miRNA-target interactions (MTIs) between these mature miRNAs and genes were identified using miRTarBase via multiMiR (Database Version: 2.2.0 Updated: 2017-08-08) (Ru et al. 2014). These MTIs were sorted into their KEGG signaling pathways (Kanehisa et al. 2017); interactions in the TGF-β, MAPK, and Wnt pathways were chosen for further analysis. GenomicRanges was used to find genes in these pathways that have MRE sequences without interruption by other genes in their upstream -7Kbp region (Supplementary Table 1).

Venn diagrams (Oliveros 2007-2015) were produced from the lists of MTIs from each signaling pathway to identify those genes and miRNAs that are shared between pathways (Supplementary Table 2). The gene cohort with experimentally verified MTIs was analyzed by the Panther GO-Slim Biological Process (Panther Overrepresentation Test release 20171205; PANTHER version 13.1 Released 2018-02-02; $\alpha = 0.05$) (Ashburner et al. 2000; Gene Ontology 2015; Tahimic et al. 2013; Zhao et al. 2017). In addition, genes from osteogenic KEGG pathways for which there was at least one experimentally verified MTI were subsequently used to query miRDB via multiMiR for predicted gene interactions (target scores ≥ 81) (Wang 2016; Wong and Wang 2015). The Panther Overrepresentation Test was performed on this cohort of predicted gene targets as well. Fisher's exact testing with false discovery rate multiple test correction was used to identify significantly overrepresented annotated gene ontologies.

The experimentally verified MTI genes that are shared between the signaling pathways were analyzed for their protein-protein interactions using the STRING database (v10.5) (Szklarczyk et al. 2015; Szklarczyk et al. 2017). The interactions were visualized using the Network viewer. The analysis was performed using the highest minimum required interaction score (0.900). Only experimentally derived and database interaction sources are reported (Supplementary Table 3).

Results

The TSSs for 86 pre-miRNA genes were predicted by the microTSS algorithm; 44 of these pre-miRNAs contain an MRE within the range specified, without interruption between the MRE motif and the MIR gene TSS by the presence of another gene. These 44 pre-miRNAs equate to 73 mature miRNAs. Within the TGF-β, MAPK, and Wnt signaling pathways, 65 of the 73 mature miRNAs have Tarbase-verified MTIs (hereafter called "verified MTIs") with 241 genes. Of the 460 genes in these three signaling pathways, 413 have an MRE in the range specified. Of those 413 genes, 213 have verified MTIs with 64 mature miRNAs which also have MREs. The TGF-β pathway contained MTIs consisting of 39 miRNAs/44 genes, the MAPK pathway contained MTIs consisting of 57 miRNAs/130 genes, and the Wnt pathway contained MTIs consisting of 43 miRNAs/61 genes. *MAPK1* exhibited 10 MTIs, the most verified MTIs in the TGF-β and MAPK signaling pathways. *CCND2* exhibited 9 MTIs, the most verified MTIs in the Wnt signaling pathway. Hsa-miR-124-3p targeted the most genes in the TGF-β (12 genes) and MAPK (32 genes) signaling pathways. Hsa-miR-20b-5p targeted the most genes, 12, in the Wnt signaling pathway (Table 1; Supplementary Table 1).

Genes with MREs and verified MTIs that are shared between two or more signaling pathways are organized in Venn diagrams (Figure 2). *MYC* is the only gene that is shared among TGF-β, MAPK, and Wnt signaling pathways. MAPK and Wnt pathways share 11 other genes; TGF-β and MAPK pathways share five genes; and TGFβ and Wnt pathways share four genes (Figure 2A; Supplementary Table 2). There are 28 miRNAs that are shared among the three pathways (Figure 2B; Supplementary Table 2). There are seven miRNAs that are experimentally verified to target *MYC* (Table 2); hsa-miR-34a-5p exhibits the most MTIs within each signaling

pathway. The seven miRNAs targeting *MYC* are within the cohort of 28 miRNAs that interact with all three pathways (Figure 2B, 2C).

The protein-protein interactions of the genes shared between the three signaling pathways that are targets for miRNAs that contain MREs upstream from their TSSs, and that also contain MREs within their own promoter regions, were identified using the String database (v10.5) (Figure 3; Supplementary Table 3). *JUN* exhibits the most protein-protein interactions (10), and *MAPK8* exhibits the second most interactions (9). *MYC*, though involved with all three signaling pathways, interacts with just five other proteins.

There are 21 genes with verified MTIs that are common to at least two signaling pathways; these were used to query the Panther GO-Slim Biological Process database to identify overrepresented gene ontologies (Figure 4). Intracellular signal transduction exhibited the highest enrichment score. This cohort is also significantly enriched in genes involved with cellular process, cell proliferation, and transmembrane receptor protein serine/threonine kinase signaling pathway. The 64 miRNAs with MREs that target genes in the osteogenic pathways exhibited 6886 predicted MTIs with 3613 genes (only target scores ≥ 81 were analyzed). This larger cohort of genes was enriched most in cellular process, followed by localization, metabolic process, and cell communication (data not shown).

Discussion

Zn is crucial for healthy bone growth and development, but the mechanism for its effects is complex and not fully elucidated. There is continued interest in understanding the translational control of osteogenesis through the nexus of miRNAs and osteogenic signaling pathway genes (Fushimi et al. 2018; Lian et al. 2012; Liu et al. 2018; Vimalraj and Selvamurugan 2013). Previous *in silico* analyses support the associations between Zn, MTF-1/MRE, and miRNAs in regulating

osteogenic signaling pathways (Grider et al. 2017). This investigation serves to expand on the previous results, further supporting the role Zn plays in osteogenesis through its interaction with the MTF-1/MRE complex and miRNAs.

TGF-β, MAPK, and Wnt signaling pathways play key roles in osteogenesis (Baron and Kneissel 2013; Chen et al. 2012; Wu et al. 2016b). The data indicate that 213 genes within these three pathways have experimentally verified MTIs with 64 miRNAs. The MTIs in these pathways share a single gene, *MYC*, a proto-oncogene involved in cell cycle progression and transformation, and apoptosis. Others have shown that *MYC* expression is increased with activation of Wnt/β-catenin, and correlated with reduced terminal differentiation of osteoblasts, increased bone mass through osteoblast proliferation, and reduced bone strength (Chen et al. 2015; Li et al. 2017). The addition of EDTA to cultured HL-60 cells, a model of Zn deficiency, decreased the expression of *MYC* and cellular proliferation; Zn addition reversed these effects (Morimoto et al. 1992). Our data indicate that *MYC* contains several MREs in its promoter, supporting the hypothesis that Zn regulates *MYC* expression via MTF-1.

We also observed that seven miRNAs formed MTIs with *MYC*; these miRNAs also contain MREs upstream from their TSSs. Hsa-miR-34a-5p targets *MYC* and additionally exhibits the largest number of experimentally verified MTIs with this gene in each signaling pathway. The expression of this miRNA is induced in mice by dietary Zn (Liuzzi 2014). This miRNA has been studied extensively and inhibits osteoblast differentiation and bone formation. In models of human stromal (skeletal, mesenchymal) stem cell (hMSC) differentiation, overexpression of hsa-miR-34a in hMSCs reduced heterotopic bone formation by 60%. Conversely, in miR-34a-deficient hMSC, in vivo bone formation was increased by 200% (Chen et al. 2014). The available data indicate that osteoblast differentiation is inhibited by both *MIR34A* and *MYC* gene products. Others have found

that the deletion of *MYC* in osteoclasts increases bone mass in mice which have undergone ovariectomy (Bae et al., 2017). The results from our *in silico* analysis suggests a complex relationship between the MRE, hsa-miR-34a-5p, and *MYC* effects on osteogenesis. MTF-$1_{(Zn)}$ activates gene expression via the MRE in both *MYC* and *MIR34A*; hsa-miR-34a-5p is expected to inhibit *MYC* but may act synergistically with the MRE in the *MYC* promoter to stimulate *MYC* transcription; the result is increased osteoblast proliferation, and decreased osteoblast differentiation and bone strength. These data suggest that the role of Zn in osteogenesis is a complex series of reactions between MTF-1/MRE, and genes in the signaling pathways that remain to be elucidated.

Genes that exhibit the most verified MTIs include *MAPK1* and *CCND2*. *MAPK1* exhibited the most MTIs within the TGF-β and MAPK signaling pathways. *MAPK1* is a member of a serine/threonine kinase family of extracellular signal-regulated kinases that are involved in numerous phosphorylation reactions associated with development and cellular differentiation (Kyosseva 2004; Martin-Blanco 2000). It has also been reported to function as a transcriptional repressor (Hu et al. 2009). The interaction that *MAPK1* and *MYC* exhibit may involve phosphorylation or transcriptional repression of *MYC* by *MAPK1*. *CCND2* encodes cyclin D2, which complexes with cyclin-dependent kinases at the beginning of the G_1 phase of the cell cycle (Sherr 1994; Vermeulen et al. 2003). *CCND2* is not shared between Wnt and the other signaling pathways. Nevertheless, it is transcriptionally upregulated by the *MYC* gene product (Bouchard et al. 2001; Bouchard et al. 1999; Mai et al. 1999). *CCND2* and *MYC* are also targeted by different sets of miRNAs.

The top five MTI miRNAs are the same among the three signaling pathways, though in different orders. Hsa-miR-124-3p is involved in the most MTIs in the TGF-β and MAPK signaling

pathways. The downregulation of this miRNA is correlated with increased tumorigenesis and poor clinical outcomes, whereas upregulation results in decreased cell proliferation and tumor suppression (Feng et al. 2016; Yang et al. 2017; Zhang et al. 2015; Zhou et al. 2017). Hsa-miR-20b-5p is involved in the most MTIs in the Wnt signaling pathway. This miRNA also functions as a tumor suppressor (Xin et al. 2016) and is GO-linked to apoptosis and autophagy (Wu et al. 2016a). Our data support a role for Zn, through its binding to MTF-1 and subsequent MRE activation, in the transcriptional regulation of these miRNAs. Their specific effects on osteogenesis, though, remain to be determined.

MAPK8 and *JUN* exhibit the most protein-protein interactions among the MTI gene products shared between the TGF-β, MAPK, and Wnt signaling pathways. The *JUN* gene product, c-Jun, is activated by phosphorylation and is a necessary component of the AP-1 DNA-binding complex (Miller et al. 2010; Vasilevskaya and O'Dwyer 2003). c-Jun also forms a complex with β-catenin and TCF4 to upregulate *MYC* transcription at the 3' enhancer region (Yochum et al. 2008). Our analysis indicates that several mitogen-activated protein kinases, also called c-Jun N-terminal kinases, are responsible for phosphorylating c-Jun, including the gene product of *MAPK8*, *JNK1* (Bubici and Papa 2014). We report that *JUN*, *MAPK8*, and *MYC* are involved in MTIs, and each MTI component contains one or more MREs upstream from its TSS.

These observations support the hypothesis that the transcriptional regulation of osteogenesis by Zn occurs at multiple nodes within osteogenic signaling pathways. MREs are located upstream from the TSSs of both components of the MTI. We propose that feed-forward loops are involved with the Zn-dependent transcriptional regulation of osteogenesis through the MTF-1/MRE/MTI interactions (Figure 5). The results from this study support the supposition that Zn regulation of bone growth and development occurs at the most fundamental levels. Future

studies are planned to validate these complex Zn-dependent regulatory interactions as they relate to bone growth and development.

References

Andreini C, Banci L, Bertini I, Rosato A (2006) Counting the zinc-proteins encoded in the human genome. J Proteome Res 5:196-201 doi:10.1021/pr050361j

Arora S, Rana R, Chhabra A, Jaiswal A, Rani V (2013) miRNA-transcription factor interactions: a combinatorial regulation of gene expression. Mol Genet Genomics 288:77-87 doi:10.1007/s00438-013-0734-z

Ashburner M et al. (2000) Gene ontology: tool for the unification of biology. The Gene Ontology Consortium. Nature Genet 25:25-29 doi:10.1038/75556

Bae, S., Lee, M. J., Mun, S. H., Giannopoulou, E. G., Yong-Gonzalez, V., Cross, J. R., Murata, K., Giguere, V., van der Meulen, M., Park-Min, K. H., 2017. MYC-dependent oxidative metabolism regulates osteoclastogenesis via nuclear receptor ERRα. J Clin Invest 127:7, 2555-2568.

Baron R, Kneissel M (2013) WNT signaling in bone homeostasis and disease: from human mutations to treatments. Nat Med 19:179-192 doi:10.1038/nm.3074

Berger PK et al. (2015) Zinc supplementation increases procollagen type 1 amino-terminal propeptide in premenarcheal girls: a randomized controlled trial. J Nutr 145:2699-2704 doi:10.3945/jn.115.218792

Bittel DC, Smirnova IV, Andrews GK (2000) Functional heterogeneity in the zinc fingers of metalloregulatory protein metal response element-binding transcription factor-1. J Biol Chem 275:37194-37201 doi:10.1074/jbc.M003863200

Bouchard C, Dittrich O, Kiermaier A, Dohmann K, Menkel A, Eilers M, Luscher B (2001) Regulation of cyclin D2 gene expression by the Myc/Max/Mad network: Myc-dependent TRRAP recruitment and histone acetylation at the cyclin D2 promoter. Genes Dev 15:2042-2047 doi:10.1101/gad.907901

Bouchard C, Thieke, K, Maier A, Saffrich R, Hanley-Hyde J, Ansorge W, Reed S, Sicinski P, Bartek J, Eilers M, (1999) Direct induction of cyclin D2 by Myc contributes to cell cycle progression and sequestration of p27. EMBO J 18:5321-5333 doi:10.1093/emboj/18.19.5321

Bougle DL, Sabatier JP, Guaydier-Souquieres G, Guillon-Metz F, Laroche D, Jauzac P, Bureau F (2004) Zinc status and bone mineralisation in adolescent girls. J Trace Elem Med Bio 18:17-21 doi:10.1016/j.jtemb.2004.03.001

Bubici C, Papa S (2014) JNK signalling in cancer: in need of new, smarter therapeutic targets. Br J Pharmacol 171:24-37 doi:10.1111/bph.12432

Cesur Y, Yordam N, Dogan M (2009) Serum insulin-like growth factor-i and insulin-like growth factor binding protein-3 levels in children with zinc deficiency and the effect of zinc supplementation on these parameters. J Pediatr Endocr Met 22:1137-1143

Chen G, Deng C, Li YP (2012) TGF-beta and BMP signaling in osteoblast differentiation and bone formation. Int J Biol Sci 8:272-288 doi:10.7150/ijbs.2929

Chen L, Holmstrom K, Qiu W, Ditzel N, Shi K, Hokland L, Kassem M (2014) MicroRNA-34a inhibits osteoblast differentiation and in vivo bone formation of human stromal stem cells. Stem Cells 32:902-912 doi:10.1002/stem.1615

Chen S et al. (2015) Adverse effects of osteocytic constitutive activation of ss-catenin on bone strength and bone growth. J Bone Miner Res 30:1184-1194 doi:10.1002/jbmr.2453

Chen X, Chu M, Giedroc DP (1999) MRE-Binding transcription factor-1: weak zinc-binding finger domains 5 and 6 modulate the structure, affinity, and specificity of the metal-response element complex. Biochem 38:12915-12925

Chen XH, Agarwal A, Giedroc DP (1998) Structural and functional heterogeneity among the zinc fingers of human MRE-binding transcription factor-1. Biochem 37:11152-11161

Cho YE, Lomeda RA, Ryu SH, Sohn HY, Shin HI, Beattie JH, Kwun IS (2007) Zinc deficiency negatively affects alkaline phosphatase and the concentration of Ca, Mg and P in rats. Nutr Res Pract 1:113-119 doi:10.4162/nrp.2007.1.2.113

Chu Y, Mouat MF, Harris RB, Coffield JA, Grider A (2003) Water maze performance and changes in serum corticosterone levels in zinc-deprived and pair-fed rats. Physiol Behav 78:569-578

Durinck S MY, Kasprzyk A, Davis S, De Moor B, Brazma A, Huber W (2005) BioMart and Bioconductor: a powerful link between biological databases and microarray data analysis. Bioinform 21:3439–3440

Durinck S SP, Birney E, Huber W (2009) Mapping identifiers for the integration of genomic datasets with the R/Bioconductor package biomaRt. Nat Protoc 4:1184–1191

Eiring AM et al. (2010) miR-328 functions as an rna decoy to modulate hnrnp e2 regulation of mrna translation in leukemic blasts. Cell 140:652-665

Feng T et al. (2016) miR-124 downregulation leads to breast cancer progression via LncRNA-MALAT1 regulation and CDK4/E2F1 signal activation. Oncotarget 7:16205-16216 doi:10.18632/oncotarget.7578

Francis M, Grider A (2018) Bioinformatic analysis of the metal response element and zinc-dependent gene regulation via the metal response element-binding transcription factor 1 in Caco-2 cells. Biometals doi:10.1007/s10534-018-0115-5

Fushimi S, Nohno T, Nagatsuka H, Katsuyama H (2018) Involvement of miR-140-3p in Wnt3a and TGFbeta3 signaling pathways during osteoblast differentiation in MC3T3-E1 cells. Genes Cells 23:517-527 doi:10.1111/gtc.12591

Gene Ontology Consortium (2015) Gene Ontology Consortium: going forward. Nucleic Acids Res 43:D1049-1056 doi:10.1093/nar/gku1179

Georgakilas G, Vlachos IS, Paraskevopoulou MD, Yang P, Zhang Y, Economides AN, Hatzigeorgiou AG (2014) microTSS: accurate microRNA transcription start site identification reveals a significant number of divergent pri-miRNAs. Nat Comm 5:5700 doi:10.1038/ncomms6700

Grider A, Bakre AA, Laing EM, Lewis RD (2017) In silico analysis of microrna regulation of bone development: metal response element-binding transcription factor 1 and bone signaling pathways. Austin J Nutr Metab 4:1042

Grider A, Lewis RD, Laing EM, Bakre AA, Tripp RA (2015) Zinc affects miR-548n, SMAD4, SMAD5 expression in HepG2 hepatocyte and HEp-2 lung cell lines. Biometals 28:959-966 doi:10.1007/s10534-015-9880-6

Grider A, Mouat MF, Scrimgeour AG (2007) Consumption of a moderately Zn-deficient and Zn-supplemented diet affects soluble protein expression in rat soleus muscle J Nutr Biochem 18:753-759 doi:10.1016/j.jnutbio.2006.11.013

Griffiths-Jones S (2004) The microRNA Registry. Nucleic Acids Res 32:D109-111 doi:10.1093/nar/gkh023

Griffiths-Jones S, Grocock RJ, van Dongen S, Bateman A, Enright AJ (2006) miRBase: microRNA sequences, targets and gene nomenclature. Nucleic Acids Res 34:D140-D144 doi:10.1093/nar/gkj112

Griffiths-Jones S, Saini HK, van Dongen S, Enright AJ (2008) miRBase: tools for microRNA genomics. Nucleic Acids Res 36:D154-D158 doi:10.1093/nar/gkm952

Guerrerio AL, Berg JM (2004) Metal ion affinities of the zinc finger domains of the metal responsive element-binding transcription factor-1 (MTF1). Biochem 43:5437-5444 doi:10.1021/bi0358418

Guntur AR, Rosen CJ (2013) IGF-1 regulation of key signaling pathways in bone. Bonekey Reports 2:437 doi:10.1038/bonekey.2013.171

Hambidge KM, Krebs NF (2007) Zinc deficiency: a special challenge. J Nutr 137:1101-1105

Hamza RT, Hamed AI, Sallam MT (2012) Effect of zinc supplementation on growth hormone insulin growth factor axis in short Egyptian children with zinc deficiency. Ital J Pediatr 38 doi:10.1186/1824-7288-38-21

Hess SY, Lonnerdal B, Hotz C, Rivera JA, Brown KH (2009) Recent advances in knowledge of zinc nutrition and human health. Food Nutr Bull 30:S5-11 doi:10.1177/15648265090301S102

Hu S et al. (2009) Profiling the human protein-DNA interactome reveals ERK2 as a transcriptional repressor of interferon signaling. Cell 139:610-622 doi:10.1016/j.cell.2009.08.037

Huber W et al. (2015) Orchestrating high-throughput genomic analysis with Bioconductor. Nat Methods 12:115 doi:10.1038/nmeth.3252

Igarashi A, Yamaguchi M (2001) Increase in bone growth factors with healing rat fractures: The enhancing effect of zinc. Int J Mol Med 8:433-438

Imamoglu S, Bereket A, Turan S, Haklar G (2005) Effect of zinc supplementation on growth hormone secretion, IGF-I, IGFBP-3, somatomedin generation, alkaline phosphatase, osteocalcin and growth in prepubertal children with idiopathic short stature. J Pediatr Endocr Met 18:69-74

International Zinc Nutrition Consultative G et al. (2004) International Zinc Nutrition Consultative Group (IZiNCG) technical document #1. Assessment of the risk of zinc deficiency in populations and options for its control. Food Nutr Bull 25:S99-203

Kanehisa M, Furumichi M, Tanabe M, Sato Y, Morishima K (2017) KEGG: new perspectives on genomes, pathways, diseases and drugs. Nucleic acids res 45:D353-D361 doi:10.1093/nar/gkw1092

Keller KA, Chu Y, Grider A, Coffield JA (2000) Supplementation with L-histidine during dietary zinc repletion improves short-term memory in zinc-restricted young adult male rats. J Nutr 130:1633-1640

Kent WJ, Sugnet CW, Furey TS, Roskin KM, Pringle TH, Zahler AM, Haussler D (2002) The Human Genome Browser at UCSC Genome Research 12:996-1006

Klug A (2010) The discovery of zinc fingers and their applications in gene regulation and genome manipulation. Annu Rev Biochem 79:213-231 doi:10.1146/annurev-biochem-010909-095056

Kozomara A, Griffiths-Jones S (2011) miRBase: integrating microRNA annotation and deep-sequencing data. Nucleic Acids Res 39:D152-D157 doi:10.1093/nar/gkq1027

Kozomara A, Griffiths-Jones S (2014) miRBase: annotating high confidence microRNAs using deep sequencing data. Nucleic Acids Res 42:D68-D73 doi:10.1093/nar/gkt1181

Kyosseva SV (2004) Mitogen-activated protein kinase signaling. Int Rev Neurobiol 59:201-220 doi:10.1016/S0074-7742(04)59008-6

Langmade SJ, Ravindra R, Daniels PJ, Andrews GK (2000) The transcription factor MTF-1 mediates metal regulation of the mouse ZnT1 gene. J Biol Chem 275:34803-34809

Lawrence M HW, Pagès H, Aboyoun P, Carlson M, Gentleman R, Morgan M, Carey V (2013) Software for computing and annotating genomic ranges. PLoS Computational Biol 9 doi:10.1371/journal.pcbi.1003118

Lee J, Li Z, Brower-Sinning R, John B (2007) Regulatory circuit of human microRNA biogenesis. PLoS Comp Biol 3:e67 doi:10.1371/journal.pcbi.0030067

Lee RC, Feinbaum RL, Ambros V (1993) The C. elegans heterochronic gene lin-4 encodes small RNAs with antisense complementarity to lin-14. Cell 75:843-854 doi:https://doi.org/10.1016/0092-8674(93)90529-Y

Li JF et al. (2017) Different bone remodeling levels of trabecular and cortical bone in response to changes in Wnt/beta-catenin signaling in mice. J Orthop Res 35:812-819 doi:10.1002/jor.23339

Lian JB, Stein GS, van Wijnen AJ, Stein JL, Hassan MQ, Gaur T, Zhang Y (2012) MicroRNA control of bone formation and homeostasis. Nature Rev Endocrinol 8:212-227 doi:10.1038/nrendo.2011.234

Liu GX et al. (2018) Hsa-let-7c controls the committed differentiation of IGF-1-treated mesenchymal stem cells derived from dental pulps by targeting IGF-1R via the MAPK pathways. Exp Mol Med 50:25 doi:10.1038/s12276-018-0048-7

Liuzzi JP (2014) Up-regulation of miR-34a by zinc deficiency. Vitam Miner 3:119 doi:10.4172/vms.1000119

Longobardi L et al. (2006) Effect of IGF-I in the chondrogenesis of bone marrow mesenchymal stem cells in the presence or absence of TGF-beta signaling. J Bone Miner Res 21:626-636 doi:10.1359/jbmr.051213

Mai S et al. (1999) Chromosomal and extrachromosomal instability of the cyclin D2 gene is induced by Myc overexpression. Neoplasia 1:241-252

Martin-Blanco E (2000) p38 MAPK signalling cascades: ancient roles and new functions. Bioessays 22:637-645

McCusker RH (1998) Controlling insulin-like growth factor activity and the modulation of insulin-like growth factor binding protein and receptor binding. J Dairy Sci 81:1790-1800 doi:10.3168/jds.S0022-0302(98)75748-0

McCusker RH, Kaleko M, Sackett RL (1998) Multivalent cations and ligand affinity of the type 1 insulin-like growth factor receptor on P2A2-LISN muscle cells. J cell physiol 176:392-401

Miller GM, Ogunnaike BA, Schwaber JS, Vadigepalli R (2010) Robust dynamic balance of AP-1 transcription factors in a neuronal gene regulatory network. BMC Syst Biol 4:171 doi:10.1186/1752-0509-4-171

Morimoto S et al. (1992) EDTA induces differentiation and suppresses proliferation of promyelocytic leukemia cell line HL-60--possible participation of zinc. Biochem Int 28:313-321

O'Halloran T (1993) Transition metals in control of gene expression. Sci 261:715-725 doi:10.1126/science.8342038

Oliveros JC (2007-2015) Venny. An interactive tool for comparing lists with Venn's diagrams. http://bioinfogp.cnb.csic.es/tools/venny/index.html. Accessed 01 Aug 2018.

Orom UA, Nielsen FC, Lund AH (2008) MicroRNA-10a binds the 5 ' UTR of ribosomal protein mRNAs and enhances their translation. Mol Cell 30:460-471

Pagès H AP, Gentleman R, DebRoy S (2018) Biostrings: Efficient manipulation of biological strings. R package version 2.48.0

Park SG, Choi HN, Yang HR, Yim JE (2017) Effects of zinc supplementation on catch-up growth in children with failure to thrive. Nutr Res Pract 11:487-491 doi:10.4162/nrp.2017.11.6.487

Ru Y et al. (2014) The multiMiR R package and database: integration of microRNA–target interactions along with their disease and drug associations. Nucleic Acids Res 42:e133-e133 doi:10.1093/nar/gku631

Ryu MS, Langkamp-Henken B, Chang SM, Shankar MN, Cousins RJ (2011) Genomic analysis, cytokine expression, and microRNA profiling reveal biomarkers of human dietary zinc depletion and homeostasis. P Natl Acad Sci USA 108:20970-20975 doi: 10.1073/pnas.1117207108

Sackett RL, McCusker RH (1998a) Multivalent cations depress ligand affinity of insulin-like growth factor-binding proteins-3 and -5 on human GM-10 fibroblast cell surfaces. J Cell Biochem 69:364-375

Sackett RL, McCusker RH (1998b) Multivalent cations depress ligand binding to cell-associated insulin-like growth factor binding protein-5 on human glioblastoma cells. Endocrinol 139:1943-1951 doi:10.1210/endo.139.4.5924

Saydam N, Adams TK, Steiner F, Schaffner W, Freedman JH (2002) Regulation of metallothionein transcription by the metal-responsive transcription factor MTF-1 - identification of signal transduction cascades that control metal-inducible transcription. J Biol Chem 277:20438-20445 doi:10.1074/jbc.M110631200

Seo HJ, Cho YE, Kim T, Shin HI, Kwun IS (2010) Zinc may increase bone formation through stimulating cell proliferation, alkaline phosphatase activity and collagen synthesis in osteoblastic MC3T3-E1 cells. Nutr Res Pract 4:356-361 doi: 10.4162/nrp.2010.4.5.356

Sherr CJ (1994) G1 phase progression: cycling on cue. Cell 79:551-555

Stuart GW, Searle PF, Chem HY, Brinster RL, Palmiter RD (1984) A 12-base-pair DNA motif that is repeated several times in metallothionein gene promoters confers metal regulation to a heterologous gene. Proc Natl Acad Sci 81:7318–7322

Szklarczyk D et al. (2015) STRING v10: protein-protein interaction networks, integrated over the tree of life. Nucleic Acids Res 43:D447-452 doi:10.1093/nar/gku1003

Szklarczyk D et al. (2017) The STRING database in 2017: quality-controlled protein-protein association networks, made broadly accessible. Nucleic Acids Res 45:D362-D368

Tahimic CG, Wang Y, Bikle DD (2013) Anabolic effects of IGF-1 signaling on the skeleton. Front Endocrinol 4:6 doi:10.3389/fendo.2013.00006

Team R (2016) RStudio: Integrated Development for R. RStudio, Inc Boston, MA URL http://www.rstudio.com/

Tuerk MJ, Fazel N (2009) Zinc deficiency. Curr Opin Gastroen 25:136-143 doi:10.1097/Mog.0b013e328321b395

Vasilevskaya I, O'Dwyer PJ (2003) Role of Jun and Jun kinase in resistance of cancer cells to therapy. Drug Resist Updat 6:147-156

Vasudevan S, Tong YC, Steitz JA (2007) Switching from repression to activation: MicroRNAs can up-regulate translation. Sci 318:1931-1934

Vermeulen K, Van Bockstaele DR, Berneman ZN (2003) The cell cycle: a review of regulation, deregulation and therapeutic targets in cancer. Cell Prolif 36:131-149

Vimalraj S, Selvamurugan N (2013) MicroRNAs: synthesis, gene regulation and osteoblast differentiation. Curr Issues Mol Biol 15:7-18

Wang X (2016) Improving microRNA target prediction by modeling with unambiguously identified microRNA-target pairs from CLIP-ligation studies. Bioinform 32:1316-1322 doi:10.1093/bioinformatics/btw002

Wong N, Wang X (2015) miRDB: an online resource for microRNA target prediction and functional annotations. Nucleic Acids Res 43:D146-152 doi:10.1093/nar/gku1104

Wu BL et al. (2016a) An integrative framework to identify cell death-related microRNAs in esophageal squamous cell carcinoma. Oncotarget 7:56758-56766

Wu M, Chen G, Li YP (2016b) TGF-beta and BMP signaling in osteoblast, skeletal development, and bone formation, homeostasis and disease. Bone Res 4:16009 doi:10.1038/boneres.2016.9

Wuehler SE, Peerson JM, Brown KH (2005) Use of national food balance data to estimate the adequacy of zinc in national food supplies: methodology and regional estimates. Public Health Nutr 8:812-819 doi:10.1079/Phn2005724

Xin Y, Cai H, Lu T, Zhang Y, Yang Y, Cui Y (2016) miR-20b inhibits T cell proliferation and activation via NFAT signaling pathway in thymoma-associated myasthenia gravis. Biomed Res Int 2016:9595718 doi:10.1155/2016/9595718

Yang Y et al. (2017) MicroRNA214 targets Wnt3a to suppress liver cancer cell proliferation. Mol Med Rep 16:6920-6927 doi:10.3892/mmr.2017.7483

Yochum GS, Cleland R, Goodman RH (2008) A genome-wide screen for beta-catenin binding sites identifies a downstream enhancer element that controls c-Myc gene expression. Mol Cell Biol 28:7368-7379

Zhang Y, Li H, Han J, Zhang Y (2015) Down-regulation of microRNA-124 is correlated with tumor metastasis and poor prognosis in patients with lung cancer. Int J Clin Exp Pathol 8:1967-1972

Zhao Y, Wang F, Chen S, Wan J, Wang G (2017) Methods of microRNA promoter prediction and transcription factor mediated regulatory network. BioMed Res Int 2017:8 doi:10.1155/2017/7049406

Zhou Y, Han Y, Zhang Z, Shi Z, Zhou L, Liu X, Jia X (2017) MicroRNA-124 upregulation inhibits proliferation and invasion of osteosarcoma cells by targeting sphingosine kinase 1. Hum Cell 30:30-40 doi:10.1007/s13577-016-0148-4

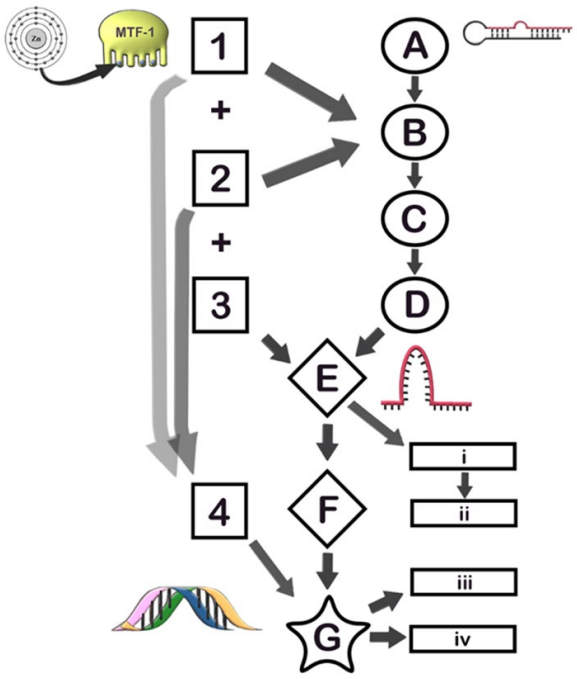

Figure 2.1. *In silico* experimental pipeline. (1) Biostrings: identified all metal regulatory element (MRE) motifs (5'-TGCRCNC-3') throughout the human genome. (2) BiomaRt: mapped the location of all HUGO Gene Nomenclature Committee (HGNC) named genes in the genome. (3) Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways used to categorize genes from osteogenic development pathways TGF-β (hsa04350), MAPK (hsa04010), and Wnt (hsa04310). (4) Output – 460 genes with an upstream MRE within -7Kbp were found in these three KEGG pathways; 413 genes with MREs not interrupted by another gene (Supplementary Table 2) continued through the pipeline. (A) 86 pre-miRNAs whose transcriptional start sites (TSSs) were predicted by the microTSS algorithm. (B) GenomicRanges: identified the 85/86 pre-miRNAs with one or more MREs in the range -7Kbp to +1Kbp from their predicted TSSs; 44 pre-miRNA genes with MREs not interrupted by another gene continued through the pipeline. (C) miRBase: converted the 44 pre-miRNAs into 73 mature miRNAs. (D) multiMiR: find TarBase-verified miRNA-target gene interactions (MTIs) of the 73 mature miRNAs. (E) 64/73 mature miRNAs

have TarBase-verified MTIs with genes from the KEGG pathways. (F) 241 gene targets from the miRNAs. (G) Of the 241 gene targets, 213 also have an MRE. (i) mirdb via multiMiR: finds all predicted gene targets of the miRNAs. (ii) Panther GO-Slim biological process for the predicted targets. (iii) Venn diagram identifies shared genes and miRNA between signaling pathways. (iv) Protein-protein interactions mapped using STRING.

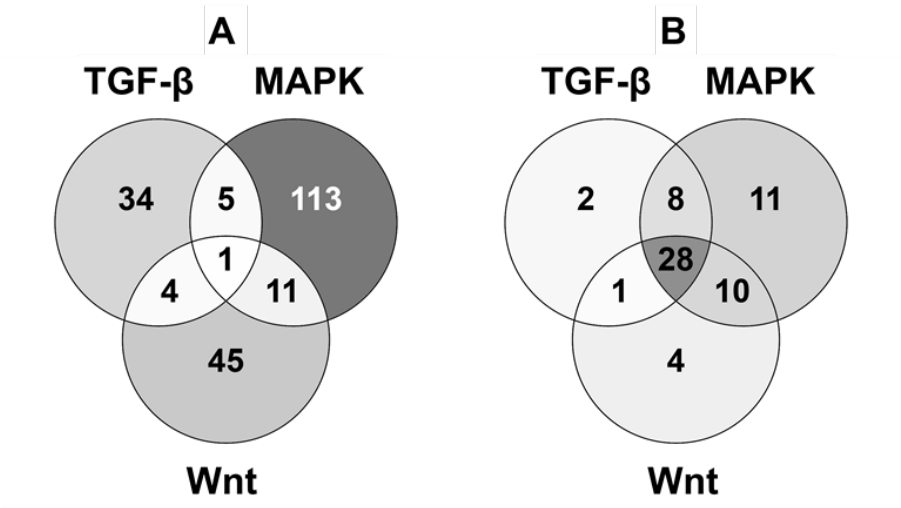

Figure 2.2. Venn diagrams of shared signaling pathway genes and miRNAs. (A) Genes with MREs in their promoter regions that are targets for miRNAs that contain MREs within their own promoter regions. (B) MiRNAs that target the genes in 2A.

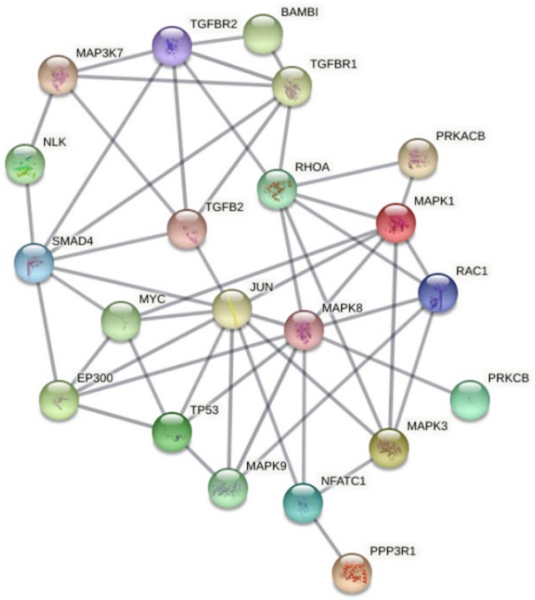

Figure 2.3. Protein-protein interactions between miRNA-target gene interactions. The sources for determining active interactions were from experiments and databases. The minimum interaction score used to generate the interactions web was 0.900.

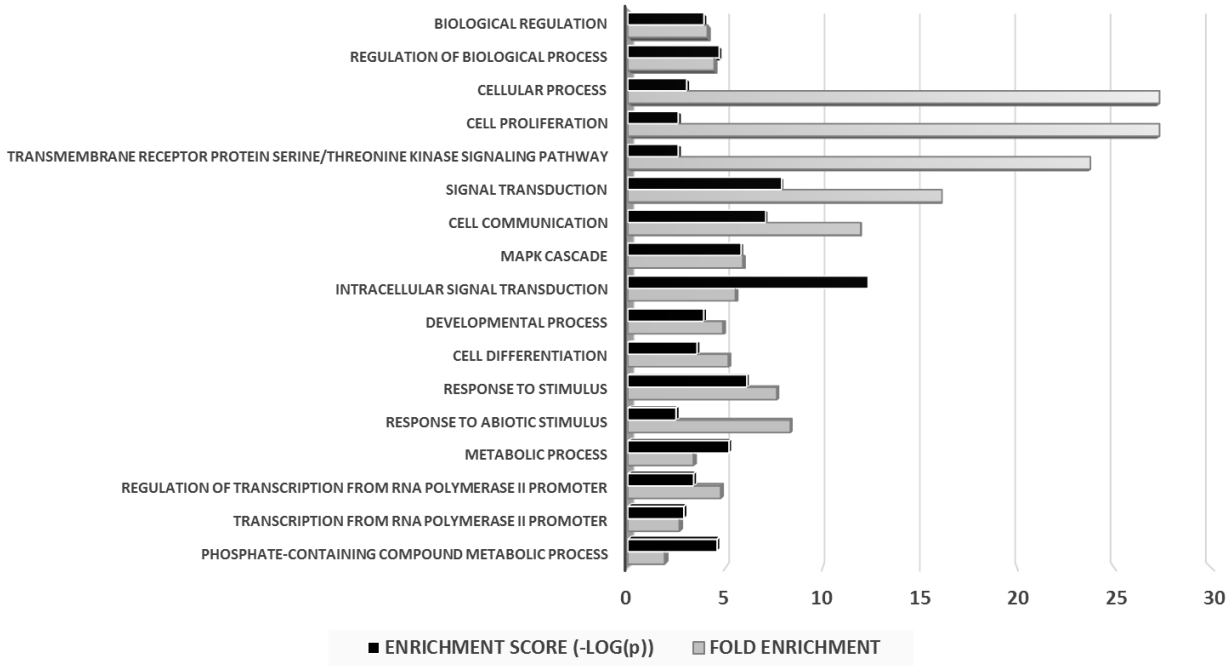

Figure 2.4. Gene ontology enrichment of the MTI genes. The MTI genes were used to query the PANTHER GO-Slim Biological Process database. Significance was determined using the Fisher's Exact test with the false discovery rate multiple test correction. All values reported in the graph exhibited a false discovery rate >0.05.

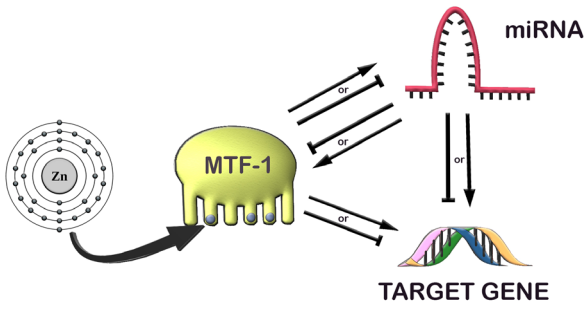

Figure 2.5. Zn activates MTF-1 and MTF-1 will bind to the MRE in promoters of genes. These genes include miRNAs, which are themselves repressors of gene activity. A feedforward loop can arise in which MTF-1 and miRNAs impact the expression of each other and of target genes.

Table 2.1. The five highest miRNA/target gene interactions (MTIs) within each signaling pathway. (A) Signaling pathway genes with the highest number of MTIs. (B) miRNAs that target most genes in each signaling pathway.

Table 2.1A

TGF-β		**MAPK**		**Wnt**	
GENE	**MTI**	**GENE**	**MTI**	**GENE**	**MTI**
MAPK1	10	MAPK1	10	CCND2	9
SMAD4	8	IGFR1	9	SMAD4	8
TGFBR2	8	TAOK1	9	CCND1	7
ACVR1	7	RPS6KA5	8	MYC	7
ACVR1B	7	TGFBR2	8	TP53	7

Table 2.1B

TGF-β		**MAPK**		**Wnt**	
Mature miRNA	**MTI**	**Mature miRNA**	**MTI**	**Mature miRNA**	**MTI**
hsa-miR-124-3p	12	hsa-miR-124-3p	32	hsa-miR-20b-5p	12
hsa-miR-20b-5p	11	hsa-miR-19b-3p	19	hsa-miR-106a-3p	11
hsa-miR-19b-3p	9	hsa-miR-34a-5p	19	hsa-miR-34a-5p	11
hsa-miR-106a-3p	8	hsa-miR-106a-3p	17	hsa-miR-124-3p	10
hsa-miR-34a-5p	7	hsa-miR-20b-5p	9	hsa-miR-19b-3p	9

Table 2.2. MYC miRNA/target gene interaction. *MYC* is shared by the TGF-β, MAPK, and Wnt signaling pathways. The list of mature miRNAs that target *MYC*. Each of these miRNAs also target other genes within the signaling pathways (MTI). Hsa-miR-34a-5p exhibits the most MTIs within each signaling pathway.

Mature miRNA	**TGF-β MTI**	**MAPK MTI**	**Wnt MTI**
hsa-miR-34a-5p	7	19	11
hsa-miR-148a-3p	5	6	4
hsa-miR-222-3p	4	9	5
hsa-miR-940	3	12	9
hsa-miR-148a-5p	2	2	2
hsa-miR-92a-2-5p	2	7	3
hsa-miR-19b-2-5p	1	4	1

CHAPTER 4

GENOMIC CHARACTERIZATION OF THE ZINC TRANSCRIPTIONAL REGULATORY ELEMENT REVEALS POTENTIAL FUNCTIONAL ROLES OF ZNF658[3]

[3] Francis, M.; Cheng, H; Ma, P; Grider, A. 2019. *Biological Trace Element Research*. Reprinted here with permission of the publisher.

Abstract

The zinc transcriptional regulatory element (ZTRE) is a newly reported binding motif for human zinc finger protein ZNF658, which alters gene expression in response to cellular zinc. The ZTRE has two nucleotide components—the reverse palindromic flanking pairs, and the bridging "N" bases between these flanks that range in number from 0 to 100. There are twelve pairs of ZTRE flanks (designated A-L). 3525 genes contain one or more ZTREs -1000 to +200bp from their transcriptional start site (TSS). ZTRE-E is observed at a greater frequency, and ZTRE containing 25 bridging bases are less frequent, within -200bp from the TSS. The genes with ZTREs in this range are enriched in processes that may compensate zinc deficiency, while other genes with ZTREs outside this range are enriched in transcriptional activation processes. The division of ZTREs into two groups may imply a dual role of ZNF658, similar to the homologous yeast protein Zap1, via binding to low or high affinity sequences dependent upon cellular zinc. The KLF/Sp1-family binding motif is prevalent within the ZTRE "N" bridging bases, suggesting ZNF658 may compete with Sp1-like transactivators to suppress transcription.

Introduction

The Cys_2His_2 class of zinc (Zn) finger proteins (ZFPs) accounts for about 3% of the human genome and is the largest class of putative transcription factors (TFs). The majority of these ZFPs have unknown functions, and those that have been characterized have diverse properties—indicating a potentially vast regulatory network that is largely unstudied [1]. ZFPs are also considered ideal for the development of certain molecular medical applications, such as induced transcriptional activation or repression of target genes, or in the fusion of ZF peptides with other functional protein domains to manipulate gene products [2]. For certain Cys_2His_2 ZFPs, some of their ZFs act as Zn sensors, and when Zn levels reach a certain threshold relative to their Zn-binding affinity, the TF will activate and bind to DNA. In eukaryotes these are Zap1 (*Saccharomyces cerevisiae*), Loz1 (*Schizosaccharomyces pombe*), and MTF-1 (*Homo sapiens*, conserved among metazoan species) [3]. To this list we may also add the recently sequenced factor ZNF658, which has been shown to bind to DNA and regulate gene transcription in response to cellular Zn levels [4].

ZNF658 has a relatively large amount of ZFs (21), which suggests a high degree of Zn sensitivity. It binds to the variable-length motif known as the Zn transcriptional regulatory element (ZTRE), whose sequence is 5'-CMCDCCY$N_{0\text{-}100}$RGGHGKG-3' (M=C or A; D=A, G, or T; Y=C or T; R=A or G; H=A, C, or T; K=T or G) [5]. The nucleotides in the 7-mer 5'- and 3'- flanks are required to be reverse palindromic for ZNF658 binding. There are no reported criteria for the middle "N" bases, which can range in number from zero to potentially 100 [5]. This variability seen in some TF binding to palindromic motifs can be a consequence of TF dimerization [6,7]. The Zn dependent activation of ZNF658 binding to the ZTRE was verified in *SLC30A5* (ZnT5), *SLC30A10* (ZnT10), and *CBWD* genes [5]. Introducing ZNF658 siRNA to the transcriptome of

Caco-2 cells had significant effects on the up- or down-regulation of 124 genes in a microarray panel, including a large number of rRNA genes [4].

ZNF658 and Zap1 in *S. cerevisiae* are homologous proteins whose essential DNA-binding ZF domains are aligned and highly similar [4]. Zap1 binds to an 11-mer reverse palindromic motif, the Zn-Responsive Element (ZRE). Zap1 is the main regulator of Zn homeostasis in *S. cerevisiae,* and has low activity in Zn-adequate cells and high activity during Zn deficiency [8]. Zap1 is known to initiate a "Zn-sparing" response by sensing deficiency and repressing levels of certain Zn-binding proteins, while simultaneously increasing expression of replacement proteins that have lower Zn requirements [9]. Severely Zn deficient cells increase levels of Zap1 via autoregulation; this increase enables the activation of target genes by allowing Zap1 to bind to lower binding affinity ZREs [10]. These lower affinity ZREs are found closer to target gene transcriptional start sites (TSSs), and are also sites of Zap1-mediated repression. This repression is thought to be a result of Zap1 blocking transcription initiation sites [11].

The two independent activation domains of Zap1, AD1 (dominant) and AD2, can sense Zn levels and initiate DNA binding, and may also be relevant to our understanding of ZNF658 [12]. Zn binding to AD1 causes a conformational change in Zap1 that inhibits the ability of AD1 to activate transcription via coactivator recruitment; a similar mechanism governs AD2 [13]. There is a region of similarity at the AD1 domain between the aligned sequences of Zap1 and ZNF658 [4].

Therefore, Zap1 is a ZFP-TF that may be an evolutionary precursor to ZNF658. The dual activator/repressor mechanism of Zap1 is related to preferential binding to high and low affinity ZREs based on cellular Zn status. Additionally, ZNF658 has been shown to repress the expression of certain genes under conditions of high Zn, and silencing its expression caused a wide variety of

in vitro transcriptomic changes [4]. Based on our genomic analysis of predicted ZNF658 binding sites in the human genome, we have identified a significant difference in composition between ZTREs that occur closest to gene TSSs and those that do not, as well as the functional annotations of these gene sets. This suggests ZNF658 may provide a similar response to Zap1 in Zn deficiency, as activator or repressor via preference for lower binding affinity ZTREs that are found closer to gene TSSs.

Materials and Methods

Analyses were performed using GENCODE reference annotation for the human genome, Release 29 (GRCh38.p12), standard chromosomes [14]. All Bioconductor packages were executed in R Studio (v1.1.463) [15-17]. Gene promoters considered as the region -1000 bp upstream and +200 bp downstream from gene transcriptional start sites (TSSs). Geneious was used to determine genomic positions of the ZTRE motif, 5'-CMCDCCYN$_{(0\text{-}100)}$RGGHGKG-3' (Geneious; v10.2.3) [18]. Background ZTREs were defined as those which fit the sequence format of ZTRE but do not occur in this defined promoter range. Overlaps between ZTRE positions and gene promoters were recorded using GenomicRanges (v1.34.0) and GenomicFeatures (v1.34.1) [19,20]. Multiple Em for Motif Elicitation (MEME, v5.0.2) and TOMTOM HOmo sapiens COmprehensive MOdel COllection (HOCOMOCO, v11) were accessed via MEME suite [21]; only motifs that occurred in one hundred or more ZTRE "N" base sequences were reported. Ggseqlogo (v0.1) was used to plot sequence logos from MEME data [22]. BLASTp (BLOSUM62 scoring algorithm) was used to identify homologous amino acid sequences [23]. DAVID Bioinformatics Resources (v6.8) functional annotation clustering was used to identify enrichment of gene cohorts [24,25]. Panther GO Biological Process (v13.1) Fisher's exact testing with Bonferroni correction for multiple

testing was used to identify significantly overrepresented annotated gene ontologies (α = 0.05) [26,27].

Statistical analyses were performed using R (v3.5.0) [15]. Chi-square test was used to determine whether there is a significant association between ZTRE Flank ID and ZTRE location, based on whether ZTREs are located in the range -200 bp to TSS vs. other promoter ranges (α = 0.05); this test assumes independence between two categorical variables in the null hypothesis. Two Proportion Z-test was used to determine significant difference between ZTRE-E in the range -200 bp to TSS vs. background, and vs. all promoter ZTREs (α = 0.05); and also between ZTREs containing 25 bridging bases in the range -200 bp to TSS versus all ZTREs in the promoter range; this test assumes no significant difference between two population proportions in the null hypothesis. Wilcoxon rank-sum tests were used to compare the number of bridging bases between independent groups of ZTREs, first between ZTREs in background vs. ZTREs in promoter, and then between ZTREs in the range -200 bp to TSS vs. other ZTREs in the promoter range (α = 0.05); this test assumes that the two groups come from the same distribution in the null hypothesis.

Results

Genomic identification of ZTREs

The ZTRE sequence, 5'-CMCDCCYN$_{0\text{-}100}$RGGHGKG-3', was used to search the human genome (M=C or A; D=A, G, or T; Y=C or T; R=A or G; H=A, C, or T; K=T or G). There a total of 85436 ZTREs, and 5200 of these were found in the promoters (-1000 to +200 bp from TSS) of 3525 genes (Supplementary Table 1). Of these 3525 genes, 61.4% (2165) are protein coding. The mean distance from the ZTRE start site to TSS for protein coding genes is -222 bp upstream. Based

on previous reports, the maximum number of bridging bases used in this study was set to 100; 41.6% of ZTREs have 0-50 bridging bases.

Analysis of palindromic flanking regions

The ZTRE consists of twelve possible pairs of 7-mer reverse palindromic 5'- and 3'-flanking sequences. We named these ZTRE Flank ID A through L based on descending background frequency (Supplementary Table 2, Figure 1). There is significantly more ZTRE-E, $CCCGCCCN_{0\text{-}100}GGGCGGG$, in gene promoters than background (Z = -79.46, P < 2.2e-16), and there is also significantly more ZTRE-E in the range -200 bp to the TSS versus all promoter ranges together (Z = 12.88, P < 2.2e-16). Overall, there is a significant association between ZTRE Flank ID and whether it is located in the range -200 bp to the TSS versus other promoter ranges (χ^2 = 439.97, df = 11, P < 2.2e-16).

Analysis of bridging "N" bases

The most frequently occurring number of promoter (and background) bridging bases is 25 (Figure 2). However, there are significantly fewer ZTREs containing 25 bridging bases in the range -200 bp to TSS versus all ZTREs in the promoter range -1000 to +200 bp (Z=-2.53, P = 0.00566). Wilcoxon rank-sum tests indicate that there are significantly more bridging bases in ZTREs found in the promoter range than in background (U = 200660000, Z = -11.725, P < 2.2e-16), but significantly fewer bridging bases in ZTRE found in the -200 bp to TSS range than in all promoter ranges (U = 5162000, Z = -1.807, P = 0.03538).

Differences in "N" base motifs

The "N" bridging base sequences were analyzed for recurring motifs to predict binding similarities of ZNF658 to other TFs, and also to define further differences between the two ZTRE location-based cohorts (Figure 3, Table 1). A version of the repeating GC-rich sequence (Motifs 2, 4 and 8) containing the Sp1/KLF TF family binding motif occurs in all sets of ZTREs and has the highest *E* values. Motifs 1 and 3, which are significant in all ZTREs taken together, do not retain their significance when the ZTREs are separated into groups. ZTREs closest to TSSs exhibit significant enrichments for binding sites similar to NFY family TFs and androgen receptors (Motifs 5 and 6). In the other group of ZTREs, Motif 7 shares similarity with the binding sites of three relatively unstudied TFs: ALX1 (associated with autosomal-recessive frontonasal dysplasia), DUX4 (associated with facioscapulohumeral dystrophy), and ZFP28 (associated with circadian rhythm).

Functional gene annotation differs with ZTRE position

A distinction in the ontologies of the DAVID functional annotation clusters can be seen based upon which of the two location-based groups of ZTREs these genes contain in their promoter (Table 2). For genes with ZTREs in the range -200 bp to the TSS (1112 mapped DAVID IDs), there is significant enrichment in pleckstrin homology (PH) domain-containing genes, microtubule/kinesin transport, and leucine zipper domain (bZIP) annotation clusters. For genes with ZTREs in other ranges, there is significant enrichment in RNA pol II transcription, transcription and activation processes, and PWWP domain-containing gene annotation clusters. Significantly enriched clusters in each group of genes are unenriched in the other. Nervous system

development was the single enriched GO biological process observed for genes with ZTREs in the range -200 bp to the TSS.

Homology of ZNF658 with Zap1, Sp1, and MTF-1

We compared the amino acid sequence of ZNF658 to Zap1 and MTF-1, and also to Sp1 since the Sp1/KLF binding motif recurred throughout the ZTRE "N" bases (Table 3). MTF-1 then Zap1 exhibit the highest total bit scores, indicating a high degree of similarity. (For reference, the total bit score between MTF-1 and Zap1 is 235. These larger total bit scores are due in part to the 21 repetitive ZF sequences in ZNF658.) MTF-1 ZFs 1-3 are homologous with ZFs 12-16 of ZNF658; these are the essential DNA binding domains of each TF. The AD1 domain of Zap1 is 26% identical and 35% similar to a corresponding region in ZNF658; the percent identity versus sequence length comparison for AD1 falls into the "twilight zone" of indeterminate homology [28], however the *E* value (0.008) indicates some statistical significance. The three ZFs of Sp1 are homologous to ZF regions of ZNF658, and when the three are queried together they are 62% similar to a continuous region of ZNF658. ZF1 of Sp1 is 76% identical to ZF16 of ZNF658 (*E* = 2e-07). The essential DNA-binding Zn finger domains in both Sp1 and MTF-1 also act as a nuclear localization signal (NLS) [29,30], and may have a similar role in ZNF658.

ZTREs associated with Ogo et. al. (2015) genes

Of 124 genes in Caco-2 cells that exhibited fold changes in the expression of their products following the introduction of ZNF658 siRNA, and under normal Zn conditions [4], 10 have ZTREs in the range -1000bp to +200bp from their TSS (*CGB5*, *DIRC2*, *F2RL1*, *IGF2*, *ITPRIPL2*, *MBNL3*, *PAK2*, *PRPF40A*, *SESTD1*, and *SOX15*). *F2RL1* and *SESTD1* both contain ZTRE-E in the range

-200bp to the TSS. There is no derivable relationship between the measured fold changes of these gene products and the characteristics of the ZTRE(s) that occur near these genes.

Discussion

The large number of Zn fingers and thus potential Zn sensitivity of ZNF658, combined with the significant bisection in ZTRE motifs that we have shown here, suggests that this TF may have a dual regulatory function that is dependent on cellular Zn concentration. The mechanism of this function may be analogous to the Zn-activated TF in *S. cerevisiae*, Zap1. The DAVID functional annotation of gene clusters (Table 2) contain several pieces of information which supports two (or more) distinct categories of ZTREs: namely, one for low Zn (in the range -200bp to TSS) and one for adequate Zn (in the other promoter locations).

During Zn deficiency, Zap1 induces *CKI1* and *EKI1* genes to maintain phospholipid synthesis [31]; this adaptation process may be analogous to the enriched set of genes that code for proteins containing Pleckstrin homology domains, which are known to bind phospholipids and are involved in their processing [32]. Zap1 also upregulates the *TSA1* gene, whose product Tsa1 is a protein chaperone that operates under conditions of Zn deficiency. Tsa1 stabilizes the accumulation of Zn-dependent apoproteins in cells, and shields them from misfolding and aggregation until Zn levels increase [31]. In human neurons, these unfolded and misfolded proteins are moved outward by anterograde transport, mediated by microtubule motor activity [33], which is also a significantly enriched functional annotation for genes with ZTREs in the range of -200 bp to the TSS. In *Arabidopsis thaliana* and *Triticum aestivum*, Zn deficiency also leads to increased expression of bZIP (basic-leucine zipper domain) transcription factors [34]. It is not known whether there is a similar Zn-dependent effect on these TFs in humans, however, the bZIP family

are well-characterized as repressors, and this further supports the idea that ZTREs closer to gene TSSs are associated with Zn deficiency and transcriptional "off" regulatory functions. Meanwhile, the top two significantly enriched DAVID annotation clusters for promoter ZTREs not in the -200 bp to the TSS range are associated with RNA Pol II activation and transcriptional activation. These genes could represent a cascading "on" signal for when Zn levels are adequate enough to resume normal cellular protein synthesis.

The "N" bases of the ZTRE match binding sites for several TFs related to Zn (Table 1). NF-Y transcription complex associated with Motif 5 (Figure 3), which has global transcription effects and occurs in 30% of gene promoters, is co-expressed with the ZFPs Sp2 and ZNF143 [35]. Motif 6 is similar to the binding site for androgen receptors, which are suppressed with elevated Zn levels in the context of prostate cancer [36]. A deletion in *DUX-4* is associated with facioscapulohumeral dystrophy; muscle strength of those afflicted has been shown to improve following zinc (and other) supplementation [37].

Sp1-like proteins are defined as having a high degree of sequence similarity with the Sp1 DNA-binding domain, comprised of three Kruppel-like Zn fingers. The KLF/Sp1-like proteins have similarity in this domain that ranges from 68-96%; ZNF658 exhibits 62% homology in this domain (Table 3). The view that Sp1 binds to all such GC-rich motifs (Table 1) may be overly simplified, and a more likely scenario is that many different TFs compete for these sites based on binding affinity that can be influenced by environmental factors, e.g. Zn levels [38]. Indeed, many KLF/Sp1-like proteins have dual activator/repressor roles under various cellular stresses, similar to the potential role of ZNF658 that we propose here.

When considering the role of Zap1, and the role of ZNF658 that we have proposed here as an initiator of cellular changes based on Zn status, it is not surprising that indirect (nonspecific)

effects could account for many of the changes in expression levels that were observed in the ZNF658 knockdown gene expression panel performed by Ogo et. al. [4]. *F2RL1* and *SESTD1* may be of particular interest because of the presence of ZTRE-E in the range -200bp to the TSS. It is of interest going forward to design experiments that specifically target the expression of genes with different permutations of the ZTRE in their promoter (e.g. ZTRE-E vs. ZTRE-A), under varied Zn concentrations.

References

1. Najafabadi HS, Mnaimneh S, Schmitges FW, Garton M, Lam KN, Yang A, Albu M, Weirauch MT, Radovani E, Kim PM, Greenblatt J, Frey BJ, Hughes TR (2015) C2H2 zinc finger proteins greatly expand the human regulatory lexicon. Nat Biotechnol 33:555. doi:10.1038/nbt.3128

2. Klug A (2010) The discovery of zinc fingers and their applications in gene regulation and genome manipulation. Annu Rev Biochem 79:213-231. doi:10.1146/annurev-biochem-010909-095056

3. Choi S, Bird AJ (2014) Zinc'ing sensibly: controlling zinc homeostasis at the transcriptional level. Metallomics 6 (7):1198-1215. doi:10.1039/c4mt00064a

4. Ogo OA, Tyson J, Cockell SJ, Howard A, Valentine RA, Ford D (2015) The zinc finger protein ZNF658 regulates the transcription of genes involved in zinc homeostasis and affects ribosome biogenesis through the zinc transcriptional regulatory element. Mol Cellular Biol 35 (6):977-987. doi:10.1128/mcb.01298-14

5. Coneyworth LJ, Jackson KA, Tyson J, Bosomworth HJ, van der Hagen E, Hann GM, Ogo OA, Swann DC, Mathers JC, Valentine RA, Ford D (2012) Identification of the human zinc transcriptional regulatory element (ZTRE): a palindromic protein-binding DNA sequence responsible for zinc-induced transcriptional repression. J Biol Chem 287 (43):36567-36581. doi:10.1074/jbc.M112.397000

6. Huang Y-H, Jankowski A, Cheah KSE, Prabhakar S, Jauch R (2015) SOXE transcription factors form selective dimers on non-compact DNA motifs through multifaceted interactions between dimerization and high-mobility group domains. Sci Rep 5:10398. doi:10.1038/srep10398

7. Peirano RI, Wegner M (2000) The glial transcription factor Sox10 binds to DNA both as monomer and dimer with different functional consequences. Nucleic Acids Res 28 (16):3047-3055

8. Lyons TJ, Gasch AP, Gaither LA, Botstein D, Brown PO, Eide DJ (2000) Genome-wide characterization of the Zap1p zinc-responsive regulon in yeast. P Natl A Sci 97 (14):7957

9. Merchant SS, Helmann JD (2012) Elemental economy: microbial strategies for optimizing growth in the face of nutrient limitation. Adv Microb Physiol 60:91-210. doi:10.1016/b978-0-12-398264-3.00002-4

10. Wu CY, Bird AJ, Chung LM, Newton MA, Winge DR, Eide DJ (2008) Differential control of Zap1-regulated genes in response to zinc deficiency in Saccharomyces cerevisiae. BMC Genom 9:370. doi:10.1186/1471-2164-9-370

11. Bird AJ, Blankman E, Stillman DJ, Eide DJ, Winge DR (2004) The Zap1 transcriptional activator also acts as a repressor by binding downstream of the TATA box in ZRT2. EMBO J 23 (5):1123

12. Bird AJ, Zhao H, Luo H, Jensen LT, Srinivasan C, Evans-Galea M, Winge DR, Eide DJ (2000) A dual role for zinc fingers in both DNA binding and zinc sensing by the Zap1 transcriptional activator. EMBO J 19 (14):3704-3713. doi:10.1093/emboj/19.14.3704

13. Frey AG, Eide DJ (2012) Zinc-responsive coactivator recruitment by the yeast Zap1 transcription factor. Microbiologyopen 1 (2):105-114. doi:10.1002/mbo3.8

14. Frankish A, Diekhans M, Ferreira A-M, Johnson R, Jungreis I, Loveland J, Mudge JM, Sisu C, Wright J, Armstrong J, Barnes I, Berry A, Bignell A, Carbonell Sala S, Chrast J, Cunningham

F, Di Domenico T, Donaldson S, Fiddes IT, García Girón C, Gonzalez JM, Grego T, Hardy M, Hourlier T, Hunt T, Izuogu OG, Lagarde J, Martin FJ, Martínez L, Mohanan S, Muir P, Navarro FCP, Parker A, Pei B, Pozo F, Ruffier M, Schmitt BM, Stapleton E, Suner M-M, Sycheva I, Uszczynska-Ratajczak B, Xu J, Yates A, Zerbino D, Zhang Y, Aken B, Choudhary JS, Gerstein M, Guigó R, Hubbard TJP, Kellis M, Paten B, Reymond A, Tress ML, Flicek P (2018) GENCODE reference annotation for the human and mouse genomes. Nucleic Acids Res:gky955-gky955. doi:10.1093/nar/gky955

15. R Core Team (2018) R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria URL https://www.R-project.org/

16. Team R (2016) RStudio: Integrated Development for R. RStudio, Inc Boston, MA URL http://www.rstudio.com/

17. Huber W, Carey VJ, Gentleman R, Anders S, Carlson M, Carvalho BS, Bravo HC, Davis S, Gatto L, Girke T, Gottardo R, Hahne F, Hansen KD, Irizarry RA, Lawrence M, Love MI, MacDonald J, Obenchain V, Oleś AK, Pagès H, Reyes A, Shannon P, Smyth GK, Tenenbaum D, Waldron L, Morgan M (2015) Orchestrating high-throughput genomic analysis with Bioconductor. Nat Methods 12:115. doi:10.1038/nmeth.3252

18. Kearse M, Moir R, Wilson A, Stones-Havas S, Cheung M, Sturrock S, Buxton S, Cooper A, Markowitz S, Duran C, Thierer T, Ashton B, Meintjes P, Drummond A (2012) Geneious Basic: An integrated and extendable desktop software platform for the organization and analysis of sequence data. Bioinformatics 28 (12):1647-1649. doi:10.1093/bioinformatics/bts199

19. Lawrence M HW, Pagès H, Aboyoun P, Carlson M, Gentleman R, Morgan M, Carey V (2013) Software for Computing and Annotating Genomic Ranges. PLOS Comput Biol 9. doi:10.1371/journal.pcbi.1003118

20. Lawrence M, Huber W, Pagès H, Aboyoun P, Carlson M, Gentleman R, Morgan MT, Carey VJ (2013) Software for computing and annotating genomic ranges. PLOS Comput Biol 9 (8):e1003118. doi:10.1371/journal.pcbi.1003118

21. Bailey TL, Boden M, Buske FA, Frith M, Grant CE, Clementi L, Ren J, Li WW, Noble WS (2009) MEME Suite: tools for motif discovery and searching. Nucleic Acids Res 37 (suppl_2):W202-W208. doi:10.1093/nar/gkp335

22. Wagih O (2017) ggseqlogo: a versatile R package for drawing sequence logos. Bioinformatics 33 (22):3645-3647. doi:10.1093/bioinformatics/btx469

23. Altschul SF, Madden TL, Schaffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res 25 (17):3389-3402

24. Huang da W, Sherman BT, Lempicki RA (2009) Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. Nat Protoc 4 (1):44-57. doi:10.1038/nprot.2008.211

25. Huang da W, Sherman BT, Lempicki RA (2009) Bioinformatics enrichment tools: paths toward the comprehensive functional analysis of large gene lists. Nucleic Acids Res 37 (1):1-13. doi:10.1093/nar/gkn923

26. Ashburner M, Ball CA, Blake JA, Botstein D, Butler H, Cherry JM, Davis AP, Dolinski K, Dwight SS, Eppig JT, Harris MA, Hill DP, Issel-Tarver L, Kasarskis A, Lewis S, Matese JC, Richardson JE, Ringwald M, Rubin GM, Sherlock G (2000) Gene ontology: tool for the unification of biology. The Gene Ontology Consortium. Nature Genet 25 (1):25-29. doi:10.1038/75556

27. The Gene Ontology Consortium (2017) Expansion of the Gene Ontology knowledgebase and resources. Nucleic Acids Res 45 (D1):D331-d338. doi:10.1093/nar/gkw1108

28. Rost B (1999) Twilight zone of protein sequence alignments. Protein engineering 12 (2):85-94

29. Ito T, Kitamura H, Uwatoko C, Azumano M, Itoh K, Kuwahara J (2010) Interaction of Sp1 zinc finger with transport factor in the nuclear localization of transcription factor Sp1. Biochem Bioph Res Co 403 (2):161-166. doi:https://doi.org/10.1016/j.bbrc.2010.10.036

30. Günther V, Lindert U, Schaffner W (2012) The taste of heavy metals: gene regulation by MTF-1. Biochim Biophys Acta 1823:1416-1425. doi:10.1016/j.bbamcr.2012.01.005

31. Wang Y, Weisenhorn E, MacDiarmid CW, Andreini C, Bucci M, Taggart J, Banci L, Russell J, Coon JJ, Eide DJ (2018) The cellular economy of the Saccharomyces cerevisiae zinc proteome. Metallomics. doi:10.1039/C8MT00269J

32. Maffucci T, Falasca M (2001) Specificity in pleckstrin homology (PH) domain membrane targeting: a role for a phosphoinositide-protein co-operative mechanism. FEBS Lett 506 (3):173-179

33. Chevalier-Larsen E, Holzbaur ELF (2006) Axonal transport and neurodegenerative disease. BBA-Mol Basis Dis 1762 (11):1094-1108. doi:https://doi.org/10.1016/j.bbadis.2006.04.002

34. Evens NP, Buchner P, Williams LE, Hawkesford MJ (2017) The role of ZIP transporters and group F bZIP transcription factors in the Zn-deficiency response of wheat (Triticum aestivum). Plant J 92 (2):291-304. doi:10.1111/tpj.13655

35. Dolfini D, Zambelli F, Pedrazzoli M, Mantovani R, Pavesi G (2016) A high definition look at the NF-Y regulome reveals genome-wide associations with selected transcription factors. Nucleic Acids Res 44 (10):4684-4702. doi:10.1093/nar/gkw096

36. To KP, Do M-H, Cho Y-S, Kwon S-Y, Kim SM, Jung C (2018) Zinc inhibits expression of androgen receptor to suppress growth of prostate cancer cells. Int J Mol Sci 19 (10). doi:10.3390/ijms19103062

37. Passerieux E, Hayot M, Jaussent A, Carnac G, Gouzi F, Pillard F, Picot M-C, Böcker K, Hugon G, Pincemail J, Defraigne JO, Verrips T, Mercier J, Laoudj-Chenivesse D (2015) Effects of vitamin C, vitamin E, zinc gluconate, and selenomethionine supplementation on muscle function and oxidative stress biomarkers in patients with facioscapulohumeral dystrophy: A double-blind randomized controlled clinical trial. Free Radical Bio Med 81:158-169. doi:https://doi.org/10.1016/j.freeradbiomed.2014.09.014

38. Lomberk G, Urrutia R (2005) The family feud: turning off Sp1 by Sp1-like KLF proteins. Biochem J 392 (Pt 1):1-11. doi:10.1042/BJ20051234

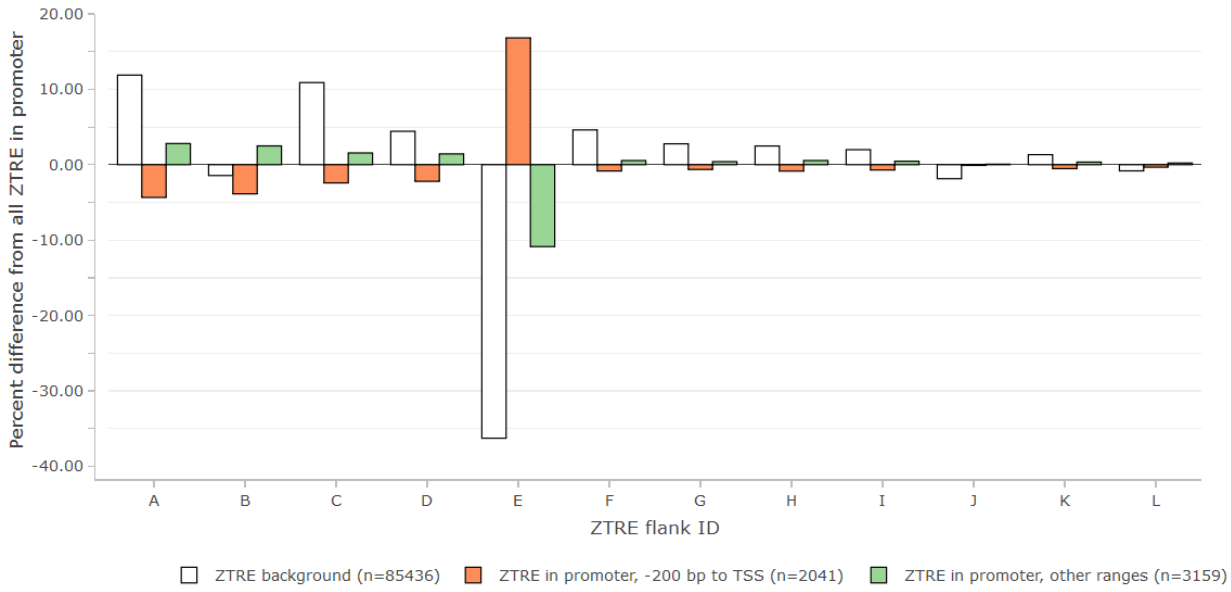

Figure 3.1. Twelve pairs of 5'- and 3'- reverse palindromic flanking sequences of the ZTRE, named ZTRE-A through L based on their background abundances. Shown are the relative abundances of these sequences as compared to all ZTREs in gene promoters (n=5200). ZTRE flank IDs sorted according to their distance from the TSS.

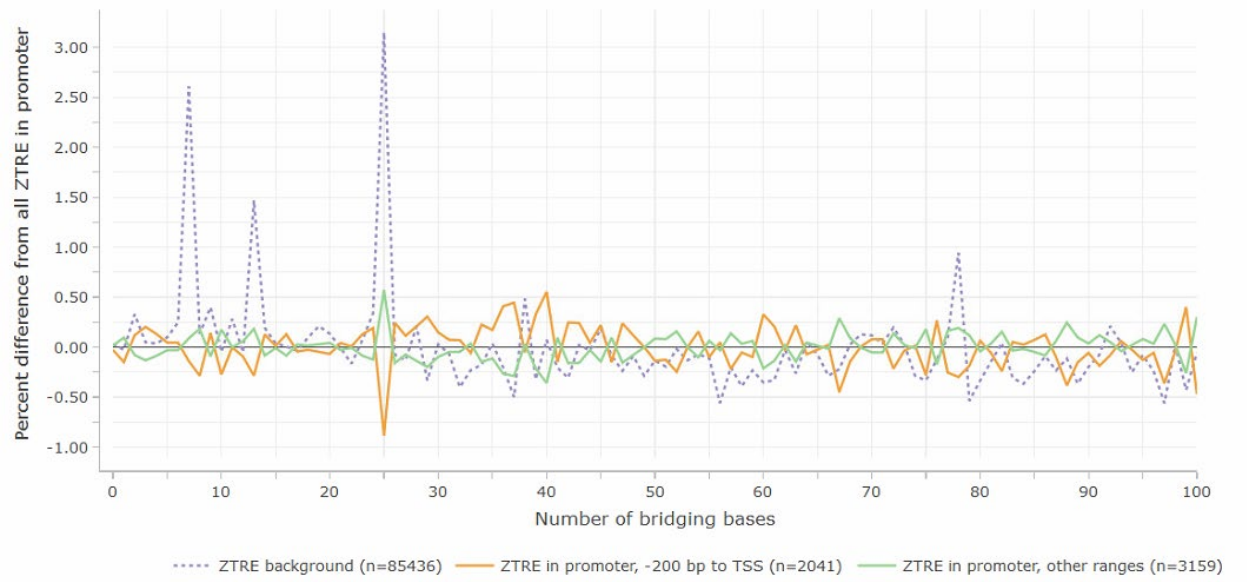

Figure 3.2. Numbers of bridging "N" bases in ZTRE sequences. There is a significant difference in the total number of bridging bases $N_{0\text{-}100}$, as well as at N_{25}, for ZTREs located in the range -200 bp to the TSS, compared to other promoter ZTREs or background ZTREs.

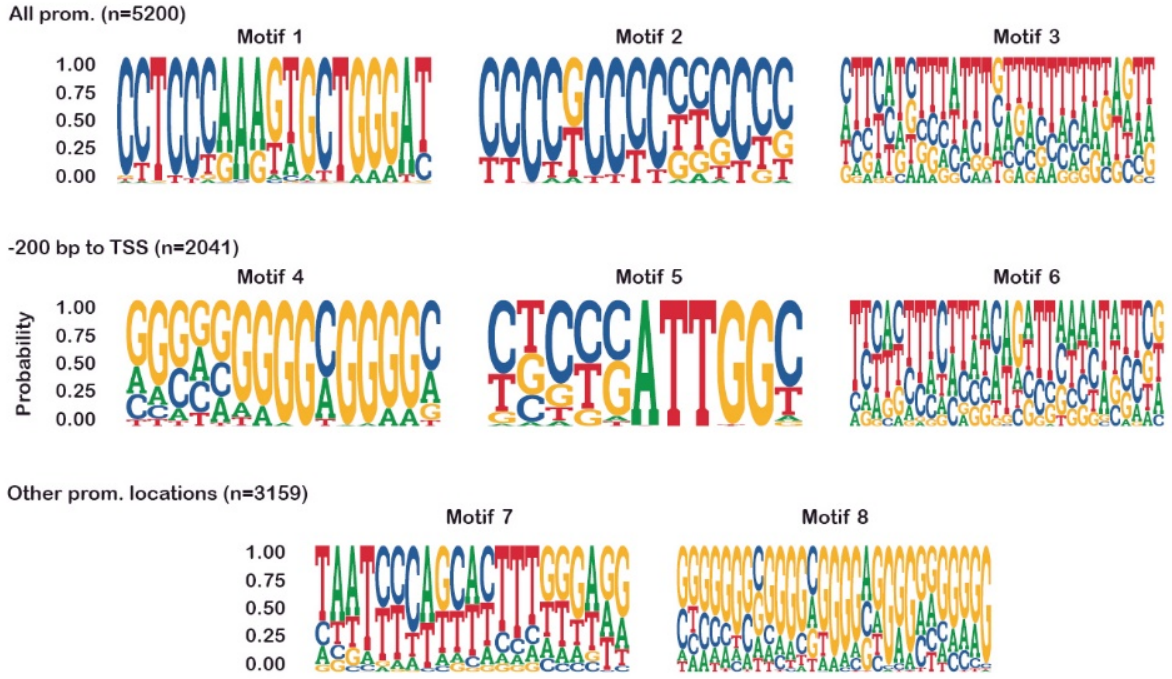

Figure 3.3. Significantly recurring motifs found in ZTRE "N" bases. Top row are all ZTREs found in the promoters of genes (n=5200). Middle row motifs found in ZTREs in the range -200 bp upstream to the TSS (n=2041). Bottom row motifs are other promoter ZTREs (n=3159). A version of the GC-rich Sp1-like binding motif (4,8, and reverse complement of 2) occurs in all three sets.

Table 3.1. Results from ZTRE "N" base analysis as depicted in Figure 3. Sp1-like GC-rich binding motifs are common to all groups and have the lowest *E* values, though high statistical significance is noted for all motifs listed. Only motifs with 100 or more sites are reported. TFs with similar binding sites to the motifs identified are also listed.

ZTRE set	Motif	Width	ZTRE "N" base motif *E* value	Sites	TOMTOM HOCOMOCO database matches
All in promoter (n=5200)	1	19	2.00E-177	127	IKZF1**
	2	15	1.80E-189	1000	PATZ1***, Sp1-TF family***, VEZF1***, KLF3***, ZN467***
	3	29	5.20E-125	385	PRDM6**, SOX2**, LEF1*, FOXJ3*, IRF1*
-200 bp to TSS (n=2041)	4	15	1.80E-284	673	Sp1-TF family***, KLF3***, KLF12***, PATZ1***, WT1***
	5	11	3.60E-138	229	NFYC***, NFYA***, FOXI1***
	6	29	5.00E-118	106	ANDR*
Other promoter locations (n=3159)	7	21	3.1e-310	415	ALX1*, DUX4*, ZFP28*
	8	29	8.20E-181	455	MAZ***, WT1***, Sp1-TF family***, KLF15***, PATZ1***

TOMTOM matches E value:
*E<0.1
**E<0.01
***E<0.001

Table 3.2. Enrichment scores for genes with a ZTRE in the range -200 bp to the TSS, compared to those with ZTREs in other promoter locations. Top DAVID annotation clusters for each cohort are reported. Annotation clusters significantly enriched for genes found in the range -200 bp to the TSS may be associated with Zn deficiency cellular responses, while enriched genes for other ZTREs are associated with transcriptional activation.

Summary: Annotation/enrichment scores	**Genes with ZTRE -200 bp to TSS**	**All other ZTRE Genes**
Number of mapped DAVID IDs	1112	1481
DAVID Pleckstrin homology domain annotation cluster	4.73**	0.54
DAVID Microtubule transport annotation cluster	1.81**	0.25
DAVID Leucine zipper domain annotation cluster	1.42*	0
DAVID RNA pol II transcriptional activation annotation cluster	0.26	3.56**
DAVID Transcription/activation annotation cluster	0.62	2.86**
DAVID PWWP domain annotation cluster	0	2.42**
GO nervous system development	1.41*	-

*p<0.01
**p<0.001

Table 3.3. Comparison of amino acid sequences of Zap1, Sp1, and MTF-1 versus ZNF658. Homologous ZF domains are found in all of these proteins. The AD1 domain of Zap1 has regulatory element-binding characteristics which may be analogous to those of ZNF658, though direct homology of the domains is indeterminate.

Subject organism	**Subject protein**	**Subject domain (position)**	**Human ZNF658 homologous region**	**E value**	**Identity**	**Posi-tives**	**Total bit score (BLOSUM 62)**
S. cerevisiae	Zap1	(all)	-	-	-	-	1649
S. cerevisiae	Zap1	AD1 (258-317)	199-253	8.00E-03	26%	35%	-
H. sapiens	Sp1	(all)	-	-	-	-	1072
H. sapiens	Sp1	Zn finger 1 (626-650)	886-902 (ZF16)	2.00E-07	76%	82%	-
H. sapiens	Sp1	Zn finger 2 (656-680)	523-540(ZF3)	4.00E-04	44%	72%	-
H. sapiens	Sp1	Zn finger 3 (686-708)	440-462(ZF2)	9.00E-06	43%	69%	-
H. sapiens	Sp1	NLS/Zn fingers 1-3 (626-708)	971-1040(ZFs 19-21)	3.00E-20	49%	62%	-
H. sapiens	MTF-1	(all)	-	-	-	-	1705
H. sapiens	MTF-1	NLS/Zn fingers 1-3 (112-202)	768-903 (ZFs 12-16)	2.00E-31	44%	58%	-

CHAPTER 5

CONCLUSION

In comparison to some of the more well-studied transcription factors (TFs), MTF-1 and ZNF658 have less gene expression data reported in the literature. There is a need for studies to determine the precise relationships between MTF-1 and ZNF658 TF binding sites (i.e. permutation, length, flanking bases) and the strength of their regulatory effects. Similarly, there has been no validation for the combinatorial regulation by these Zn-activated TFs and microRNAs (miRNAs). The work contained herein provides the computational framework for future investigations designed to validate these phenomena.

Bioinformatics give us tools to make functional predictions from DNA structure, on a scale that was previously unimaginable. Our studies reported here illustrate a merger between nutrition and genome-wide analysis techniques, called nutrigenomics. Here we have shown three different types of analyses performed with these techniques: secondary analysis of gene expression data, regulatory network prediction, and functional predictions based on homology. These studies pave the way for novel studies designed to increase our understanding of Zn-activated transcription factors. Based on our analyses, their influence may be much greater than anticipated, and beyond simply the regulation of cellular Zn homeostasis.