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Letter

Systematic Characterization of the Zinc-Finger-Containing Proteins in the Mouse Transcriptome

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Zinc-finger-containing proteins can be classified into evolutionary and functionally divergent protein families that share one or more domains in which a zinc ion is tetrahedrally coordinated by cysteines and histidines. The zinc finger domain defines one of the largest protein superfamilies in mammalian genomes; 46 different conserved zinc finger domains are listed in InterPro (http://www.ebi.ac.uk/InterPro). Zinc finger proteins can bind to DNA, RNA, other proteins, or lipids as a modular domain in combination with other conserved structures. Owing to this combinatorial diversity, different members of zinc finger superfamilies contribute to many distinct cellular processes, including transcriptional regulation, mRNA stability and processing, and protein turnover. Accordingly, mutations of zinc finger genes lead to aberrations in a broad spectrum of biological processes such as development, differentiation, apoptosis, and immunological responses. This study provides the first comprehensive classification of zinc finger proteins in a mammalian transcriptome. Specific detailed analysis of the SP/Krüppel-like factors and the E3 ubiquitin-ligase RING-H2 families illustrates the importance of such an analysis for a more comprehensive functional classification of large protein families. We describe the characterization of a new family of C2H2 zinc-finger-containing proteins and a new conserved domain characteristic of this family, the identification and characterization of Sp8, a new member of the Sp family of transcriptional regulators, and the identification of five new RING-H2 proteins.

[Supplemental material is available online at www.genome.org. To facilitate future characterization of this superfamily, we generated a Web-based interface, http://cassandra.visac.uq.edu.au/zf, containing the structural classification of the entire zinc finger data set discussed in this study.]

Zinc-finger-containing proteins constitute the most abundant protein superfamily in the mammalian genome, and are best known as transcriptional regulators. They are involved in a variety of cellular activities such as development, differentiation, and tumor suppression. The first zinc finger domain to be identified in *Xenopus laevis*, basal transcription factor TFIIIA (Miller et al. 1985), is the archetype for the most common form of zinc finger domain, the C2H2 domain. The three-dimensional structure of the basic C2H2 zinc finger is a small domain composed of a β-hairpin followed by an α-helix held in place by a zinc ion. Zinc fingers generally occur as tandem arrays, and in DNA-binding modules the number of sequential fingers determines specific binding to different DNA regions. One zinc finger binds the major groove of the double helix and interacts with 3 bp, and the minimal number of fingers required for specific DNA binding is two (Choo et al. 1997). One of the best characterized families of DNA-binding zinc fingers is the Sp/Krüppel-like factor. Members of this family share in common three highly conserved C2H2-type fingers in their C-terminal ends combined with transcriptional activator or repressor domains in the N terminus. Other families of DNA-binding zinc fingers differ from the C2H2-type basic module in the spacing and nature of their zinc-chelating residues (cysteine–histidine or cysteine–cysteine; Laity et al. 2001; Table 1). Additional families of zinc finger domains have been implicated in protein–protein interactions and lipid binding (Table 1; Bach 2000; Tucker et al. 2001).

Association of many zinc finger proteins with DNA- and/or protein-binding domains allows the formation of multiprotein complexes in which DNA-binding motifs recognize a target sequence in a specific manner or protein–protein interaction domains allow the assembly of multiprotein regulatory complexes, commonly involved in chromatin remodeling (Aasland et al. 1995; David et al. 1998). Other zinc finger proteins lack DNA- or RNA-binding activity. For example, the

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Table 1. Zinc Finger Domains Listed in the IterPro Database

InterPro	Name	Function	Specificity
IPR000882	C2H2 type	Nucleic acid-binding	DNA/RNA
IPR001841	RING finger	Protein–protein interactions	Proteins
IPR001909	KRAB	Protein–protein interactions	Proteins
IPR001781	Zn-binding protein, LIM	Protein–protein interactions	Proteins
IPR001965	PHD finger	Protein–protein interactions	Proteins
IPR001628	C_4-type steroid receptor	Nucleic acid-binding	DNA
IPR002219	Protein kinase C phorbol ester/diacylglycerol binding	Lipids	Diacylglycerol and phorbol esters
IPR000315	B-box	Protein–protein interactions	Proteins
IPR001878	Knuckle, CCHC type	Nucleic acid-binding	DNA
IPR000571	C-x8-C-x5-C-x3-H type	Nucleic acid-binding	RNA
IPR000306	FYVE type	Lipids	Phosphatidylinositol-3-phosphate
IPR001876	Ran-binding protein	Protein–protein interactions	RanGDP
IPR001164	GCS-type	Protein–protein interactions	Protein
IPR001594	DHHC type	Protein–protein/nucleic acid-binding	Protein/DNA
IPR003604	U1-like	Nucleic acid-binding	RNA/DNA
IPR000379	GATA type	Nucleic acid-binding	DNA
IPR000433	ZZ type	Protein–protein interactions	Unknown
IPR002893	MYND type	Nucleic acid-binding	DNA
IPR001293	TRAF type	Protein–protein interactions	Protein
IPR000058	AN1-like	Nucleic acid-binding	DNA
IPR001562	Tec/Btk domain	Protein–protein interactions	Protein
IPR004217	Tim10/DDP type	Protein–protein interactions	Protein
IPR002653	A20-like	Protein–protein interactions	Protein
IPR004181	MIZ type	Nucleic acid-binding	DNA
IPR001275	DM DNA-binding	Nucleic acid-binding	DNA
IPR003000	Silent information regulator protein Sir2	Nucleic acid-binding	DNA
IPR000465	XPA protein	Nucleic acid-binding	DNA
IPR001510	NAD^+ ADP-ribosyltransferase	Nucleic acid-binding	ssDNA
IPR003071	Orphan nuclear receptor, HMR type	Nucleic acid-binding	DNA
IPR003656	BED finger	Nucleic acid-binding	DNA
IPR003957	Histone-like transcription factor/archaeal histone/topoisomerase	Nucleic acid-binding	DNA
IPR004198	C5HC2 type	Nucleic acid-binding	DNA
IPR000380	Prokaryotic DNA topoisomerase I	Nucleic acid-binding	DNA
IPR001529	DNA-directed RNA polymerase, M/15 kD subunit	Nucleic acid-binding	DNA
IPR002515	C2HC type	Nucleic acid-binding	DNA
IPR002857	CXXC type	Nucleic acid-binding	DNA/methyl cytosine
IPR003126	Zn-finger (putative), N-recognin	Protein–protein interactions	Protein
IPR000197	TAZ finger	Protein–protein interactions	Protein
IPR000678	Nuclear transition protein 2	Protein–protein interactions	Protein
IPR000967	NF-X1 type	Nucleic acid-binding	DNA/X-box motif
IPR000976	Wilm's tumor protein	Nucleic acid-binding	DNA
IPR002735	Translation initiation factor IF5	Nucleic acid-binding	DNA
IPR002906	Ribosomal protein S27a	Nucleic acid-binding	RNA/DNA
IPR003079	Nuclear receptor ROR	Nucleic acid-binding	DNA
IPR003655	KRAB-related	Nucleic acid-binding	DNA
IPR004457	ZPR1 type	Protein–protein interactions	Protein
Domains commonly associated with zinc finger domains			
IPR000210	BTP/POZ domain	Protein–protein interactions	Proteins
IPR001214	SET-domain of transcriptional regulator	Protein–protein interactions	Protein
IPR003309	SCAN domain	Protein–protein interactions	Protein
IPR003879	Butyrophylin C-terminal DUF	Protein–protein interactions	Protein
IPR002999	Tudor domain	Unknown	Unknown
IPR001258	NHL repeat	Protein–protein interactions	Protein

RING-H2-finger-containing proteins are implicated in the ubiquitination signal pathway. They function as ubiquitin-ligase (E3), and interact with the ubiquitin-conjugating enzymes (E2) to facilitate the transfer of a ubiquitin group to target proteins that can then be recognized and degraded by the proteosome (Lorick et al. 1999).

Zinc fingers are among the most common structural motifs in the proteome predicted from the genome sequences of *Saccharomyces cerevisiae*, *Drosophila melanogaster*, and *Caenorhabditis elegans* (Rubin et al. 2000) as well as the draft human genomic sequences (Lander et al. 2001). However, genome sequence annotation provides an incomplete and imperfect prediction and description of the full-length transcripts and splice variants that can be transcribed from the genome.

The RIKEN Mouse Gene Encyclopedia Project has provided the most comprehensive collection of full-length mammalian complementary DNAs (cDNAs; Okazaki et al. 2002).

Table 2. Frequencies of the Zinc Finger Domains in the Mouse Transcriptome

InterPro	Name	Total in the RTPS	Novel	Homolog to other organisms
IPR000882	C2H2 type	506	126	82
IPR001841	RING finger	196	64	25
IPR001909	KRAB	134	61	22
IPR001781	LIM	60	10	2
IPR001965	PHD finger	52	19	5
IPR002219	Protein kinase C phorbol ester/diacylglycerol binding	46	7	9
IPR001628	C_4-type steroid receptor	44	1	0
IPR000315	B-box	37	7	1
IPR000571	C-x8-C-x5-C-x3-H type	32	14	3
IPR001878	Knuckle, CCHC type	27	13	3
IPR000306	FYVE type	21	7	1
IPR003604	U1-like	20	8	1
IPR001164	GCS-type	18	4	4
IPR001876	Ran-binding protein	17	2	3
IPR001594	DHHC type	17	13	2
IPR002893	MYND type	14	5	3
IPR000379	GATA type	12	0	1
IPR000433	ZZ type	12	2	0
IPR001293	TRAF type	9	1	1
IPR001562	Tec/Btk domain	9	1	0
IPR000058	AN1-like	7	2	0
IPR004217	Tim10/DDP type	6	0	3
IPR002857	CXXC type	6	2	0
IPR003000	Silent information regulator protein Sir2	5	0	4
IPR002653	A20-like	4	0	0
IPR004181	MIZ type	4	0	0
IPR003957	Histone-like transcription factor	4	0	0
IPR001275	DM DNA-binding	3	2	0
IPR004198	C5HC2 type	3	0	0
IPR002515	C2HC type	3	0	1
IPR003126	Zn-finger (putative), N-recognin	3	1	1
IPR003079	Nuclear receptor ROR	3	1	0
IPR003655	KRAB-related	3	3	0
IPR001510	NAD^+ ADP-ribosyltransferase	2	0	0
IPR000380	Prokaryotic DNA topoisomerase I	2	0	0
IPR001529	DNA-directed RNA polymerase, M/15 kD subunit	2	1	0
IPR000197	TAZ finger	2	0	0
IPR000967	NF-X1 type	2	1	0
IPR002735	Translation initiation factor IF5	2	0	1
IPR004457	ZPR1 type	2	1	0
IPR000465	XPA protein	1	0	0
IPR003071	Orphan nuclear receptor, HMR type	1	0	0
IPR003656	BED finger	1	0	0
IPR000678	Nuclear transition protein 2	1	0	0
IPR000976	Wilm's tumor protein	1	0	0
IPR002906	Ribosomal protein S27a	1	0	0
Domains commonly associated with zinc finger domains				
IPR000210	BTB/POZ domain	124	50	25
IPR001214	SET-domain of transcriptional regulator	30	13	7
IPR003309	SCAN domain	22	6	2
IPR003879	Butyrophylin C-terminal DUF	14	2	1
IPR002999	Tudor domain	16	6	2
IPR001258	NHL repeat	10	3	3
TOTAL		1573	459	218

Combined with the mouse genome sequence and annotation at ENSEMBL (http://www.ensembl.org), MGC (http://www.informatics.jax.org/mgihome), NCBI (http://www.ncbi.nlm.nih.gov), and EST assemblies at TIGR (http://www.tigr.org), for the first time we now have a comprehensive coverage of the mouse transcriptome. The present version of the mouse transcriptome is composed of ~20,000 representative protein-coding functional transcripts produced from distinct transcriptional units (Representative Transcripts and Proteins set, RTPSv6). In this study we have produced a zinc finger full-length protein set (ZFPS), based on the mouse transcriptome generated by the FANTOM consortium (Okazaki et al. 2002; http://fantom2.gsc.riken.go.jp/).

A total of 1573 protein sequences were extracted based on the presence of one or more zinc finger domains as recognized by InterPro (http://www.ebi.ac.uk/InterPro). We first grouped protein sequences according to conserved domain composition, which generally correlates with function, and then analyzed the different groups in more detail. Because the zinc finger is a modular domain that occurs commonly in

Table 3. Zinc Finger Protein Clusters Generated by All-Against-All BLAST Analysis

Clusters	Domains	Description	Proteins
Cluster 1	C2H2/KRAB/BTB	Transcriptional regulators with C2H2 core-binding domain	296
Cluster 2	C_4 zinc finger	Steroid hormone receptors	44
Cluster 3	BTB/POZ/Kelch repeat	Actin-binding proteins	35
Cluster 4	RING-CH/B-box/coiled coil	Tripartite motif family	26
Cluster 5	Three C2H2 zinc finger	Sp/Krüppel-like factor family	25
Cluster 6	LIM/Homeobox	LIM/homeobox family	18
Cluster 7	RING-H2	E3 ubiquitin-ligase family	14
Cluster 8	Four LIM domains	Four and a half LIM domain family	14
Cluster 9	Protein kinase C/Ser/Thr-type protein kinases	Protein kinase C	12
Cluster 10	C5HC2/JmJN	Jumonji family	7
Cluster 11	FYVE/PH	FYVE family	7
Cluster 12	RING-CH/B-box/coiled coil/fibronactin type III	B-box family	6
Cluster 13	Three LIM domain	Ajuba family	6
Cluster 14	C1/Rho/GAP	Myosin IX family	6
Cluster 15	GATA type zinc finger	GATA family	6
Cluster 16	Four N-terminal C2H2 and two C-terminal C2H2	Ikaros family	5
Cluster 17	TRAF/RING-CH/MATH	TNF-receptor-associated factors	5
Cluster 18	C_2/PH/Btk	Ras GTPase family	5
Cluster 19	Zinc finger ZZ	Dystrophin family	5
Cluster 20	C1/PHD/EF hand/DAGKc/GAGka	Diacylglycerol kinase family	5
Cluster 21	PHD	Chromatin regulator family	5
Cluster 22	C2H2/Homeodomain	Transcription factors developmentally related	4
Cluster 23	One N-terminal C2H2, six central C2H2, two C-terminal C2H2	Novel C2H2 family (NFTR)	4
Cluster 24	RING-H2/PA	G1-related family	4
Cluster 25	SET/Chromodomain	Heterochromatin component proteins family	4
Cluster 26	MIZ/Sap	Inhibitor of STAT family	4
Cluster 27	NHL repeat/EGF	Oz/ten-m homolog family	4
Cluster 28	RING-CH/C2H2 zinc finger	Novel C2H2/RING finger family	4
Cluster 29	RING-CH/WWE	Deltex family	4
Cluster 30	PHD finger	Novel PHD-containing family	4
Cluster 31	LIM/PDZ	C-terminal LIM domain family	4
Cluster 32	Two PHD finger	Novel PHD-containing family	4
Cluster 33	CXXC finger/PHD/FYRN/bromodomain/SET	Chromatin regulator family	4
Cluster 34	C2H2 zinc finger/PH	Forkhead-related transcription factors	4
Cluster 35	C_3H_1 zinc finger/RNA recognition domain	U2 small nuclear ribonucleoprotein auxiliary factor	4
Cluster 36	Three C2H2 C-terminal zinc fingers	EGR family	4
Cluster 37	Tyr Kinase/Sh2/Sh3/Btk/PH	Tyrosine protein kinase BTK family	4
Cluster 38	RING-CH	Polycomb family	4
Cluster 39	C2H2 zinc finger/Gag-p24	Gag family	3
Cluster 40	Three C2H2 zinc fingers	Novel C2H2 family	3
Cluster 41	ArfGap/PH	Centaurin family	3
Cluster 42	RHO/BTB	Novel BTB family	3
Cluster 43	DHHC zinc family	DHHC family	3
Cluster 44	Fourteen C2H2 zinc finger	Novel C2H2 family	3
Cluster 45	GCS-type zinc finger/Arfgap/Ank repeat	GCS-type zinc finger family	3
Cluster 46	GATA/SANT/ELM2/BAH	Metastasis-associated proteins	3

tandem arrays encoded by single exons, we have also studied the incidence of splice variants in the zinc finger data set compared with the incidence in the RTPS. In particular, we present a detailed analysis of the Sp/Krüppel-like factors and E3 ubiquitin-ligase RING-H2 families, and we report the characterization of a possible new family of C2H2 zinc-finger-containing transcriptional regulators.

RESULTS AND DISCUSSION

Generation of Nonredundant Zinc Finger Protein Set

The RIKEN Genome Science Center in collaboration with the FANTOM consortium (http://genome.gsc.riken.go.jp) generated a nonredundant full-length protein sequence data set (Representative Transcripts and Protein Set, RTPS) by combining the collection of 60,770 full-length cDNA sequences from the Functional Annotation of the Mouse Genome (FANTOM) with various sequences in the public domain (Okazaki et al. 2002). The RTPS contains ~20,000 protein sequences (http://fantom2.gsc.riken.go.jp/).

InterPro searches for 46 conserved zinc finger domains against the RTPS-extracted 1573 zinc-finger-containing proteins. These represent 7.5% of the entire RTPS (Table 2). All 46 classifications of zinc finger domains were represented in the RTPS, with the five most frequent zinc finger domains being the C2H2 (506), RING finger (196), KRAB-box (134), LIM domain (60), and the PHD finger (52). Comparative analysis with other eukaryotes confirms similar frequencies of the zinc finger domains in other genomes (Supplementary Table 1; available online at www.genome.org).

A comparison of the profiles with nonmammalian genomes revealed lineage-specific evolution in the zinc-finger-

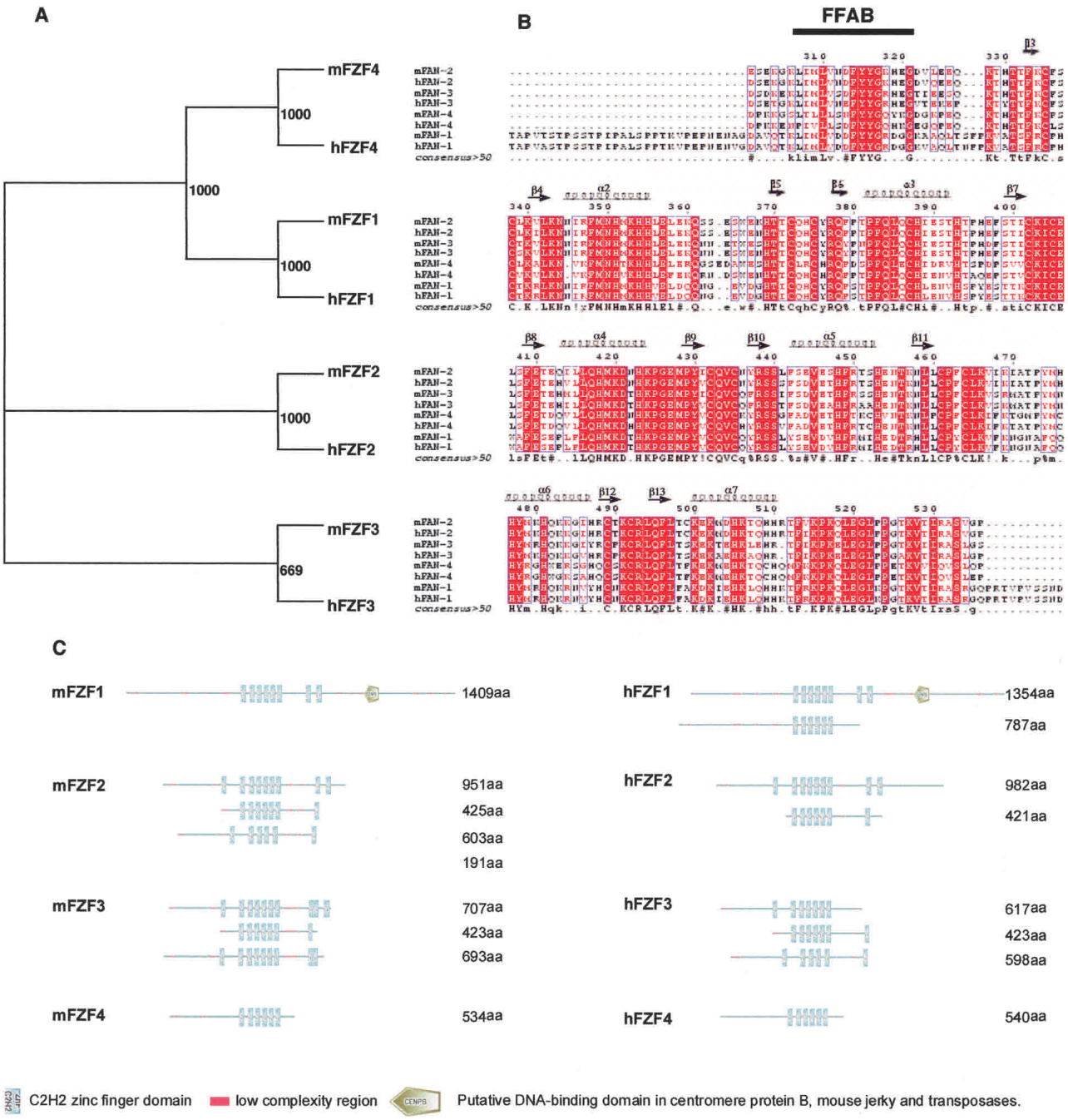

Figure 1 (*A*) Unrooted phylogeny among the Fzf family. The entire mouse and human protein sequences of the Fzf family (Table 3) were aligned and subjected to Neighbor Joining with 1000 bootstrap analysis. (*B*) Protein sequence alignment of the six C2H2 core zinc finger domains of the FZF proteins. The secondary structure of the six fingers is shown *above* the alignment; the domain consensus is shown *below* the alignment, the FFAB domain is indicated by the black bar. (*C*) Domain architecture of the mouse and human Fzf proteins. Protein structural representation was generated using Simple Modular Architecture Research Tool (SMART; http://smart.embl-heidelberg.de).

containing proteins. Certain zinc finger domains are vertebrate-specific. The KRAB (IPR001909), KRAB-related (IPR003655), Nuclear transition protein 2 (IPR000678), SCAN domain (IPR003309), and the subfamily of Nuclear receptor ROR (IPR003079) have not been identified in the *D. melanogaster* (http://www.fruitfly.org/), *C. elegans* (http://www.wormbase.org/), and *S. cerevisiae* (http://genome-www.stanford.edu/Saccharomyces) predicted proteomes (Supplementary Table 1).

In contrast, comparison of the predicted mouse and human zinc finger sets shows minimal lineage-specific evolution, although there are some examples of structural domain differences in putative mouse and human ortholog pairs. RNF6, RNF13, and G1RP1 are such examples (protein archi-

Table 4. Gene Structure of the Mouse and Human Fantom Zinc Finger Family (Cluster 23)

Proposed mouse gene symbols	IPR domains	Mouse protein accessions	Mouse Chr/band	Locus length (kb)	Exons/introns	Human protein accessions	Human Chr/band
Fzf1	9×C2H2/CENPB	9530006B08Rik	3f2	15.85	19/18	XP_047883	1q21.3
Fzf2	9×C2H2	B130043A04Rik	9d	63.63	19/18	NP_060131	15q21.3
Fzf3	9×C2H2	XM_135874	Xa3.2	34.99	1617	AK000102	Xq26.1
Fzf4	6×C2H2	6030407P18Rik	10b5.3	10.3	3/2	NP_542778	22q11.22

tectures of the mouse and human RNF6, RNF13, and G1RP1 are shown in Supplementary Fig. 4).

Cluster Analysis of the Zinc-Finger-Containing Proteins

In order to subdivide the zinc finger superfamily into likely functional clusters, we performed two different classifications of the entire set of the mouse zinc-finger-containing proteins (see Methods). This enabled separation of the superfamily into clusters of structurally and functionally related zinc finger families. The seven major clusters were: C2H2/KRAB type zinc finger (296), steroid receptors, C4 type (44), BTB/BOZ-containing proteins (35), tripartite motif proteins (26), Sp/KLF family (26), LIM/homeobox family (25), and the E3 ubiquitin-ligase RING-H2 family (18; Table 3). The complete list and architectural structure of each of the zinc-finger-containing clusters can be found at http://cassandra.visac.uq.edu.au/zf.

The ZFPS contains 677 proteins that have not been identified previously. In this analysis, we consider as novel all those proteins that were annotated in the MATRICS computational pipeline (Kawai et al. 2001; Okazaki et al. 2002), as "hypothetical protein," "weakly similar to," "related to," "protein containing motifs," "RIKEN clone number," and "unclassifiable transcripts." Proteins annotated as "homolog to [gene name]-organism" are likely to be the mouse homologs or orthologs of a protein with known functions in other organisms that have not previously been identified in mouse.

We found that 33 of the 46 zinc finger families we have analyzed have at least one new member in the RTPS (Table 2). The majority of new proteins belong to the C2H2 family. Among 506 C2H2-containing-proteins, 208 are new mouse transcripts (41%). The zinc finger family that presents the highest proportion of newly described proteins is the recently discovered DHHC-type zinc finger (IPR001594; Putilina et al. 1999). Of 17 DHHC-containing proteins, 15 (88%) are new to the mouse. A high rate of novelty was also found in proteins containing the transcriptional repressor KRAB-box. Of 134 KRAB-containing proteins, 83 (61%) are new mouse transcripts (Supplementary Table 2).

In our classification we noted a small group of structurally related newly described proteins that appear entirely novel. An example is cluster 24, which contains four new proteins sharing in common a central array of six C2H2 zinc fingers, one N-terminal C2H2 zinc finger, and an array of two to three C-terminal C2H2 zinc fingers. BLAST analysis of the proteins in cluster 24 (http://www.ncbi.nlm.nih.gov/BLAST) reveals no homologous proteins with functional annotation (Fig. 1). The name Fzf (Fantom zinc finger protein) has been proposed for this new family of C2H2 zinc fingers. The murine *Fzf1* (9530006B08Rik) encodes a 1409-amino-acid protein with a predicted molecular mass of 155 kD. The murine *Fzf2* (B130043A04Rik) encodes a 951-amino-acid protein with a predicted molecular mass of 88.5 kD. The murine *Fzf3* (AAH28839Rik) encodes a 707-amino-acid protein with a predicted molecular mass of 77.8 kD. Finally, *Fzf4* (6030407P18Rik) encodes a 534-amino-acid protein with a predicted molecular mass of ~60 kD. The four genes of this family have been mapped to the ENSEMBL mouse genome (http://www.ensembl.org) using the correspondent RIKEN full-length complementary DNA (Table 4).

Although there is no functional or structural information regarding these proteins, there are human orthologs of the Fzf family, and proteins with sequence similar to the Fzf family are also evident in other eukaryotes such as *Xenopus laevis*, *D. melanogaster*, and *C. elegans*. We also identified a conserved stretch of 16 amino acids immediately N-terminal to the central zinc finger array that does not show similarity with other previously described conserved domains, KLIMLV-[D/N/S]-[D/N/S]-FYYG-[K/R/Q]-[H/Y/D]-[E/K/G]-G (Fig. 1B). This new conserved domain, named Fantom family associated box (FFAB), is highly conserved in all FZF proteins and together with the characteristic distribution of C2H2 zinc finger domains can be considered as the signature domain of this new family.

The ENSEMBL gene prediction program Genscan (http://www.ensembl.org) predicted functionally different splice variants for the murine *Fzf2* (three) and *Fzf3* (two) genes. Similar variants are predicted also for the human orthologs (Fig. 1C). The C2H2 zinc finger domains have been extensively demonstrated to be involved in DNA/RNA binding and are usually associated with transcription regulatory proteins. The presence of this domain in the FZF family indicates that this family may be involved in transcriptional regulation.

To determine substructures within the major clusters and better characterize the new genes present in this data set, Neighbor Joining phylogenetic trees were calculated from multiple sequence alignments (see Methods; Figs. 1A, 2A, and 3A). To illustrate the importance of this analysis in gene discovery and annotation, clusters 5 and 7, containing proteins of the Sp/Krüppel-like factors and RING-H2, E3 ubiquitin-protein ligase families, respectively, are discussed in detail below.

The Sp/Krüppel-Like Factors Family: Identification of a New Sp Family Member

Sp/Krüppel-like factors are transcriptional regulators involved in development, cell growth, and differentiation (Lania et al. 1997; Dang et al. 2000). Proteins of this family are characterized by a highly conserved array of three C2H2 zinc fingers in their C-terminal region. As a result, all members of this family bind preferentially to "GC-box" or "CACCC elements" on DNA (Fig. 2C; Supplementary Fig. 3). In addition to the conserved amino acid sequence of the zinc fingers, these proteins

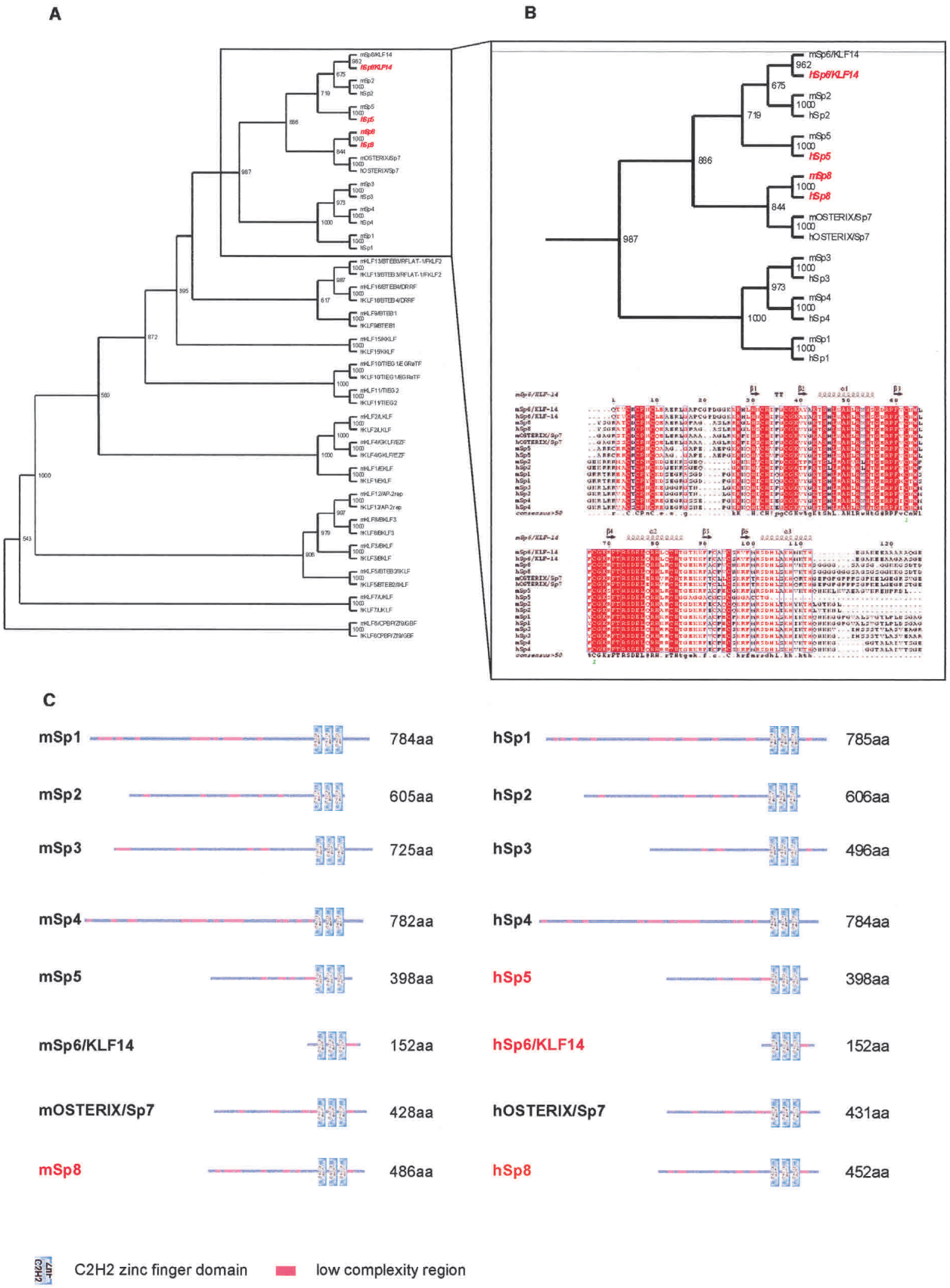

Figure 2 (*A*) Unrooted phylogeny among the Sp/Krüppel-like factors. The entire mouse and human protein sequences of the Sp/Krüppel-like factors (Table 4) were aligned and subjected to Neighbor Joining with 1000 bootstrap analysis. (*B*) Magnification of the Sp branch of the phylogeny tree and alignment of the zinc finger region of the Sp proteins. The secondary structure of the six fingers is shown *above* the alignment; the domain consensus is shown *below* the alignment. (*C*) Mouse and human Sp protein domain architecture. Those highlighted in red are the newly described proteins.

Table 5. Gene Structure of the Mouse and Human Krüppel-Like Factors Family (Cluster 5)

Proposed mouse gene symbols	IPR domains	Mouse protein accessions	Mouse Chr/band	Human nucleotide accessions	Human Chr/band
Sp1	3×C2H2	NP_038700	15f3	AF252284	12q13.13
Sp2	3×C2H2	AAH21759	11d	NM_003110	17q21.32
Sp3	3×C2H2	AAC16322	2c3	AJ310752	2q31.1
Sp4	3×C2H2	NP_033265	12f2	AC004595	7p15.3
Sp5	3×C2H2	NP_071880	2c3	ENSG00000172168	2q31.1
Sp6/KLF14	3×C2H2	Q9ESX2	11d	ENSG00000159127	17q21.32
Sp7/Osterix	3×C2H2	NP_569725	15f3	AAL84281	12q13.13
Sp8	3×C2H2	5730507L14Rik	12f2	AK056857	7p21.1
KLF1/EKLF	3×C2H2	P46099	8c3	NM_006563	19p13.2
KLF2/LKLF	3×C2H2	Q60843	8c1	NM_016270	19p13.2
KLF3/BKLF	3×C2H2	Q60980	5c3.3	NM_016531	4p13
KLF4/GKLF/EZF	3×C2H2	Q60793	4b3	NM_004235	9q31.2
KLF5/BTEB2/IKLF	3×C2H2	Q9Z0Z7	14e2.1	NM_001730	13q22.1
KLF6/CPBP/Zf9/GBF	3×C2H2	O08584	13a1	NM_001300	10p15.2
KLF7/UKLF	3×C2H2	NP_291041	1c2	NM_03709	2q33.3
KLF8/BKLF3	3×C2H2	A830097P10Rik	Xf3	NM_007250	Xp11.22
KLF9/BTEB	3×C2H2	O35739	19c1	NM_001206	9q21.12
KLF10/TIEG1/EGRaTF	3×C2H2	O89091	15c	NM_005655	8q22.3
KLF11/TIEG2	3×C2H2	CAC06699	12a3	NM_003597	2p25.1
KLF12/AP-2rep2	3×C2H2	O35738	14e2.2	NM_016285	13q22.1
KLF13/BTEB3/RFLAT1	3×C2H2	Q9JJZ6	7c	NM_015995	15q13.1
KLF15/KKLF	3×C2H2	NP_075673	6d2	NM_014079	3q21.3
KLF16/BTEB4/DRRF	3×C2H2	P58334	10c1	NM_031918	19p13.3

share a highly conserved interfinger spacer, TGEKP(Y/F) X, also called the H/C link.

Sequence-based hierarchical clustering segregates the Sp proteins from the Krüppel-like factors to form a clearly distinct subfamily of transcriptional regulators (Fig. 2A). This segregation revealed a new member of the Sp subfamily, named Sp8 (Bouwman and Philipsen 2002). Sp8 protein has a clear human ortholog, AK056857 (Fig. 2B,C). Tissue expression profile studies using the RIKEN 60K array chips (Bono et al. 2002) indicate that murine Sp8 is tissue-restricted. It is expressed mainly in thymus, skin, and testis (Supplementary Fig. 1). It might therefore be a candidate regulator of cellular differentiation.

The 13.30-kb-long murine *Sp8* locus is found at Chromosome 12 band f2 with a structure of 4 exons and 3 introns, and encodes a 486-amino-acid protein with a predicted molecular mass of 48 kD (Table 5).

The N-terminal part of Sp1 can be divided into five domains: the Sp-box (Harrison et al. 2000), the activator domains A and B, the domain C rich in charged amino acids including the Buttonhead-box (Harrison et al. 2000), and the domain D in the very C terminus of the protein. Domains A and B can be subdivided into an N-terminal serine/threonine-rich region and a C-terminal glutamine-rich region (Kolell and Crawford 2002). Similar modular structures can be found in Sp2, Sp3, and Sp4. These four proteins occur on a separate branch from Sp5, Sp6, Sp7, and Sp8, which, in turn, lack similar sequence outside the zinc finger region (Fig. 2B).

BLAST analysis reveals that the three C-terminal zinc fingers of Sp8 have 95% homology with Sp5 and 97% with the *D. melanogaster* Sp1 (NP_572579). Outside the zinc finger domain, Sp8 has a serine/alanine-rich region in the very N terminus of the protein (amino acids 11–116) and a glycine-rich region in the central region (amino acids 132–149). This region of the protein shows 23% homology with osterix/Sp7 with which Sp8 clusters in the hierarchical tree. Osterix/Sp7 has been shown to be a transcription factor required for osteoblast differentiation and hence for bone formation (Nakashima et al. 2002). Sp8 also resembles Sp6/KLF14 (Scohy et al. 2000) and the *D. melanogaster* zinc finger proteins, scribbler (NP_524678; Senti et al. 2000; Yang et al. 2000).

The mouse and human protein architectures of the Sp/KLF family including different isoforms generated by alternative splicing are shown in Supplementary Figure 3.

Treichel et al. (2001) suggested that Sp5 is the evolutionary link between the Sp and KLF subfamilies of zinc finger proteins. In the zinc finger region, Sp5 shares high homology with other Sp proteins, but in the N-terminal region, Sp5 is more similar to Krüppel-like factors (Treichel et al. 2001). Based on the hierarchical cluster, we suggest that Sp8 may have been the first Sp protein evolutionarily differentiated from a common ancestor. Sp5 has probably been generated during evolution by domain swapping between Sp8 and a member of the evolutionarily related Krüppel-like factor subfamily.

The different homologies of the zinc finger domain and the non-zinc finger domain found in the Sp/KLF family is evidence of their different evolutionary history. This family of transcriptional regulators most likely evolved novel proteins by modular evolution in which domains were created by gene duplication and translocated by domain shuffling events (Morgenstern and Atchley 1999; Kolell and Crawford 2002).

RING-H2 and the E3 Ubiquitin-Protein Ligase Family

The RING finger (IPR001841) is a zinc-binding domain of 40–60 amino acids. It binds two zinc ions and is involved in protein–protein interactions in the formation of macromolecular scaffolds. There are two different variants, the C4HC3-type and the C3H2C3-type, that are clearly related despite the different cysteine/histidine pattern.

Cluster analysis identified a group of 14 proteins that share in common a C-terminal RING-H2-type finger (Table 3,

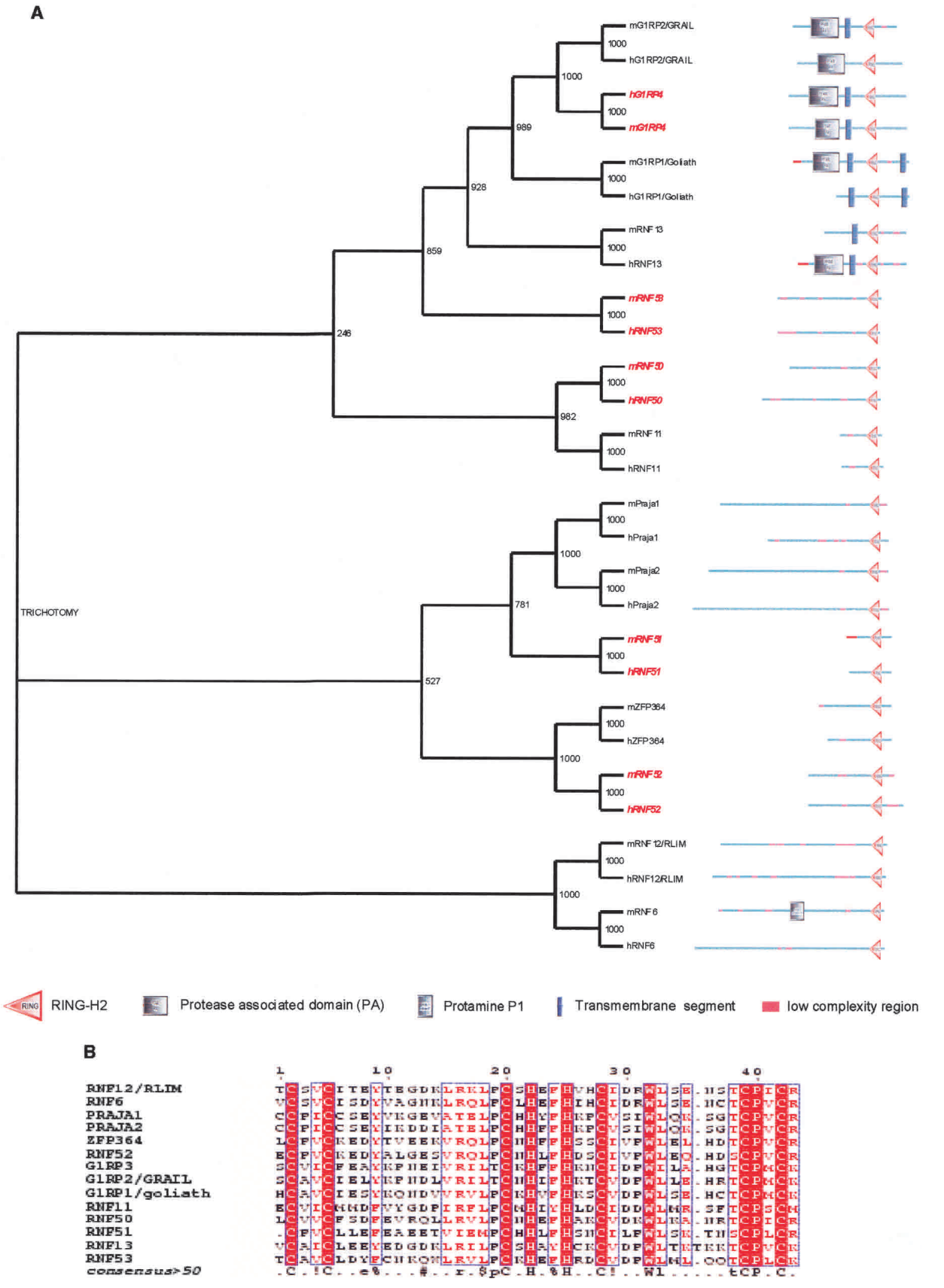

Figure 3 (*A*) Unrooted phylogeny of the cluster 7. The entire mouse and human protein sequences of the RING-H2 proteins (Table 5) were aligned and subjected to Neighbor Joining with 1000 bootstrap analysis. Domain architecture of the RING-H2 proteins is also shown in the picture. (*B*) RING-H2 zinc finger alignment of the C2H2 zinc finger consensus sequence is shown *below* the alignment. In red are highlighted the proteins described for the first time in this study.

cluster 7; Fig. 3A,B). Five of the 14 proteins are newly identified mouse proteins. *RNF50* (NP_598825) encodes a 339-amino-acid protein with a predicted molecular mass of 37.9 kD with a central proline-rich region (56–228). *RNF51* (2500002L14Rik) encodes a 166-amino-acid protein with a predicted molecular mass of 19.1 kD. *RNF52* (AAH16543) encodes a 313-amino-acid protein with a predicted molecular mass of 34.08 kD, with a C-terminal serine-rich region (293–313). *RNF53* (0610009J22Rik) encodes a 380-amino-acid protein of 41.57 kD with a predicted molecular mass of 1.59 kD. A proline-rich region is present in the very N-terminal part of the protein (7–33). Names for these four proteins are proposed based on the conventional nomenclature for ring finger proteins (RNFX; Table 6).

The fifth newly identified mouse protein 1700042K15Rik shares 61% of protein identity with the g1-related protein (G1RP1), a homolog to the *D. melanogaster* g1 (Baker and Reddy 2000). Along with G1RP2, these present a subfamily of this cluster (Fig. 3A). They are characterized by the C-terminal RING-H2 finger and by an N-terminal protease-associated domain (IPR003137). The newly identified murine G1RP3 is a 340-amino-acid-long protein with a predicted molecular mass of 38.14 kD. In contrast to G1RP1 and G1RP2, there is no prediction of a transmembrane region in the G1RP3 protein sequence. Expression analysis shows that its expression is restricted to testis (data not shown; Table 6). The mouse and human protein architectures of this family, including the Ring finger protein 13 (RNF13) isoforms A to F generated by alternative splicing, are shown in Supplementary Figure 4.

An emerging role of RING-finger-containing proteins is in ubiquitination pathways, where they play a central role in the transfer of ubiquitin (Ub) to a heterologous substrate, thereby targeting the substrate for destruction by the proteosome (Joazeiro and Weissman 2000). Protein ubiquitination begins with the formation of a thiol-ester bond between the C terminus of Ub and a cysteine of an Ub-activating enzyme (E1). Ub is then transferred to an Ub-conjugating enzyme (E2), again through a thiol-ester bond. Ub-protein ligases (E3) are responsible for specificity during ubiquitination. They recognize the target proteins and promote the transfer of the Ub from E2 either to a reactive lysine of target proteins or to the last Ub of the Ub chain already attached to the target proteins.

The ubiquitination pathway is crucial for cells to maintain protein homeostasis and to allow proteins that are folded incorrectly to be targeted for degradation. Ubiquitination is also important in chromatin remodeling and transcriptional regulation by histone ubiquitination. Ubiquitination of histones H2A and H2B might work as tagging them for the recruitment of the histone acetyl-transferases necessary for chromatin remodeling during transcriptional activation or histone displacement by protamines during spermatogenesis (Jason et al. 2002). Interestingly, Bach et al. (1999) showed that RNF12/RLIM is, indeed, necessary for the recruitment of the Sin3A/histone deacetylase corepressor complex during inhibition of LIM homeodomain transcription factors (Bach et al. 1999). Hence, the five new RING-H2 zinc finger proteins identified here are also candidate regulators of transcription and chromatin remodeling.

Alternative Splicing in the Zinc-Finger-Containing Proteins Set (ZFPS)

One aspect that became apparent when examining the zinc-finger-containing proteins was the high number of proteins present in different isoforms. The frequency of alternative splicing in the mouse transcriptome was analyzed elsewhere (Okazaki et al. 2002; Zavolan et al. 2002; http://genomes.rockefeller.edu). Among transcription units with multiple transcripts mapped to the mouse genome, we found 655 clusters annotated as zinc fingers. Of these, 311 (47.5%) have multiple splice forms (Table 7). This frequency is significantly greater than is apparent for the rest of the transcription units (TU; 4439 TUs with variants/11022 total TUs = 41.1%, *p*-value = 0.0002). The average number of transcripts sampled from each transcription unit is very similar between zinc fingers (4.0) and the rest of TUs (4.04), indicating that the difference in the frequency of splice variation is not caused by deeper sampling of transcripts encoding zinc finger proteins. The frequency increased even further when ESTs from dbEST were included in the analysis of splice variation (data not shown), indicating that many variants are yet to be discovered. The frequency of specific types of variation (cryptic exons, intron inclusions) is also higher among zinc finger proteins (Supplementary Table 3). Furthermore, for 334 (51%) of the 655 TUs, we found at least one transcript that would generate a truncated protein. Truncated protein forms may have

Table 6. Gene Structure of the Mouse and Human RING-H2 Family (Cluster 7)

Proposed mouse gene symbols	IPR domains	Mouse protein accessions	Mouse Chr/band	Exons/introns	Human nucleotide accessions	Human Chr/band
Praja1	RING	NP_032879	Xc2	3/2	NM_022368	Xq13.1
Praja2	RING	AAH17130	17e1.2	9/8	NM_014819	5q21.2
RNF11	RING	NP_038904	4c7	4/3	NM_014372	1p33
RNF50	RING	NP_598825	13b2	11/10	NM_014901	5q35.2
RNF51	RING	2500002L14Rik	6c3	5/4	NM_016494	2p13.3
RNF52	RING	AAH16543	10c1	9/8	NM_017876	19p13.3
RNF53	RING	0610009J22Rik	11a1	9/8	AAD43187	22q12.2
ZFP364	RING	NP_080682	3f2	9/8	CAB45280	1q12
RNF12/RLIM	RING	Q9WTV7	Xc3	5/4	NM_016120	Xq13.2
G1RP1/goliath	RING/PA	NP_067515	11b1.2	8/7	NM_018434	5q35
G1RP2/GRAIL	RING/PA	NP_075759	Xf1	8/7	NM_024539	Xq22.3
G1RP3	RING/PA	1700042K15Rik	6a3	2/1	NM_139175	7q31.32
RNF13a	RING/PA/EGF	AF037205	3d	10/9	XP_017311	3q25.1
RNF6	RING/protamine P1	1200013I08Rik	5g2	5/4	NM_005977	13q12.13
RNF25	RINGR/WD	NP_067288	1c3	10/9	NP_071898	2q35

Table 7. Frequencies of the Splice Variants in the RTPS and RTPS + ESTs Zinc Finger Datasets

Category	Clones		Clusters
FANTOM2			
All ZNF	2263		NA
Well-mapped	1533	67.74%	NA
In multitranscript clusters	1286	56.83%	655
In variant clusters	703		311
In nonvariant clusters	583		344
FANTOM2 + ESTs			
All ZNF	2263		NA
Well-mapped	1533	67.74%	NA
In multitranscript clusters	1394	61.59%	750
In variant clusters	995		510
In nonvariant clusters	399		240

important regulatory functions (Yang et al. 2002), for example, negative regulation of STAT92E by an N-terminally truncated STAT derived from an alternative promoter site.

The high rate of alternative splicing in the zinc finger superfamily could reflect the modular domain architecture, and the fact that individual domains commonly occur as single exons within a gene.

Detailed analysis of individual transcripts confirmed that isoforms generated by alternative splicing are likely to have different functions (Supplementary Figs. 3–6). For example, the murine transcription factor Krüppel-like factor 13 (mKLF13; Scohy et al. 2000) has a domain structure in which three C-terminal C2H2-type zinc fingers are responsible for the DNA binding. In this study, we identified a new variant in which exon 1 is skipped (Modrek et al. 2001; Modrek and Lee 2002) and an alternative cryptic exon (Hanawa et al. 2002) is used to generate an isoform with only two C2H2-type zinc fingers (IPR000822), where the N-terminal zinc finger is spliced out (Supplementary Figs. 3 and 5). This isoform is likely to have a different DNA-binding affinity compared with the three-finger isoform as shown in Supplementary Figure 3 (variant cluster scl9359 "mKLF13"; Zavolan et al. 2002).

Another example of likely functional plasticity is found in the RIKEN transcript C330026E23Rik, which encodes a protein with a C-terminal C2H2-type finger and an N-terminal KRAB-repressor domain (IPR001909). Two isoforms were identified, encoding proteins that contain only the C2H2 fingers and lack the KRAB domain (variants cluster scl11314). The two different structural isoforms could compete with the full-length protein to relieve transcriptional repression, because they lack the repressor domain KRAB (Friedman et al. 1996).

In the RING finger family, alternative splicing may modulate the cellular localization of different isoforms. In the case of the membrane-bound protein Ring finger protein 13 (RNF13; NM_011883; variants cluster scl7546), we found six isoforms of this transcript (Supplementary Fig. 6), encoding proteins from 381 to 200 amino acids long. The 200-amino-acid isoform f (C230033M15Rik) generated by alternative use of a cryptic exon lacks a membrane domain and is presumably soluble (Supplementary Fig. 4).

Conclusion

The zinc finger domains are not only one of the most abundant domains in the eukaryotic genomes but are also one of the best examples of protein structure modularity. The abundance of zinc finger proteins in eukaryotic transcriptomes is believed to be a consequence of the high structural stability of the zinc-binding domains, the redox stability of the zinc ion to the ambient reducing conditions in a cell. These features make this domain a perfect structure for the formation of protein–protein and protein–nucleic acid complexes (Laity et al. 2001; Nomura and Sagiura 2002).

The evolution of the zinc finger proteins has occurred in a modular fashion (Morgenstern and Atchley 1999). New proteins not only evolve by point mutation but rather are generated by adding or swapping domains to already structured proteins. This is confirmed by several cases of vertebrate-specific zinc finger domains (KRAB, KRAB-related, SCAN domain, Nuclear receptor ROR, and Nuclear transition protein 2) with different evolutionary histories in the zinc finger and non-zinc-finger domains of the Sp/KLF family. The gene structure of many zinc finger proteins facilitates a modular evolution. Normally, a zinc finger domain is contained in a single exon, which increases the probability of domain duplication and swapping. The exonic structure of the domains may explain also the higher frequencies of splice variation that we found in zinc finger proteins compared with the other protein families in the mouse transcriptome. In this study, we also found that splice variation can generate structurally and functionally distinct zinc finger proteins.

The RIKEN full-length, Representative Transcript and Protein Set (RTPS), represents the most complete transcriptome available in higher eukaryotes. The full-length cDNA and protein sequences allow us to better map each individual transcript to the mouse genome and define human homologs and possible splice variants generated from a single genetic locus. Gene prediction algorithms used in the mouse and human genome projects are imperfect. The availability of large full-length sequence sets reduces this imprecision in gene structure prediction. The high incidence of newly described genes present in the RTPS will allow a more thorough and systematic approach in characterizing protein families.

In overview, we have analyzed 46 structurally related zinc finger families in the mouse transcriptome, and placed the first part of the analysis in the public domain. We have looked in detail at three of these families and started to suggest nomenclature based on family relationships. Annotation of the remaining families may provide a rationale basis for future nomenclature, and also a basis for prioritization of functional characterization of members of this key family.

To facilitate future characterization of this superfamily, we generated a Web-based interface (http://cassandra.visac.uq.edu.au/zf) containing the structural classification of the entire zinc finger data set discussed in this study.

METHODS

Zinc Finger Classification

Zinc-finger-containing proteins were identified in the RTPS of 21,019 protein sequences using the InterPro protein domain searching tool version 5.0, resulting in a data set of 1573 proteins having at least one zinc finger domain. Specific subsets were selected from this data set based on two different classifications. The first classification is by distinct zinc finger domains as defined by the 46 distinct PROSITE sequence signatures. Obviously, a protein with more than one zinc finger domain can be present in more than one class, and proteins in

the same class may have completely different domain compositions and are not necessarily functionally related.

The second classification was much more rigorous and attempted to identify protein families that are truly functionally related. An all-against-all sequence comparison was performed using the BLASTP 2.1.3 program (Altschul et al. 1990), and a graph was constructed in which all pairs of proteins are connected when their BLAST expectation value is less than a given threshold of 10^{-25} or 10^{-8}, respectively. Pairs of sequences below that similarity threshold were regarded as unconnected in the graph. From this graph, all isolated connected subgraphs were computed. It is this collection of subgraphs that naturally describes a classification of the data set, and the edges of a subgraph are members of that class. Unlike with the PROSITE classification, a sequence is assigned to a single class only. It is important to understand when looking at classes from this approach, however, that two sequences in the same class are not necessarily similar with an expectation value below the above given BLAST threshold, but rather the evolutionary link between these two sequences may come from several intermediate sequences, each pair linked with the high likelihood to be evolutionarily related. The fasta files of these data sets can be downloaded at http://cassandra.visac.uq.edu.au/zf/.

Alignments and Phylogenetic Construction

Protein GenBank accession nos. used for alignments and phylogenetic trees for the, NFTR, Sp/KLF, and RING-H2 families are listed, respectively, in Tables 4, 5, and 6.

CLUSTALX version 1.6.6 (Thompson et al. 1997) was used for the generation of the family alignments and Bootstrap (1000 replicates) Neighbor Joining trees (NJ tree). ESPript 2.0 beta was used for the protein alignments visualization (http://prodes.toulouse.inra.fr/ESPript). TreeView software (http://taxonomy.zoology.gla.ac.uk/rod/treeview.html) was used for the NJ trees visualization.

Mapping of the New Mouse and Human Zinc-Finger-Containing Proteins

The genomic mapping of the new mouse and human proteins characterized in this study was done using Sequence Search and Alignment by Hashing Algorithm (SSAHA; http://www.sanger.ac.uk/Software/analysis/SSAHA/), against ENSEMBL mouse and human genome browsers (http://www.ensembl.org/). The murine cDNA sequences used for this mapping are *Fzf1*, 9530006B08Rik; *Fzf2*, B130043A04Rik; *Fzf3*, BC028839; *Fzf4*, 6030407P18Rik; *Sp8*, 5730507L14Rik; *rnf20*, NM_134064; *rnf21*, 2500002L14Rik; *rnf22*, BC016543; *rnf23*, 0610009J22Rik; *G1RP3*, 1700042K15Rik. The names of these newly described proteins have been proposed during this study.

Alternative Spliced Variants in the Zinc Finger Data Set

The cDNA sequences of the zinc finger data set used in this study combined the RIKEN 60,000 full-length cDNA collection and the mouse RefSeq (ftp://ftp.ncbi.nih.gov/refseq/). These were mapped to the draft of the mouse genome (Assembly v3) and used for the prediction of the splice variants as described by Zavolan (2002).

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