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Structural Metal Sites in Nonclassical Zinc Finger Proteins Involved in Transcriptional and Translational Regulation

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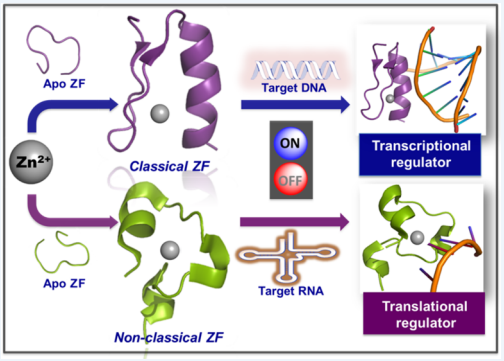
CONSPECTUS: Zinc finger (ZF) proteins are a large family of metalloproteins that utilize zinc for structural purposes. Zinc coordinates to a combination of cysteine thiol and histidine imidazole residues within the ZF polypeptide sequence resulting in a folded and functional protein. Initially, a single class of ZFs were identified. These ZFs, now referred to as the "classical" ZFs, utilize a Cys_2His_2 (CCHH) ligand set to bind zinc. Upon Zn coordination, the classical ZFs fold into a structure made up of an α helix and an antiparallel β sheet. When folded, classical ZFs recognize and bind to specific DNA targets and function as transcription factors. With the advent of genome sequencing and proteomics, many additional classes of ZFs were identified based upon their primary amino acid sequences. At least 13 additional classes of ZFs are known, and collectively these "nonclassical" ZFs differ in the ligand set involved in Zn(II) coordination, the organization of the ligands within the polypeptide sequence and the macromolecular targets. Some nonclassical ZFs are DNA binding "transcription factors", while others are involved in RNA regulation and protein recognition. Much less is known about these nonclassical ZFs with regards to the roles of metal coordination in fold and function. This Account focuses on our laboratory's efforts to characterize two families of "nonclassical" ZFs: the Cys_3His (or CCCH) ZF family and the Cys_2His_2Cys (or CCHHC) ZF family.



Our work on the CCCH ZF family has focused on the protein Tristetraprolin (TTP), which is a key protein in regulating inflammation. TTP contains two CCCH domains that were proposed to be ZFs based upon their sequence. We have shown that while this protein can coordinate Zn(II) at the CCCH sites, it can also coordinate Fe(II) and Fe(III). Moreover, the zinc and iron bound forms of TTP are equally adept at discriminating between RNA targets, which we have demonstrated via a fluorescence anisotropy based approach. Thus, CCCH type ZFs appear to be promiscuous with respect to metal preference and a role for iron coordination in CCCH ZF function is proposed.

The CCHHC family of ZFs is a small family of nonclassical ZFs that are essential for the development of the central nervous system. There are three ZFs in this family: neural zinc finger factor-1 (NZF-1), myelin transcription factor-1 (MyT1), and suppressor of tumorgenicity 18 (ST18). All three proteins contain multiple clusters of "CCHHC" domains, which are all predicted to be Zn binding domains. We have focused on a tandem-CCHHC domain construct of NZF-1, which recognizes β-RARE DNA, and we have identified key residues required for DNA recognition. Unlike classical ZFs, for which a few conserved residues are required for DNA recognition, the CCHHC class of ZFs utilize a few nonconserved residues to drive DNA recognition leading us to propose a new paradigm for ZF/DNA binding.

INTRODUCTION

Metal ions play important roles in gene regulation, both at the transcriptional and translational levels.[1−3] Metal ions can be associated directly with genetic material (e.g., DNA or RNA) or with proteins involved in gene regulation.[1−7] In the latter role, the interaction of specific metal ions with proteins affects the macromolecular interactions between the protein and its biological targets. Proteins that utilize metal ions to control transcription or translation are often called "metalloregulatory" proteins, particularly when these proteins regulate metal ion homeostasis in bacteria.[2,3,8]

The paradigm for metalloregulatory protein function is that upon metal binding the protein undergoes a conformational change that affects the protein's ability to recognize and bind specific DNA or RNA targets, thereby affecting transcription or translation.[3,5,9] In this sense, the metal ion functions as a regulatory "switch".[3,5,10] Metalloregulatory proteins are found in both eukaryotes and prokaryotes and many different types of metal ions serve as "switches" including biologically "essential" metal ions (e.g., Zn(II) and Fe(II)) and toxic metal ions (e.g., As(II) and Cd(II)).[3,11−14] The conformational changes that accompany metal ion coordination to allow the protein to recognize DNA and RNA are similarly diverse. In some

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instances, metal ion coordination leads to a large structural change in the protein architecture, while in other cases metal ion coordination results in a subtle, local change.[15,16] Regardless of whether the structural change is significant or subtle, the change is essential for oligonucleotide recognition.

In eukaryotes, zinc finger (ZF) proteins are the most common metalloregulatory protein. These proteins require zinc coordination to fold and function. ZFs were first discovered more than 25 years ago.[17] Recent bioinformatics studies have highlighted the importance of ZFs in biology, and identified an increasingly large number of these types of proteins.[18,19] It is estimated that at least 3% of the coding proteins in the human genome are ZFs.[20] Thus, ZFs are critically important to eukaryotic transcriptional and translational regulation.

CLASSICAL VERSUS NONCLASSICAL ZFs

Collectively, ZFs are proteins that utilize Zn coordination to fold and function (Figure 1). Since the discovery of the first ZF,

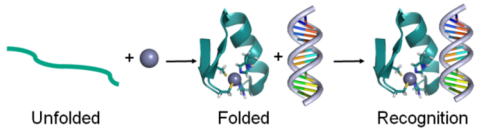

Figure 1. Zinc coordination results in a folded ZF protein (PDB 1M9O).

which is now referred to as the "classical ZF", at least 13 other classes of "nonclassical" ZFs have been identified.[5,19,21] As a group, ZFs share several features: (i) the amino acids that serve as coordinating ligands are always four cysteine and/or histidine residues, (ii) the coordination number is always four, and (iii) the geometry at the Zn(II) center is always close to tetrahedral.[5,22,23] Another common property is that ZFs contain multiple domains that bind zinc and fold individually, and in some cases even function individually.[15,19] ZFs can be divided into classes delineated by (i) the ligand set involved in zinc coordination (e.g., Cys_4 versus Cys_2His_2), (ii) the three-dimensional fold adopted by the ZF, and (iii) the macromolecular target (e.g., DNA, RNA or another protein).

Classical ZFs

The best-studied family of ZFs are the "classical" ZFs. Classical ZFs contain the sequence $CysX_{2-5}CysX_{12-18}HisX_{3-5}His$, where X is any amino acid.[5] These proteins utilize the two cysteine and two histidine residues as zinc ligands and fold into structures composed of an α helix and a β sheet.[15,24] The fold of the classical ZF was predicted by Berg in 1988, who compared the newly identified ZF sequence to known structurally characterized metalloproteins and presciently predicted that it would fold into an α helix and β sheet in the presence of zinc.[17] This prediction was borne out by structural studies.[24] Since these initial studies, classical ZFs have been identified in a range of eukaryotes where they are involved in myriad functions, ranging from regulation of cell development to cancer proliferation.[21] Classical ZFs recognize DNA via specific amino acid side chains located on the α helical portion of the protein that form hydrogen bonds with specific bases on the DNA target.[6,24] These protein/DNA recognition properties are so well understood that "designer" ZFs as artificial transcription factors for gene therapy based upon classical ZFs are being developed.[25,26]

In contrast, the mechanisms of metal mediated DNA or RNA recognition for many of the families of nonclassical ZFs are poorly understood, in many cases because these proteins have only recently been identified.[19] Our laboratory has focused on two families of nonclassical ZFs: the Cys_3His (or CCCH) family, which are involved in RNA regulation, and the Cys_2His_2Cys (or CCHHC) family, which are involved in neuronal regulation (Figure 2).[5] In this Account, we review our

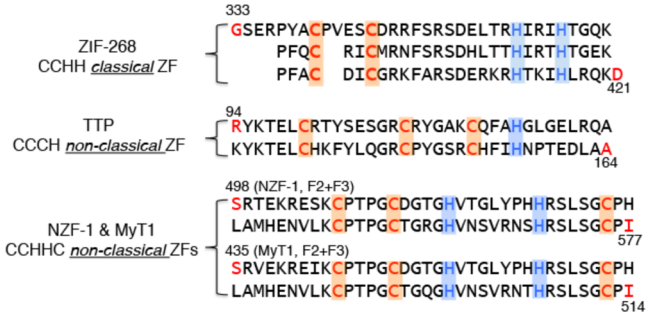

Figure 2. Sequence alignment of the classical ZF ZIF-268 (CCHH) with the nonclassical ZF TTP (CCCH) and the nonclassical ZFs NZF-1 and MyT1 (CCHHC).

work in this area and propose some general principles that define the relationships between metal ion coordination, protein folding, and target DNA or RNA recognition for these ZFs.

NONCLASSICAL ZF FAMILY #1: Cys_3His (OR CCCH) TYPE ZFs

Tristetraprolin (TTP): The CCCH Archetype

Over 20 years ago, three research groups identified a protein that was upregulated in response to growth factors including serum and insulin in mice fibroblasts.[27−29] The protein was named Nup475, Tristetraprolin, Tis11, and ZFP36 and is now referred to as Tristetraprolin (or TTP). Initially thought to be a transcription factor, TTP was subsequently determined to be involved in RNA regulation. Specifically, TTP regulates mRNAs involved in inflammation and misregulation of TTP is implicated in inflammatory diseases such as arthritis and cancer.[30] The mechanism of regulation involves binding of the two ZF domains of TTP directly to AU-rich element (ARE) sequences located at the 3′ end of mRNA targets.[5,31,32] The TTP/mRNA complex is degraded by exosomes.[33] TTP is a general regulator of inflammation, and a large number of cytokines (proteins that regulate inflammation) as well as several enzymes (cyclooxygenase, nitric oxide synthase) are regulated directly by TTP.[5,34] An example of this regulation is shown in Figure 3. As such, TTP is considered a potential drug target for down-regulation of cytokines as a means to treat inflammation.[34] TTP has homologues in a number of other eukaryotes; however, less is known about these proteins' structure/function relationships, including the role(s) of metal ion coordination.

A Role for Zn(II) Coordination in CCCH Protein Function

Within the primary sequence of TTP, there are two repeats of a Cys_3His motif, $CysX_{7-10}CysX_{4-5}CysX_3His$, that were predicted to coordinate zinc.[35] Initial studies to test this hypothesis involved preparation of peptides that corresponded to either a single Cys_3His domain (named TTP-1D) or both of the Cys_3His domains (named TTP-2D) followed by metal binding

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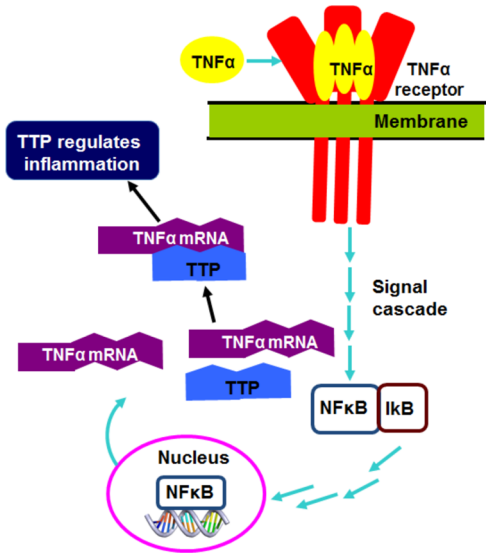

Figure 3. Cartoon depicting TTP regulation of the cytokine tumor necrosis factor alpha (TNFα).

and folding studies utilizing UV−visible and NMR spectroscopies.[35−38] This is an approach that has successfully been utilized to measure metal binding and folding for other types of ZFs, most notably classical ZFs.[39] The UV−visible studies use Co(II) as a spectroscopic probe for Zn(II) and involve titrating apo-ZF with Co(II) followed by titration with Zn(II). The direct titration with Co(II) results in the appearance of d−d transitions indicative of tetrahedral coordination by Co(II) (Figure 4) and the appearance of ligand-to-metal charge

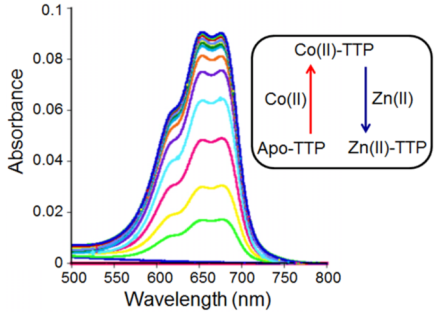

Figure 4. UV−visible spectrum of the d−d bands that appear upon Co(II) coordination and disappear upon Zn(II) coordination to TTP.

transfer bands (LMCT) indicative of Co(II) to sulfur (from cysteine) coordination.[37] The subsequent titration of Zn(II) with Co(II)-ZF results in a decrease of these spectroscopic features as the spectroscopically silent Zn(II) replaces the spectroscopically active Co(II) in binding to the ZF. The Co(II)/Zn(II) competition studies with TTP-1D and TTP-2D revealed that these proteins bind Co(II) and Zn(II) in a tetrahedral geometry with upper limit dissociation constants, K_d of 2×10^{-6} M (Co^{2+}) and 2.0×10^{-10} M (Zn^{2+}) for TTP-1D and 3.3×10^{-6} M (Co^{2+}) and 6.2×10^{-11} (Zn^{2+}) for TTP-2D.[32,36,37] NMR studies in which Zn(II) was titrated with the apo forms of these peptides demonstrated that the peptides fold in the presence of Zn(II) with a stoichiometry of 1 Zn(II) per 1 Cys_3His domain.[35,40] Multidimensional NMR studies of single ZF domain of TTP and of a two domain construct of Tis11d (a TTP homologue) revealed that the protein adopts a minimal fold made up of a series of loops when zinc is coordinated (Figure 5).[38,41] Thus, the CCCH domain found in TTP is a novel ZF binding domain that adopts a unique fold.

Figure 5. NMR structure of a two-ZF domain of Tis11d (TTP homologue, PDB 1RGO).

A Role for Fe(II)/Fe(III) Coordination in TTP Function

New families of ZF proteins are typically identified based upon similar sequence elements. In the genome databases, any protein that contains a Cys/His motif is annotated as a ZF. Thus, TTP and homologues that contain a $CysX_{2-5}CysX_{12-18}HisX_{3-5}His$ motif are all annotated as ZFs; however, there is no direct in vivo evidence that Zn is the native metal ion for any of these proteins. TTP's biological role is to regulate mRNA during inflammation, when iron and reactive oxygen species (ROS) are elevated, thus we hypothesized that there may be a role for iron coordination in TTP function.[36] This hypothesis was supported by our experimental observation that TTP turns red when overexpressed.[36] To test whether TTP coordinates iron, direct titrations of Fe(III) with apo-TTP-2D (the construct made up of the two CCCH domains) were performed. In the UV−visible spectrum, bands centered around 270, 308, and 500 nm were observed (Figure 6a). We proposed these were LMCT bands based upon similarities to the LMCT bands observed for Rubredoxin, a protein that binds Fe(II)/Fe(III) in a tetrahedral geometry utilizing four cysteine ligands.[36] Fe(II) also binds to TTP-2D and exhibits an absorbance spectrum that resembles that of Fe(II)-Rubredoxin.

Our finding that iron can coordinate TTP-2D as well as zinc led to the question: which is the functional form? Our hypothesis was that the functional form would be the form that selectively bound RNA. TTP recognizes ARE sequences located on the 3′end of mRNA and the canonical RNA target for TTP had been identified as UUUAUUUAUUU, with each ZF recognizing a UAUU repeat.[32,36,41,42] Using fluorescence anisotropy (FA) to measure protein/RNA binding, we found that when Zn(II) is bound to TTP-2D the protein preferentially recognizes a UUUAUUUAUUU sequence (K_d ~ 16.0 nM); by modifying a single A to a G (UUUGUUUAUUU), the measured affinity decreased to a K_d of 44 nM, and when both A's were modified to either G or U (UUUGUUUGUUU or UUUUUUUUUUU), the measured affinities decreased to K_d's of 258 and 415 nM, respectively.[36] Wilson

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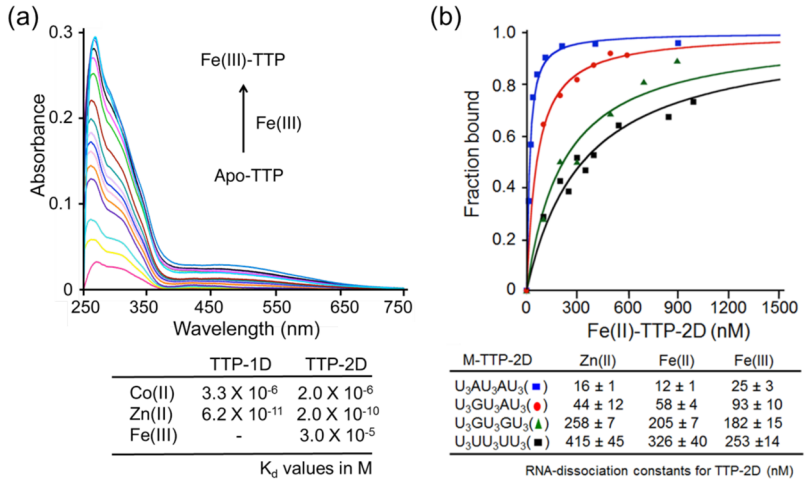


	TTP-1D	TTP-2D
Co(II)	3.3×10^{-6}	2.0×10^{-6}
Zn(II)	6.2×10^{-11}	2.0×10^{-10}
Fe(III)	-	3.0×10^{-5}

K_d values in M

M-TTP-2D	Zn(II)	Fe(II)	Fe(III)
$U_3AU_3AU_3$(■)	16 ± 1	12 ± 1	25 ± 3
$U_3GU_3AU_3$(●)	44 ± 12	58 ± 4	93 ± 10
$U_3GU_3GU_3$(▲)	258 ± 7	205 ± 7	182 ± 15
$U_3UU_3UU_3$(■)	415 ± 45	326 ± 40	253 ±14

RNA-dissociation constants for TTP-2D (nM)

Figure 6. (A) UV−visible spectrum as Fe(III) is titrated with TTP-2D. Table of measured binding affinities for Zn(II), Co(II), and Fe(III) coordination. (B) RNA binding of TTP-2D measured by FA. Adapted with permission from ref 36. Copyright 2006 American Chemical Society.

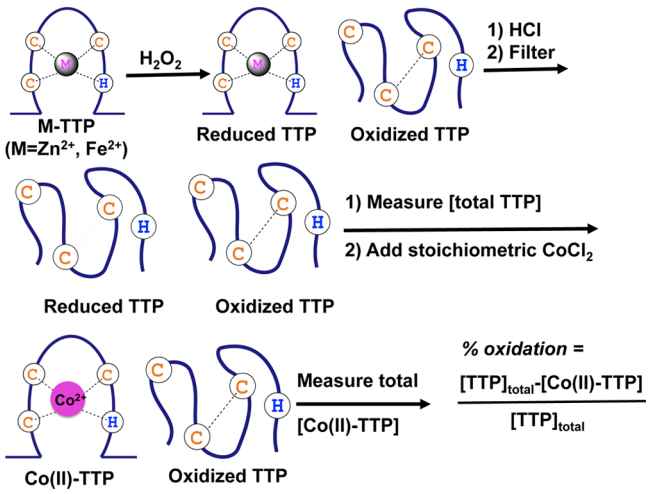
and co-workers independently reported similar results.[42] We then repeated the FA studies using Fe(II)-TTP-2D and Fe(III)-TTP-2D with the same RNA sequences, and the affinities and trends in RNA binding matched those determined for Zn(II)-TTP-2D (Figure 6b).[36] These findings indicated that the identity of the metal ion is *flexible* for TTP function. This principle is not true for all ZFs: for example, the classical ZFs (Cys_2His_2), transcription factor IIIA is inactive (no DNA binding) when iron is coordinated, while the nonclassical estrogen receptor (Cys_4) ZF binds its target DNA more tightly when Fe is coordinated.[36] We suggest that the annotation of proteins with Cys/His domains as "ZFs" must be interpreted with caution: in some instances, other metal ions are capable of activating these proteins.

A Role for Oxidation in TTP Function

It has long been proposed that reactive oxygen species (ROS) play a role in ZF function as a means to control activity.[43−45] During inflammation when TTP is upregulated, ROS concentrations are elevated.[46] We hypothesized that ROS may control TTP function by oxidizing coordinating cysteine residues resulting in metal ion ejection and loss of fold and function.[46] We sought a rapid and reproducible assay to monitor oxidation of TTP to test this prediction. We developed a novel spectroscopic assay based upon the well-understood Co(II) binding properties of ZFs (Scheme 1).[46] In the assay, the ZF is exposed to an oxidant and then ratio of total ZF versus ZF that binds Co(II) is measured via UV−visible spectroscopy. Only the fully reduced ZF will bind to Co(II), and thus, the ratio of Co(II) bound ZF/total ZF provides a measure of oxidation. We determined that when Fe(II) is coordinated to TTP-2D, the protein is more rapidly oxidized than when Zn(II) is coordinated, with apo-TTP-2D exhibiting an intermediate rate (Figure 7a). Addition of EMPO (2-ethoxycarbonyl-2-methyl-3,4-dihydro-2*H*-pyrrole-1-oxide), which serves as a spin trap, followed by electron paramagnetic resonance (EPR) analysis identified radical formation by Fenton chemistry as the source of the increased oxidation rates for Fe(II)-TTP-2D (Figure 7b). The oxidized proteins exhibited weaker RNA binding than the fully reduced proteins indicated that TTP function is modulated by oxidation.

This spectroscopic assay has the potential to measure oxidation rates for any type of ZF.[46] As such, we utilized this

Scheme 1



assay to measure oxidation of a classical ZF, ZIF-268, as a function of metal ion coordination.[47] Remarkably, this protein, which has a more significant secondary structure (well-defined antiparallel two β-strands followed by an α-helix compared to TTP which contains limited secondary structure), is more rapidly oxidized by H_2O_2 than TTP.[46,47] Moreover, the metal ion coordinated to the protein does not appear to play a significant role in ZIF-268 oxidation.[47] Thus, the susceptibility of cysteine residues in ZFs to oxidation does not appear to be directly related to protein structure.

Cd(II) Toxicity via TTP Coordination

We also investigated the functional consequences of cadmium coordination to TTP.[40] Cadmium is a toxic and potent carcinogen that targets proteins with thiol groups, such as the zinc storage protein metallothionein and several ZFs (i.e., the classical ZFs TFIIIA and Sp-1 and the nonclassical Cys_4 estrogen receptor ZF).[40] Cadmium had been shown to affect TTP expression levels in hepatocytes, and we hypothesized that may be a consequence of Cd coordination to the Zn sites of TTP. Co/Cd competition titrations with apo-TTP2D monitored by UV−visible spectroscopy along with NMR titrations of Cd with apo-TTP-2D were performed. Cd was found to bind TTP-2D at the two zinc sites in a 2:1 Cd/TTP-2D

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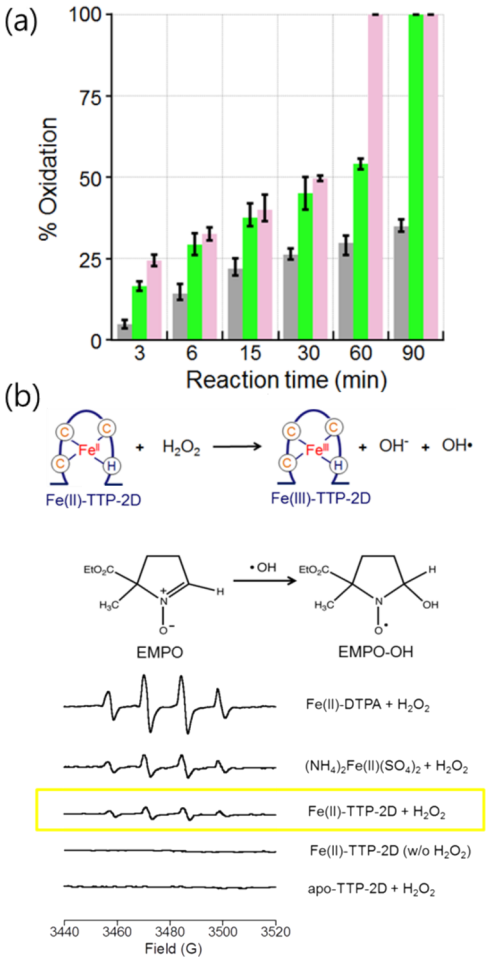

Figure 7. (a) Plots of the measured oxidation of M-TTP-2D [M = Zn (gray), apo (green), Fe(II) (pink)] with H_2O_2 as a function of time. (b) EPR spin−trap experiments with EMPO. Adapted with permission from ref 46. Copyright 2010 American Chemical Society.

stoichiometry with a K_d of 3.5 ± 0.1 nM. To determine the functional consequences of Cd coordination, FA titrations of Cd(II)-TTP-2D with RNA (UUUAUUUAUUU-F, UUUG-UUUAUUU-F, UUUGUUUGUUU-F, and UUUUUUUU-UUU-F) were performed. Strikingly, Cd bound to the canonical RNA target (UUUAUUUAUUU) with higher affinity than Zn(II)-TTP-2D and to modified RNA targets with weaker affinity than Zn(II)-TTP-2D. This increased selectivity for RNA by Cd(II)-TTP-2D suggests a possible mechanism for Cd induced toxicity in which the inflammatory response is prematurely turned off.[40]

The Paradigm of Metal Induced Folding for TTP

TTP requires metal coordination to function, that is, bind to RNA. Our studies have revealed that the identity of the metal ion is flexible: Zn(II), Fe(II), and Fe(III) coordination all result in a functional protein, as does the toxic metal ion Cd(II). NMR structures of a single CCCH domain of TTP and two CCCH domains of a homologue, Tis11d, show that the protein adopts limited secondary structure upon metal binding. How this "folding" upon metal coordination drives RNA recognition is still not clear; although studies probing folding via intrinsic fluorescence have indicated that RNA binding may induce additional secondary structure at the second CCCH domain.[48] Our current model for TTP function involves metal coordination inducing small structural changes that are enhanced upon RNA binding. We hypothesize that other CCCH family members will utilize a similar mechanism for RNA recognition.

NONCLASSICAL ZF FAMILY #2: Cys_2His_2Cys (CCHHC) TYPE ZFs

Neural Zinc Finger Factor 1 (NZF-1), Myelin Transcription Factor 1 (MyT1), and Suppressor of Tumorgenicity 18 (ST18)

The Cys_2His_2Cys (CCHHC) family of nonclassical ZFs is a small, but important family of proteins with three members: neural zinc finger factor-1 (NZF-1), myelin transcription factor 1 (MyT1), and suppression (or suppressor) of tumorigenicity 18 (ST18).[5,49] NZF-1 and MyT1 are involved in neuronal development and regulate neurons and oligodendrocytes, respectively (Figure 8).[5] The biological role of ST18 has not

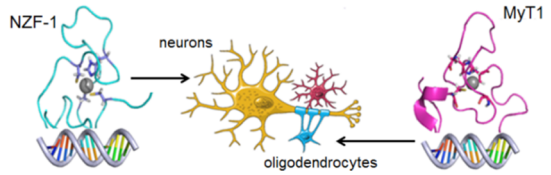

Figure 8. Cartoon depicting the types of cells that NZF-1 and MyT1 regulate (neurons and oligodendrocytes, respectively).

yet been delineated; however, it has been shown to be associated with the suppression of tumor cells, particularly in brain and breast cancers and also to localize in the prostrate.[50−52] All three CCHHC ZFs contain multiple zinc binding domains arranged in clusters. The sequences of these metal-binding domains are unusually homologous, with upward of 99% sequence homology between domains. Classical ZFs typically only show sequence conservation in the metal binding ligands and a few additional residues that are important for protein fold.[5]

Identification of Metal Binding Ligands. Early biochemical studies on NZF-1 focused on identifying which of the five potential zinc-binding ligands were involved in metal coordination.[53,54] ZFs typically utilize four ligands to bind zinc in a tetrahedral coordination geometry; however, the presence of five ligands suggested the possibility of five-coordinate geometry. A simple way to assess geometry of ZF sites is to examine the Co(II)-bound UV−visible spectra; the wavelength at which d−d transitions appear along with their measured extinction coefficient can be correlated with the geometry (Figure 9). Initial studies on a peptide that corresponded to the third ZF domain of NZF-1 resulted in a UV−visible spectrum for the Co-bound species that had an absorbance between 520 and 720 nM indicative of four-coordinate tetrahedral geometry, indicating that this domain uses only four ligands for metal ion coordination.[54] More recently, Wilcox and co-workers reported analogous studies of the second ZF domain of MyT1 as part of a larger comprehensive study that detailed the thermodynamics of metal binding to various ZF sites and observed an equivalent UV−visible spectrum for the Co(II) bound complex.[55] The shape of the absorbance envelope is indicative of a 3Cys/1His ligand set (Figure 9). NMR structures of the second ZF domain of NZF-1 and the fifth ZF domain of MyT1 have been reported, and a 3Cys/1His ligand set for Zn(II) that matches

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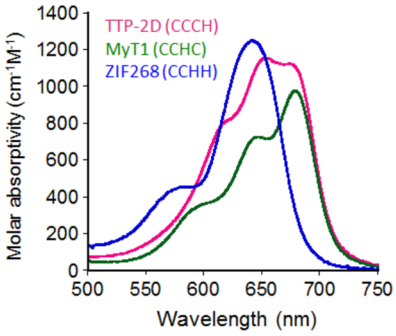

Figure 9. Shape of the Co(II)-ZF d-d transitions as a function of ligand set.

that predicted by the Co(II) optical spectra was observed, with the second histidine involved in Zn coordination.[56,57]

DNA Targets for NZF-1, MyT1, and ST18. NZF-1 regulates the beta retinoic acid receptor (β-RARE), a critical factor for the development of neurons; MyT1 regulates the proteolipid protein (PLP) which is involved in myelin formation and as well as opalin (Tmem10) and CNP (2′3′-cyclic nucleotide 3′-phosphodiesterase), while ST18 is involved in liptoxicity and cytokine induced β-cell death, although the exact genes it regulates have not yet been defined.[5] A DNA target sequence has only been identified for a two-domain construct (ZF2 and ZF3) of NZF-1. The sequence, from the β-RARE promoter, includes an AAGTT motif that is present in some, but not all, of the promoters of genes regulated by MyT1. It has been proposed that AAGTT is the recognition sequence for CCHHC type ZFs. We utilized FA to measure the binding affinity of a two-domain construct of NZF-1 (F2 + F3) as well as a two domain construct of MyT1 (F2 + F3) for this DNA sequence. The NZF-1 construct bound to β-RARE with a K_d of 11 nM, while MyT1 bound to this same target with a K_d of 16 μM.[58,59] In addition, Mackay and co-workers have reported that a two domain construct of MyT1 (F4 + F5) binds to the β-RARE sequence with affinities ranging from 0.3 to 1 μM when measured by SPR and ITC, respectively.[57,60] β-RARE is not the physiological DNA target for MyT1, likely explaining why weaker DNA binding affinities are measured for MyT1 constructs with β-RARE, compared to the affinities of NZF-1 with β-RARE. We propose that the AAGTT motif itself it not the only factor recognized by MyT1 and further studies are needed to decipher the DNA recognition sequence.

How NZF-1 and MyT1 interact with their target DNA partners is not well understood. A modeling study of MyT-1 with the β-RARE promoter target suggested that each ZF domain fits into the major groove of the DNA with several serine and threonine residues at the C-terminus interacting with the DNA.[57,60] This is an intriguing proposal as would be an entirely new way of DNA recognition by a ZF protein wherein zinc coordination results in a structured domain that "fits" in the major groove rather than a domain involved in specific protein/DNA recognition events.

Determining the Role(s) of the Noncoordinating His in DNA Recognition by NZF-1. In the structure of NZF-1-F2, the noncoordinating histidine is positioned such that it appears to π-stack with a tyrosine residue. This π-stacking was proposed to be important for the fold and function of the protein. If π-stacking is important, a mutation of the histidine to should retain function. We mutated this histidine to a phenylalanine in a two domain construct of NZF-1 (F2 + F3).[59] The resultant construct had two mutations, one per domain, and was capable of binding Co(II) and Zn(II). However, the mutant did not bind β-RARE DNA, suggesting that the role of the noncoordinating histidines is greater than just in π-stacking (Figure 10).

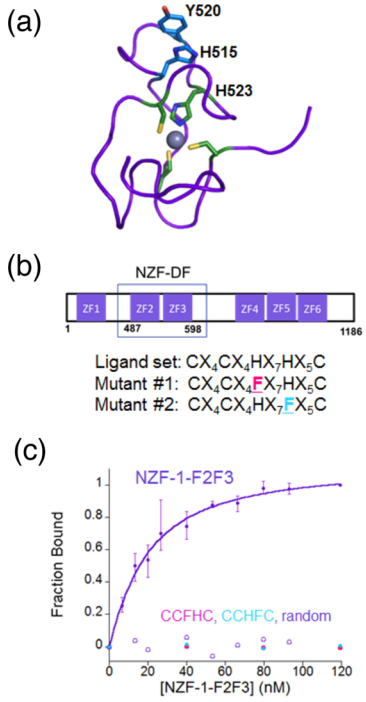

Figure 10. (A) Single ZF domain of NZF-1 (PDB 1PXE). (B) Mutations of H/F were made to the two H ligands of NZF-1. (C) Effects of mutations on DNA binding, measured by FA. Adapted with permission from ref 59. Copyright 2013 American Chemical Society.

Functional Effects of Switching the Coordinating Histidine in NZF-1. When the coordinating histidine is mutated to an alanine in a single domain construct of NZF-1, the noncoordinating histidine replaces the coordinating histidine as a ligand to achieve a tetrahedral site. The ability of NZF-1 to switch ligands led to the question, can NZF-1 bind DNA when the coordinating ligand is switched? The initial metal binding studies were performed on a single domain construct, which does not bind DNA. Thus, we mutated the two coordinating histidines in our two-domain construct of NZF-1, which does bind DNA, and measured the effect of the mutation on DNA binding. The mutant protein did not bind to β-RARE DNA, indicating that switching the histidine ligands results in a protein that can still bind Zn(II), but the overall fold of the protein is disrupted such that it cannot bind to DNA (Figure 10).[59]

Switching the DNA Binding Properties of Myt1 to That of NZF-1. Our finding that two ZF domains of Myt1 bind to β-RARE weakly while the analogous two ZF domains of NZF-1 bound tightly led us to carefully inspect the sequences of the two constructs. This revealed only two differences within the ZF domains: S/T and R/Q (NZF-1-F2F3 vs Myt1-F2F3). Arginine is often important for DNA binding, and we hypothesized that mutation of the Q in Myt1-F2F3 to R would improve DNA binding. Remarkably, we found that this mutant bound β-RARE with almost identical affinity to the native NZF-1-F2F3.[59] Thus, a single residue appears to be

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important for DNA recognition in the CCHHC family of ZFs. We proposed a new paradigm for DNA recognition for the CCHHC family of ZFs. Unlike the classical ZFs, in which only a handful of amino acids are conserved and drive recognition, in the CCHHC family most of the amino acids are conserved and the handful of nonconserved amino acids appear to drive DNA binding.

A Role for Fe(II) and Fe(III) Coordination in NZF-1 Function. NZF-1 is present during neuronal development when iron levels are elevated, and we sought to understand whether iron could coordinate to NZF-1, in place of Zn, and determine the functional consequences. Following the approach utilized for TTP, we determined that Fe(II) binds NZF-1-F2F3 and that when iron is coordinated, the protein binds to β-RARE DNA with an equal affinity at the Zn(II) bound form. Thus, NZF-1 can function with either zinc or iron coordinated.[58]

■ CONCLUSIONS

Since the identification of the "classical ZFs" in the late 1980s, many new families of "nonclassical ZFs" have been identified. The nonclassical ZFs differ in sequence, fold, and function, and we are learning that ZF function is not a "one-size fits all" proposition based upon the classical ZF model. Rather each family of ZFs utilizes a unique mixture of metal coordination to adopt novel structural folds and achieve specific oligonucleotide recognition. We are just beginning to unravel the nuances in metal mediated oligonucleotide recognition for these proteins.

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Notes

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