A Novel Subfamily of Zinc Finger Genes Involved in Embryonic Development

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Abstract C_2H_2 zinc finger proteins make up one of the largest protein families in eukaryotic organisms. Recent study in several different systems has identified a set of novel zinc finger proteins that appear to form a distinct subfamily that we have named the NET family. Members of the NET family (Noc, Nlz, Elbow, and Tlp-1) share two protein motifs—a buttonhead box and an Sp motif—with zinc finger proteins from the Sp family. However, the NET family is uniquely characterized by a single atypical C_2H_2 zinc finger, in contrast to the Sp family that contains three tandem C_2H_2 fingers. Here, we review current information about the biochemical function and in vivo role for members of this subfamily. In general, NET family proteins are required during embryonic development. They appear to act by regulating transcription, most likely as repressors, although they are unlikely to bind DNA directly. In the future, it will be important to directly test if NET family proteins control transcription of specific target genes, perhaps via interactions with DNA-binding transcription factors, as well as to further explore their function in vivo. J. Cell. Biochem. 93: 887–895, 2004.   © 2004 Wiley-Liss, Inc.

Key words: NET family; Sp motif; transcriptional repressor; buttonhead corepressor

A NOVEL SUBFAMILY OF ZINC FINGER GENES

Recent analyses of embryogenesis in both vertebrates and invertebrates have demonstrated important roles for the *noc*, *elbow*, *tlp-1*, and *nlz* genes [Cheah et al., 1994; Dorfman et al., 2002; Zhao et al., 2002; Runko and Sagerstrom, 2003, 2004; Hoyle et al., 2004; Weihe et al., 2004], which encode related zinc finger proteins. Other similar genes have been reported (e.g., *Nolz-1* in the mouse [Chang et al., 2004]) and detected in databases (see for instance [Runko and Sagerstrom, 2003]), but the function of these additional genes has not yet been determined. Sequence analyses demo-

nstrated that *noc*, *elbow*, *tlp1*, and *nlz* are most closely related to the *sp/buttonhead* zinc finger family. A phylogenetic analysis (Fig. 1A) revealed that the Noc, Elbow, Tlp-1, and Nlz proteins form a subgroup that is separate from the other Sp/Buttonhead family proteins. We also note that the original *Drosophila* Buttonhead protein does not cluster with any of the other proteins in this family. In contrast, a *Drosophila* protein named D-Sp1 [Wimmer et al., 1996] clusters with vertebrate Sp8. This suggests that D-Sp1 represents an invertebrate Sp ortholog, while direct orthologs of *Drosophila* Buttonhead may not yet have been found in vertebrates, or may have been lost, although the accumulation of glutamine rich stretches in *Drosophila* Buttonhead (as well as in D-Sp1) complicates the phylogenetic analysis. Further, although zebrafish Bts1 and mouse mBtd are reportedly related to *Drosophila* Buttonhead [Tallafuss et al., 2001; Treichel et al., 2003], these cluster with vertebrate Sp5 and Sp8, respectively, suggesting that they are more closely related to vertebrate Sp proteins than to *Drosophila* Buttonhead. Thus, there are at least two distinct subfamilies within this group of zinc finger proteins. One consists of the Sp proteins and one of the Noc, Elbow, Tlp-1 and

Nlz related proteins, herein referred to as the NET (Noc/Nlz, Elbow, Tlp-1) subfamily. *Drosophila* Buttonhead forms a potential third subfamily. Several additional related genes have been identified in the *Drosophila* and *C. elegans* genomes, but these do not fall into either of the clusters, suggesting that additional subfamilies may exist. It also remains to be seen if there are vertebrate orthologs to these additional invertebrate genes.

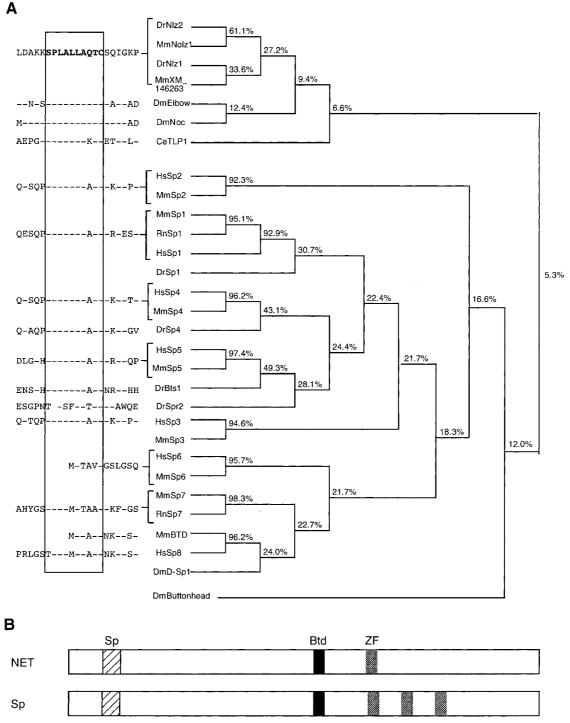As expected, the Sp and NET subfamilies share several sequence features (Fig. 1B). First,

Fig. 1.

all NET and Sp proteins contain a Buttonhead (Btd) box [Wimmer et al., 1993], defined as a 7–10 amino acid motif with the consensus R-X_{0-4}-C-X-C/D/N-P-N/Y-C. The Btd box is somewhat more divergent in the NET family, where the initial R is often missing and the N/Y at the second to last position is occasionally replaced with an A. The function of the Btd box is not clear, but it appears required for transcriptional activation in some instances [Athanikar et al., 1997] and its high degree of conservation is consistent with an important role. Second, an N-terminal domain named the 'Sp motif' is also shared between the Sp and NET subfamilies [Zhao et al., 2002; Runko and Sagerstrom, 2003]. The Sp motif has a consensus core sequence of S-P-L-A-L/M-L-A-A/Q-T-C and is found in vertebrate NET and Sp family proteins, but not in *Drosophila* Buttonhead or D-Sp1. Proteins in the Sp6, 7, 8 subgroup show a more divergent Sp motif and in some instances only part of the motif appears to be present. The role of the Sp motif is not clear, but it may regulate protein degradation [Su et al., 1999], or transcriptional activity [Murata et al., 1994]. Interestingly, sequence variations within the Sp motif show a strong correlation with a proteins position in the phylogenetic tree (Fig. 1A). For instance, position 8 is generally an A in the Sp subfamily and a Q in the NET subfamily. Similarly, position 5 is an M in Sp7 and 8 (and possibly Sp6), but an L in most other family members. Subfamily-specific conserved domains also extend N- and C-terminal to this core sequence such that D-A-K-K is found N-terminal to the core in the NET subfamily (with some variations in Elbow and TLP-1) and Q-E/D-S/A-Q-P is found N-terminal to the core in Sp1, 2, 3, and 4. Similarly, S-Q-I-G-K/A-P/D is found C-terminal to the core in the NET subfamily (except TLP-1) and S-R/K-I-G is found in Sp1–Sp4. Such subfamily-specific sequences might indicate that each subfamily carries out unique functions, but this remains to be determined experimentally. Since Sp motifs are found in vertebrate and invertebrate NET proteins, as well as in most vertebrate Sp proteins, but not in *Drosophila* Buttonhead or D-Sp1, it is not clear if an ancestral gene contained an Sp motif that was subsequently lost from Sp and Buttonhead proteins in the lineage leading to flies, or if an ancestral gene lacked the Sp motif and it was subsequently independently gained by the NET and Sp families at different points during evolution.

Differences between the Sp and NET subfamilies are also readily apparent in the primary protein sequence (Fig. 1B). In particular, the Sp subfamily contains three C_2H_2 zinc fingers [Kadonaga et al., 1987] while the NET subfamily contains only a single zinc finger. Furthermore, although the single zinc finger in NET proteins appears to belong to the C_2H_2 class, it is atypical. C_2H_2 zinc fingers fall into the consensus F/Y-X-C-X_{2-5}-C-X_3-F/Y-X_5-ψ-X_2-H-X_{3-5}-H (where ψ indicates a hydrophobic residue), but NET family proteins contain eight residues between the two cysteines. Thus, although both subfamilies contain zinc finger domains, the NET subfamily differs markedly from the Sp subfamily in zinc finger sequence. Since the related KLF (Krüppel-like factor) family has three zinc fingers, it is likely that the NET family derives from a three zinc finger ancestor, but lost two zinc fingers and underwent significant divergence of the remaining finger. Both the Sp and NET subfamilies also have multiple serine/threonine- and glutamine-

Fig. 1. Nlz, Noc, Elbow, and Tlp-1 form a novel zinc finger protein subfamily. **A**: Phylogenetic tree showing sequence relationship between zinc finger proteins. Protein names are given at left of the tree and are preceded by the two-letter code for each species (Dr, *Danio rerio*; Mm, *Mus musculus*; Dm, *Drosophila melanogaster*; Ce, *Caenorhabditis elegans*; Hs, *Homo sapiens*; Rn, *Rattus norvegicus*). Accession numbers are as follows: DrNlz1 (NP_571897), DrNlz2 (AAQ72694), MmNolz1 (NP_663434), DmElbow (AAM48283), DmNoc (A55929), CeTLP1 (NP_502647), HsSp1 (NP_612482), MmSp1 (O89090), RnSp1 (NP_036787), DmD-Sp1 (CAB55429), DrSp1 (NP_997827), HsSp2 (NP_003101), MmSp2 (NP_084496), DrSpr2 (AAR01215), HsSp3 (NP_003102), MmSp3 (AAC16322), HsSp4 (NP_003103), MmSp4 (NP_033265), DrSp4 (AAH53313), HsSp5 (XP_371581), MmSp5 (NP_071880), HsSp6 (NP_954871), MmSp6 (NP_112460), MmSp7 (NP_569725), RnSp7 (NP_852039), HsSp8 (NP_874359), MmBTD (NP_796056), DmButtonhead (Q24266), DrBts1 (AAK83353). The tree was generated using the Higgins and Sharp algorithm in the DNASIS software (Hitachi). Numbers at nodes indicate percent sequence similarity. The core Sp motif (boxed) and surrounding sequence is shown to the left for each protein. **B**: Schematic representation of conserved domains in NET and Sp subfamily proteins. Cross-hatched domain represents Sp motif that is found in all NET proteins and most Sp proteins (with the exception of DmButtonhead, DmD-Sp1 and possibly some members of the Sp6-8 subgroup). Black domain represents the Buttonhead box (Btd) present in all NET and Sp family proteins. Stippled domains represent C_2H_2 zinc fingers (ZF) present in NET proteins (one finger) as well as Sp proteins (three fingers).

rich regions, but these have undergone extensive divergence. In summary, Sp and NET family members share the Btd box and Sp motif, but differ in whether they have one (NET subfamily) or three (Sp subfamily) C_2H_2 zinc fingers.

MEMBERS OF THE NET SUBFAMILY MAY ACT AS TRANSCRIPTIONAL REPRESSORS

Proteins in the Sp subfamily bind GC-rich DNA sequences using three tandem zinc finger domains, but it is not clear if the single zinc finger in NET subfamily proteins binds DNA. First, usually 2–4 C_2H_2 zinc fingers are required for efficient binding to DNA (e.g., [Berg, 1990; Iuchi, 2001]). Although single zinc fingers in GATA and GAGA family proteins reportedly interact with DNA, they are not sufficient for binding, but require adjacent basic domains [Pedone et al., 1996, 1997; Omichinski et al., 1997]. The single zinc finger of NET family proteins lacks adjacent basic domains, suggesting that it cannot bind DNA. Furthermore, although Nlz proteins form homomeric and heteromeric complexes, thereby potentially bringing together several zinc fingers, such complex formation does not seem to be required for Nlz function [Runko and Sagerstrom, 2004]. Second, the sequence of the NET family C_2H_2 finger differs from the consensus for DNA-binding C_2H_2 fingers. Specifically, although at least four residues of the consensus are involved in contacting DNA (-1, 2, 3, and 6; reviewed in [Wolfe et al., 2000]) only two of these (positions 2 and 3) are conserved in the NET family, while the other two have diverged. Taken together, this information suggests that the C_2H_2 zinc finger of NET family proteins does not bind DNA. Instead, the single zinc finger of NET family proteins may mediate protein-protein interactions, as reported for other C_2H_2 zinc finger proteins [Yang and Evans, 1992; Merika and Orkin, 1995; Gregory et al., 1996].

Sp proteins play broad roles as regulators of transcription (Table I). Some family members appear to function primarily as activators while others act as repressors and some may have both functions depending on cellular context (reviewed in [Suske, 1999; Kaczynski et al., 2003]). For instance, Sp1 efficiently activates transcription from the SV40 early promoter and this effect is mediated by two N-terminal glutamine-rich regions [Courey and Tjian, 1988; Courey et al., 1989] that interact with components of the transcription machinery [Hoey et al., 1993; Gill et al., 1994]. Sp2, Sp3, and Sp4 also appear capable of activating transcription, at least under some circumstances [Hagen et al., 1995; Udvadia et al., 1995; Dennig et al., 1996; Ihn and Trojanowska, 1997; Bakovic et al., 2000]. However, Sp family

TABLE I. Summary of Sp and NET Family Functions

	Transcriptional activity	Expression pattern	Loss of function phenotype	References
Sp1	Activator	Ubiquitous	Embryonic lethal	[Courey and Tjian, 1988; Courey et al., 1989; Saffer et al., 1991; Marin et al., 1997]
Sp2	Activator/repressor	Unknown	Unknown	[Bakovic et al., 2000; Phan et al., 2004]
Sp3	Activator/repressor	Ubiquitous	Reduced viability, defects in tooth, skeletal, and hematopoietic development	[Hagen et al., 1992, 1994; Udvadia et al., 1995; Ihn and Trojanowska, 1997; Kennett et al., 1997; Bouwman et al., 2000; Gollner et al., 2001b; Van Loo et al., 2003]
Sp4	Activator/repressor	Broad, enriched in heart and brain	Arrhythmia, reduced viability, and fertility	[Zhu et al., 1993; Hagen et al., 1995; Supp et al., 1996; Kwon et al., 1999; Nguyen-Tran et al., 2000; Gollner et al., 2001a; Wong et al., 2001]
Sp5	Unknown	Broad	No defect	[Harrison et al., 2000; Treichel et al., 2001]
Sp6	Unknown	Ubiquitous	Unknown	[Scohy et al., 2000]
Sp7	Unknown	Osteoblasts	Abnormal bone formation	[Nakashima et al., 2002]
Sp8	Unknown	Apical ectodermal ridge, brain, tailbud	Limb, neural tube, and tail defects	[Bell et al., 2003; Treichel et al., 2003; Beermann et al., 2004]
Nlz1/Nlz2	Repressor?	Caudal embryo	Hindbrain defects	[Sagerström et al., 2001; Runko and Sagerstrom, 2003, 2004]; Hoyle et al., 2004
Noc/Elbow	Repressor?	Trachea, appendages	Neural, tracheal, and appendage defects	[Cheah et al., 1994; Davis et al., 1997; Dorfman et al., 2002; Weihe et al., 2004]
Tlp-1	Unknown	Caudal embryo	Tail defects	[Zhao et al., 2002]

proteins are also capable of repressing transcription. For instance, some forms of Sp3 can repress gene expression [Hagen et al., 1994; Kennett et al., 1997], at least in part via competition for DNA and/or cofactor binding [Kwon et al., 1999; Kennett et al., 2002], although Sp3 may also recruit histone deacetylase 1 (HDAC1) to create a repressive chromatin structure [Doetzlhofer et al., 1999]. In addition, Sp2 also appears to repress transcription under some conditions [Bakovic et al., 2000].

Some NET family members (e.g., Nlz1 [Runko and Sagerstrom, 2004]) contain a glutamine-rich region, but this region is less extensive than in Sp1−4 and Sp1−4 also contain two glutamine-rich domains (with the possible exception of Sp2) [Suske, 1999]. These observations suggest that NET proteins may not activate transcription via glutamine-rich regions and, accordingly, we find that the glutamine-rich region is not required for Nlz1 function in an in vivo misexpression assay [Runko and Sagerstrom, 2004]. Instead, Nlz1, Nlz2, and Elbow bind the corepressor Groucho and Nlz1 and Nlz2 also bind HDAC1 and HDAC2 [Dorfman et al., 2002; Runko and Sagerstrom, 2003, 2004], suggesting that NET family proteins may repress transcription. Elbow binds Groucho via an N-terminal FKPY motif that is found at a similar position also in Noc. Although such a motif is found also in the other NET family members (except TLP-1), it is in a slightly different position and may not be required for Groucho binding since deleting a domain containing the FKPY motif does not affect binding of Groucho to Nlz1 [Runko and Sagerstrom, 2004]. Instead, Groucho appears to bind Nlz1 via a domain between the Btd box and the C_2H_2 zinc finger [Runko and Sagerstrom, 2003]. Notably, HDAC1 and HDAC2 bind Nlz1 via the same domain as Groucho [Runko and Sagerstrom, 2004], in contrast to Sp1 and Sp3 that bind HDAC1 via their C-termini [Doetzlhofer et al., 1999]. The domain in Nlz1 required for Groucho and HDAC binding appears essential for normal function since deleting it generates a dominant negative form of Nlz1 [Runko and Sagerstrom, 2003].

In vivo experiments also support a role for NET family proteins as transcriptional repressors. In particular, ectopic Elbow abolishes the expression of tracheal genes in *Drosophila* [Dorfman et al., 2002] and ectopic Nlz1 or Nlz2 results in loss of gene expression within the rostral hindbrain of zebrafish [Runko and Sagerstrom, 2003, 2004; Hoyle et al., 2004]. Conversely, *elbow* and *noc* mutants exhibit an expansion in the expression of tracheal branch-specific genes [Dorfman et al., 2002] and expression of a dominant negative form of Nlz1 leads to expansion of rhombomere 5-specific gene expression [Runko and Sagerstrom, 2003]. Further, fusion of Nlz to the VP16 transactivation domain mimics the effect of the dominant negative Nlz construct [Runko and Sagerstrom, 2003], suggesting that the dominant negative construct permits activation of target genes normally repressed by Nlz. These results are consistent with NET family proteins acting as transcriptional repressor. Accordingly, Elbow [Dorfman et al., 2002], TLP-1 [Zhao et al., 2002], Nlz1 [Runko and Sagerstrom, 2003], and Nlz2 [Runko and Sagerstrom, 2004] localize to the nucleus and optimal Nlz1 function is dependent on nuclear localization [Runko and Sagerstrom, 2004].

Importantly, these findings only provide a circumstantial case for NET family proteins acting as repressor and a concrete answer awaits direct analysis of NET family proteins in transcription assays. Such efforts have been hampered to date by a lack of known NET-regulated promoters. Indeed, as discussed, it is likely that NET family proteins do not bind DNA directly, but are instead recruited to target promoters by sequence specific transcription factors. Another important issue is therefore the identification of NET-interacting proteins. There are indications that such partner proteins exist. For instance, C-terminal sequences are required for Nlz1 and Nlz2 to enter the nucleus [Runko and Sagerstrom, 2004], but there is no apparent nuclear localization signal at the C-terminus of either protein, suggesting that an as yet unknown protein binds a C-terminal domain and directs Nlz1 and Nlz2 to the nucleus. Furthermore, disruption of the Nlz1 Sp motif creates a form that is functionally indistinguishable from the form lacking the corepressor binding site [Runko and Sagerstrom, 2004]. The Sp motif therefore appears essential for repressor activity, perhaps because it interacts with a partner protein. Since *noc* and *elbow* interact genetically [Davis et al., 1997] and mutations in these genes give similar phenotypes in tracheal development [Dorfman et al., 2002], it has been suggested that Noc and Elbow function as heterodimers [Dorfman et al.,

2002]. Accordingly, zebrafish Nlz1 forms homodimers as well as heterodimers with Nlz2 [Runko and Sagerstrom, 2004]. However, *elbow* and *noc* appear to act redundantly in appendage development [Weihe et al., 2004] and dimerization does not seem required for Nlz function in vivo, suggesting that intra-family dimerization is not required for all NET protein functions.

NET FAMILY PROTEINS ACT DURING EMBRYOGENESIS

Sp family proteins play roles in a number of different biological processes (Table I). In particular, germ line knock-out of *sp1* or *sp3*, which are ubiquitously expressed in vivo [Saffer et al., 1991; Hagen et al., 1992], leads to retarded growth and reduced viability [Marin et al., 1997; Bouwman et al., 2000], although *sp3* knock-out mice also have defects in tooth formation, skeletal ossification, and hematopoiesis [Bouwman et al., 2000; Gollner et al., 2001b; Van Loo et al., 2003]. Similar to *sp3*, *sp7* is expressed in developing bone and is also required for bone formation [Nakashima et al., 2002]. In contrast, *sp4* expression is enriched in the heart and central nervous system and mice lacking *sp4* function exhibit low postnatal survival rates, impaired growth and fertility defects [Supp et al., 1996; Gollner et al., 2001a] as well as cardiac arrhythmia [Nguyen-Tran et al., 2000]. *sp5* is dynamically expressed during mouse development and while mice homozygous for a *sp5* null mutation do not exhibit an overt phenotype, the *sp5* mutation interacts genetically with the T mutation (a null allele of *brachyury*) [Harrison et al., 1999]. Accordingly, one zebrafish *sp5*-related gene, *spr2*, appears to regulate expression of the *no tail* gene [Zhao et al., 2003] (the zebrafish ortholog of *brachyury* [Schulte-Merker et al., 1994]). In contrast, anti-sense mediated 'knock-down' of another *sp5*-related gene, *bts1*, disrupts gene expression at the midbrain-hindbrain boundary of the zebrafish central nervous system [Tallafuss et al., 2001]. Lastly, the recently identified *sp8* gene is required for limb development both in the mouse and the beetle [Bell et al., 2003; Treichel et al., 2003; Beermann et al., 2004] and *sp6*, which is dynamically expressed during embryogenesis, may promote cell proliferation although it has not yet been analyzed by loss of function approaches [Nakamura et al., 2004]. In addition, the two *Drosophila* Sp family proteins (Buttonhead and D-Sp1) are required for head segmentation and formation of mechanosensory organs as well as the ventral imaginal disks [Wimmer et al., 1993, 1996; Schock et al., 1999; Estella et al., 2003]. Thus, although members of the Sp family have divergent functions, each of the members plays important roles during embryogenesis, raising the possibility that NET family members also act during embryonic development.

This possibility is supported by analyses to date. The first members of the NET family to be identified were *Drosophila elbow* and *noc*. Both were identified based on their mutant phenotypes and found to reside in a 200 kb region on chromosome 2 near the *Adh* gene (see [Davis et al., 1990, 1997] for details). Alleles of these genes vary in strength, but in general *elbow* mutant flies have small bent wings [Davis et al., 1997], while *noc* mutant flies display defects in the light sensitive organs (the ocelli) [Woodruff and Ashburner, 1979a,b] and the supraesophageal ganglion [Cheah et al., 1994], demonstrating that both genes act during embryogenesis. Subsequent analyses have also revealed roles for *elbow* and *noc* in tracheal [Dorfman et al., 2002] and appendage [Weihe et al., 2004] development of *Drosophila*. Mutations in the *C. elegans tlp-1* gene lead to abnormal tail morphology, apparently as a result of improper cell polarity specification [Zhao et al., 2002], also indicating a role during embryonic development. Lastly, the zebrafish *nlz1* and *nlz2* genes are required for proper development of the hindbrain. Disrupting *nlz* function using a dominant negative construct leads to an expansion of rhombomere 5-specific gene expression, apparently at the expense of rhombomere 4-specific gene expression [Runko and Sagerstrom, 2003], while anti-sense mediated knock-down of *nlz1* and *nlz2* leads to complete loss of rhombomere 4-specific gene expression [Hoyle et al., 2004]. The difference between these phenotypes may stem from residual *nlz* function in embryos expressing the dominant negative construct. Thus, as for the Sp family, NET family members play distinct roles in various aspects of embryonic development.

FUTURE DIRECTIONS

Taken together, experiments to date indicate that members of the NET family may function

as transcriptional repressors during embryogenesis. However, several important issues remain to be resolved in order to confirm this hypothesis. First, it will be important to directly test if NET proteins act as repressor. While it might be possible to accomplish this in standard cell-culture based reporter assays, it would also be useful to identify target genes directly regulated by NET proteins. If NET proteins regulate transcription, it will also be interesting to determine how they are recruited to the appropriate target gene and if they interact with other known sequence-specific transcription factors. Second, NET family members are broadly expressed during embryogenesis, but to date only a few functions have been ascribed to them in vivo. It is therefore possible that various family members act redundantly and that simultaneous disruption of several NET genes will be required to get a complete picture of their roles in vivo. Addressing these and related questions will not only reveal a role for NET family members, but also further explore the remarkable diversity of functions carried out by members of the zinc finger family.

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