FEZ1 Dimerization and Interaction with Transcription Regulatory Proteins Involves Its Coiled-coil Region*

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The fascification and elongation protein ζ1 (FEZ1) is a mammalian orthologue of the Caenorhabditis elegans protein UNC-76, which is necessary for axon growth in that nematode. In previous studies FEZ1 has been found to interact with protein kinase Cζ, DISC1, the agnoprotein of the human polyomavirus JC virus, and E4B, a U-box-type ubiquitin-protein isopeptide ligase. We reported previously that FEZ1 and its parologue FEZ2 are proteins that interact with NEK1, a protein kinase involved in poly cystic kidney disease and DNA repair mechanisms at the G2/M phase of the cell cycle. Here we report the identification of 16 proteins that interact with human FEZ1-(221–396) in a yeast two-hybrid assay of a human fetal brain cDNA library. The 13 interacting proteins of known functions take part either in transcription regulation and chromatin remodeling (6 proteins), the regulation of neuronal cell development (2 proteins) and cellular transport mechanisms (3 proteins) or participate in apoptosis (2 proteins). We were able to confirm eight of the observed interactions by in vitro pull-down assays with recombinant fusion proteins. The confirmed interacting proteins include FEZ1 itself and three transcription controlling proteins (SAP30L, DRAPI, and BAF60a). In mapping studies we found that the C-terminal regions of FEZ1, and especially its coiled-coil region, are involved in its dimerization, its heterodimerization with FEZ2, and in the interaction with 10 of the identified interacting proteins. Our results give further support to the previous speculation of the functional involvement of FEZ1 in neuronal development but suggest further that FEZ1 may also be involved in transcriptional control.

FEZ1 (fasciulation and elongation protein ζ1) was initially identified as a mammalian orthologue of the Caenorhabditis elegans protein UNC-76, which is necessary for normal axonal outgrowth, bundling, and elongation in this nematode (1). FEZ1 mRNA is expressed abundantly in the rat adult brain and throughout all developmental stages of the brain in mouse embryos (2, 3). The human FEZ1 includes 392 amino acid residues, and its predicted structural organization shows that the protein possesses three glutamine-rich regions and a coiled-coil region (4) (Fig. 3). A mammalian homologue of FEZ1, the protein FEZ2, which shows ubiquitous tissue expression, was described in rat and human and has 48% amino acid sequence identity to FEZ1 (3).

Interestingly, FEZ1 was identified as an interacting protein partner in several yeast two-hybrid screens with independent protein baits. The first bait shown to interact with FEZ1 was the regulatory domain of the protein kinase Cζ (PKCζ) (2). It was further found that FEZ1 is a cellular substrate for phosphorylation through PKCζ and that phosphorylated FEZ1 promotes neurite extension of PC12 cells in the absence of nerve growth factor. In a second study, using the C terminus of DISC1 (disrupted-in-schizophrenia 1) as bait, FEZ1 was also identified as an interacting protein. The DISC1 gene has been implicated as a candidate gene for the etiology of schizophrenia (5–7). The interaction of FEZ1 and DISC1 was found to be up-regulated in PC12 cells during neuronal differentiation, and this caused an enhanced extension of neurites in the presence of nerve growth factor (6). Third, the agnoprotein of the human polyomavirus JC virus, the causative agent of a fatal demyelinating disease, showed direct interaction with FEZ1 and microtubules (4).

It was further verified that FEZ1 associated with the microtubules and that the agnoprotein induced FEZ1 dissociation from the microtubules leading to inhibited neurite outgrowth in PC12 cells. Furthermore, FEZ1 was found to interact with E4B, a U-box-type ubiquitin-protein isopeptide ligase, again via yeast two-hybrid system studies (8). This interaction is enhanced in the presence of PKCζ, and phosphorylation and/or ubiquitination of FEZ1 may contribute to neurite extension.

We found FEZ1, as well as its parologue FEZ2, as NEK1 protein kinase interacting proteins in a previous study (9). Members of the NEKs (Nima-related kinases) take part in the regulation of the cell cycle and meiosis and constitute the kinase family so far less well characterized functionally (10). Further NEK1 interactors include kinesin family member 3A (KIF3A), which had been described to also interact with FEZ1 (4). The FEZ1 orthologue UNC-76 has also been reported to interact with kinesin (11). Together, this may suggest that UNC-76/FEZ1 could play a role in kinesin-mediated transport pathways (4).

We set out to use FEZ1 itself as bait in a yeast two-hybrid assay and screened a human fetal brain cDNA library for potential FEZ1-interacting proteins. We found that FEZ1 interacts with itself and were able to confirm both FEZ1 homodimerization as well as its heterodimerization with FEZ2 by a series of in vitro experiments. In total we identified 16 FEZ1-interacting proteins that are either involved in transcriptional regulation (6 proteins), neuronal cell development (2 proteins), intra-cellular transport processes (3 proteins) or apoptosis (2 proteins), or are of unknown function (3 proteins), and we were also able to confirm 8 of these interactions by in vitro pull-down assays with recombinant proteins. In summary, our results further support previous findings that FEZ1 may be a regulatory protein with important functions during neu-

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4 The abbreviations used are: PKC, protein kinase C; GST, glutathione S-transferase; 3-AT, 3-amino 1,2,4-triazole; PBS, phosphate-buffered saline; PVDF, polyvinylidene difluoride.
Characterization of FEZ1 Protein Interactions

Molecular characterization of FEZ1 is important for understanding its role in cell development. Our study aimed to investigate the protein interactions of FEZ1 and identify potential binding partners. We used yeast two-hybrid screening to screen a human fetal brain cDNA library and confirmed interactors by restriction endonuclease analysis and automated DNA sequencing.

**EXPERIMENTAL PROCEDURES**

**Plasmid Constructions**—Several sets of oligonucleotides were designed for PCR amplification of complete FEZ1 or deletion constructs thereof, which were then inserted into vectors pBTM116 and pGAD424 in fusion with the LexA DNA binding domain or in vectors pACT2 or pGAD424 (Clontech) in fusion with the Gal4 activation domain (Fig. 1A). The nucleotide sequence coding residues 207–353 of human FEZ2 were PCR-amplified using a specific primer set, 5′-AGGAAATTCGGCAGTTATGAAAGAGAGAAGGTG-3′ and 5′-AGGTCGACGTTACACTTCTCTATCATAC-3′, and then cloned into EcoRI and Sall restriction site in vector pBTM116. To express full-length FEZ1 or its different indicated deletions fused to a His tag, the corresponding nucleotide sequences were amplified by PCR and inserted into bacterial expression vector pET28a (Novagen/EMD Biosciences, San Diego, CA). FEZ1-(131–392) represents a FEZ1 clone obtained in a yeast two-hybrid screening using the regulatory domain of NEK1 (9) and was subcloned and expressed using the vector pProEX-HTc (Invitrogen). For expression of BAF60a-(404–515) and complete FEZ1 as well as its different deletion constructs fused to GST, the corresponding nucleotide sequences were cloned into a modified vector pET28a-GST that codifies GST protein upstream of the protein to be inserted. All nucleotide sequences encoding the proteins identified to interact with the FEZ1-(221–392), except that encoding BAF60a-(404–515), were subcloned from the vector pACT2 to the bacterial expression vector pGEX-4T-2 (GE Healthcare, Waukesha, WI), which allows the expression of the proteins in the form of a GST fusion. The orientation, frame, and correctness of sequence of each insert DNA were confirmed by restriction endonuclease analysis and automated DNA sequencing.

**Yeast Two-hybrid Screen and DNA Sequence Analyses**—The yeast two-hybrid screen (12) of a human fetal brain cDNA library (Clontech) was performed by using the yeast strain L40 (trp1-901, his3Δ200, leu2-3, ade2 lys2::(lexAop)4·His3 uri3::(lexAop)8·lac GAL4) and human FEZ1-(221–392) as a bait fused to the yeast LexA DNA binding domain in vector pBTM116 (13). This fragment of FEZ1 does not activate the yeast reporter genes (see Fig. 1 and “Results”). The autonomous activation test for His3 was performed in minimal medium plates without tryptophan and histidine but containing 0, 5, 10, 20, 30, or 50 mM of 3-amin-1,2,4-triazole (3-AT). Furthermore, the autonomous activation of LacZ was measured by the β-galactosidase filter assay described below. Yeast cells were transformed according to the protocols supplied by Clontech. The screening was performed in minimal medium plates without tryptophan, leucine, and histidine. Half of the transfected cells were plated in selective medium containing 10 mM 3-AT, and the other half was plated on selective medium without addition of 3-AT. Recombinant pACT2 plasmids of positive clones were isolated and their insert DNAs sequenced with a DNA sequencing model 377S (Applied Biosystems, Foster City, CA). The obtained DNA sequence data were translated using the TRANSLATE Tool of ExPaSy (Expert Protein Analysis System), available online, and compared with sequences in the NCBI data bank using the BLASTP 2.2.12 program (14). The prediction of coiled-coil structures in the analyzed protein sequences was performed by the software COILS available on line (Swiss Institute for Experimental Cancer Research).

**Assay for β-Galactosidase Activity in Yeast Cells**—β-Galactosidase activity in yeast cells was determined by the filter assay method. Yeast transformants (Leu+, Trp+, and His+) were transferred onto nylon membranes, permeabilized in liquid nitrogen, and placed on Whatman 3MM paper previously soaked in Z buffer (60 mM NaHPO4, 40 mM NaH2PO4, 10 mM MgCl2, 50 mM 2-mercaptoethanol, pH 7.0) containing 1 mg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal). After incubation at 37 °C for 1 h, the yeast cells forming dark blue colonies were taken from replica plates for further analysis.

**Mapping the Protein Interaction Sites**—One of the deletion constructs of FEZ1-(269–392) had the coiled-coil region removed. This construct was co-transformed in Saccharomyces cerevisiae strain L40 with the “bait”-plasmid DNAs isolated from the two-hybrid screening. After transformation, yeast clones were streaked on minimal medium plates without tryptophan, leucine, and histidine for testing their growth capacity under interaction-selective conditions. The presence of both types of plasmids was controlled by growth on plates with minimal medium plates without tryptophan and leucine (15). To further test if the paralogue of FEZ1, FEZ2, interacts with the proteins that were isolated in the yeast two-hybrid screen using FEZ1, the corresponding bait-plasmid DNAs were co-transformed in L40 with the construct pBTM116-FEZ2-(207–353). FEZ2-(207–353) alone does not transactivate the reporter genes, and the co-transformed L40 clones were selected on minimal medium without tryptophan, leucine, and histidine.

**Protein Expression and Purification**—The nucleotide sequences in the library vector pACT2, which are inserted between restriction sites EcoRI and Xhol and code for the interacting proteins identified in the yeast two-hybrid system screen, were subcloned into the bacterial expression vector pGEX-4T-2 (GE Healthcare, Waukesha, WI) to allow expression of recombinant GST fusion proteins in Escherichia coli BL21 (DE3) cells. Soluble FEZ1 (complete or deletions), fused to the His tag or the GST tag, was purified for *in vitro* analyses from 1 liter of culture of *E. coli* BL21 (DE3) cells that were induced for 3 h to protein expression at 30 °C using 0.4 mM isopropyl 1-thio-β-D-galactopyranoside. For size exclusion chromatography, the nucleotide sequence of the pACT2 clone (Fig. 1A), containing FEZ1-(131–392), was subcloned into expression vector pProEXHTB (Invitrogen) using the restriction sites EcoRI and Xhol, as described (9).

All His-tagged proteins used in this study were purified using a HiTrap chelating column in an ÄKTA™ FPLC™ (GE Healthcare) as follows. Cells were harvested by centrifugation at 4,500 × g for 10 min, and the cell pellet was resuspended and incubated for 30 min with 10 volumes of lysis buffer (137 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4, 1.8 mM KH2PO4, pH 7.4, 1 mg/ml lysozyme, 1 mM phenylmethylsulfonyl fluoride, and 0.05 mg/ml DNase). After three cycles of sonication, soluble and insoluble fractions were separated by centrifugation at 28,500 × g for 30 min at 4 °C. The cleared supernatant was then loaded onto a HiTrap chelating column (GE Healthcare) pre-equilibrated with lysis buffer (lacking lysozyme and DNase), followed by extensive wash of the column with the same buffer. Bound proteins were eluted in a gradient of 0–100% of elution buffer (137 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4, 1.8 mM KH2PO4, 1 mM phenylmethylsulfonyl fluoride, and 500 mM imidazole, pH 7.4). Aliquots of each eluted fraction obtained were analyzed by SDS-PAGE, and peak fractions containing FEZ1 were dialyzed with buffer (137 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4, 1.8 mM KH2PO4, 0.5 mM dithiothreitol, and 5% glycerol, pH 7.4).

The FEZ1 proteins (complete or deletions) fused to GST were induced for expression as described above. After sonication the lysate was cleared by centrifugation at 28,500 × g for 30 min at 4 °C. The...
resulting supernatant was incubated with glutathione-Uniflow resin (Clontech) and used for in vitro binding assay. The cDNA coding the proteins identified by yeast two-hybrid assay were cloned into the vector pGEX-4T-2 with GST, and fusion proteins were expressed in E. coli BL21 (DE3) cells at 37°C using 0.5 mM isopropyl β-D-galactopyranoside for 4 h.

**In Vitro Binding Assay**—Expressed GST, GST-KIBRA, GST-SAP30L, GST-CLASP2, GST-RAI14, GST-Bamacan, GST-DRAP1, and GST-BAF60a proteins were allowed to bind to 25 μl of glutathione-Uniflow resin (Clontech) in 1 ml of total bacterial protein extract in PBS for 1 h at 4°C. After incubation, the beads containing bound recombinant proteins were washed three times with PBS (0.14 M NaCl, 2.7 mM KCl, 10 mM Na2HPO4, 1.8 mM KH2PO4, pH 7.4) at 4°C. 25 μg of purified full-length 6xHis-FEZ1 fusion protein were added to the resins containing GST or GST fusion proteins and incubated in 0.1 ml of PBS 1 × for 4 h at 4°C to allow protein-protein interactions to occur. The beads were then washed three times with 0.5 ml of PBS, followed by three washings with 0.5 ml of PBS containing 0.1% Triton X-100, then three washes with 0.5 ml of PBS only. Resin-bound proteins were resolved on two separate 10% SDS-polyacrylamide gels. After electrophoresis, the proteins were transferred to PVDF membranes by semi-dry electroblotting. After saturation with unspecific protein (5% bovine serum albumin) in TBS (0.15 M NaCl, 20 mM Tris-HCl, 0.05% Tween 20, pH 7.2), one of the membranes was incubated with a mouse anti-His tag (1:5000) and the other with mouse monoclonal anti-GST antibody 5.3.3 (hybridoma supernatant 1:5) for 1 h each. The anti-GST monoclonal antibody 5.3.3 had been generated by immunizing BALB/c mice with a GST-CGI-55 recombinant fusion protein (16). Selection of hybridoma producing anti-CGI-55 or anti-GST antibodies was tested by enzyme-linked immunosorbent assay with purified recombinant 6xHis-CGI-55 or GST protein. Specificity of the recloned hybridoma 5.3.3 was confirmed by anti-GST Western blot. After three washes with TBS, 0.05% Tween 20, the membranes were incubated with the secondary horseradish peroxidase-conjugated anti-mouse IgG antibody (1:5000; Santa Cruz Biotechnology) for 1 h and washed again three times with TBS. The membranes were then developed by chemiluminescence using the reagent Luminol (Santa Cruz Biotechnology) for detection of His-tagged or GST fusion proteins.

To confirm the in vitro interaction of FEZ1 with itself, GST-FEZ1-(1–392), GST-FEZ1-(1–227), GST-FEZ1-(221–392), or GST-FEZ1-
(269–392), which lacks the coiled-coil region, were all allowed to bind to glutathione-UNiflow resin as described above. For each preparation of loaded beads, we added in separate reactions 25 μg of each of the following three different purified 6His-FEZ1 fusion proteins, 6His-FEZ1(1–392), 6His-FEZ1(1–227), or 6His-FEZ1(221–392), which were incubated, washed, and analyzed for protein interaction by Western blot as described above.

Size Exclusion Chromatography—Five milligrams of purified 6His-FEZ1(131–392) were loaded on a Superdex™ 75 10/30 Prep Grade column (GE Healthcare) that had been equilibrated previously with 20 mM Tris, pH 7.5, 150 mM NaCl and calibrated with the following standard proteins: aldolase (158 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), and chymotrypsinogen A (25 kDa). 0.5-ml fractions were collected, and 10-μl samples of each fraction of the different peaks observed were analyzed by 12.5% SDS-PAGE.

RESULTS

Analysis of the Auto-activation of Different FEZ1 Constructs in the Yeast Two-hybrid System—Before the yeast two-hybrid screening of the human fetal brain cDNA library, the construction encoding full-length FEZ1 was tested for autonomous activation of the reporter gene HIS3, i.e. capacity of growth in minimal medium (lacking tryptophan and histidine) and with addition of 0, 5, 10, 20, 30, or 50 mM 3-AT, an inhibitor of HIS3, that suppresses background growth of the yeast on minimal medium lacking histidine. To further investigate the autonomous activation of the second reporter gene lacZ, we also performed the β-galactosidase filter assay. The results of both assays revealed a strong and autonomous activation of the reporter genes HIS3 and lacZ by the full-length protein FEZ1. To solve this problem, a set of oligonucleotides was designed to subclone different regions of FEZ1 into the pBTM116 vector and to test them for autoactivation. Only the two constructions FEZ1(221–392) and FEZ1(269–392), which lack the coiled-coil region, did not show autonomous activation of the reporter genes.

Identification of Proteins that Interact with FEZ1—To identify proteins interacting with FEZ1, we employed the yeast two-hybrid system (13) and screened a human fetal brain cDNA library. FEZ1-(221–392) was chosen for the yeast two-hybrid screening of a human fetal brain cDNA library. Interestingly, all N-terminal constructs of FEZ1 showed strong autoactivation (1–129, 122–227, 122–392, and 1–227). This may indicate that FEZ1 is able to function as a transcriptional activation domain located at its N terminus seems to have a conserved function in respect to the activation of the reporter genes.

### TABLE 1
Human FEZ1-interacting proteins identified by the yeast two-hybrid system screen

| Protein interacting with FEZ1 (aliases) | Coded protein residues (complete sequence/retrieved) | Accession no. | Domain composition (native protein)* | Function† | Ref. |
|----------------------------------------|-----------------------------------------------------|---------------|--------------------------------------|-----------|-----|
| DRAP1 (NC2a)                           | 205/1–205                                           | NP_006433     | Histone domain, coiled-coil region   | Transcriptional control | 23  |
| BAF60a (SMARCD1)                       | 515/82–515 and 515/404–515                          | AAH09368      | SWIB domain, coiled-coil region      | Transcriptional control; recruitment of chromatin-remodeling complex by specific transcription factors | 27, 28 |
| SAP30L                                 | 183/1–183                                           | NP_078908     | Transcriptional control; recruitment of the Sin histone deacetylase complex by specific transcription factors | 29       |
| Bromodomain containing protein 1       | 1058/1002–1058                                      | NP_055392     | Bromodomain; PWW domain; coiled-coil region | Transcriptional control; chromatin remodeling | 26, 49 |
| Tlk2                                   | 830/33–830                                          | AAM44925      | Catalytic domain of a serine/threonine kinase; coiled-coil region | Transcriptional control; chromatin remodeling; DNA damage checkpoint | 17   |
| Zinc finger protein 251                | 293/25–293                                          | AAM06258      | Znf C2H2                              | Transcriptional control? | 18   |
| Bamacan (CSPG6/SMC3)                   | 1217/881–1217                                       | NP_009436     | Coiled-coil regions; SMC-hinge       | Sister chromatid cohesion; DNA repair; microtubule dynamics; tumorigenesis | 44   |
| CLASP2                                 | 1362/1046–1300                                      | NP_055912     | HEAT repeats; coiled-coil region     | Microtubule dynamics | 50   |
| RAB3 GAP                               | 981/515–981                                         | NP_036365     | Coiled-coil region                   | Neuronal motor transport; neurotransmitter release | 19, 51 |
| FEZ1 (Zyg in 1)                        | 392/238–392 and 392/131–392                        | NP_005094     | Coiled-coil region                   | Neuronal development; microtubule dynamics | 2    |
| KIBRA                                  | 1113/869–1113                                       | NP_056053     | WW, coiled-coil regions              | Neuronal development? | 39   |
| Retinoic acid-induced 14               | 983/720–983                                         | AAP84319      | Ankyrin repeats, coiled-coil regions | Apoptosis? | Unpublished* |
| Programmed Cell Death 7 (ES18)         | 485/272–430                                         | NP_005698     | Coiled-coil regions                  | Apoptosis | 35   |
| Similar to short coiled-coil protein   | 115/1–115                                           | AAM616511     | Coiled-coil region                   | Unknown   | 18   |
| Hypothetical Protein FLJ13909          | 266/50–266                                          | AAM18719      | Unknown                              | Unknown   | 18   |
| Similar to OAT-like 1                  | 366/258–366                                         | AAM26050      | TBC domain                           | Transaminase activity | 18   |

* Other domains may be present.
† Other functions may be known.
* GenBank™ data deposited by R. Huo, X. Y. Huang, H. Zhu, Z. Y. Xu, L. Lu, M. Xu, L. L. Yin, J. M. Li, Z. M. Zhou, and J. H. Sha.

### Notes
- Retinoic acid-induced 14
- Programmed Cell Death 7 (ES18)
- Similar to short coiled-coil protein
- Hypothetical Protein FLJ13909
- Similar to OAT-like 1
was used as bait, and a total of about $1.5 \times 10^6$ co-transformed clones were assayed in two groups. Although the first half of transformants was plated on selective minimal medium plates (without tryptophan, leucine, and histidine), the second half was plated on selective minimal medium (without tryptophan, leucine, and histidine) with the addition of 10 mM 3-AT. All grown colonies that showed a strong blue color in the subsequent \(-\)-galactosidase filter assay had their plasmid DNA extracted and sequenced. A total of 101 plasmid DNAs from clones positive for both \(\text{HIS3}\) and \(\text{LacZ}\) reporters were sequenced. 16 different proteins were identified using FEZ1-(221–392) as bait (Table 1), which can be organized into the following groups according to the major described function attributed to them: 1) proteins involved in transcriptional control and chromatin organization (6 proteins); 2) proteins that take part in the regulation of neural cell development, microtubule dynamics, and transport (5 proteins); 3) proteins taking part in apoptosis processes (2 proteins) or tumorigenesis; and 4) proteins with little or no functional information available as yet (3 proteins). Table 1 summarizes the domain organization and functional characteristics of the proteins found to interact with FEZ1.

The first group of FEZ1-interacting proteins is involved with transcriptional control and chromatin organization and includes the subunit BAF60a of the chromatin-remodeling complex SWI/SNF, DRAP1 (DR1-activating protein), also known as NC2 (negative cofactor 2, subunit a), a component of NC2 a transcriptional repressor complex. Furthermore, this group contains SAP30L (Sin3-associated protein 30-like), the bromodomain-containing protein, the Tlk2 (tousled-like kinase 2), a nuclear serine/threonine kinase, described to be a target of the DNA damage checkpoint (17), and finally a transcriptional regulator of unknown specific function called zinc finger protein 251 (18).
Characterization of FEZ1 Protein Interactions

The second group includes FEZ1 itself, thereby suggesting that FEZ1 could form dimers. Two different clones of FEZ1 were isolated in the screen; the first encodes amino acid residues 131–392 and the second residues 238–392 of FEZ1. Also found in this group of proteins, involved in neural cell development and function and microtubule dynamics and transport, is the protein “RAB3 GTPase-activating protein,” which is necessary for efficient anchoring of synaptic vesicles to the presynaptic membrane (19–21). Also, this group containstheproteins CLASP2 and Bamacan, and both have also been implicated in microtubule dynamics. Another protein included in this group is KIBRA, a WW domain-containing protein, with functional implications in the cytoskeleton and possibly neuronal functions (Table 1).

The proteins of the third group of interacting proteins include the Programmed Cell Death 7 (ES18), RAI14 isoform, and three proteins already cited in first two groups that have some participation in tumor formation and transformation cellular, such as Bamacan, SAP30L, and Tlk2. In the last group of proteins with unknown function, we find the short coiled-coil protein, the hypothetic protein FLJ13909, and a protein similar to OAT-like 1.

**In Vitro Confirmation of the FEZ1 Protein-Protein Interactions**—To confirm the interactions observed in the yeast two-hybrid system, all retrieved cDNA sequences encoding the FEZ1-interacting proteins were subcloned into bacterial expression vectors and expressed as GST or His, fusion proteins in E. coli. However, excluding the soluble FEZ1 itself (see below), only seven of the subcloned cDNAs resulted in the expression of soluble proteins. The interaction of FEZ1 with these proteins in fusion with GST (KIBRA, SAP30L, CLASP2, RAI14 isoform, Bamacan, DRAP1, and BAF60a) was then tested by in vitro pull-down assays (Fig. 2), and all seven interactions could be confirmed. The high number of washes suggests that the interactions were strong. The specificity of the observed interactions was demonstrated because no interaction of constructs FEZ1 was observed with free GST under the same conditions (Fig. 2).

**FEZ1 Interacts with FEZ2**—FEZ1 has a human paralogue called FEZ2, whose amino acid sequence has an identity of 60% (76% similarity) in the highly conserved C-terminal region (amino acids 1101–1220) (Fig. 3). The N terminus of the two proteins is less conserved (28% sequence identity and 60% similarity).

Because we observed FEZ1 homodimerization in the yeast-two hybrid assay, we were interested to test whether FEZ1 and FEZ2 are also capable of forming heterodimers, again using the yeast two-hybrid system (Fig. 4A). Our results indicate that two clones of FEZ2 (207–353 and 128–353) are both capable of engaging in interactions with three of the tested FEZ1 constructs (131–392, 221–392, and 238–392), but FEZ2-(128–353) failed to interact with FEZ1-(269–392) (Fig. 4A). This result indicates that the coiled-coil region of FEZ1 is important for the
heterodimeric interaction (Fig. 3 and Fig. 4A) because FEZ1-(269–392) lacks the coiled-coil region. Conversely, a partial deletion of the coiled-coil as in FEZ1-(238–392) still allows interaction with FEZ2-(207–353).

The coiled-coil region is very conserved between the two proteins FEZ1 and FEZ2 (76% amino acid sequence identity, 92% similarity). On the other hand, the region seems to be important for the interaction of FEZ1 with the majority of the proteins found to interact with it in the yeast two-hybrid screen (Table 2), because 11 of the 16 tested proteins (including FEZ1 itself) no longer interact with FEZ1 when its coiled-coil region has been deleted.

Curiously, however, FEZ1 still homodimerizes when the coiled-coil region in one of the two interacting FEZ1 constructs is missing (Fig. 4B). FEZ1-(269–392), which lacks the coiled-coil, still interact normally with FEZ1-(131–392) containing the full-length coiled-coil. However, FEZ1-(269–392), which completely lacks the coiled-coil motif, interacts significantly less with the constructs FEZ1-(238–392), which lack the first 10 amino acids of the coiled-coil region (Fig. 4B and Table 2).

This result may suggest that the coiled-coil region between 231 and 266 is critical in this interaction.

Size Exclusion Chromatography—The finding that FEZ1 interacts with itself in the yeast two-hybrid assay suggested that FEZ1 forms dimers. To confirm the FEZ1 dimerization in vitro, we submitted the purified protein 6xHis-FEZ1-(131–392) to a size exclusion chromatography. Three protein peaks were eluted (Fig. 5A). The first and highest peak corresponds to a protein of ~90.4 kDa and probably represents a dimer of 6xHis-FEZ1-(131–392). The SDS-PAGE analysis of fractions 17–23, which correspond to the elution volume of this first peak, confirmed a protein of the expected monomer molecular mass of ~45 kDa. The other two peaks observed correspond to proteins of 32.2 kDa (fractions 22–24, Fig. 5B) and 10.3 kDa (not shown) and probably represent proteolytic degradation products of 6xHis-FEZ1-(131–392).

In Vitro Confirmation of the FEZ1 Dimerization—To confirm the FEZ1 homodimerization and to map which protein regions may be
involved in this interaction, we performed in vitro pull-down assays with GST and His<sub>6</sub> fusion proteins of FEZ1 (Fig. 6). Full-length GST-FEZ1 as well as all three deletion constructs of FEZ1 tested were able to interact with full-length 6xHis-FEZ1 in the pull-down assay. In this experiment we observed that the N-terminal region of FEZ1-(1–227), when fused to GST, can interact with full-length 6xHis-FEZ1 (Fig. 6, lanes 4–6). Furthermore, it is surprising that in vitro neither 6xHis-FEZ1-(1–127) nor 6xHis-FEZ1-(221–392) was able to interact with any of the tested GST-FEZ1 fusion proteins, including the full-length FEZ1 fusion GST-FEZ1-(1–392). These rather unexpected results could be explained either by a spatial blockage of the interaction depending on the size or nature of the fusion protein partner or by the possibility that the N- and C-terminal FEZ1 fragments are not folded properly, when fused to a His<sub>6</sub> tag. However, because of the autoactivation of the reporter genes by the control plasmid pBTM116-6xHis-FEZ1-(221–392), or pBTM116-Galactosidase filter assay was performed. ++++, very strong coloration; +++, strong coloration; ++, weak coloration; +, no coloration.

**DISCUSSION**

In a previous study, we identified interacting protein partners for the large regulatory domain of human NEK1 by using the yeast two-hybrid assay (9). Among several other interacting proteins, we also identified FEZ1 and FEZ2. At that time little information about the function of FEZ1 was available, so we proceeded to screen a human fetal brain cDNA library using FEZ1 as bait to discover proteins that interact with it. However, because of the autoactivation of the reporter genes by the full-length protein FEZ1, we used the nonself-activating fragment FEZ1-(221–392) as a bait in the yeast two-hybrid screen.

We were able to identify 16 proteins that interacted with FEZ1 and that participated in cellular processes ranging from transcriptional control and chromatin organization (6 proteins), regulation of neuronal cell development (2 proteins), and microtubule organization and transport.
functions (3 proteins) to apoptosis (2 proteins) (Table 1 and Fig. 7). Three of the 16 proteins have no functional information available.

The most interesting group of six proteins we found to interact with FEZ1 consists of those that are involved in transcription regulation, chromatin organization, and other nuclear functions, and to date no data from the literature has suggested that FEZ1 may be associated with nuclear functions. These proteins include SAP30L, a protein involved in the recruitment of the Sin3-histone deacetylase complex that participates in transcriptional repression when tethered to DNA. Furthermore, we found BAF60a (BRG1-associated factor 60a), a subunit of the SWI/SNF chromatin-remodeling complex, and DRAP1 (also termed NC2α), a subunit of the negative cofactor 2 (NC2) that acts in an inhibitory way on the RNA polymerase II/RNA polymerase (22, 23). Additional proteins include the Tlk2 (Tousled-like kinase 2), a nuclear serine/threonine kinase that plays a role in cell cycle progression through processes involved in regulation of chromatin dynamics (24), and the bromodomain-containing protein 1. In the latter case, little is known of its function, but it is well know that the bromodomain in general is an evolutionary conserved protein module, which is found in many chromatin-associated proteins and in nearly all known histone acetyltransferases. Several groups proposed a role of bromodomains in transcriptional regulation on chromatin substrates (25, 26). The function of the zinc finger protein 251 is also not known, but its sequence clearly allows to group it into zinc finger-like transcription factors (22).

The selective expression of genes that are packaged into repressive chromatin structures is a fundamental process that controls gene regulation during development. Genetic and biochemical studies have defined several mechanisms that relieve nucleosomal repression and increase the accessibility of DNA for protein interactions that establish appropriate patterns of gene expression. The mammalian SWI/SNF complexes consist of about 15 subunits and fall into two broad classes depending on whether they contain hBMR or BRG1 as ATPase. The glucocorticoid receptor interacts with non-core subunits of the BRG1
complex, including BAF60a, and a direct interaction between glucocorticoid receptor and the DNA binding domain region within the N-terminal region of BAF60a is required for the recruitment of the BRG1 complex and efficient chromatin remodeling (27). BAF60a is also a determinant for the transactivation potential of Fos/Jun. Heterodimers of the latter recruit the SWI/SNF complex, again via BAF60a interaction, to AP-1-binding sites to initiate transcription programs that eventually regulate cellular growth, differentiation, and development (28).

The interaction of FEZ1 with BAF60a may suggest that FEZ1 could represent yet another regulatory molecule that targets the SWI/SNF complex and possibly directs it to still unknown chromatin elements. Future studies must address whether the regulation of these supposed target regions could be involved in the differentiation program of neuronal cells, where FEZ1 could have its predominant function in the context of the neurite outgrowth.

The mRNA of SAP30L codes for a nuclear protein with 70% amino acid sequence identity to SAP30 (Sin3-associated protein 30), which is a component of another multiprotein complex involved in chromatin remodeling and transcriptional regulation, called Sin3/histone deacetylase (29). SAP30 binds to the motif PAH3 (paired amphipathic helix motif 3) of mSin3A and is capable of repressing transcription when tethered to DNA (30). For instance, it has been shown that SAP30 is required for the N-CoR-mediated repression through antagonist-bound estrogen receptor (30). In fact, histone deacetylation by the mSin3A/histone deacetylase co-repressor complex has been linked to transcriptional silencing of genes regulated through several of the retinoid and thyroid hormone receptor families, including Ikaros, EeF, and Myc/Max/Mad proteins (31). Furthermore, the p53-binding protein p33ING1b also interacts with the Sin3 complex via direct interaction with SAP30. Two distinct Sin3/p33ING1-containing complexes were isolated. Interestingly, only one of them associates with sub-units of the Brg1-based SWI/SNF chromatin-remodeling complex (32, 33), described above. The interaction of FEZ1 with two different multiprotein chromatin-remodeling complexes, which are functionally and physically connected, is striking.

DRAP1 is another transcription regulatory protein we found to interact with FEZ1. DRAP1 (or NC2α/H9251) is one of the two subunits of the NC2 (negative acting co-factor 2), which regulates the eukaryotic activator of RNA polymerase II. In vitro, the NC2αβ heterodimer acts as a molecular clamp, gripping the upper and lower surfaces of the TATA-binding protein-DNA complex, and NC2β exerts its negative regulatory function by blocking out the recognition of TATA-binding protein-DNA by transcription factor IID (23). For now, we do not know what the influence of the FEZ1 interaction on this intricate protein complex may be, but it is noteworthy that all interacting proteins or their complexes have either negative regulatory functions or promote transcriptional repression.

In the context of interactions with transcriptional regulators, it is noteworthy that the sequence of FEZ1 at the beginning of its coiled-coil motif contains a short signature motif “LXXLL” (where X is any amino acid), which is frequently found in transcriptional co-activators (34). Most interestingly, the same motif is also found in the interacting protein Programmed Cell Death 7 (ES18) (35). This motif has been described at first in the co-activators of transcription RIP-140, SRC-1, and CBP, which bind to liganded retinoid or lipophilic hormone receptors through this short sequence motif (34).
A second large group of proteins identified to interact with FEZ1 in this work is functionally related to neuronal development, to the cytoskeleton, and to transport processes and includes the RAB3 GAP (RAB3 GTPase-activating protein), which participates in membrane trafficking, KIBRA, a WW domain containing protein with some association to the cytoskeleton, and CLIP-associating protein 2 (CLASP2), which participates in microtubule stabilization. Bamacan (or chondroitin sulfate proteoglycan 6 or SMC3) may also be associated with this group, because it is involved in diverse roles, including microtubule dynamics, chromosome organization (cohesion, assembly, and segregation), and DNA repair (36).

Bamacan is localized either as a secreted proteoglycan in the basal membrane (chondroitin sulfate proteoglycan 6) or as an intracellular protein known to be involved in the structural maintenance of chromosome 3 (SMC3). The multimeric complex cohesin is composed by the heterodimer SMC1/SMC3, two non-SMC components termed SA1/SA2 (stromal antigen 1/2) and RAD21, and localizes to the spindle poles during mitosis (37). SMC3 has been shown to play essential roles during sister chromatid separation, in DNA repair and recombination, and microtubule-mediated intracellular transport processes (36).

Among the proteins involved in neuronal development and transport functions, we find RAB3 GAP a member of the Rab family of proteins that play a pivotal role in controlling membrane trafficking along the endo- and exocytosis pathways (20). In the brain these proteins cycle on and off synaptic vesicles in concert with exocytosis (21). Rab3 proteins specifically participate in the exocytosis of hormones and neurotransmitters (20) and are also involved in the formation of long term potentiation in the hippocampus (19). Interestingly, another protein identified in our two-hybrid screen with FEZ1 was a protein similar to OAT-like 1 that possess a TBC domain (Table 1), which is found as a domain also in yeast RAB GAPs (38).

The function of the FEZ1 interactor KIBRA is not known yet, but it contains WW domains and interacts with human Dendrin, a putative modulator protein of the postsynaptic cytoskeleton (39), and with PKCζ, which interacts through its catalytic domain with KIBRA and phosphorylates its glutamic acid-rich C terminus (40). Interestingly, FEZ1 itself was also found to interact with PKCζ and is a substrate thereof (2). CLASP proteins stabilize microtubules by retaining their plus ends in the peripheral region of the cell, where they are either pausing or undergoing short polymerization-depolymerization cycles. A short repetitive region in the central region of CLASP1 and -2 can bind to EB1 and EB3 and recognize growing microtubular tips, whereas the C-terminal domains of CLASP2 associates with the Golgi apparatus and cellular cortex (41). FEZ1 itself was among the proteins interacting and also belongs to this group of proteins involved in neuronal development.

The third group of FEZ1 interactors includes proteins involved in either tumorigenesis or apoptotic processes. The function of RAI14 is still unknown. However, retinoic acids are metabolites of vitamin A, the regulative functions of which on proliferation, differentiation, tumor growth and apoptosis are long known (42, 43). Bamacan/SMC3 can cause cellular transformation, when overexpressed (44). Proteins that participate in apoptotic processes are RAI14 and Programmed Cell Death 7 (or PCD7 or ES18). The function of the latter is still poorly understood but is related to specific apoptotic processes in mouse T-cells, where its expression is selectively regulated by distinct apoptotic stimuli (35).

Recently, a large scale two-hybrid map was reported that consists of more than 3000 putative human protein-protein interactions (45). The protein FEZ1-(131–392) was used as a bait, and 21 FEZ1-interacting proteins were identified, most of which are involved in transcription regulation (2 proteins), nervous system development and signaling...
Characterization of FEZ1 Protein Interactions

(4 proteins), apoptosis (1 protein), mitochondrial transport (3 proteins), unknown functions (3 proteins), or other functions (2 proteins), or represent secreted, extracellular proteins (6 proteins), including the parathyroid hormone. Interestingly, PDCD7 (Programmed Cell Death 7) was the only protein in common identified by both that study and our own two-hybrid assay.

It may have been expected that the longer FEZ1 bait used by Stelzl et al. (45) should have also picked up the same pre-y proteins that we identified in our screen. However, the upper limit of tested proteins in their automated yeast two-hybrid mating screen was 5632. In fact, we only found 5 of the 16 FEZ1-interacting proteins we identified here in their list of tested proteins (DRAP1, KIBRA, Programmed Cell Death 7, OAT, and FEZ1).

In our classical yeast two-hybrid screen, we screened over 1.5 million clones of a human fetal brain cDNA library, which encodes fusion proteins potentially representing the majority of cDNAs expressed in the fetal human brain. Human fetal brain is a physiologically and developmentally very complex and active tissue, expected to contain a very large number of different expressed cDNAs. Certainly, the two sets of identified FEZ1-interacting proteins represent complementary and not contradictory groups, because the majority (16:30) of identified proteins participate in the same three functional contexts as follows: transcription regulation, nervous system development, and signaling or apoptosis.

Another explanation for the different result may be related to the fact that Stelzl et al. (45) used a larger bait molecule (FEZ1, residues 131–392) than we did (FEZ1, residues 221–392). We excluded region 1–220 from our bait, because we observed a strong autoactivation of two clones spanning amino acids 1–129 and 122–227. FEZ1-(131–392) contains two glutamic acid-rich regions, which may represent additional protein-docking sites, with higher affinity to those proteins identified as baits by Stelzl et al. (45). In case of the prey proteins identified by Stelzl et al. (45), further domain mapping studies are required to clarify the importance of the Glu-rich region versus the coiled-coil region, which we identified here as critical for the interaction between FEZ1 and the prey proteins identified here.

Interestingly, 15 of the 21 FEZ1 preys identified by Stelzl et al. (45) were also predicted to contain coiled-coil regions, as do 12 of the 16 proteins we found to interact with FEZ1. It is further noteworthy, that Stelzl et al. (45) classified the 1705 interacting proteins of their screen in two large groups. The first group consists of the majority of proteins that represent only one or few interactions with other proteins, and the second group, which they call hubs, shows a large number of protein interactions, typically more than 30. Proteins acting as hubs are three times more likely to be essential for cells than proteins with only a small number of links (46). Our results together with those of Stelzl et al. (45) suggest that FEZ1 has at least 36 interacting protein partners as follows: 21 identified by Stelzl et al. (45) and another 15 different identified by us. This may suggest that FEZ1 falls also into the group of hub proteins and is therefore likely to have an important function in the cell.

A recent study by Naghavi et al. (47) indeed shows an important function of FEZ1 in the post-entry block of retrovirus replication. They found that FEZ1 is overexpressed in retrovirus-resistant fibroblasts and that its overexpression is functionally relevant for the observed retroviral resistance. Naghavi et al. (47) speculate that FEZ1 may operate at multiple points in the viral life cycle but that it is likely that FEZ1, previously proposed to function as a transport protein (2, 11), may be relevant for the observed retroviral inhibition.

There are many proteins containing coiled-coil regions taking part in signaling networks and growth control regulation processes via protein-protein interactions. The coiled-coil roles include dynamic motions, rearrangements, and regulation of dimerization. For example in several families of transcriptional activators, the coiled-coil regions mediate dimerization, and the combinatorial rearrangement of factors by selective heterodimerization can direct the DNA binding regions to different DNA target regions (48).

It was already predicted that FEZ1 and FEZ2 contain conserved coiled-coil regions in the central region of their amino acid sequences. After the identification of 16 interacting proteins, we were interested in checking if the identified proteins would also possess coiled-coil regions in their sequences. It was no surprise to find that 12 of 16 interacting proteins were predicted to contain one to several coiled-coil regions in their sequences (Table 1). Furthermore, 11 of these 12 proteins had at least one coiled-coil region in the identified interacting protein fragments encoded in the sequenced cDNA region.

These results seemed to suggest that the coiled-coil region of FEZ1 is both necessary and sufficient for the majority of observed interactions. However, when we tested deletion construct FEZ1-(269–392), which lacks the coiled-coil region, in the two-hybrid system against all interacting proteins, we were able to identify three groups of proteins (Table 2).

The first group of nine proteins only interacts with FEZ1 when the latter contains its coiled-coil region (KIBRA, DRAP1, CLASP2, Rab3 GAP, similar to short coiled-coil, Tlk2, FLJ13909, and bromodomain containing protein 1, similar to OAT-like).

The second group contains five proteins, which do not depend on the presence of the coiled-coil region in FEZ1, and still interacts with FEZ1 when the coiled-coil is absent (RAI14, Zinc Finger Protein 251, Bamacan, Programmed Cell Death 7, and SAP30L).

The third group contains FEZ1 and BAF60a and showed clone-dependent interaction with FEZ1 lacking the coiled-coil region. The FEZ1 prey clone, which contains the coiled-coil region, still interacted with the FEZ1 bait lacking the coiled-coil region, whereas the other FEZ1 prey clone, which lacks the coiled-coil region, did not interact with it. This suggests that for the homodimerization of FEZ1 to occur, at least one of the interacting molecules must have a coiled-coil region. On the other hand, this result seems to indicate that aside from the coiled-coil region, other protein regions are involved in the dimerization.

This interpretation finds certain support by the fact that two fragments of BAF60a (82–515 and 404–515), can both interact with FEZ1-(221–392), but only the longer one that contains the chromatin remodeling SWIB domain is able to interact with FEZ1-(269–392), which lacks the coiled-coil region. Thus, as with the FEZ1 dimerization, this result suggests that two independent regions of BAF60a interact with two different regions of FEZ1, whereas only one of the supposed contact points involves the coiled-coil region.

In summary, we performed a yeast two-hybrid assay using FEZ1-(221–392) as a bait, which resulted in the identification of 16 interacting proteins. Most of these confirm the role of FEZ1 as a regulatory protein involved in neuronal development, axonal outgrowth, and cellular transport. However, the fact that we were able to identify several nuclear proteins, six of which are involved in transcriptional regulation or chromatin-remodeling, could suggest that FEZ1 may also have nuclear regulatory functions. In the future, more detailed studies must test whether FEZ1 itself is a general or specific transcriptional regulator in human cells, identify which are the possible target genes that may be regulated by FEZ1, and analyze whether these genes could contribute to neuronal development and differentiation.
Characterization of FEZ1 Protein Interactions

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