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Proteínas da família FEZ (*Fasciculation and Elongation protein Zeta*) como adaptadoras bivalentes do transporte: aspectos funcionais, estruturais e evolutivos

Tese apresentada ao Instituto de Biologia da Universidade Estadual de Campinas para a obtenção do Título de Doutor em Biologia Funcional e Molecular, na área de Bioquímica

Orientador: Prof. Dr. Jörg Kobarg

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Índice

Agradecimentos v
Lista de Abreviações e Siglas viii
Resumo ix
Abstract x
1. Introdução 1
1.1. Descoberta do gene e relação com o desenvolvimento neuronal 1
1.2. FEZ1 e esquizofrenia 3
1.3. Relação com a maquinaria de transporte 4
1.4. Super-expressão de FEZ1: atividade anti-viral e núcleo com morfologia "*flower-like*" 7
1.5. Organização molecular de FEZ 9
2. Objetivos 11
2.1. Objetivos gerais 11
2.2. Objetivos específicos 11
3. Resultados 13
3.1. Artigo I – *FEZ1 Dimerization and Interaction with Transcription Regulatory Proteins Involves Its Coiled-coil Region* 15
3.2. Artigo II – *FEZ2 Has Acquired Additional Protein Interaction Partners Relative to FEZ1: Functional and Evolutionary Implications* 31
3.2.1. Artigo II – Material Suplementar 45
3.3. Artigo III – *Human FEZ1 Protein Forms a Disulfide Bond Mediated Dimer: Implications for Cargo Transport* 53
3.4. Manuscrito I – *Structural studies by SAXS and NMR of the FEZ1 dimer and the tetramer FEZ1 / SCOCO* 65
4. Discussão 84
4.1. Análise global da duplicação gênica de *unc-76* e do interactoma das proteínas homólogas 85
4.1.1. Duplicação gênica de *unc-76* originando *FEZ1* e *FEZ2* 85
4.1.2. Aspectos evolutivos: padrão de interação entre os homólogos 85
4.1.3. Padrão diferencial das interações entre FEZ1 e FEZ2 – tendências 87
4.1.4. Hipótese para a interação diferencial entre as homólogas 89
4.2. Análise Pontual do Interactoma 91
4.2.1. Parceiros de interação envolvidos com esquizofrenia 91
4.2.2. Parceiros de interação envolvidos no desenvolvimento do sistema nervoso 93
4.2.3. Parceiros de interação envolvidos com infecção viral 95
4.2.4. Parceiros de interação envolvidos com a maquinaria de transporte 96
4.3. Análise estrutural da homodimerização e da formação de heterotetrâmeros com implicações na função 97
4.3.1. FEZ1 como molécula adaptadora bivalente do transporte mediado por kinesinas 97
4.3.2. Bases moleculares da homodimerização de FEZ1 e fenótipo *flower-like* 98
5. Conclusões 99
6. Referências 101

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O que fizemos apenas por nós mesmos morre conosco.
O que fizemos pelos outros e pelo mundo permanece e é imortal.
Albert Pike

6xHis – cauda de 6 histidinas em proteínas recombinantes
APPL – *Amyloid precursor protein-like*
cDNA – *complementary DNA* (DNA complementar)
CLASP2 – *CLIP-associating protein 2*
CLIPs – *Cytoplasmic Linker Proteins*
DISC1 – *Disrupted-in-Schizophrenia 1*
DNA – *Deoxyribonucleic acid*
F-actina – actina filamentosa
FEZ – Fasciculation and Elongation protein Zeta (gene)
FEZ – *Fasciculation and Elongation protein Zeta* (proteína)
GST – *Glutathione S-transferase* (cauda das proteínas recombinantes)
HIV – *Human immunodeficiency virus*
JC virus – *John Cunningham Virus*
JIP – *c-Jun N-terminal kinase-interacting protein 1*
KHC – *Kinesin Heavy Chain*
KLC – *Kinesin Light Chain*
MK-801 – *Dizocilpine*
MLV – *Moloney Murine Leukemia Viruses*
mRNA – *messenger RNA*
mRNP – *mRNA/Protein complex*
NGF – *Nerve Growth Factor*
PAGE – *Polyacrilamide gel electrophoresis*
PKCζ – *Protein Kinase C Zeta*
PMA – *phorbol 12-myristate 13-acetate*
PPI – *protein-protein interaction*
RMN – Ressonância Magnética Nuclear
RNA – *Ribonucleic acid*
RNAi – *RNA interference*
SAXS – *Small Angle X-ray Scattering*
SCOCO – *Short Coiled-Coil protein*
SYD – *Sunday Driver protein*
TrKA – *neurotrophic tyrosine kinase, receptor*
unc – uncoordinated (gene)
UNC – *uncoordinated* (proteína)

Resumo

As proteínas humanas FEZ1 e FEZ2 (*fasciculation and elongation protein zeta*) são ortólogas da proteína UNC-76 de *C. elegans* e estão envolvidas no crescimento e na fasciculação dos axônios através de interações que envolvem kinesinas, mitocôndrias e vesículas sinápticas. Além disso, algumas evidências sugerem a participação de FEZ1 na etiologia da esquizofrenia, no ciclo viral, além da resistência à quimioterápicos. Sua estrutura intrinsecamente desordenada, com *coiled-coil* ao longo da sequência, pode contribuir para sua função. Nós exploramos a evolução molecular da família de proteínas FEZ com ênfase no ramo dos vertebrados. Através do perfil do interactoma comparado entre FEZ1 e FEZ2 de *Homo sapiens* e UNC-76 de *C. elegans* foi observado um padrão de conservação das interações proteína-proteína entre FEZ1 e UNC-76, que explicam a capacidade de *FEZ1* resgatar os defeitos causados por mutações em *unc-76* em nematoides, de acordo com o descrito por Bloom e colaboradores em 1997. Além disso, caracterizamos a interação entre FEZ1 e SCOCO (*short coil-coiled*) por SAXS (*Small Angle X-ray Scattering*). Essa interação já foi descrita previamente entre os seus ortólogos UNC-76 e UNC-69, que cooperam no crescimento axonal. Um estado de heterotetramérico foi observado, consistindo de duas moléculas GST-SCOCO interagindo com duas moléculas de 6xHis-FEZ1 dimerizadas. Por PAGE (*Polyacrylamide Gel Electrophoresis*, eletroforese em gel de poli-acrilamida), SAXS, Espectrometria de Massas e Ressonância Magnética Nuclear, constatamos que FEZ1 dimeriza envolvendo a formação de ponte dissulfeto. *In vivo*, este estado dimérico de forma covalente pode ser importante para o transporte mediado por kinesinas de proteínas ao longo dos microtúbulos. Assim, FEZ1 pode ser classificada como uma proteína adaptadora do transporte, dimérica e bivalente, essencial para o crescimento axonal e organização pré-sináptica normal e transporte de cargas. A agregação de novos parceiros de interação encontrada para a proteína FEZ2 poderia ser interpretada como aquisição de novas funções moleculares e pode ter ocorrido nos primeiros estágios da evolução dos cordados.

Abstract

The human proteins FEZ1 and FEZ2 (fasciculation and elongation protein zeta 1) are orthologs of the protein UNC-76 from *C. elegans*, involved in growth and fasciculation of axons, through interactions that involve kinesins, mitochondria and synaptic vesicles. Moreover, some evidence suggests involvement of FEZ1 in the etiology of schizophrenia, in addition to the viral cycle and resistance to chemotherapy. Its structure intrinsically disordered, with coiled-coil along the sequence, can contribute to its function. We have explored the molecular evolution of the FEZ protein family with emphasis on the vertebrata branch. Analyzing the interactome profile of the FEZ1 and FEZ2 from *Homo sapiens* and UNC-76 from *C. elegans* we observed a conserved pattern of protein-protein interactions among FEZ1 and UNC-76 that explain the ability of *FEZ1* to rescue the defects caused by *unc-76* mutations in nematodes, according to Bloom and co-workers in 1997. Furthermore, we characterized the interaction between FEZ1 and SCOCO (short coiled-coil protein) by SAXS (Small Angle X-ray Scattering). This interaction has been previously reported between their orthologs UNC-76 and UNC-69 that cooperate in axonal outgrowth. A heterotetrameric state was observed, which consists of two GST-SCOCO molecules attached to two FEZ1 molecules. By PAGE (Polyacrylamide Gel Electrophoresis), SAXS, Mass Spectrometry and Nuclear Magnetic Resonance we defined that FEZ1 dimerizes involving formation of disulfide bond. *In* vivo this covalent mediated dimeric state could be important for kinesin mediated protein transport along the microtubule. Thereby, FEZ1 may be classified as a dimeric and bivalent transport adaptor, essential to axon outgrowth and normal pre-synaptic organization and transport of cargoes. The aggregation of new interaction partners found for the FEZ2 protein could be interpreted as the acquisition of new molecular functions and may have occurred in the early stages of chordate evolution.

1.1. Descoberta do gene e relação com o desenvolvimento neuronal

A designação família de proteínas FEZ (*Fasciculation and Elongation protein Zeta*) foi inicialmente proposta por Bloom & Horvitz (1997) no primeiro estudo de caracterização funcional do gene de *C. elegans unc-76* (ortólogo de *FEZ1* humana). O gene *unc-76* foi descoberto através de uma triagem genética, onde *C. elegans* que apresentavam deficiências de locomoção possibilitaram a identificação de diversos genes, sendo então referidos como *uncs* (*uncoordinated*), muitos deles relacionados ao crescimento e fasciculação axonal[1]. O trabalho de Bloom & Horvitz demonstrou que o gene *unc-76* é essencial para o crescimento normal do axônio ao longo de superfícies neuronais, assim como para a normal fasciculação e adesão destes axônios no verme. A conservação da função do gene *unc-76* de *C. elegans* ao longo da história evolutiva começa também a ser explorada. Quando o gene humano *FEZ1* foi introduzido em células germinativas de linhagens de *C. elegans* mutados em *unc-76* não se observou mais o fenótipo de locomoção descoordenada, resgatando-se também a normalidade do crescimento e da fasciculação axonal (figura 1). Desta forma, a função e até mesmo a estrutura foram propostas como serem conservadas ao longo da evolução. Descreve-se neste trabalho pioneiro a presença de *FEZ2* no genoma humano, evidenciando-se duas cópias de genes da família FEZ em *H. sapiens* (*FEZ1* e *FEZ2*) e de apenas uma cópia do gene da família FEZ em *C. elegans* (*unc-76*)[1]. A função de *FEZ2* até a presente data permanece pobremente conhecida. Enquanto o mRNA de *FEZ1* é expresso exclusivamente no sistema nervoso, o mRNA de *FEZ2* é expresso ubiquamente nos tecidos, demonstrando que os membros da família FEZ possuem função não tão somente no sistema nervoso mas também em outros tecidos[1,2,3] (função até então desconhecida e que seria atribuída a *FEZ2*). Em relação à nomenclatura dos genes da família FEZ, de uma forma em geral, aqueles presentes em invertebrados são referidos como *unc-76* e os presentes em vertebrados como *FEZ1* e *FEZ2*.

Através dos estudos de Bloom & Horvitz as proteínas da família FEZ ganharam visibilidade em pesquisas sobre o desenvolvimento neuronal. Uma triagem em biblioteca de cDNA de cérebro de rato através da técnica de duplo híbrido em levedura com o domínio regulatório da PKCζ (*protein kinase C zeta*), visando identificar parceiros de interação para esta cinase também importante para o desenvolvimento neuronal, identificou-se FEZ1 como parceira de interação assim como substrato desta cinase[4]. Kuroda e colegas (1999) demonstraram também que FEZ1 é translocada da membrana plasmática para o citoplasma quando da ativação da PKCζ em células renais COS-7. A ativação de PKCζ é requerida

para a diferenciação neuronal de células PC12, oriundas de glândula adrenal de rato, induzidas com NGF (*Nerve Growth Factor*)[5]. O NFG também é capaz de estimular a síntese do mRNA de *FEZ1*[6]. Células PC12 são estimuladas para diferenciação neuronal com NGF, contudo, a diferenciação, com a elongação do neurito, não ocorre quando o gene de *FEZ1* é silenciado por RNAi. Assim, pode-se estipular que a função da proteína FEZ1 pode estar relacionada com a via estimulada por NGF e ativação da PKCζ para a diferenciação neuronal[6], corroborando com os níveis de expressão do seu mRNA em camundongos: durante a fase embrionária do camundongo, o nível de expressão do mRNA de FEZ1 aumenta progressivamente durante o desenvolvimento cerebral, com um nível máximo 11 dias pós-coito[2,7], correspondendo ao período de crescimento axonal – de 9,5 a 12,5 dias[8].

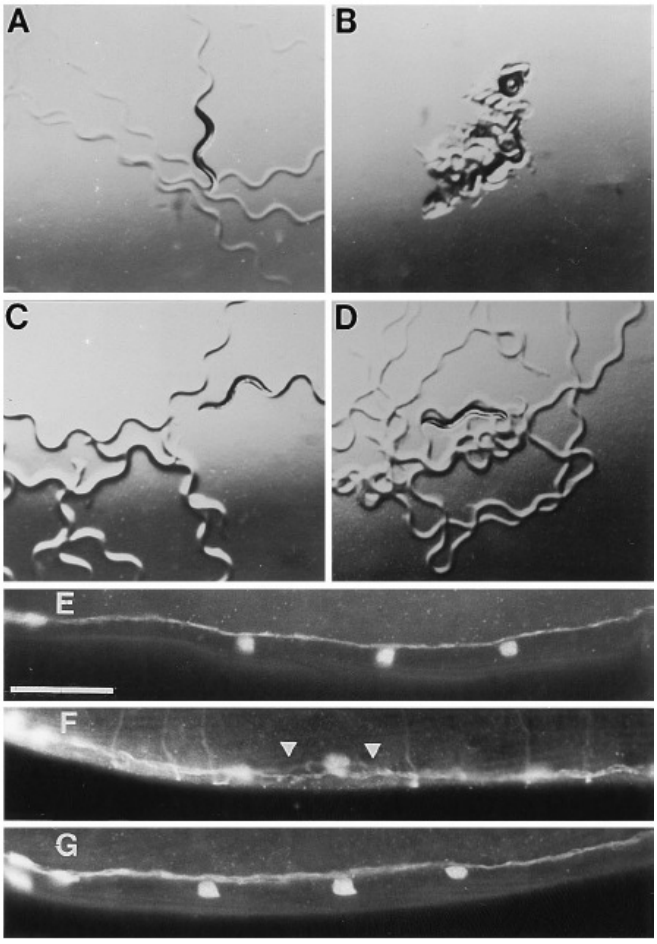

Figura 1. Restauração da locomoção e fasciculação do cordão nervoso ventral em vermes mutados em *unc-76* por *FEZ1*. (A – D) Animais foram fotografados (detectando-se as distâncias percorridas e os movimentos corporais) no período de 1 hora em placa contendo ágar; (A) tipo selvagem; (B) mutado em *unc-76*; (C) mutado em *unc-76* complementado com o gene de *C. elegans unc-76*; (D) mutado em *unc-76* complementado com o gene humano *FEZ1*. (EG) Cortes histológicos de cordões nervosos ventrais posteriormente localizados à cabeça de adultos marcados com anti-GABA. A porção anterior está à esquerda. (E) Tipo selvagem; (F) mutado em *unc-76*; (G) mutado em *unc-76* e complementado com o gene humano *FEZ1*. Pontas de seta indicam as regiões de desfasciculação. (Barra: 5 μm.). Reproduzido de Bloom & Horvitz (1997)[1].

1.2. FEZ1 e esquizofrenia

Embora mutações no gene *unc-76* em *C. elegans* e em *D. melanogaster* evidenciarem fenótipos de comprometimento locomotor e desenvolvimento neuronal[1,9], camundongos *knockout* para *FEZ1* não apresentaram quaisquer anomalias em relação à morfologia cerebral nem ao crescimento de axônios, tampouco comprometimento da função motora[3]. Contudo, vários testes comportamentais revelaram um fenótipo caracterizado por hiperatividade mediante condições de interações sociais em novos ambientes ou de estresse. A hiperatividade é um proeminente endofenótipo de muitas condições psiquiátricas, incluindo a esquizofrenia. Alguns psico-estimulantes são utilizados para o estudo da esquizofrenia por mimetizarem seus sintomas, como o MK-801[10]. Os camundongos *knockout* para *FEZ1* exibiram uma maior sensibilidade a este psico-estimulante em relação ao *wild-type*, apresentando uma maior hiperatividade locomotora após sua administração. O mesmo foi observado quando da administração da metanfetamina. Uma maior atividade locomotora é associada com o aumento da transmissão dopaminérgica. Através de microdiálise *in vivo* do *nucleus accumbens* verificou-se níveis aumentados de dopamina após a administração de metanfetamina nos camundongos *knockout* para *FEZ1* em relação ao *wild type*. Assim, supõe-se que a deficiência em *FEZ1* acarreta em maiores níveis de dopamina no *nucleus accumbens*.

Trabalhos anteriores a este já vinham correlacionando a proteína FEZ1 com esquizofrenia, como o que descreve sua interação com a proteína DISC1[11]. O gene *DISC1* é geneticamente ligado à esquizofrenia[12,13]. A descoberta veio de uma análise de *linkage* em uma família escocesa com uma infinidade de doenças mentais, onde fora observada a translocação no gene *DISC1* em vários membros desta família. Esta translocação resulta em uma proteína truncada, que perdeu a região C-terminal, e não é mais capaz de interagir com FEZ1[11]. FEZ1 e DISC1 co-localizam-se em cones de crescimento de culturas de neurônios do hipocampo, onde elas interagem com F-actina[11,14]. FEZ1 e DISC1 também cooperam para a diferenciação neuronal de células PC12[15]. Assim, a interação molecular de FEZ1 com DISC1 sugere uma estreita relação com a patologia neuronal. Evidências genéticas independentes para *FEZ1* como gene de susceptibilidade à esquizofrenia são fracas, com apenas um de três estudos[16,17,18] relatando uma significância nominal de dois marcadores polimórficos em uma população do Japão[18]. Os outros dois estudos relataram associações negativas para *FEZ1* em populações japonesa, caucasiana norte-americana e afro-americana[16,17,18]. Embora as análises de SNP (*single nucleotide polymorphism* – polimorfismos de nucleotídeo único) sejam controversas, um outro estudo demonstra que a expressão do mRNA de *FEZ1* (assim como a de outros parceiros de interação de DISC1) é significativamente reduzida em amostras de cérebro pós-morte de indivíduos com esquizofrenia[19,20].

A exata função da interação entre DISC1 e FEZ1 no estado normal e no patológico ainda não é bem compreendida. Contudo, dois novos estudos direcionam a discussão para a sinalização via receptores de dopamina, centrossomo e formação do cílio primário. Marley e colegas (2010)[21] demonstraram que DISC1 é essencial para a formação do cílio primário e que ela também está localizada na região pericentriolar (região localizada na base do cílio e intimamente envolvida na sua formação). Mostraram também, pela primeira vez, a presença de receptores dopaminérgicos, dentre outros já demonstrados, nesta estrutura celular. É importante salientar que mutações em algumas classes de receptores dopaminérgicos também são correlacionadas à etiologia da esquizofrenia. Também em 2010, um trabalho publicado pelo nosso grupo, a co-localização centriolar ou pericentriolar de FEZ1 com outras proteínas com as quais interage torna-se evidente. Esta co-localização é interrompida após a ativação de PKC por PMA[22]. Os dados são congruentes com o modelo de camundongo *knockout* para *FEZ1*, onde há maiores níveis de dopamina após estimulação com metanfetamina, de tal forma que, se FEZ1 atua na mesma via que DISC1, provavelmente, sua função também está relacionada com a formação do cílio primário e presença de receptores dopaminérgicos nesta estrutura. Pode-se especular que camundongos deficientes para *FEZ1* apresentaram alguma alteração de receptores dopaminérgicos no cílio primário, o que poderia explicar o aumento de dopamina na região extra-celular destes animais, mas experimentos necessitam ser realizados para se comprovar esta hipótese.

1.3. Relação com a maquinaria de transporte

Foi a partir do trabalho de Gindhart e colegas (2003) que a função das proteínas da família FEZ passou a ser relacionada com a maquinaria de transporte intracelular, utilizando-se *Drosophila melanogaster* como modelo[9]. A partir de uma triagem em busca de parceiros de interação para o domínio cauda da cadeia pesada da proteína motora kinesina-I (KHC – *kinesin heavy chain*), em uma biblioteca de cDNA de embrião de *D. melanogaster*, a proteína UNC-76 foi identificada como parceira de interação. A proteína motora kinesina-I move-se em direção *plus* do microtúbulo e facilita o movimento de vesículas, ribonucleoproteínas mensageiras (mRNP - *messenger ribonucleoproteins*) e organelas, sendo essencial para o transporte em tipos celulares muito alongados (como neurônios)[9,23]. Em *C. elegans*, mutações no gene *unc-116* (gene homólogo à KHC) também foram relacionadas com deficiências de locomoção e desenvolvimento normal dos axônios nestes vermes, assim como mutações em regiões não traduzidas do gene de *unc-76* resultaram em letalidade para larvas de *D. melanogaster* na transição do segundo para o terceiro *instar* e duplicações do gene foram capazes de complementar o gene mutado. Ainda, mutações

em outras proteínas que interagem com a cadeia leve da kinesina 1 (*Amyloid precursor protein-like* [APPL] e *Sunday driver* [SYD]) acarretaram no mesmo fenótipo que mutações em *unc-76*, que se caracterizava por interrupção do transporte axonal em nervos de segmentos larvais de *D. melanogaster*, evidenciada por surgimento de agregados ligados à membranas[9].

Diante destes achados, começa-se o estabelecimento de um modelo no qual a interação entre kinesina-1 e UNC-76 seria essencial para o transporte intracelular durante o desenvolvimento neuronal. Este modelo é reforçado através da inibição por RNAi da expressão de *FEZ1*, em neurônios hipocampais de rato, resultando em falha no estabelecimento da polaridade desta linhagem celular (mais de 60% das células). Os defeitos na polarização das células foram correlacionados com deficiências na morfologia e na distribuição, com retardo na velocidade do movimento anterógrado, de mitocôndrias. A mitocôndria é proposta então como uma primeiro carga descrita à qual a proteína FEZ1 poderia estar associada, relacionando a proteína FEZ1 a uma possível função de proteína adaptadora do transporte mediado por kinesina[15].

A proteína kinesina 1 quando não está transportando cargas é mantida numa conformação estrutural inativa, através de um mecanismo de auto-inibição da região motora N-terminal pela região cauda C-terminal. A interação de FEZ1 com KHC e de JIP (*JNK Interacting Protein*) com KLC (*Kinesin Light Chain*) simultaneamente foram capazes de liberar a kinesina 1 do estado auto-inibitório, permitindo sua associação com o microtúbulo e a execução de sua função motora. A proteína FEZ1, portanto, não atuaria tão somente como uma proteína adaptadora no transporte mediado por kinesina, mas também teria uma função essencial no mecanismo de ativação desta proteína motora[24].

A regulação de todo o sistema relacionado à maquinaria de transporte é essencial para o desenvolvimento de células altamente polarizadas, como os neurônios. Neste tipo celular uma importante adição de membrana deve ocorrer para que a célula desenvolva axônios e dendritos. Contudo, a composição de membranas e proteínas entre as diversas regiões de um neurônio é diferente. A entrega de membranas e receptores demanda de uma intensiva rede de transporte pós-Golgi. A coordenação de todo o processo ocorre na classificação de proteínas (*sorting*), vesiculação, transporte ao longo do citoesqueleto e fusão de vesículas. Muitos estudos demonstram que a cinase UNC-51 auxilia na distribuição de receptores essenciais para a orientação da extensão do axônio. O NGF estimula a poli-ubiquitinação de UNC-51, permitindo sua associação com o TrkA, que é o receptor do NGF, e com p62, que interagem com PKC atípicas (como a PKCζ), e é requerida para a internalização do TrkA. Proteínas da família FEZ (UNC-76 e FEZ1), como já mencionado, se ligam a kinesinas e contribuem para o transporte de precursores de vesículas sinápticas. A proteína de *D. melanogaster* DUnc-51 se liga e fosforila DUnc-76. DUnc-76 fosforilada se liga diretamente à proteína de vesícula sináptica

Synaptotagmin-1[25], sugerindo que proteínas adaptadoras adquirem especificidade por ligarem-se diretamente à cargas de um modo que pode ser regulado pós-traducionalmente (figura 2)[26].

Destaca-se a via de sinalização mediada por NGF, com posterior ativação da PKCζ, influenciando na montagem/regulação dos elementos da maquinaria de transporte através de modificações pós-traducionais dos adaptadores do transporte. Proteínas da família FEZ emergem como importantes adaptoras para o processo de crescimento axonal, podendo auxiliar na composição diferencial dos elementos transportados pela maquinaria de transporte, em especial no transporte mediado por kinesinas, o que pode refletir na composição dos elementos de membrana importantes para o desenvolvimento neuronal, assim como na distribuição de receptores. Observa-se assim uma consonância entre a importância dos receptores na esquizofrenia, a influência de sua distribuição por DISC1 e a participação de FEZ1 no processo.

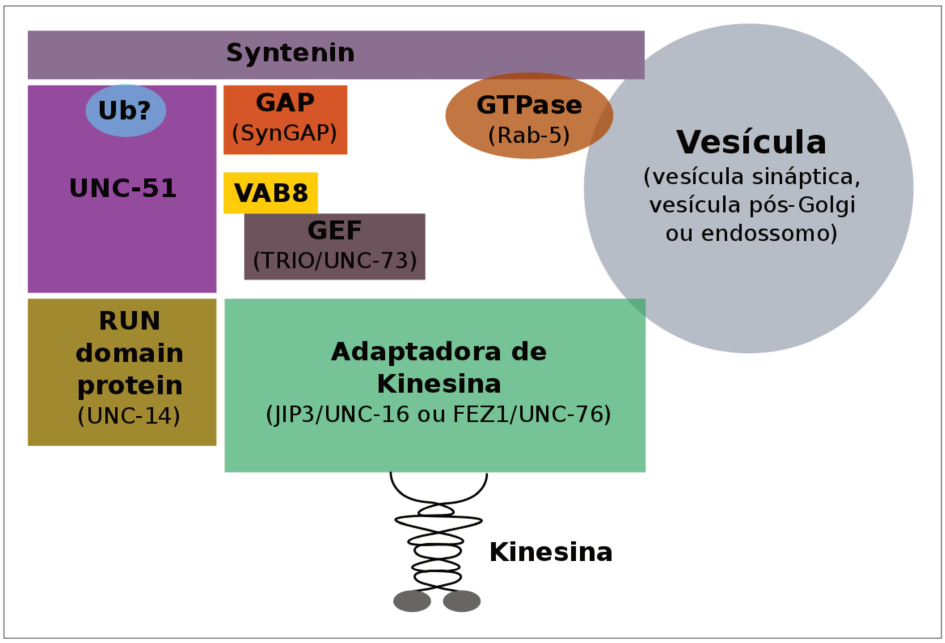

Figura 2. UNC-51 medeia a formação de vesículas e dos transportes em crescimento de neuritos. Esquema generalizado das conexões entre UNC-51 e seus parceiros de ligação. UNC-51, direta ou indiretamente, interage com proteínas de domínio RUN e proteínas adaptadoras, tais interações provavelmente ajudam a ligar a proteína motora kinesina em vesículas. Proteínas que servem de plataforma, tais como *Syntenin*, estabelecem contatos entre UNC-51 e Rab GTPases-5 e vesículas, por exemplo. A ubiquitinação de UNC-51 pode regular o complexo, assim como a proteína adaptadora FEZ1. Figura adaptada de Sann e colegas (2009)[26].

1.4. Super-expressão de FEZ1: atividade anti-viral e núcleo com morfologia "*flower-like*"

A função de FEZ1 relacionada à maquinaria de transporte também é destacada ante sua atividade anti-viral quando de sua super-expressão. Em 2005, Suzuki e colegas identificaram FEZ1 como parceira de interação da agnoproteína do vírus JC[27]. A agnoproteína é uma proteína viral expressa na fase tardia da infecção, sendo importante para a liberação da progênie de virions do núcleo para a superfície celular[28]. Os resultados obtidos por Suzuki demonstraram que tanto a agnoproteína quanto a proteína FEZ1 têm a capacidade de interagir com microtúbulos e que a agnoproteína é capaz de dissociar FEZ1 destes elementos, de forma a inibir a extensão de neuritos em células PC12. Contudo, células super-expressando FEZ1 foram resistentes à infecção pelo vírus JC. O número de células infectadas pelo vírus JC não foi diferente após 3 dias de inoculação entre células estavelmente expressando FEZ1 e células controle. Após 7 dias da inoculação a proporção já é alterada. O número de células positivas para VP1 (proteína do capsídeo viral) foi muito menor em células expressando FEZ1, indicando que FEZ1 influencia na fase tardia da infecção. Outro achado importante refere-se à localização intracelular de VP1, que foi presente tanto no núcleo quanto no citoplasma de células controle e restrita ao núcleo em células super-expressando FEZ1, demonstrando que FEZ1, quando super-expressa, prejudica a função da agnoproteína no transporte dos virions, provavelmente por favorecer o transporte de elementos neuronais ao invés de elementos virais pela agnoproteína. Linhagens celulares permissivas à infecção pelo vírus JC (SVG-A ou IMR-32) apresentaram um nível de expressão de FEZ1 muito menor do que células neuronais não permissivas (SH-SY5Y) e ensaios de RNAi comprovaram a eficiência de FEZ1 em sua atividade anti-viral[27].

Ao passo que o vírus JC possui DNA dupla-fita circular, MLV (*Moloney murine leukemia virus*) é um retrovírus. Linhagens de fibroblastos de rato R3-2 e R4-7 foram isoladas por serem resistentes à infecção por MLV (100 e 1000 vezes, respectivamente) e apresentaram um nível de expressão de FEZ1 trinta vezes mais elevado do que em células permissivas. Os níveis de expressão do mRNA de *FEZ1* em células HEK293 e Rat2 transfectadas com *FEZ1* também foram correlatos ao grau de resistência. Nas células resistentes à infecção viral e com super-expressão de FEZ1 observou-se a normalidade dos níveis de síntese de DNA viral, mas uma reduzida formação do DNA circular, indicando que FEZ1 atuaria após a transcrição reversa, mas antes da entrada no núcleo do genoma viral. Desta forma, propõe-se que FEZ1 teria a habilidade de bloquear o transporte do DNA viral para o núcleo[29]. Basicamente através da mesma estratégia, verificou-se que a super-expressão de FEZ1 em cultura de células primárias de macrófagos cerebrais (CHME3) bloqueia a infecção pelo HIV (*human immunodeficiency virus* – vírus da imunodeficiência humana) [30].

Muitos genes que podem estar envolvidos na etiologia da esquizofrenia estão implicados também no ciclo de patógenos, como vírus. DISC1, por exemplo, controla a rede de microtúbulos que é utilizada por vírus como rota para o núcleo. Anteriormente, como citado, a própria FEZ1 pode estar envolvida no ciclo viral. As vias traçadas por genes de susceptibilidade à esquizofrenia dizem respeito tanto às redes de sinalização intrínsecas quanto às vias usadas especificamente por patógenos para comandar a fisiologia da célula hospedeira. Os genes e proteínas do hospedeiro e patógeno, juntos, formam uma rede transgenômica integrada que pode reger o risco de se desenvolver esquizofrenia[31].

Outro aspecto relacionado à super-expressão da proteína FEZ1 é a formação de um núcleo multi-lobulado (fenótipo "*flower-like*"). O fenótipo *flower-like* ocorre em decorrência da atuação de FEZ1 nos microtúbulos, e possivelmente é causado pela formação de *loops* de microtúbulos mediados pelo excesso de FEZ1, que constringem o núcleo, formando lóbulos. Este fenótipo é observado células presentes em alguns tipos de leucemia e está relacionado à resistência à quimioterápicos (figura 3)[32].

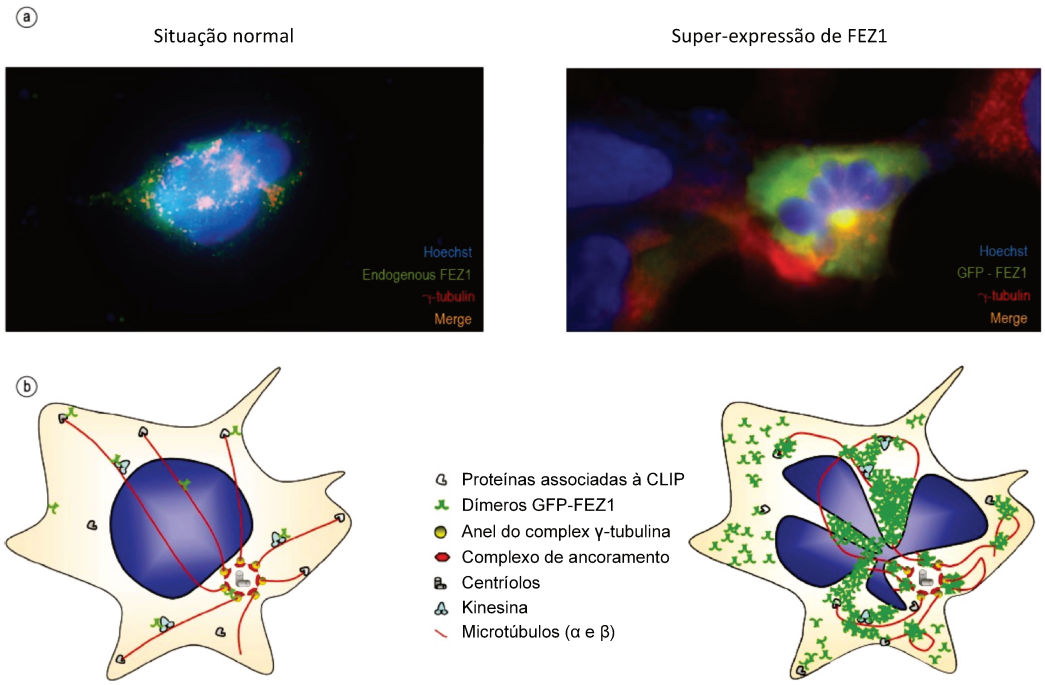

Figura 3. Fenótipo *flower-like* e reorganização do citoesqueleto causados pela super-expressão de GFP-FEZ1. A) Direita: GFP-FEZ1 (verde) co-localiza (amarelo) com gama-tubulina (vermelho) no centro organizador de microtúbulos (MTOC) em células HEK293 com núcleo *flower-like*. Esquerda: células HEK293 normais (sem super-expressão de FEZ1) para comparação. B) Representação esquemática hipotética sobre como a super-expressão de FEZ1 acarretaria na formação do núcleo *flower-like*. Painel da esquerda: célula normal. Painel da direita: célula super-expressando GFP-FEZ1, que seria capaz de interagir com vários componentes do citoesqueleto (tubulina, CLASP2) e consigo própria. Sua super-expressão possibilitaria a formação de pontes artificiais entre os microtúbulos com consequente formações de *loops*. A associação com CLASP2, localizada na ponta dos microtúbulos, poderia promover interações entre gamma-tubulina nos MTOCs, promovendo a formação dos *loops*. O encurtamento dos loops poderia promover a constrição do núcleo, causando a formação do fenótipo *flower-like*. Figura adaptada de Lanza e colegas (2007)[33].

1.5. Organização molecular de FEZ

As proteínas da família FEZ, de uma forma em geral, podem ser classificadas como intrinsecamente desestruturadas. Além de dados obtidos através de softwares de predição de organização estrutural, experimentos de SAXS com a proteína FEZ1 e dicroísmo circular, evidenciaram seu caráter intrinsecamente desestruturado. Dados de SAXS demonstraram também que FEZ1 dimeriza pela região N-terminal, de forma permissiva à projeção da região C-terminal[34] (figura 4). A região C-terminal da proteína FEZ1, e das proteínas da família FEZ em geral, possui regiões com significativa propensão à formação de estruturas em *coiled-coils* e que estão relacionadas com a maioria das interações proteína-proteína aqui relatadas, com exceção das tubulinas, que interagem no extremo C-terminal, em uma região conservada, mas sem predição de *coiled-coil* [27].

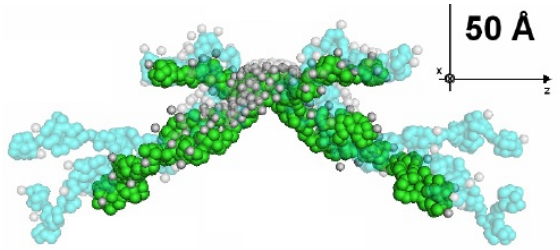

Figura 4. FEZ1 dimeriza pelo N-terminal expondo as regiões C-terminais com *coiled-coil*. Modelos baseados em dados de SAXS das proteínas 6xHis-FEZ1 (1-227) (verde) e 6xHis-FEZ1 (1-392) (azul). As esferas cinzas representam moléculas de água, presentes em regiões com elevado índice de aminoácidos ácidos.

2.1. Objetivos gerais

Estudar a estrutura e função das proteínas da família FEZ, correlacionando os dados com uma abordagem evolutiva.

2.2. Objetivos específicos

- Identificar proteínas parceira de interação (PPI – *protein-protein interaction*) de FEZ1 e FEZ2 humanas. Confirmar as PPIs identificadas com FEZ1 e FEZ2 humanas e UNC-76 de *C. elegans*, visando padrões de interação entre os homólogos que explicassem os fenótipos observados nos animais mutantes e *knockouts*.

- Avaliar a capacidade de FEZ1 se ligar a duas proteínas/cargas ao mesmo tempo para confirmar a hipótese de proteína adaptadora bivalente

- Definir os mecanismos bioquímicos envolvidos em sua dimerização pela região N-terminal.

3. Resultados

Os resultados que conduzem à discussão final da presente tese serão apresentados na forma de 3 artigos científicos publicados e 1 manuscrito.

O primeiro artigo, intitulado "*FEZ1 Dimerization and Interaction with Transcription Regulatory Proteins Involves Its Coiled-coil Region*", descreve, através de técnicas de duplo híbrido em levedura e co-preciptação *in vitro*, 16 novas interações proteína-proteína utilizando-se como isca a proteína FEZ1 (221-392) humana no processo de triagem em biblioteca de cDNA de cérebro fetal humano. Estas interações foram confirmadas com FEZ2 (207-353) humana, muitas delas relacionadas com o processo transcricional. Neste artigo, demonstra-se também a homodimerização de FEZ1 pela região C-terminal, também por duplo-híbrido em levedura e co-preciptação *in vitro*.

O segundo artigo, intitulado "*FEZ2 Has Acquired Additional Protein Interaction Partners Relative to FEZ1: Functional and Evolutionary Implications*", sugere, através de análises filogenéticas, que o gene *unc-76* duplicou-se no ramo dos cordados, originando *FEZ1* e *FEZ2*. Através da técnica de duplo híbrido em levedura, 59 interações proteína-proteína foram identificadas utilizando-se como isca a proteína FEZ2 (207-353) humana no processo de triagem (sendo 8 destas já descritas no artigo anterior). Verificou-se a interação destas 59 proteínas com FEZ1 (221-392) humana e UNC-76 (242-378) de *C. elegans*. Destas 59, 40 interações foram confirmadas para FEZ1 e UNC-76, restando 19 específicas para FEZ2. Acredita-se que muitas das proteínas descobertas interagindo com proteínas da família FEZ sejam cargas desta. Teorias sobre possível ganho e manutenção de função são discutidas.

O terceiro artigo, intitulado "*Human FEZ1 Protein Forms a Disulfide Bond Mediated Dimer: Implications for Cargo Transport*", através de técnicas de gel não-desnaturante, SAXS (*Small Angle X-ray Scattering*), espectrometria de massas e análises por SDS-PAGE seguido por *western blot* de proteína endógena de células HEK293, demonstra que FEZ1 homodimeriza pelo N-terminal através de ponte dissulfeto envolvendo a cisteína 133, além da homodimerização pela região C-terminal. Neste artigo, demonstrou-se também que a proteína FEZ2 pode dimerizar pelo N-terminal através de ponte dissulfeto. A natureza covalente da homodimerização pode ser crucial nos processos de transporte de cargas e formação no núcleo multi-lobulado com morfologia *flower-like*.

O manuscrito, intitulado "*Structural studies by SAXS and NMR of the FEZ1 dimer and the tetramer FEZ1 / SCOCO*", através de co-purificação e SAXS, demonstra a natureza heterotetramérica da proteína FEZ1 complexada com a proteína SCOCO. Refinando o modelo, análises por RMN (Ressonância Magnética Nuclear) indicam que a homodimerização de FEZ1 ocorre de forma paralela. Em conjunto, estes dados demonstram que proteínas da família FEZ poderiam atuar como adaptadoras bivalentes no processo de transporte, interagindo com 2 cargas simultaneamente.

3.1. Artigo I

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

Assmann EM, Alborghetti MR, Camargo MER, Kobarg J

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

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The fasciculation 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 paralogue FEZ2 are proteins that interact with NEK1, a protein kinase involved in polycystic kidney disease and DNA repair mechanisms at the G_2/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, DRAP1, 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 (fasciculation and elongation protein ζ -1) was initially identified as a mammalian orthologue of the *Caenorhabditis elegans* UNC-76 protein, 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 C ζ (PKCζ)[4] (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-schizophenia 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 paralogue 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), intracellular 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.

ronal development, possibly through its involvement in intracellular transport processes. However, our new finding that FEZ1 interacts with nuclear proteins, six of which are functionally involved in transcription regulation and chromatin remodeling, opens the intriguing new possibility that FEZ1 may in addition to the previous predicted functions also have regulatory functions in the nucleus.

EXPERIMENTAL PROCEDURES

Plasmid Constructions—Several sets of oligonucleotides were designed for PCR amplification of complete FEZ1 or deletion constructs thereof, which were then inserted in vector pBTM116 in fusion with the LexA DNA binding domain or in vectors pACT2 or pGAD424 (Clontech) in fusion with the Gal4 activation domain (Fig. 1*A*). The nucleotide sequence coding residues 207–353 of human FEZ2 were PCR-amplified using a specific primer set, 5′-AGGAATTCGGCAGTTATGAAGAGAGAGTG-3′ and 5′-AGGTCGACGTTACACTCTCTCTTCATAACT-3′, and then cloned into EcoRI and SalI 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 URA3*::(*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 autoactivate 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-amino-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 sequencer 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 Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM $MgCl_2$, 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-transfected 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 XhoI 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. 1*A*), containing FEZ1-(131–392), was subcloned into expression vector pProExHTb (Invitrogen) using the restriction sites EcoRI and XhoI, 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 Na_2HPO_4, 1.8 mM KH_2PO_4, 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 Na_2HPO_4, 1.8 mM KH_2PO_4, 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 $Na_2HPO_4 \cdot 7H_2O$, 1.8 mM KH_2PO_4, 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

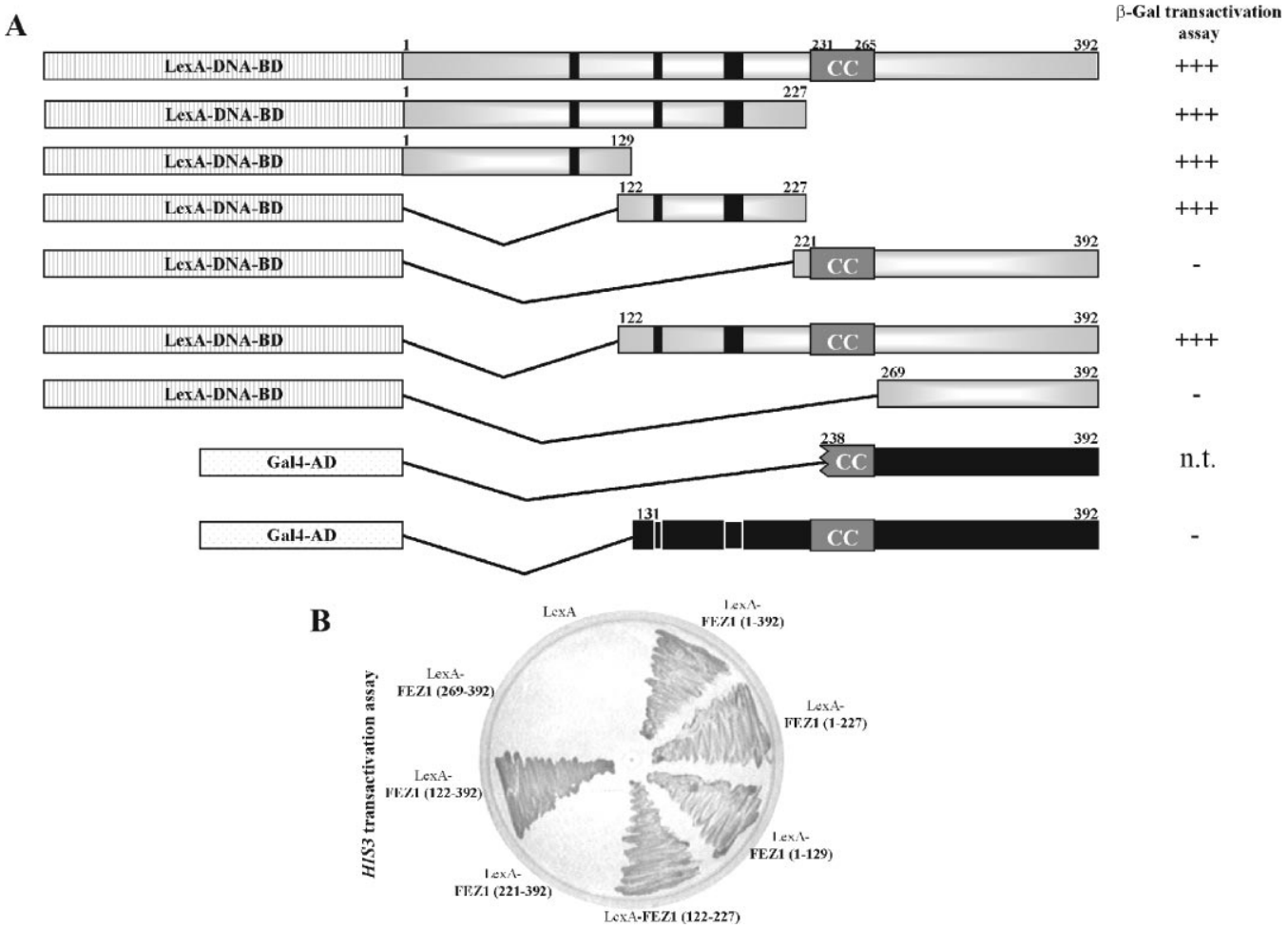

FIGURE 1. **Mapping of the FEZ1 deletion constructs for autoactivation in the yeast two-hybrid system.** *A,* schematic representation of full-length human FEZ1 and different truncation constructs of the proteins fused to either the LexA DNA binding domain in plasmid pBTM116 or the Gal4 activation domain in plasmid pACT2. The construct FEZ1-(131–392) was isolated from a human fetal brain cDNA library in a yeast two-hybrid assay with the regulatory domain of NEK1 protein used as a bait in a previous study of our group (9). FEZ1 contains a coiled-coil region (*gray box* marked *CC*) predicted to span residues 231–266. In its N-terminal region FEZ1 contains three motifs each consisting of 4–8 glutamic acid residues (*black boxes*), which are partially conserved in FEZ2 (see also Fig. 3). *B,* results of the test for autoactivation of the reporter gene activation by different FEZ1 constructs. The L40 yeast strain was transfected with the indicated pBTM116-FEZ1 vector constructs and plated onto minimal medium without tryptophan and histidine. This test was performed to assay the capacity of these FEZ1 constructs to autoactivate the reporter genes. As a negative control we used the empty vector pBTM116. The presence of the plasmid in the L40 cells was verified by growth on plates lacking tryptophan alone (not shown). *n.t.,* not tested.

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 in fusion with GST, and fusion proteins were expressed in *E. coli* BL21 (DE3) cells at 37 °C using 0.5 mM isopropyl 1-thio-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 Na_2HPO_4, 1.8 mM KH_2PO_4, 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-

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

Protein interacting with FEZ1C (aliases)	Coded protein residues (complete sequence/retrieved)	Accession no.	Domain composition (native protein)[a]	Function[b]	Ref.
DRAP1 (NC2α)	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	AAH44925	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	AAH06258	ZnF C2H2	Transcriptional control?	18
Bamacan (CSPG6/SMC3)	1217/881–1217	NP_005436	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 (Zygin I)	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[c]
Programmed Cell Death 7 (ES18)	485/272–430	NP_005698	Coiled-coil regions	Apoptosis	35
Similar to short coiled-coil protein	115/1–115	AAH16511	Coiled-coil region	Unknown	18
Hypothetic Protein FLJ13909	266/50–266	AAH18719		Unknown	18
Similar to OAT-like 1	366/258–366	AAH26050	TBC domain	Transaminase activity	18

[a] Other domains may be present.
[b] Other functions may be known.
[c] 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.

(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 6xHis-FEZ1 fusion proteins, 6xHis-FEZ1-(1–392), 6xHis-FEZ1-(1–227), or 6xHis-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 6xHis-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 a 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 *HIS3* and *lacZ* reporter genes (Fig. 1*B*). The construction FEZ1-(221–392) was chosen for the screening of the 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 could be a transcriptional activation protein, and its putative transcriptional activation domain located at its N terminus seems to have a conserved function in respect to the activation of the reporter genes in yeast. It is noteworthy that the three glutamic acid-rich motifs are found in the N-terminal region of FEZ1 (Fig. 3*B*), the second and third of which are partially conserved also in FEZ2.

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)

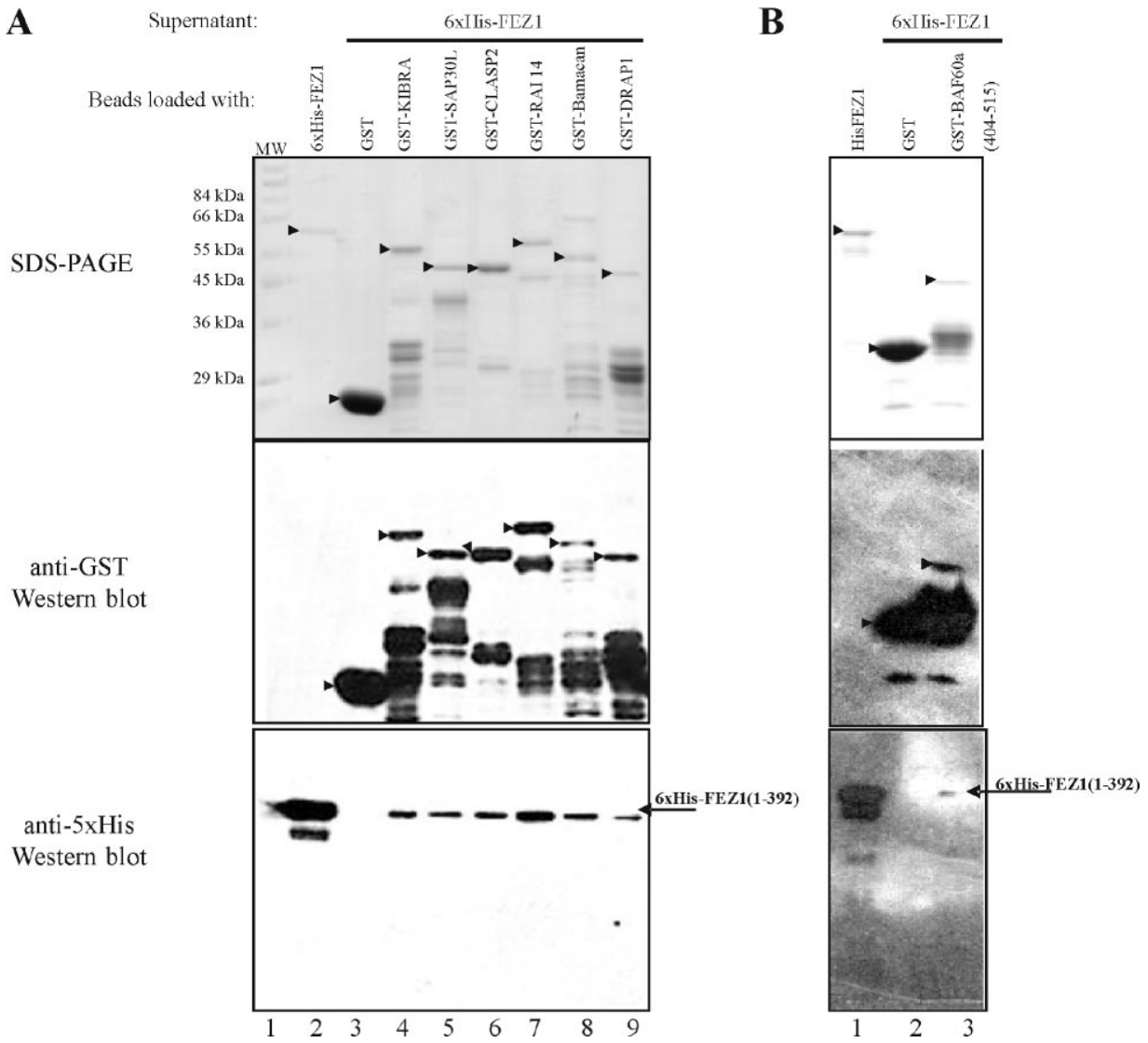

FIGURE 2. ***In vitro* confirmation of the interaction between FEZ1 and selected proteins retrieved in the yeast two-hybrid screen.** *A, in vitro* pull-down assays between full-length 6xHis-FEZ1 and the protein fragments of KIBRA, SAP30L, CLASP2, RAI14 isoform, Bamacan, and DRAP1 all expressed as GST fusion proteins (see Table 1 for details). *B*, BAF60a-(404–515) retrieved from the human fetal brain cDNA library and subcloned in bacterial expression vector for its expression as a GST fusion. Free GST control protein or the indicated GST fusion proteins were loaded on glutathione-Sepharose beads, and after washing the beads they were incubated with the purified full-length 6xHis-FEZ1 fusion protein. After high stringency washes using PBS/Triton X-100, the samples were loaded in three replicate SDS-polyacrylamide gels. One was stained with Coomassie Blue, and the other two were transferred to PVDF membranes that were then developed with either monoclonal mouse anti-GST antibody 5.3.3 (anti-GST Western blot) or a mouse anti-5xHis-tag antibody (anti-5xHis Western blot) for detection of GST- or His-tagged fusion proteins, respectively. All proteins tested interacted strongly with 6xHis-FEZ1, except free GST, used as a control protein. The *arrows* indicate the positions of the proteins indicated above the lanes in the SDS-PAGE and anti-GST Western blot.

was used as bait, and a total of about 1.5×10^6 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 another 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 β-galactosidase filter assay had their plasmid DNA extracted and sequenced. A total of 101 plasmid DNAs from clones positive for both *HIS3* and *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 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 NC2α (negative cofactor 2, subunit α), 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).

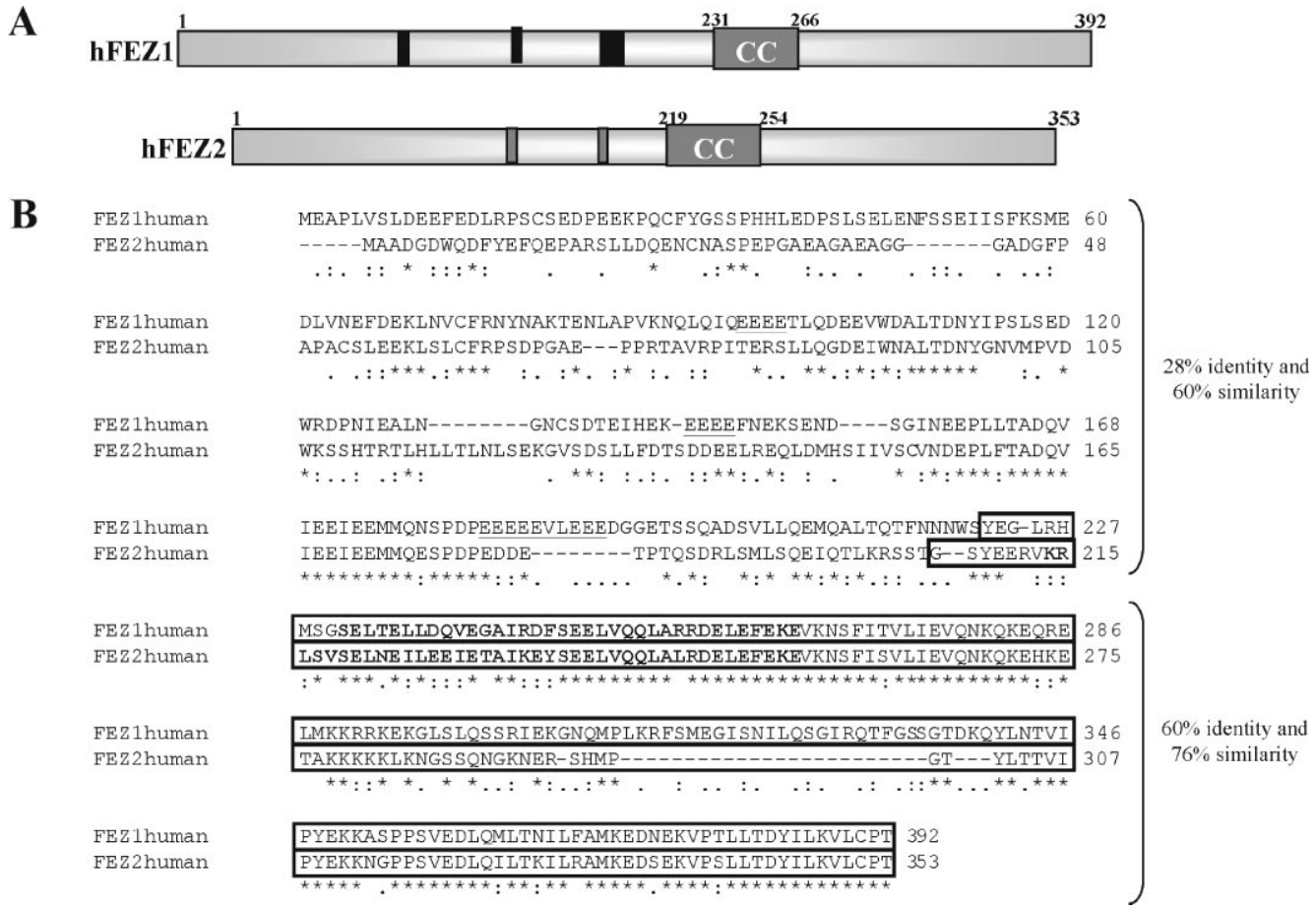

FIGURE 3. **Amino acid sequence alignment and domain organization of the human proteins FEZ1 and FEZ2.** *A,* predicted structural organization of human proteins FEZ1 and FEZ2. In FEZ1 the *black rectangles* show three glutamic acid-rich regions, which are partially conserved in FEZ2 (*gray rectangles*). The *gray boxes* marked with *CC* represent the predicted coiled-coil regions. *B,* FEZ1 and FEZ2 amino acid alignment. The proteins sequences of human FEZ1 (GenBank™ accession number NP_005094) and human FEZ2 (GenBank™ accession number BAD06207) were aligned using the ClustalW (1.82) multiple sequence alignment program. Identical residues are marked with *asterisks*; conserved residues are marked with *colons*; and semi-conserved residues are marked with *periods*. The coiled-coil regions (*CC*) were predicted by the SMART (Simple Modular Architecture Research Tool) program available on line. Glutamic acid-rich regions are *underlined*. The values of identity and similarity were obtained using the ClustalW program.

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 pre-synaptic membrane (19–21). Also, this group contains the proteins 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_6 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 ~220–392) (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. 4*A*). 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. 4*A*). This result indicates that the coiled-coil region of FEZ1 is important for the

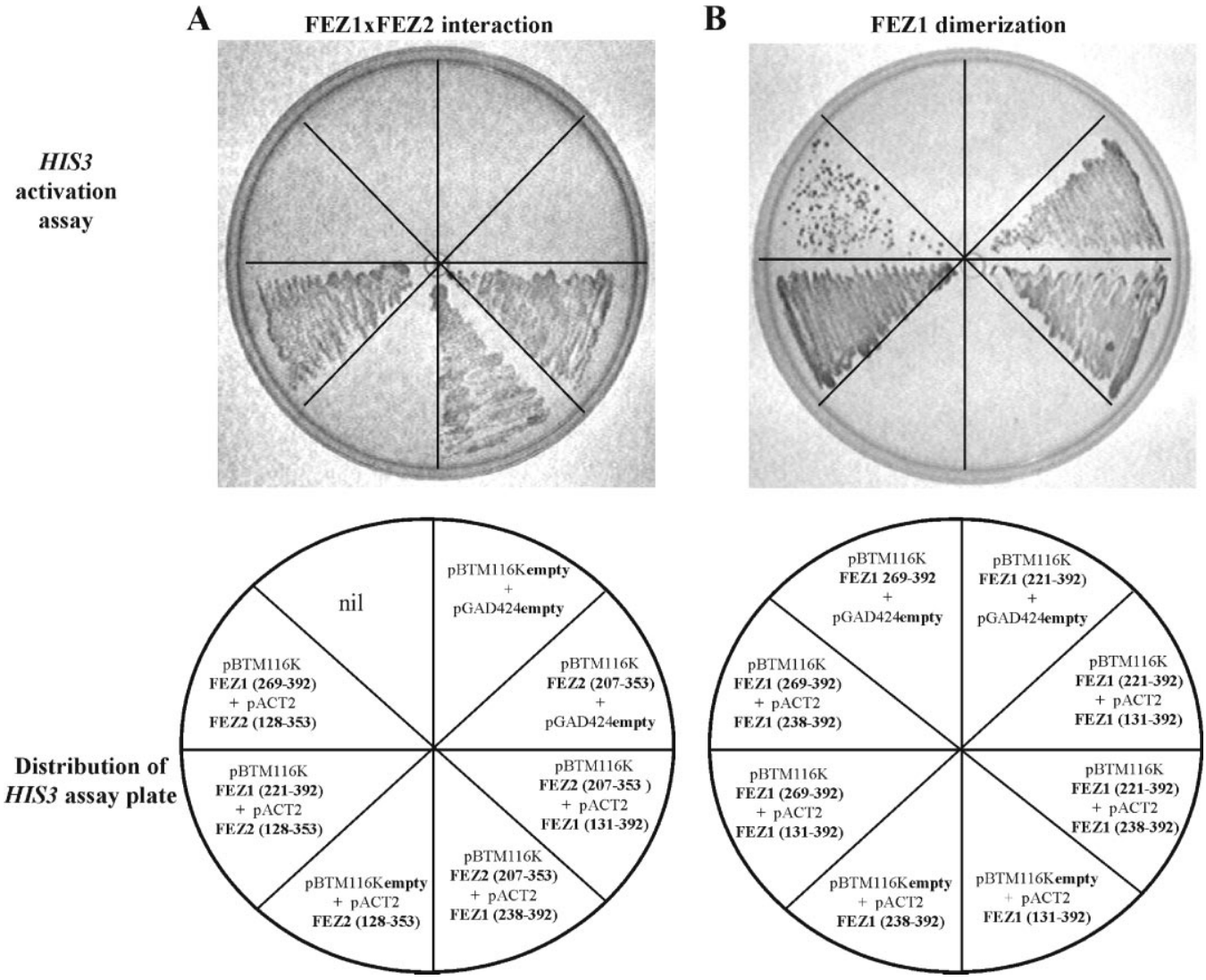

FIGURE 4. **Homodimerization of FEZ1 and heterodimerization of FEZ1 and FEZ2 as assayed in the yeast two-hybrid system.** *A*, mapping of heterodimerization of FEZ1 and FEZ2. Interaction of the indicated FEZ1 regions cloned in either vector pBTM116 or pACT2 and the indicated regions of FEZ2 cloned in either pACT2 or pBTM116 are shown. The indicated plasmid constructs were co-transfected into yeast L40 strain. Interaction was determined by the ability of the cells to grow on plates with minimal medium lacking Trp, Leu, and His but containing 20 mM 3-AT for suppression of unspecific reporter activation. Presence of bait and prey plasmids was controlled by growth on minimal plates lacking only Trp and Leu (not shown). Empty vectors pBTM116 or pGAD424 served as negative controls. *B*, mapping of the homodimerization of FEZ1. Interaction of the indicated FEZ1 regions cloned in either vector pBTM116 or pACT2 is shown. The indicated plasmid constructs were co-transfected into yeast L40 strain. Interaction was determined as described in *A* above. All transformants that grew on these plates always stained blue after 1 h of incubation in the β-galactosidase filter assay.

heterodimeric interaction (Fig. 3 and Fig. 4*A*) 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-coiled 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. 4*B*). 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. 4*B* 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) with an estimated molecular mass of ~45 kDa to a size exclusion chromatography. Three protein peaks were eluted (Fig. 5*A*). 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. 5*B*) and 10.3 kDa (not show) 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

TABLE 2

Test for interaction of FEZ2-(207–353) and FEZ1-(221–392) with or without (residues 269–392) the coiled region and the plasmids encoding the interacting proteins as identified in the yeast two-hybrid system

The recombinant plasmids pACT2 containing the cDNAs encoding the identified FEZ1 interacting proteins were transfected into yeast strain L40, which had been previously transfected with pBTM116-FEZ1-(221–392), pBTM116-FEZ1-(269–392), or pBTM116-FEZ2-(207–353). β-Galactosidase filter assay was performed. +++, very strong coloration; ++, strong coloration; +, weak coloration; −, no coloration.

Tested interacting protein (in pACT2 vector)	β-Galactosidase activity of yeast two-hybrid assay co-transformants using the following		
	pBTM116K-FEZ1-(221–392) (with coiled-coil region)	pBTM116K-FEZ1-(269–392) (without coiled-coil region)	pBTM116K-FEZ2-(207–353) (orthologue of FEZ1)
Retinoic acid-induced 14	+++	+++	+++
Zinc finger protein 251	++	+++	++
Bamacan	++	++	++
Programmed Cell Death 7	++	++	++
SAP30L	++	+	++
FEZ1-(131–392)	++	+	++
FEZ1-(238–392)	++	−	++
BAF60a-(82–515)	+++	+	+++
BAF60a-(404–515)	+++	−	++
KIBRA	+++	−	++
CLASP2	+	−	+
DRAP1	++	−	++
RAB3 GAP	+++	−	+++
Similar to short coiled-coil protein	+++	−	+++
Bromodomain containing protein 1	+	−	++
Tousled-like kinase 2	+	−	++
Hypothetic Protein FLJ13909	++	−	+
Similar to OAT-like 1	++	−	++

involved in this interaction, we performed *in vitro* pull-down assays with GST and His_6 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_6 tail. However, when fused to a larger fusion protein component such as GST, the C- and N-terminal regions of FEZ1 may be structurally stabilized. Additional experiments are required to fully elucidate these observations.

Mapping Protein Regions of FEZ1 Involved in Protein-Protein Interactions—To analyze whether the conserved coiled-coil region of FEZ1 is important for the observed interactions, we generated the construct FEZ1-(269–392) lacking the coiled-coil region, in fusion with the LexA binding domain, and we tested it in yeast two-hybrid assays against the prey plasmids retrieved from the yeast two-hybrid screen (Fig. 1 and Table 2).

The results show that the coiled-coil domain is important for the majority of the interactions (11 of 16). An equally strong interaction of FEZ1-(269–392) was only observed with the proteins RAI14 isoform, Bamacan, Programmed Cell Death 7, and zinc finger protein 251, all of which maintain the same intensity of coloration, suggesting that there was no loss of affinity in these interactions. In case of SAP30L, the coloration was weaker suggesting a certain loss in affinity. Two different clones were tested for BAF60a. The longer construct BAF60a-(82–515) still interacted with FEZ1-(269–392), whereas the shorter construct BAF60a-(404–515) ceased to do so, probably indicating that in the latter construct of BAF60a a region critically involved in the interaction has been deleted. In the case of FEZ1 itself, an interaction was no longer observed with FEZ1-(269–392) if part of the coiled-coil region was also removed from the prey FEZ1-(238–392).

FEZ2 Also Interacts with All Retrieved FEZ1-interacting Proteins—Next, all recombinant pACT2 plasmids encoding proteins that showed interaction with FEZ1-(221–392) were also assayed against the recombinant plasmid pBTM116-FEZ2-(207–353) to see if FEZ2 is also capable of interacting with the proteins found to interact with FEZ1. Bait and then prey plasmids were sequentially co-transfected into the yeast L40 strain, and clones were analyzed for interaction in the β-galactosidase filter assay (Table 2). FEZ2 is an orthologue of FEZ1, and its function is not yet known. As shown in Fig. 3, the protein FEZ2 consists of 353 residues and also has a coiled-coil region at position 219–254. Different from FEZ1, only two of the three glutamic acid-rich regions are partially conserved in FEZ2. In the second and third glutamic acid-rich regions, two of the four Glu have been substituted with the other negatively charged amino acid Asp. Overall these two proteins show an identity of 41% and a similarity of 68%. However, the C-terminal region shows a much higher degree of conservation (60% amino acid sequence identity and 76% similarity) than the N-terminal region (Fig. 3*B*). The coiled-coil region, which is found at the beginning of the conserved C-terminal domain, shows even a higher sequence identity between FEZ1 and FEZ2 (76% identity and 92% similarity). The yeast two-hybrid analysis using FEZ2-(207–353) as a bait revealed that all proteins that interact with FEZ1-(221–392) also interact with FEZ2-(207–353), and the β-galactosidase filter assay showed a comparable coloration or in some cases even a stronger 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

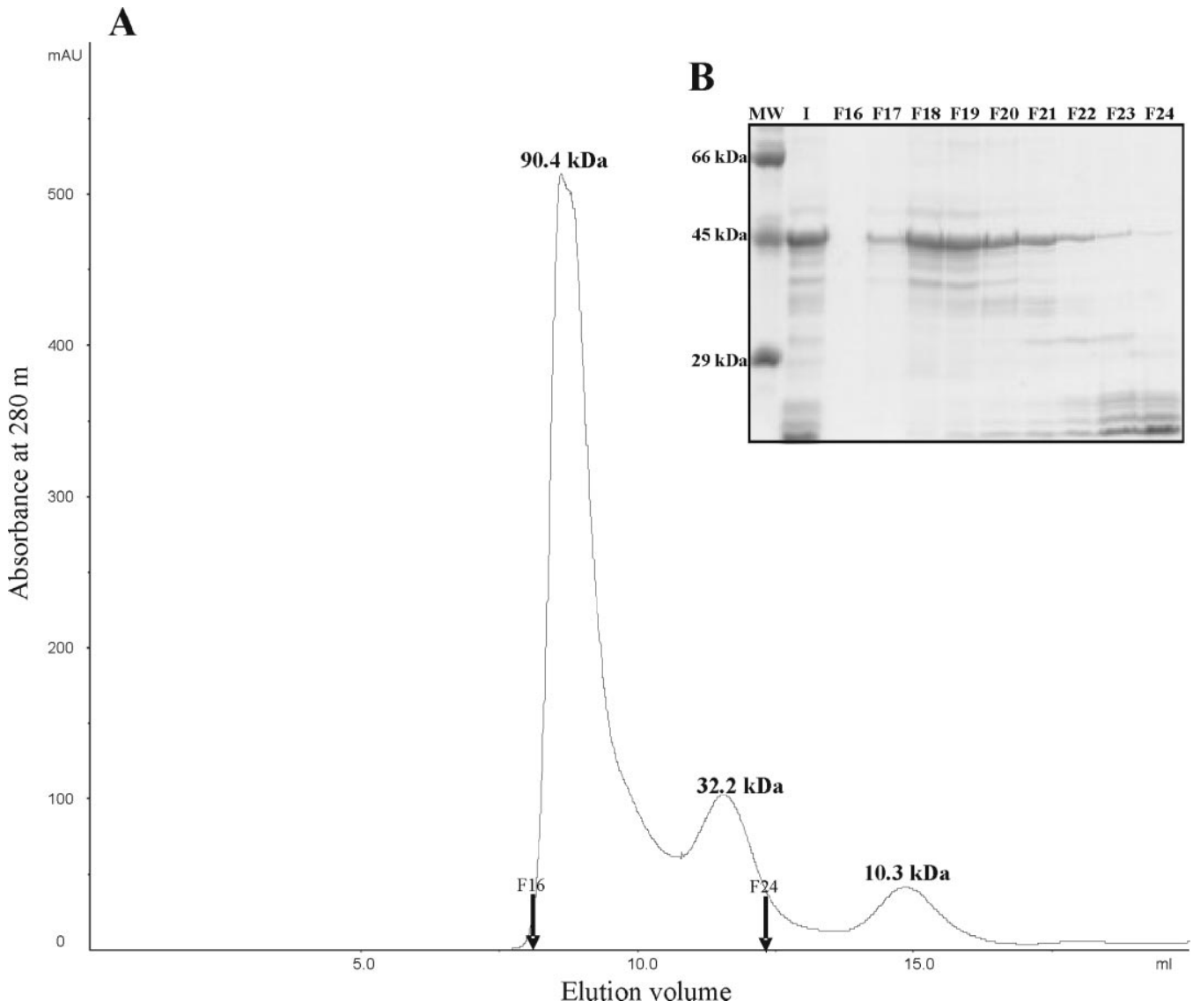

FIGURE 5. **Analytical size exclusion chromatography of 6xHis-FEZ1-(131–392).** *A*, spectrophotometric profile of purified protein 6xHis-FEZ1-(131–392) as eluted from the Superdex^{TM} 75 gel filtration column. The corresponding estimated molecular weights are indicated as determined by calibration of the column with the marker proteins. *B*, fractions eluted from the size exclusion column were analyzed on a 12.5% SDS-PAGE. The *arrows* in *A* indicate the range of eluted fractions analyzed in the SDS-polyacrylamide gel shown in *B*.

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

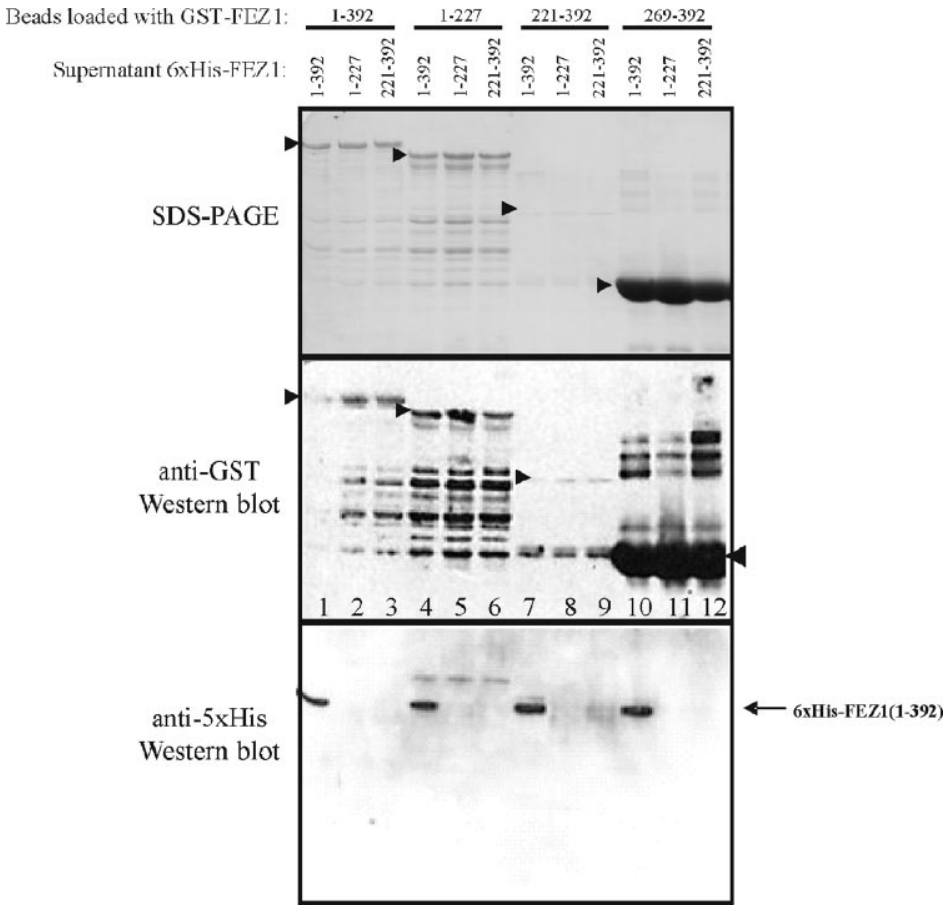

FIGURE 6. ***In vitro* confirmation and mapping of FEZ1 homodimerization.** Pull-down binding assay of the interactions between full-length 6xHis-FEZ1 or the indicated regions of FEZ1 also fused to a His_6 tag, and the indicated full-length or deletion FEZ1 constructs fused to GST protein. The indicated GST fusion proteins were loaded on glutathione-Sepharose beads. After washes the loaded beads were allowed to interact with the indicated His_6-tagged FEZ1 fusion protein constructs followed by high stringency washes using PBS/Triton X-100. Samples were then loaded on three replica SDS-polyacrylamide gels. The first was stained by Coomassie Blue, and the proteins of the other two were transferred to PVDF membranes. These were then developed with either the monoclonal mouse anti-GST antibody 5.3.3 (anti-GST Western blot) or a mouse anti-5xHis tag antibodies (anti-5xHis Western blot) to detect GST- or His-tagged proteins, respectively. The *arrows* indicate the positions of GST-FEZ1 proteins in the SDS-PAGE and anti-GST Western blots. In the Western blot performed to detect the His-tagged protein bound to GST-tagged proteins, an unspecific labeling of GST-FEZ1-(1–227) protein (*lanes 4–6*) occurred by the mouse anti-His tag antibody for unknown reasons.

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 subunits 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) 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 NC2α/β 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 IIB (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 "L*XX*LL" (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).

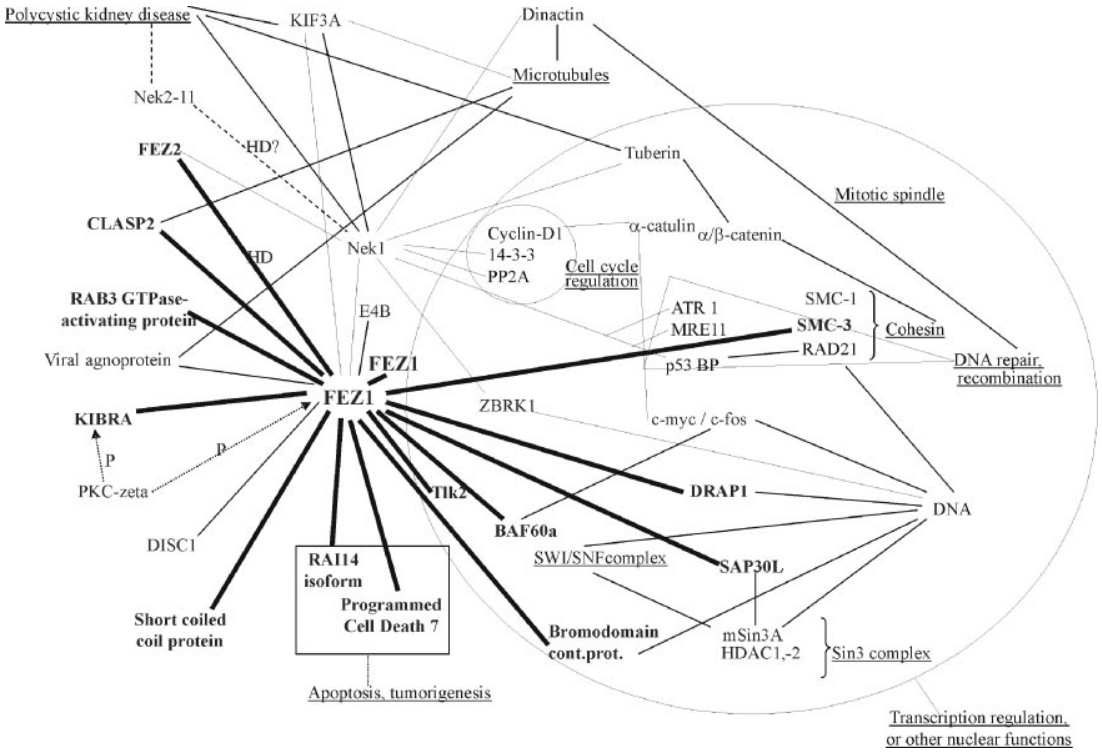

FIGURE 7. **Schematic figure of a putative protein-protein interaction network involving the protein FEZ1 as detected by the yeast two-hybrid system in this study together with other described interactions from the literature.** *HD,* heterodimerization; *P,* protein phosphorylation (also *dot-lined arrows*); *dark lines,* newly identified FEZ1 protein interactions (this study); *thin lines,* interaction previously described in the literature (see Introduction and "Discussion" for details); protein names, see text for details; *underlined words* and *boxes/circles/triangles*, and *thin lines*, possible attributed functional contexts or association with cellular structures, proteins, or groups thereof.

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 antigen1/2) 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

(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 prey-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.

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3.2. Artigo II

FEZ2 Has Acquired Additional Protein Interaction Partners Relative to FEZ1: Functional and Evolutionary Implications

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FEZ2 Has Acquired Additional Protein Interaction Partners Relative to FEZ1: Functional and Evolutionary Implications

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Abstract

Background: The FEZ (fasciculation and elongation protein zeta) family designation was purposed by Bloom and Horvitz by genetic analysis of *C. elegans unc-76*. Similar human sequences were identified in the expressed sequence tag database as *FEZ1* and *FEZ2*. The *unc-76* function is necessary for normal axon fasciculation and is required for axon-axon interactions. Indeed, the loss of UNC-76 function results in defects in axonal transport. The human FEZ1 protein has been shown to rescue defects caused by *unc-76* mutations in nematodes, indicating that both UNC-76 and FEZ1 are evolutionarily conserved in their function. Until today, little is known about FEZ2 protein function.

Methodology/Principal Findings: Using the yeast two-hybrid system we demonstrate here conserved evolutionary features among orthologs and non-conserved features between paralogs of the FEZ family of proteins, by comparing the interactome profiles of the C-terminals of human FEZ1, FEZ2 and UNC-76 from *C. elegans*. Furthermore, we correlate our data with an analysis of the molecular evolution of the FEZ protein family in the animal kingdom.

Conclusions/Significance: We found that FEZ2 interacted with 59 proteins and that of these only 40 interacted with FEZ1. Of the 40 FEZ1 interacting proteins, 36 (90%), also interacted with UNC-76 and none of the 19 FEZ2 specific proteins interacted with FEZ1 or UNC-76. This together with the duplication of unc-76 gene in the ancestral line of chordates suggests that FEZ2 is in the process of acquiring new additional functions. The results provide also an explanation for the dramatic difference between C. elegans and D. melanogaster unc-76 mutants on one hand, which cause serious defects in the nervous system, and the mouse FEZ1 -/- knockout mice on the other, which show no morphological and no strong behavioural phenotype. Likely, the ubiquitously expressed FEZ2 can completely compensate the lack of neuronal FEZ1, since it can interact with all FEZ1 interacting proteins and additional 19 proteins.

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Introduction

The FEZ (fasciculation and elongation protein zeta) family designation was first purposed by Bloom and colleagues in 1997 by genetic analysis of *C. elegans unc-76*. The 376 and 385 amino acid containing isoforms of the UNC-76 protein arise by alternative splicing of the same gene and showed no strong similarity to any previously characterized proteins, but similar human sequences were identified in the expressed sequence tag database, dbEST [1,2] and Bloom & Horvitz named the two identified human genes *FEZ1* and *FEZ2*.

The *unc-76* function is necessary for normal fascicle structure and is required specifically for axon-axon interactions in *C. elegans*. The human *FEZ1* gene was able to restore partial locomotion and axonal fasciculation in the *C. elegans unc-76* mutants in germ-line transformation experiments, indicating that both the function and the structure of the FEZ proteins have been conserved in evolution [1]. Loss of *Drosophila Unc-76* function results in locomotion and axonal transport defects reminiscent of phenotypes observed in kinesin mutants and thereby suggesting that UNC-76 is required for kinesin-dependent axonal transport [3]. The FEZ1-deficient mice however, did not exhibit any obvious abnormal brain architecture, although they manifest slight behavioral abnormalities, including a hyperlocomotion phenotype and enhanced responsiveness to psychostimulants [4].

The homologous proteins UNC-76, FEZ1 and FEZ2, share a conserved predicted coiled-coil region at their C-terminal regions [1,5,6]. Coiled-coils are autonomous folding units consisting of two to four α-helices that wrap around each other with a slight left-handed super-helical twist [7] and thereby mediate sub-unit oligomerization [8] as well as protein-protein interactions [9]. Up to now 4 interactions were reported for *C. elegans* UNC-76, 45 interactions for human FEZ1 and 17 interactions for human FEZ2 according to BIND, HPRD, BioGRID data and previous research published by our group [10]. All of these interactions occur at the C-terminus with the predicted coiled-coil region. Some of these

interactions implicate FEZ1 protein in the context of schizophrenia (DISC1) [11,12], aspects of viral infection such as post-entry block of retroviral infection [13] and inhibition of the release of progeny virions (agnoprotein of the human polyomavirus JC virus) [14], and naturally in neuritogenesis (NBR1, PKCζ, DISC1) [5,11,15]. Furthermore, the association between FEZ1 and E4B was enhanced by co-expression of a constitutively active form of protein kinase Cζ, and phosphorylation of FEZ1 by this kinase and its subsequent ubiquitylation by E4B resulted in neurite extension in PC12 cells [4,16]. FEZ1 also contributes to the polarization of hippocampal neurons by controlling mitochondrial motility [4,17].

Here, we report that nucleotide sequences belonging to the FEZ family can be found in many eukaryotic species, but that most of the putative homologs are not only functionally uncharacterized but even remain unrecognized as FEZ family members in the databases. Our phylogenetic analysis of the FEZ family of proteins indicates that the ancestral gene of the FEZ family is found only in the animal kingdom. The gene duplication of *unc-76* to *FEZ1* and *FEZ2* is predicted to have occurred during the two rounds of whole-genome duplication in the chordate ancestral line, after the divergence of cephalochordates but before the splitting of the teleosts and tetrapods taxa.

The most conserved region in FEZ proteins is its C-terminus, and we and others showed previously that this region is involved in the association with other proteins [10,18]. Here, yeast two-hybrid assays with *C. elegans* UNC-76 (248–372), human FEZ1 (221–392) and human FEZ2 (207–353) as baits and human prey clones from cDNA libraries demonstrated that the pattern of protein-protein interactions (PPIs) is highly conserved between UNC-76 and FEZ1. These orthologues did however not interact with 19 proteins that interacted specifically with FEZ2, although FEZ2 interacted with all 40 proteins that interact with FEZ1/UNC-76. In summary, we found largely overlapping PPI patterns for FEZ1 and UNC-76 and extended PPIs for FEZ2. Interestingly, our data provide an explanation for the ability of *FEZ1* to rescue the defects caused by *unc-76* mutations in nematodes and to the lack of strong defects in *FEZ1* deficient mice, since the continuous presence of the ubiquitous expressed paralogue FEZ2 may be able to compensate lack of FEZ1 because its PPI pattern contains all the interactions found for FEZ1.

Methods

Protein sequence analysis and multiple sequence alignments

Protein Psi-Blast [19] searches with the full length *C. elegans* UNC-76 sequence were performed at the NCBI Web site http://www.ncbi.nlm.nih.gov/BLAST/ using the non-redundant protein sequence database available at August 16, 2009. After six rounds of iteration, all UNC-76, FEZ1 and FEZ2 orthologs with an E-value of 0.005 or below were selected and all redundant sequences were excluded (except to *B. floridae*). All sequences were collected in FASTA format for further analysis as shown in Table S1. The identification and posterior naming of the protein sequences as either UNC-76, FEZ1 or FEZ2 is based on the phylogenetical analyses shown in Figure 1 and Figure S1. The sequences were aligned using the ClustalW2 Web Site http://www.ebi.ac.uk/Tools/clustalw2/index.html at default settings. The alignments were then shaded using the multiple sequence alignment editor GENEDOC http://www.nrbsc.org/gfx/genedoc/index.html. The identity and similarity were calculated using default settings by NPS@: Network Protein Sequence Analysis trough the alignments of human proteins FEZ1 and FEZ2 and *C. elegans* UNC-76 protein (http://npsa-pbil.ibcp.fr/cgi-bin/align_clustalw.pl) [20].

Phylogenetical analyses

PHYLIP version 3.5c [21] was used for the phylogenetical analyses at the web site http://mobyle.pasteur.fr/cgi-bin/portal.py?-jobs = http://mobyle.pasteur.fr/data/jobs/protpars. Parsimony analyses were performed using the protein alignment as input. Bootstrap values were obtained by using SEQBOOT and creating 1000 Bootstrap data sets. Analysis was then performed using PROTPARS with standard parameters. The "M" option was invoked for the analysis of the multiple data sets generated by SEQBOOT.

Plasmid constructions

The plasmid constructions of pBMT116-FEZ1 (221–392) (NM_005103.3) and pBTM-FEZ2 (207–353) (NM_005102.2) have been described previously [10]. The nucleotide sequence coding residues 248–372 of *C. elegans* UNC-76 (NM_074311.3) was PCR-amplified using a specific primer set (5′- GAATTCGATAATCTTCAAGAGCTCTCC-3′ , 5′- GTCGACCTAACACGATATATTTTTTGG-3′) as well as the template plasmid pSU001, that had been generously provided by Dr. Hengartner [4]. Subsequently the fragment was cloned in pGEM®-T vector (Promega), and subcloned via *Eco*RI and *Sal*I restriction sites into the vector pBTM116. The orientation, frame, and correctness of sequence of each insert DNA was confirmed by restriction endonuclease analysis and automated DNA sequencing. These are no new cell lines but only cDNA clones obtained by *in vitro* experiments as described.

Yeast two-hybrid screen and DNA sequence analysis

Using FEZ1 as a bait in a screening, Assmann et al. (2006) [10] identified 16 proteins interacting with FEZ1 and all of them interacted with FEZ2 protein (Fig. 2A). In this work, yeast two-hybrid screens [22] of human fetal brain and bone marrow cDNA libraries (Clontech) were performed as described previously by using the yeast strain L40 (trp1-901, his3Δ200, leu2-3, ade2 LYS2::(lexAop)4-HIS3 URA3::(lexAop)8-lac GAL4) and human FEZ2 (207–353) as a bait in fusion with the LexA protein as encoded in the recombinant vector pBTM116 [23]. This fragment of FEZ2 does not auto-activate the yeast reporter genes (as FEZ proteins full length), neither did so the C-terminal constructs of FEZ1 and UNC-76 used in one-to-one confirmation. The autonomous activation test for HIS3 was performed in minimal medium plates without tryptophane and histidine but containing 10, 20, 30, or 50 mM of 3-amino-1,2,4-triazole (3-AT). Furthermore, the autonomous activation of LacZ was measured by the beta-galactosidase filter assay as described below. Yeast cells were transformed according to the protocols supplied by the cDNA library manufacturer (Clontech). The screening with FEZ2 (207–353) as bait was performed in minimal medium plates without tryptophan, leucine, and histidine and with addition of 10 mM of 3-AT to repress unspecific background growth.Recombinant pACT2 plasmids of positive clones were isolated and their insert DNAs were sequenced with a DNA sequencer model 377S (Applied Biosystems, Foster City, CA). The obtained DNA sequence data were compared with sequences in the NCBI data bank using the BLASTX 2.2.12 program [19]. The corresponding Accession numbers of the DNA sequences identified are given in the Table S1. As no new sequences have been obtained no new sequence data have been deposited in the GenBank. After identification, one representative clone of each prey protein was selected to yeast two-hybrid one-to-one confirmation.

Yeast Two-hybrid assays one-to-one confirmation with the homologues FEZ1, FEZ2 and UNC-76

Yeast two-hybrid assays were performed with human FEZ1 (221–392) and *C. elegans* UNC-76 (248–372) as additional baits

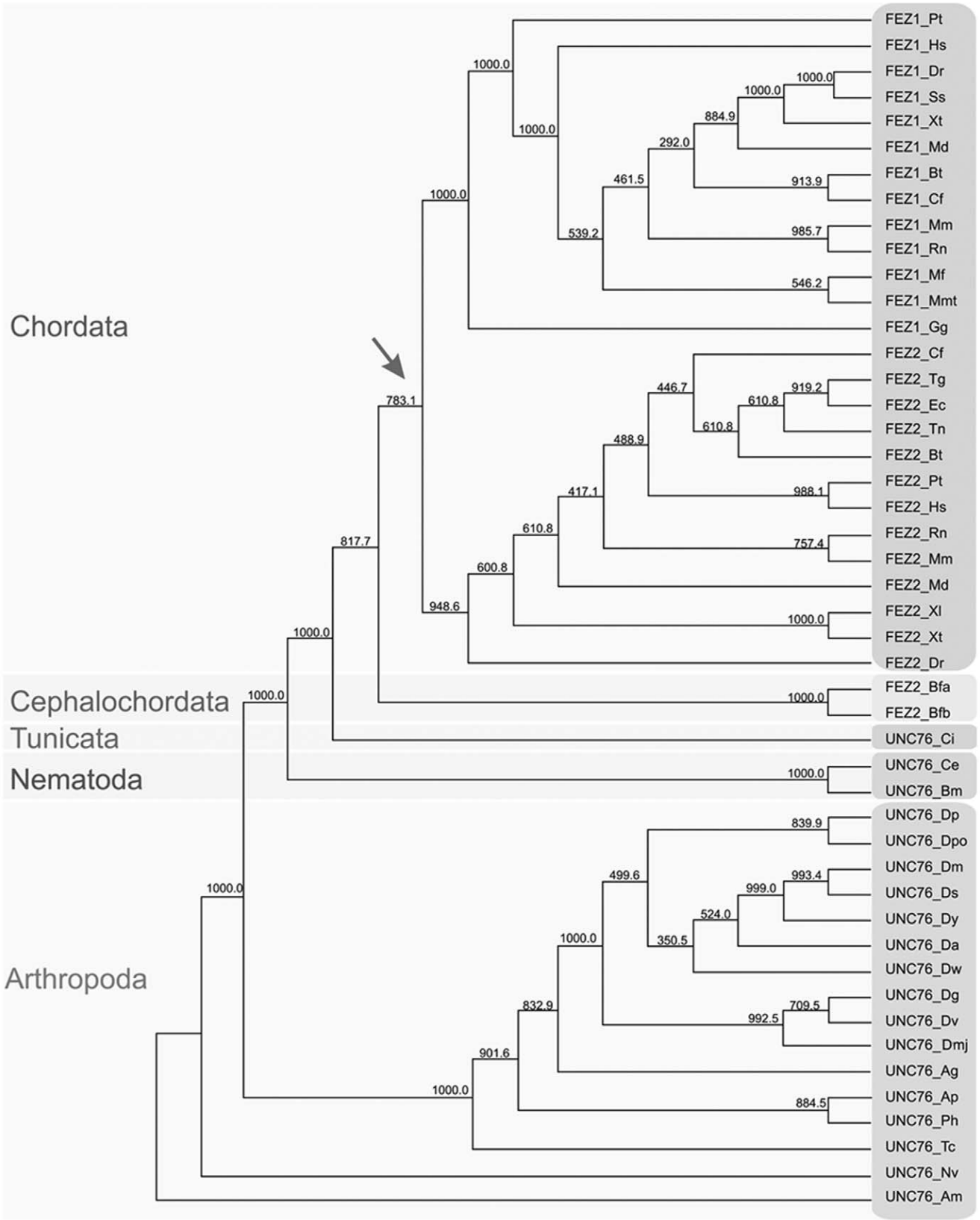

Figure 1. A phylogenetic tree of the FEZ protein family. The tree was derived by the parsimony method (Phylip protpars). The bootstrap multidataset and resampling method was employed and the numbers on the branches indicate the number of times the partition of the species into the two sets which are separated by that branch occurred among the trees out of 999.96 trees. The arrow indicates the probable point of FEZ gene duplication. Ag – *Anopheles gambiae*, Am – *Apis mellifera*, Ap – *Acyrthosiphon pisum*, Bf – *Branchiostoma floridae* (Bfa and Bfb refer to two sequences [polymorphisms] of selected hypothetical genes in this species), Bm – *Brugia malayi*, Bt – *Bos taurus*, Ce – *Caenorhabditis elegans*, Cf – *Canis familiaris*, Ci –

Ciona intestinalis, Da – *Drosophila ananassae*, Dg – *Drosophila grimshawi*, Dm – *Drosophila melanogaster*, Dmj – *Drosophila mojavensis*, Dp – *Drosophila persimilis*, Dpo – *Drosophila pseudoobscura*, Dr – *Danio rerio*, Ds – *Drosophila sechellia*, Dv – *Drosophila virilis*, Dw – *Drosophila willistoni*, Dy – *Drosophila yakuba*, Ec – *Equus caballus*, Gg –*Gallus gallus*, Hs – *Homo sapiens*, Md – *Monodelphis domestica*, Mf – *Macaca fascicularis*, Mm – *Mus musculus*, Mmt – *Macaca mulatta*, Nv – *Nasonia vitripennis*, Ph – *Pediculus humanus*, Pt – *Pan troglodytes*, Rn – *Rattus norvegicus*, Ss – *Salmo salar*, Tc – *Tribolium castaneum*, Tg –*Taeniopygia guttata*, Tn – *Tetraodon nigroviridis*, Xl – *Xenopus laevis*, Xt – *Xenopus tropicalis*.
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fused to LexA in vector pBTM116, as described above and previously [10]. The recombinant pACT2 plasmids of positives clones isolated from the library screens with bait FEZ2 (207–353) were used as preys and the interactions with FEZ1 and UNC-76 (and control FEZ2) were analyzed by growth in minimal medium plates and activation of the LacZ using the standard beta-galactosidase filter assay described in the following section (Fig. 2B).

Assay for beta-galactosidase activity in yeast cells

beta-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 3 MM paper previously soaked in Z buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM $MgCl_2$, 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 (primary screen).

Results

Identification of members of the FEZ family and of conserved regions in their amino acid sequences

A database of sequences judged to be members of the FEZ protein family was compiled (Table S1, see Material and Methods section). Altogether 47 members for the FEZ family were identified by Psi-Blast searches with the *C. elegans* UNC-76 sequence in the non-redundant protein sequence database. FEZ1 family sequences were found in a variety of species ranging from Nematodes, via Arthropods to Mammals but not in Plants, Fungi or Protists. Multiple Clustal W alignments of sequences of the FEZ family identified a highly conserved region both at the N-terminus (ca. aa 160–180 in FEZ1) and at the C-terminus (Figure S1). The latter consists mainly in coiled-coil regions [10,24,25] located at the C-terminus which mediate the majority of the protein-protein interactions (ca. aa 230–307 in FEZ1) [10]. The alignment revealed, that the nuclear localization signal (NLS) (KKRRK, ca. aa 290–294 in FEZ1) [26], which consists of basic amino acids, is quite conserved in all FEZ1 genes that resulted

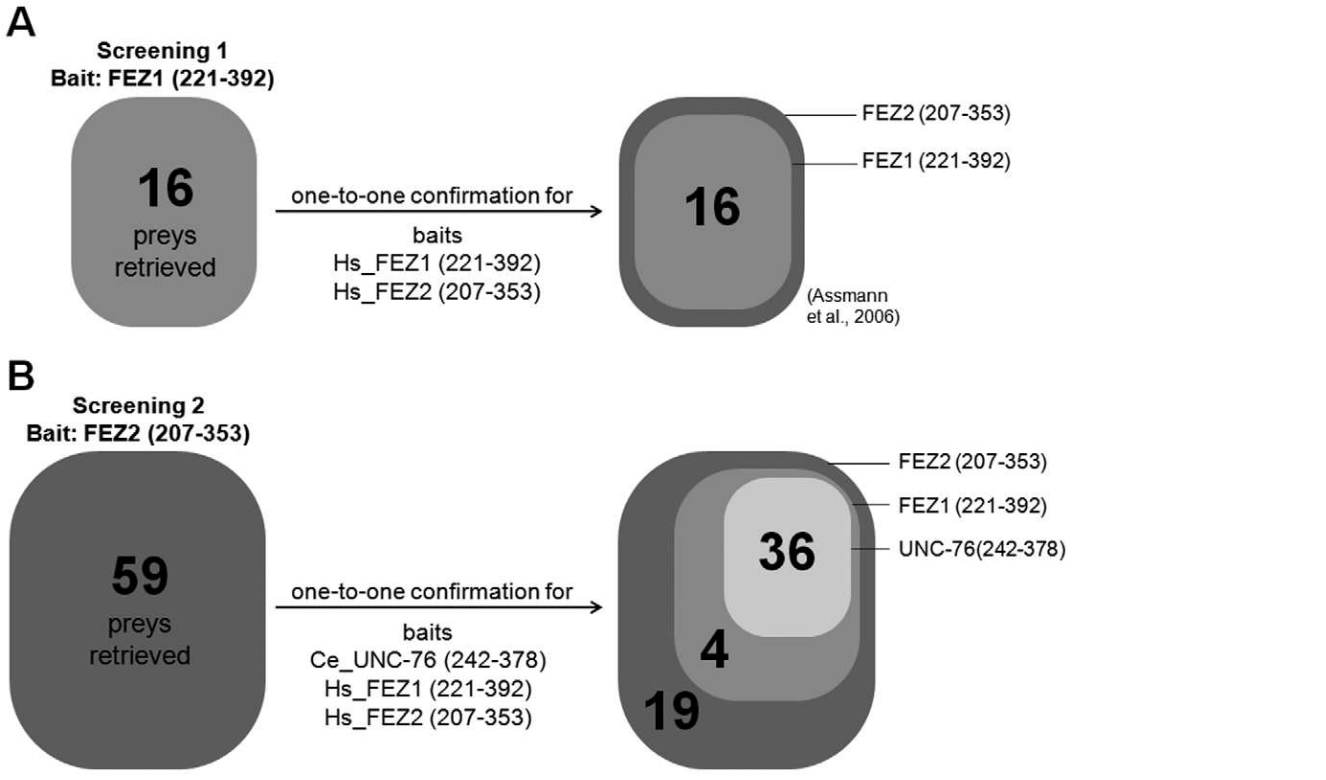

Figure 2. Schematic representation summarizing the primary yeast two-hybrid screens with UNC-76, FEZ1 and FEZ2 as baits. A) Yeast two-hybrid screen performed with FEZ1 (221–392) as a bait using a human fetal brain cDNA library retrieved 16 proteins that were confirmed to interact all with both FEZ1(221–392) and FEZ2 (207–353) in the one-to-one assay. B) Yeast two-hybrid screen performed with FEZ2 (207–353) as a bait using human fetal brain and bone marrow cDNA libraries retrieved 59 proteins as preys. In the one-to-one confirmation, 19 of these proteins interacted only with FEZ2 (207–353) protein, 40 with both FEZ2 (207–353) and FEZ1 (221–392) and 36 interacted with all FEZ2 (207–353), FEZ1 (221–392) and UNC-76 (242–378).
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from the putative *unc-76* gene duplication (see details below), suggesting that new nuclear functions may have been acquired in FEZ1 afterwards. Although there is no striking NLS in FEZ2 sequences, they contain a conserved polybasic region (e.g. KKKKK) with conserved amino acid substitutions in the FEZ1 related NLS. The NLS is less well conserved in all UNC-76 sequences.

Phylogenetic analysis of the FEZ gene duplication

A phylogenetic tree (Fig. 1) was generated from the Clustal W alignment (Figure S1) of the all identified FEZ protein sequences (Table S1) using the parsimony method. Bootstrap options to resampling methods were defined to contain 1000 replications. The presence of FEZ1 and FEZ2 in all analyzed Actinopterygii (ray-finned fishes) genomes suggests that the *FEZ1* and *FEZ2* genes originated from the ancestral *unc-76* by gene or whole genome duplications before the radiation of the Actinopterygii but after the divergence of the amphioxus branch and hence concomitant origin of the chordata. In fact, according to an amphioxus-human synteny analysis by Putnam [27] two rounds of whole-genome duplication seem to have occurred in the chordate stemline, after the divergence of the cephalochordates but before the split into teleosts and tetrapods (Fig. 1). Hence, the *unc-76* gene seems to have duplicated in the chordate stemline into *FEZ1* and *FEZ2* genes.

Identification of proteins that interact with FEZ2

So far, only interactions for C-terminal of FEZ proteins are described, as by our screenings using C termini of FEZ proteins (due to auto-activation of full length and N-terminal constructs of the FEZ proteins as baits in these assays), as well as by other publications describing FEZ proteins recovered as prey in other screens (in this case, the interaction region was mapped to be the C-terminal region) [5,14]. In a previous study, we had identified proteins that interact with human FEZ1 using its C-terminal region (221–392) as a bait in a yeast two-hybrid screen of a human fetal brain cDNA library [10]. We further found that all of the 16 interacting proteins identified were also able to interact with FEZ2 (207–353). The interacting proteins are functionally involved in transcriptional regulation (6 proteins), neuronal cell development (2 proteins), intracellular transport processes (3 proteins), apoptosis (2 proteins), or were of unknown function (3 proteins). Although these data suggested that FEZ1 and FEZ2 have largely overlapping protein interaction profiles we wanted to deepen our insights by performing another two-hybrid screen with the human paralogue FEZ2 as well as by introducing an evolutionary component by comparing the interaction profiles of both proteins with the orthologous *C. elegans* protein UNC-76, which may have preserved ancient features.

To this end we started by employing the yeast two-hybrid system [23] to screen humans fetal brain and bone marrow cDNA libraries. The truncated FEZ2 (207–353), which showed no auto-activation (see Materials and Methods), was used as a bait (Fig. 2). All grown colonies that showed a strong blue color in the subsequent β-galactosidase filter assay had their plasmid DNA extracted and sequenced. A total of 166 plasmid DNAs (69 from human bone marrow and 97 from human fetal brain cDNA libraries) from clones positive for both *HIS3* and *LacZ* reporters were sequenced. 59 different proteins were identified using FEZ2 (207–353) as a bait (Table 1, Fig. 3). These can be organized into the following groups according the major described GO (Gene-Ontology) process attributed to them: transcription (10 proteins), translation (6 proteins), apoptosis (5 proteins), signal transduction (5 proteins), neuronal cell development (5 proteins), cytoskeleton/centrosome (3 proteins), unknown function (10 proteins) and other functions (15).

Most interestingly, of these 59 FEZ2 interactors only 40 interacted also with FEZ1 (Fig. 3, Table 1) and indeed 8 of the 40 were identical with proteins that had been identified in the screen with FEZ1 [10]. This result may suggest that FEZ2 gained in respect to FEZ1 additional 19 new interactors, within the set of preys analyzed, which are specific to FEZ2. Such an evolutionary gain of function is compatible with the histological expression patterns of both proteins. Whereas FEZ1 (as its ortholog UNC-76) is expressed only in the nervous system, FEZ2 shows a ubiquitous expression pattern. Thinking of the role of FEZ proteins as transport adaptor proteins, FEZ2 may have evolved to transport additional cargo proteins, which may be expressed in non-neuronal tissues.

UNC-76 and FEZ1 share a conserved protein-protein interaction pattern

Next we were interested to involve UNC-76 as a third component in our analysis, to try to understand how the protein-protein interaction pattern may have evolved during evolution of the FEZ family proteins. To this end we employed a C-terminal construct of UNC-76 (242–378) in a direct one-on-one analysis against all 59 FEZ2 interacting proteins (Fig. 3, Table 1). The cDNA for *C. elegans* UNC-76 was a kind gift from Dr. Hengartner.

To our great surprise, the pattern of interaction of UNC-76, in relation to the proteins identified in the screening assay of two-hybrid and FEZ2 as bait, was almost identical to that observed for FEZ1 (Table 1, Fig. 3). This suggests that the protein-protein interaction profile of the orthologs FEZ1 and UNC-76 is highly conserved. Only 4 of the 40 proteins that interacted with FEZ2 and FEZ1 did not interact with UNC-76. In conclusion the protein interaction pattern for both human FEZ1 and *C. elegans* UNC-76 seemed to be mostly conserved since the divergence of the deuterostomes (represented by the human FEZ1) from the "protostomes" (represented by *C. elegans* UNC-76), which occurred around 650 million years ago.

In this context it is worthwhile to differentiate between nuclear and cytoplasmic proteins that interact with the FEZ family proteins. A significant proportion of the interacting proteins are nuclear or predicted to be nuclear (18 of 59, 30.51%). Among these 18 nuclear proteins identified in the screen with FEZ2, a high proportion of 15 (ca. 83%) also interacted with FEZ1 (Fig. 4). This may be of potential relevance because we have shown in previous cell fractionation experiments that GFP-FEZ1 can be found in the nuclear fraction [26]. The exact role of FEZ1 in the nucleus remains however to be established. Interestingly, of the 41 cytoplasmic FEZ2 interacting proteins identified, a significant fraction of 16 (ca. 40%) interact exclusively with FEZ2. This again may suggest the acquisition of new interaction partners and thereby possibly the aggregation of new "cytoplasmic functions" for FEZ2 relative to FEZ1/UNC-76.

In 2000, Walhout and collaborators [28] introduced the concept of an "interologue", referring to a protein-protein interaction pattern that is conserved between pairs of orthologs. This notion is intuitive in molecular biology and has been documented by many interactions and may be conserved even in distantly related organisms, such as bacteria and animals. Our results show not only a high degree of preservation of the interactions between FEZ1, FEZ2 and UNC-76 but also the acquisition of new PPIs for FEZ2. Makino and coworkers [29] suggested that the rate of evolution of a protein that has more partners PPI is much smaller than the one that has few partners

Table 1. Human FEZ2-interacting proteins identified by the yeast two-hybrid system screen.

Gene	Acession no.	Protein Description[1]	Coded protein residues (complete)	Coded protein residues (retrieved)[2]	UNC76[3]	FEZ1[3]	FEZ2[3]	Biological Process (GO)[4]
AATF	NP_036270	apoptosis antagonizing transcription factor	560	11–319	+	+	+	apoptosis
ATP6V1H	NP_057078	H(+)-transporting two-sector ATPase	247	63–234	+	+	+	ion transport
C1orf216	NP_689587	hypothetical protein LOC127703	229	20–229	+	+	+	UNKNOWN
C10orf54	Q9H7M9	Platelet receptor Gi24 precursor	311	215–311			+	UNKNOWN
C10orf78	NP_001002759	hypothetical protein LOC119392 isoform a	245	6–245	+	+	+	UNKNOWN
C12orf51	NP_001103132	AF-1 specific protein phosphatase	3996	2878–3161		+	+	protein modification
C14orf94	CAD62584	HAUS augmin-like complex, subunit 4	387	16–333	+	+	+	centrosome organization
CAP1	EAX07240	CAP, adenylate cyclase-associated protein 1 (yeast)	468	163–468	+	+	+	cytoskeleton/ cell polarity/ signal transduction
CDKN1	EAW96276	cyclin-dependent kinase inhibitor 1B (p27, Kip1)	53	1–53	+	+	+	apoptosis/cell growth
CHERP	BAD92967	calcium homeostasis endoplasmic reticulum protein	399	301–399		+	+	nervous development
DEFA3	AAA35753	neutrophil peptide 3 precursor	65	50–65	+	+	+	xenobiotic metabolism
DNAJB11	NP_057390	DnaJ (Hsp40) homolog, subfamily B, member 11 precursor	358	277–358			+	protein folding
DNML1	EAW88521	dynamin 1-like, isoform CRA_c	789	549–789	+	+	+	endocytosis
DRAP1	NP_006433	DR1-associated protein 1	205	1–133	+	+	+	transcription
DRG2	BAD92577	developmentally regulated GTP binding protein 2 variant	259	51–259	+	+	+	signal transduction
EEF1G	AAH21974	H sapiens eukaryotic translation elongation factor 1 gamma	355	19–346			+	translation
EI2B5	EAW78300	eukaryotic translation initiation factor 2B, subunit 5 epsilon	442	327–434	+	+	+	translation/ hormone mediated signaling
FEZ1	EAW67636	fasciculation and elongation protein zeta 1 (zygin I)	392	131–371	+	+	+	nervous development
FEZ1	NP_072043	zygin 1 isoform 2	104	1–89			+	nervous development
GNAS	AAH89157	GNAS complex locus	380	293–380			+	signal transduction
GOLPH3L	EAW53527	golgi phosphoprotein 3-like	299	159–299	+	+	+	UNKNOWN
GPRASP2	NP_612446	G protein-coupled receptor associated sorting protein 2	838	496–812	+	+	+	UNKNOWN
GTF2IRD2	AAQ19673	general transcription factor II i repeat domain 2	949	565–603			+	transcription
GTPB6	NP_036359	pseudoautosomal GTP-binding protein-like protein	403	98–347			+	UNKNOWN
HSP90B1	EAW97723	heat shock protein 90 kDa beta (Grp94), member 1	367	1–287			+	apoptosis/ protein folding/ muscular contraction
HSPA1A	BAD93055	heat shock 70 kDa protein 1A variant	709	75–163			+	response to stress

Table 1. Cont.

Gene	Acession no.	Protein Description[1]	Coded protein residues (complete)	Coded protein residues (retrieved)[2]	UNC76[3]	FEZ1[3]	FEZ2[3]	Biological Process (GO)[4]
IGL@	AAH71804	IGL@ protein	236	1–236			+	UNKNOWN
IK	EAW62028	IK cytokine, down-regulator of HLA II	557	119–333	+	+	+	cell-cell signaling
INPP1	NP_002185	inositol polyphosphate-1-phosphatase	399	355–399		+	+	signal transduction
INTS8	NP_060334	integrator complex subunit 8	995	508–816	+	+	+	snRNA proccessing
MCM7	NP_877577	minichromosome maintenance complex component 7	543	410–543	+	+	+	transcription/ cell cycle
MED7	NP_004261	mediator complex subunit 7	233	116–233	+	+	+	transcription
MLF1IP	NP_078905	MLF1 interacting protein	418	243–263	+	+	+	transcription
NFS1	BAD96959	NFS1 nitrogen fixation 1 isoform a precursor variant	457	417–457			+	metabolic proccess
NQO2	CAI23293	NAD(P)H dehydrogenase, quinone 2	172	1–115			+	oxidation reduction
OS9	NP_006803	amplified in osteosarcoma isoform 1 precursor	667	389–635		+	+	endoplasmatic reticum stress
PDCD7	AAI31705	PDCD7 protein	270	57–270	+	+	+	apoptosis
PTN	EAW83871	Pleiotrophin	246	105–223	+	+	+	nervous development
RARA	EAW60657	retinoic acid receptor, alpha, isoform CRA_e	520	111–327	+	+	+	transcription
RPL27	EAW60910	ribosomal protein L27, isoform CRA_b	80	53–80	+	+	+	translation
RPL38	NP_000990	RPL38 ribosomal protein L38	70	1–70			+	translation
RPS25	NP_001019	ribosomal protein S25	125	1–114			+	translation
SAP30	NP_003855	Sin3A-associated protein, 30 kDa	220	1–220	+	+	+	transcription
SAP30L	NP_078908	SAP30-like	183	1–183	+	+	+	transcription
SCOC	EAX05102	short coiled-coil protein, isoform CRA_a	122	42–122	+	+	+	UNKNOWN
SERPINF1	P36955	Pigment epithelium-derived factor precursor (PEDF)	418	16–267			+	nervous development
SFRS8	EAW98526	splicing factor, arginine/ serine-rich 8	951	263–511	+	+	+	transcription
SHMT1	BAD97272	serine hydroxym ethyltransferase 1 (soluble) isoform 1 variant	483	364–483			+	hormone mediated signaling
SLC25A37	AAF71063	PRO1584	81	1–58	+	+	+	muscular constraction
SLTM	EAW77552	SAFB-like, transcription modulator	1168	389–723		+	+	transcription/ apoptosis
SMC3	BAF98736	structural maintenance of chromosomes 3	1217	766–1059	+	+	+	sister chromatides cohesion/signal transduction
STRA13	NP_659435	stimulated by retinoic acid 13	63	3–63	+	+	+	UNKNOWN
TBC1D25	NP_002527	TBC1 domain family, member 25	688	498–677	+	+	+	regulation Rab GTPase activity
TLOC1	Q99442	Translocation protein SEC62	399	2–95			+	cotranslational protein targeting to membrane
TNNC2	NP_003270	fast skeletal muscle troponin C	160	1–137			+	muscular constraction
TSNAX	NP_005990	translin-associated factor X	290	1–276	+	+	+	cell differentiation

Table 1. Cont.

Gene	Acession no.	Protein Description[1]	Coded protein residues (complete)	Coded protein residues (retrieved)[2]	UNC76[3]	FEZ1[3]	FEZ2[3]	Biological Process (GO)[4]
TUBA1B	BAF82043	tubulin, alpha 1b	451	285–432			+	cytoskeleton
WWC1	EAW61508	WW, C2 and coiled-coil domain containing 1	1018	775–1018	+	+	+	UNKNOWN
"SIH"	A61065	sucrase-isomaltase homolog – human	48	1–44	+	+	+	UNKNOWN

[1]Results obtained from BLASTX (GenBank);
[2]It is depicted the mininum length of the retrieved sequences which could be visualized by forward DNA sequencing only,
[3]Interaction confirmed (+) by yeast two hybrid system with UNC-76, FEZ1 or FEZ2 proteins;
[4]Biological process based on the GO database (other functions may be known).
doi:10.1371/journal.pone.0017426.t001

PPI. This is in good agreement with the high degree of sequence similarity among FEZ1/FEZ2/UNC-76, which is especially pronounced in the C-terminal coiled-coil region, which is essential for most protein-protein interactions (Figure S2). Only 12.8% of the amino acids in the coiled-coil regions of FEZ1 and FEZ2 are different.

Three different fates have been proposed after the duplication of a genes [29]. First, one of the copies can be silenced by the accumulation of deleterious mutations and eventually become indistinguishable from genomic non-coding neighboring regions, while the other copy retains the original function. Second, while one copy maintains the original function, the other acquires a new function possibly by aggregation of advantageous mutations. Third, both copies accumulate mutations that alter the original function, but the original function is retained and compensated cooperatively. Our results seem to strongly suggest that for the FEZ family the second pathway is in progression. All interactors identified for FEZ1 in our laboratory have been confirmed to interact with FEZ2, too [10], but only 60% of the interactions identified for FEZ2 were confirmed for FEZ1 and UNC-76. Thus, FEZ2 seems to have acquired additional new functions but still preserved the majority of interactions found for FEZ1/UNC-76.

In this context it is also interesting to analyze the origin of the proteins fished with the FEZ2 bait from either bone marrow or fetal brain cDNA libraries and to correlate this with the supposed expression patterns (FEZ1: brain, FEZ2: ubiquitous) and function (FEZ1: neuron specific, FEZ2: ubiquitous) (Table 1). Of the 17 proteins that were of origin from either bone marrow or brain (not from both, like 2 additonal proteins) and that were specific to interact with FEZ2, 13 were from bone marrow and only 4 from brain. Of the 52 proteins that were of origin from either bone marrow or brain only (7 additional proteins were fished from both libraries!) and that interacted with both FEZ1 and FEZ2, 32 were from the bone marrow library and 20 from the brain. Thus, FEZ1 interactions are relatively enriched in clones from the brain, while FEZ2 interactions are of more ubiquitous origin. In fact the ratio bone marrow proteins/brain protein for FEZ2 is almost three times higher (3.25; 13/4) than for FEZ1 (1.19; 19/16). This tendency may already indicate the acquisition of additional interacting partners and functions in the more ubiquitously expressed FEZ2 relative to the brain specific FEZ1 since the candidate cargo proteins for transport of FEZ1 can be mostly found in brain tissue, whereas those of FEZ2 preferentially in other tissues, including bone marrow.

Discussion

The large number of proteins that were identified to interact FEZ1, led to its classification as a hub protein [10]. Most proteins initially characterized as hubs tend to maintain this status after additional studies [30]. Indeed, here we were able to confirm the hub status for human FEZ1 and even extend it to the entire family of FEZ proteins, since both human FEZ2 as well as *C. elegans* UNC-76 interacted also with a large number of proteins. Genome-wide studies have shown that deletions of hub protein encoding genes are three times more likely to be lethal than deletions of non-hubs, a phenomenon known as the centrality-lethality rule [31]. This is confirmed in *C. elegans* mutations in the *unc-76* gene, which

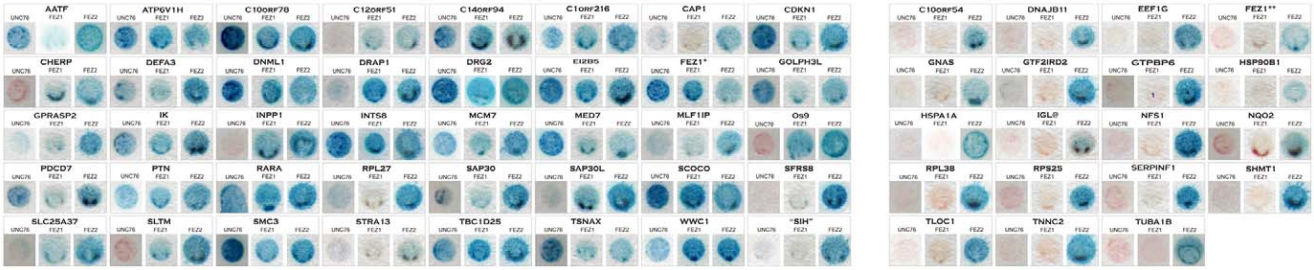

Figure 3. Protein-protein interaction pattern among homologs by beta-galactosidase assay. Result of the yeast two-hybrid assay with the C-terminal of the three homologous proteins UNC-76 (242–378), FEZ1 (221–392) and FEZ2 (207–353) as baits. All 59 clones obtained here by our yeast two-hybrid library screens with the C-terminal of FEZ2 (207–353) were tested against all three bait constructs one-to-one. The pattern of interaction between the orthologous FEZ1 and UNC-76 was almost identical, indicating a conservation of the UNC-76 function in human FEZ1. The clones that showed specific interactions with FEZ2 only, are separated on the right side of the figure. The tests were performed in duplicate, but only one representative result is shown.
doi:10.1371/journal.pone.0017426.g003

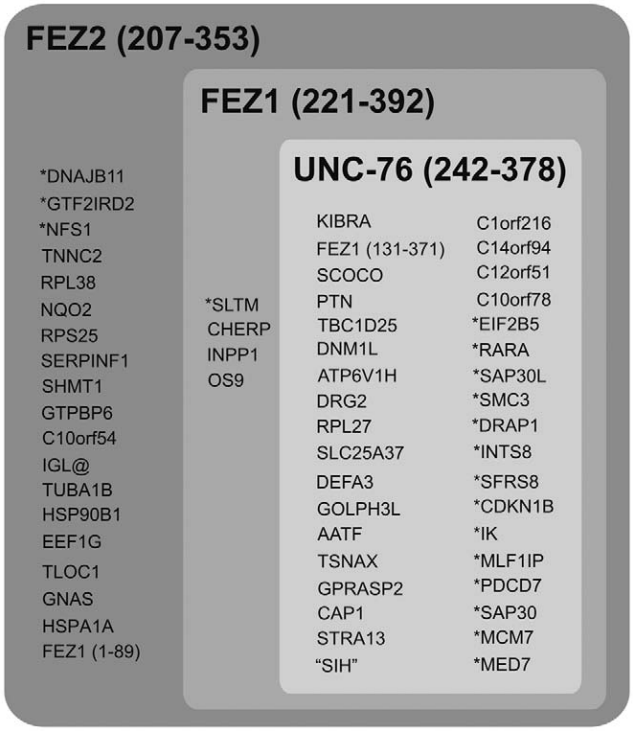

Figure 4. Schematic summary of the individual interactors of FEZ1, FEZ2 and UNC-76. On top of the boxes, highlighted by larger print and in bold, are the bait proteins used in the yeast two-hybrid assays. Sets are represented by gray boxes. The darker the gray the larger the set of proteins. Nuclear proteins are indicated by an asterisk preceeding the official protein name.
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although not lethal, cause deleterious abnormalities in the elongation of neuronal axons along other axonal surfaces (but not over non-neuronal surfaces) during the animals development, which results in a "paralyzed" phenotype, where the worm has severly reduced body movements (therefore the designation unc for uncoordinated movements). These abnormalities can be complemented by the expression of human FEZ1 [1]. This result already shows the high functional conservation which we confirmed and detailed here: the protein interaction profiles of FEZ1 and UNC-76 are highly overlapping, since 36 of 40 FEZ1 interactors also interact with UNC-76 (90%).

In this context it is also interesting to note that although the knockout of UNC-76 had severe and deleterious effects in the worm a recently described knockout mice for FEZ1 showed no dramatic changes in the brains anatomy or, the morphology of neuronal cell bodies, dendrites or axons, nor of the general locomotion or behavior of the animals [32]. Only after new social interactions or under stress conditions a slight hyper locomotion behavior was observed as well as a greater response to certain psycho-stimulants [32]. Our comparative analysis of the FEZ1 and FEZ2 interactomes becomes interesting at this point, because in mammals, and chordates in general, we have two FEZ family gene members in contrast to the *C. elegans* genome, which contains only *unc-76*. The duplication of the *unc-76* gene occurred most likely in the stemline of the chordates (Table S1, Fig. 1) [6]. Our current interpretation of the lack of any greater neurophysiological or behavioural abnormalities in the *FEZ1-/-* mice, may be due to a compensatory expression of the ubiquitously expressed FEZ2, which - as we demonstrated here - not only acquired additional new interactors in respect to FEZ1, but at the same time also maintained **all** of the interactors of FEZ1. From our point of view it is therefore not at all surprising that the FEZ1-/- mice had such a mild phenotype in comparison to the UNC-76 -/- worm [32]. It may even be that expression of FEZ2 in the FEZ1 k.o. mice, by hitherto unknown mechanisms, may be up-regulated in comparison to wild-type mice. It is interesting to note that in Figure S1A of the article of Sakae and co-workers, we can observe a new band at the height of the FEZ1 protein in extracts from the stomach of FEZ1-/- but not wild type mice (anti-FEZ1 Western blot). Since FEZ2 has almost the same MW as FEZ1, this band may represent up-regulated FEZ2 protein, in response to the lack of FEZ1. The observed relatively mild phenotype in the *FEZ -/-* mice may be as well attributed to additional proteins interacting with FEZ2, which may interfere in the way of a dominant negative in FEZ1 specific functions, as to specific but not yet identified PPI for FEZ1.

Although FEZ2 has more interactors than FEZ1 there is not a 100% functional redundancy. This would explain why both genes are still maintained in the gene pool (from a population genetics point of view). The reasons why both are maintained may be manifold, including dosage effects or a promoter specific regulation that must be maintained in the neurons (FEZ1). Data from the literature suggest that FEZ1 expression is increased upon NGF treatment, a neuronal tissue specific factor [6,33]. It may be even speculated that FEZ1 is somehow involved in neurogenesis per se. FEZ1 mRNA expression shows a peak that coincides with neurogenesis in rats, and decreases with the progress of adulthood. The mRNA expression FEZ2, although with higher levels in embryogenesis, is relatively constant, always less than that of FEZ1. Additional data on FEZ2 however are not available so far but one could speculate that its more ubiquitous expression depends on internal genetic programs rather than on external stimuli, like in the case of FEZ1.

Many of the proteins identified by our screen with FEZ2 interact with proteins that also interact with each other, increasing therefore the likelihood that these are true interactions of biological relevance. We like to highlight here the interactions of pleiotrophin (PTN), protein tyrosine phosphatase, receptor type S (PTPRS) ([18], and retinoic acid receptor alpha (RARA). In 2005, Stelzl et al. [18] performed a yeast two-hybrid screen on a large scale, reporting more than 3000 interactions between human proteins. The human protein FEZ1 interacted with 21 proteins. Of these 21 proteins, 4 interact with pleiotrophin (general transcription factor IIF, protocollagen hydroxylase, neurokinin beta, translocase of outer mitochondrial membrane 20 homolog). FEZ1 interacted further with a receptor protein tyrosine phosphatase (PTP) (sigma) of the same family of receptors of pleiotrophin (beta and zeta) [34]. Pleiotrophin is also known as neurite growth-promoting factor 1 (NEGF1), has mitotic activity and influences the growth of neuritis. It acts in the developing nervous system [35] and signals via the inactivation of a receptor-dependent ligand of RPTP beta/zeta (or PTPRS). It increases the level of tyrosine phosphorylated signaling proteins in the cell, promoting thereby downstream signaling events [34]. In the nervous system, PTN is expressed in both neurons and glia cells [36] as well as in neuronal stem cells [37]. SAGE analysis indicated a 10-fold elevation of PTN transcripts in response to long-term NGF treatment of PC12 cells. It is of interest, that in P19 neuroprogenitor cells, PTN was also induced by retinoic acid during neuronal differentiation [38,39]. Retinoic acid is essential for the differentiation and morphogenesis of various tissues, including the nervous system [40]. Silencing of FEZ1 by RNA interference (RNAi) strongly inhibited the NGF-induced differentiation and efficiently reduced the anterograde transport of

mitochondria in PC12 cells, suggesting that FEZ1 is essential for NGF-induced neuronal differentiation of PC12 cells [33,41].

Retinoic acid receptors (RARs) are transcriptional activators which are activated by binding of their ligands and act in concert with a combination retinoid X type receptors (RXR alpha, beta and gamma). Even in the absence of ligand, RAR-RXR heterodimers bind to DNA sequences known as response elements for RA, but in this state they recruit co-repressors. These co-repressors mediate the negative modulation of transcription by recruitment of the histone deacetylase complex and the transfer of methyl radicals to the DNA bound histones, thereby stabilizing the nucleosome. The binding of the ligand retinoic acid causes however conformational changes in the binding domain of RAR, which in return promote the release of the co-repressors and the recruitment of co-activators. While some co-activators interact with factors of the basal transcription machinery, others induce chromatin remodeling and specific transcriptional activation. Data from our previous two-hybrid and pull-down assays confirmed the interaction of FEZ1 and FEZ2 proteins related to activation of transcription (BAF60a) and the repression of transcription (SAP30L) [10]. Furthermore, RXR/RAR interacts with N-Cor, thereby recruiting the complex of repression of transcription [42]. It is known that SAP30 also interacts with N-Cor [43] promoting the compaction of chromatin. These data gain potential relevance by the observation that both RAR and FEZ1/FEZ2 interact with protein complexes of chromatin remodeling, and with each other. Thus, FEZ1 may be also involved in the regulation of gene transcription mediated by transcription factors.

In summary, we have described here the molecular expansion of the FEZ protein family in the chordate stemline, most likely after two rounds of whole-genome duplication. We have identified many new members of the FEZ family from different species and a highly conserved C-terminal domain, which is responsible to mediate the majority of the protein-protein interactions. Our analyses indicate that the ancestral UNC-76 protein function and protein interaction profile is surprisingly conserved in animals, evolutionary distinct as vertebrates and nematodes. The analysis of the phylogeny and the detailed protein interaction profiles uncovered a likely functional divergence between FEZ1 and FEZ2, since FEZ2 acquired in respect to FEZ1/UNC-76 additional new interactors and is ubiquitously expressed and not restricted to neuronal cells, as is FEZ1/UNC-76. Our data provide an explanation for the ability of human FEZ1 to rescue the defects caused by *unc-76* mutations in nematodes, since the PPI pattern of FEZ1 and UNC-76 are highly similar. Furthermore, no strong defects are caused in mice lacking the *FEZ1*, probably due to the compensatory presence of its paralogue FEZ2, which interacted with all FEZ1 interacting proteins, and additional new interactors that are FEZ2 specific. Our new data will hopefully stimulate and facilitate further studies on the functional role of proteins of the FEZ family in the development and function of both neuronal and non-neuronal cells. Furthermore, studies such as ours may contribute to our ever growing understanding of how protein networks function and specially how they came about and were modified and adapted during evolution. In the future we may devise new and more complete ways to visualize and comprehend how these networks were shaped throughout time.

Supporting Information

Figure S1 Amino acid sequence alignment of the members of the FEZ family. Names of the sequences are given in the Table S1. The residues in the alignment are shaded light grey, grey, or black to indicate shared identity in 40%, 70% or 100% of the analyzed sequences, respectively. The bars indicate regions predicted to form coiled-coil.
(PDF)

Figure S2 Identities and similarities between human proteins FEZ1 and FEZ2 and *C. elegans* UNC-76. A general scheme of the FEZ family proteins is shown at the top. The identity and similarity comparisons were made of two-by-two proteins both for the complete protein alignment as well as for local alignment of FEZ fragments by NPS@ (http://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page = npsa_clustalw.html). I = identity, S = strongly similar, W = weakly similar, D = different.
(TIF)

Table S1 FEZ family proteins used for amino acid sequence alignment.
(DOC)

Acknowledgments

We thank Maria Eugenia R. Camargo for technical assistance and Prof. Dr. Michael O. Hengartner for the kind gift of the pSU001 plasmid (containing *unc-76*).

Author Contributions

Conceived and designed the experiments: JK MRA. Performed the experiments: MRA ASF. Analyzed the data: MRA ASF JK. Wrote the manuscript: MRA JK.

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3.2. Artigo II

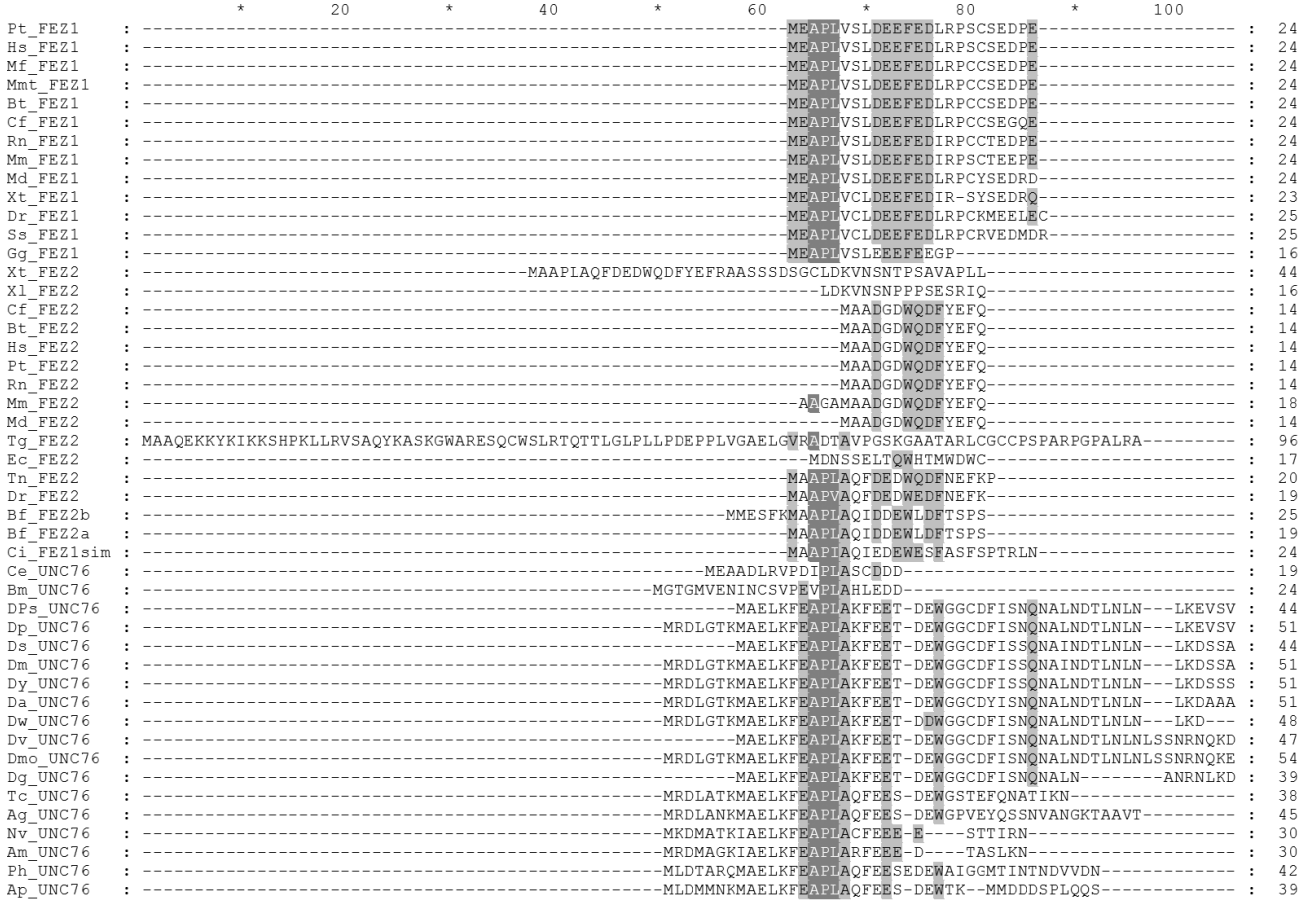
3.2.1. Material suplementar

FEZ2 Has Acquired Additional Protein Interaction Partners Relative to FEZ1: Functional and Evolutionary Implications

Supplemental Table 1: FEZ family proteins used for amino acid sequence alignment.

Number	Name	Identifier	Organism	Taxonomy	Description
1	Cf_FEZ2	gi|73980130|	Canis familiaris	Eu., mt., vt., mam.	PREDICTED: similar to FEZ2
2	Hs_FEZ2	gi|114205570|	Homo sapiens	Eu., mt., vt., mam.	Fez2 protein
3	Bt_FEZ2	gi|76629046|	Bos taurus	Eu., mt., vt., mam.	PREDICTED: similar to FEZ2
4	Rn_FEZ2	gi|72679487|	Rattus norvegicus	Eu., mt., vt., mam.	Fez2 protein
5	Pt_FEZ2	gi|114576932|	Pan troglodytes	Eu., mt., vt., mam.	PREDICTED: FEZ2
6	Md_FEZ2	gi|126303164|	Monodelphis domestica	Eu., mt., vt., mam.	PREDICTED: FEZ2
7	Mm_FEZ2	gi|148706509|	Mus musculus	Eu., mt., vt., mam.	Fez2 protein
8	Md_FEZ1	gi|126327241|	Monodelphis domestica	Eu., mt., vt., mam.	PREDICTED: hypothetical protein
9	Mf_FEZ1	gi|67970547|	Macaca fascicularis	Eu., mt., vt., mam.	unnamed protein product
10	Bt_FEZ1	gi|66792834|	Bos taurus	Eu., mt., vt., mam.	zygin 1
11	Mmt_FEZ1	gi|109109139|	Macaca mulatta	Eu., mt., vt., mam.	PREDICTED: zygin 1
12	Cf_FEZ1	gi|73954536|	Canis familiaris	Eu., mt., vt., mam.	PREDICTED: similar to FEZ1
13	Pt_FEZ1	gi|114641075|	Pan troglodytes	Eu., mt., vt., mam.	PREDICTED: zygin 1 isoform 1
14	Rn_FEZ1	gi|13994121|	Rattus norvegicus	Eu., mt., vt., mam.	FEZ1
15	Hs_FEZ1	gi|4826724|	Homo sapiens	Eu., mt., vt., mam.	FEZ1
16	Mm_FEZ1	gi|148693469|	Mus musculus	Eu., mt., vt., mam.	FEZ1
17	Ec_FEZ2	gi|194220815|	Equus caballus	Eu., mt., vt., mam.	PREDICTED: similar to zygin 2
18	Tg_FEZ2	gi|224047659|	Taeniopygia guttata	Eu., mt., vt., av.	PREDICTED: similar to zygin 2
19	Gg_FEZ2	gi|118101818|	Gallus gallus	Eu., mt., vt., av.	PREDICTED: hypothetical protein isoform 1
20	Xt_FEZ2	gi|62858603|	Xenopus tropicalis	Eu., mt., vt., amp.	Fez2 protein
21	Xl_FEZ2	gi|67678016|	Xenopus laevis	Eu., mt., vt., amp.	LOC733280 protein
22	Xt_FEZ1	gi|118405066|	Xenopus tropicalis	Eu., mt., vt., amp.	zygin 1
23	Tn_FEZ1	gi|47208647|	Tetraodon nigroviridis	Eu., mt., vt., acti.	unnamed protein product
24	Dr_FEZ2	gi|116812571|	Danio rerio	Eu., mt., vt., acti.	zygin 2
25	Dr_FEZ1	gi|54261791|	Danio rerio	Eu., mt., vt., acti.	FEZ1
26	Ss_FEZ1	gi|213514730|	Salmo salar	Eu., mt., vt., acti.	FEZ1
27	Ci_FEZ2	gi|198425368|	Ciona intestinalis	Eu., mt., cph., tun	PREDICTED: similar to FEZ2
28	Bf_FEZ2a	gi|219489321|	Branchiostoma floridae	Eu., mt., cph., bra	hypothetical protein BRAFLDRAFT_103854
29	Bf_FEZ2b	gi|219411184|	Branchiostoma floridae	Eu., mt., cph., bra	hypothetical protein BRAFLDRAFT_202324
30	Ce_UNC76	gi|115534730|	Caenorhabditis elegans	Eu., mt., nem., chr.	UNC-76
31	Bm_UNC76	gi|170594585|	Brugia malayi	Eu., mt., nem., chr.	UNC-76
32	Dpo_UNC76	gi|198467847|	Drosophila pseudoobscura	Eu., mt., art., ins.	GA17820
33	Ds_UNC76	gi|195347821|	Drosophila sechellia	Eu., mt., art., ins.	GM19195
34	Dy_UNC76	gi|195477805|	Drosophila yakuba	Eu., mt., art., ins.	GE16233
35	Dm_UNC76	gi|18543271|	Drosophila melanogaster	Eu., mt., art., ins.	UNC-76
36	Da_UNC76	gi|194764284|	Drosophila ananassae	Eu., mt., art., ins.	GF21457
37	Dw_UNC76	gi|195447704|	Drosophila willistoni	Eu., mt., art., ins.	GK25735
38	Dv_UNC76	gi|195397079|	Drosophila virilis	Eu., mt., art., ins.	GJ16936
39	Dc_UNC76	gi|91081223|	Tribolium castaneum	Eu., mt., art., ins.	PREDICTED: similar to AGAP003014-PA
40	Dmj_UNC76	gi|195129810|	Drosophila mojavensis	Eu., mt., art., ins.	GI15282
41	Nv_UNC76	gi|156548791|	Nasonia vitripennis	Eu., mt., art., ins.	PREDICTED: similar to ENSANGP00000018262
42	Ag_UNC76	gi|118781797|	Anopheles gambiae	Eu., mt., art., ins.	AGAP003014-PA
43	Pn_UNC76	gi|242012918|	Pediculus humanus corporis	Eu., mt., art., ins.	Fasciculation and elongation protein zeta, putative
44	Am_UNC76	gi|48103592|	Apis mellifera	Eu., mt., art., ins.	PREDICTED: similar to Unc-76
45	Dg_UNC76	gi|195046162|	Drosophila grimshawi	Eu., mt., art., ins.	GH24577
46	Ap_UNC76	gi|193586919|	Acyrthosiphon pisum	Eu., mt., art., ins.	PREDICTED: similar to AGAP003014-PA
47	Dp_UNC76	gi|195170071|	Drosophila persimilis	Eu., mt., art., ins.	GL18226

acti. = Actinopterygii, amp. = Amphibia, art. = Arthropoda, cph = Cephalochordata, eu. = Eukaryota, ins. = Insecta, invt = Invertebrata, mam. = Mammalia, mt = Metazoa, nem = Nematoda, vt = Vertebrata, av. = Aves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Mm_FEZ1 : 24
Md_FEZ1 : 24
Xt_FEZ1 : 23
Dr_FEZ1 : 25
Ss_FEZ1 : 25
Gg_FEZ1 : 16
Xt_FEZ2 : 44
Xl_FEZ2 : 16
Cf_FEZ2 : 14
Bt_FEZ2 : 14
Hs_FEZ2 : 14
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Md_FEZ2 : 14
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Bf_FEZ2b : 25
Bf_FEZ2a : 19
Ci_FEZ1sim : 24
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Bm_UNC76 : 24
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Ds_UNC76 : 44
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Dy_UNC76 : 51
Da_UNC76 : 51
Dw_UNC76 : 48
Dv_UNC76 : 47
Dmo_UNC76 : 54
Dg_UNC76 : 39
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Ap_UNC76 : 39
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Hs_FEZ1 : 51
Mf_FEZ1 : 51
Mmt_FEZ1 : 51
Bt_FEZ1 : 51
Cf_FEZ1 : 51
Rn_FEZ1 : 51
Mm_FEZ1 : 51
Md_FEZ1 : 51
Xt_FEZ1 : 50
Dr_FEZ1 : 53
Ss_FEZ1 : 60
Gg_FEZ1 : 39
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Xl_FEZ2 : 43
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Bt_FEZ2 : 38
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Pt_FEZ2 : 42
Rn_FEZ2 : 38
Mm_FEZ2 : 42
Md_FEZ2 : 36
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Ec_FEZ2 : 50
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Bf_FEZ2b : 53
Bf_FEZ2a : 47
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Ds_UNC76 : 128
Dm_UNC76 : 135
Dy_UNC76 : 137
Da_UNC76 : 133
Dw_UNC76 : 130
Dv_UNC76 : 121
Dmo_UNC76 : 139
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Ph_UNC76 : 110
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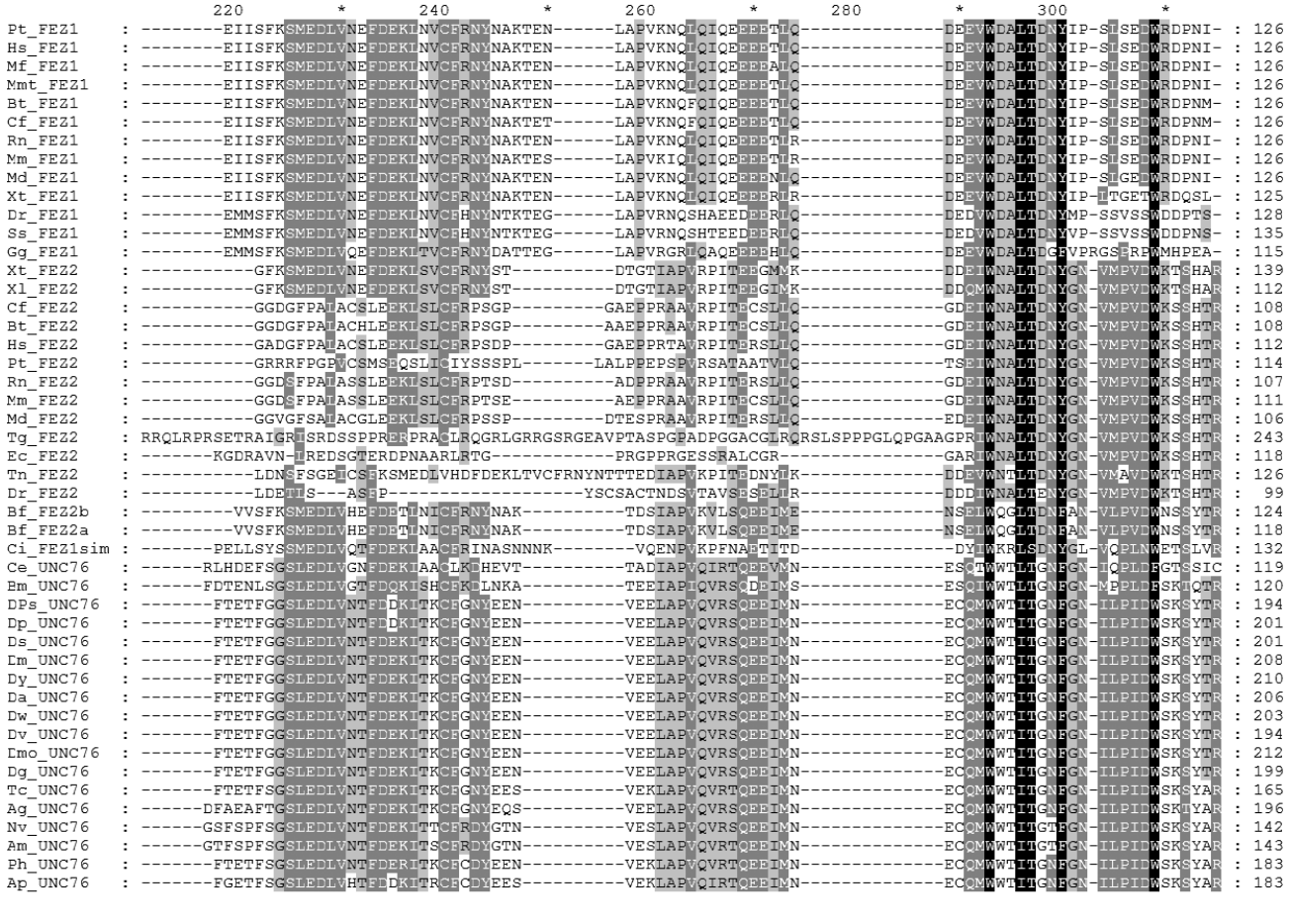
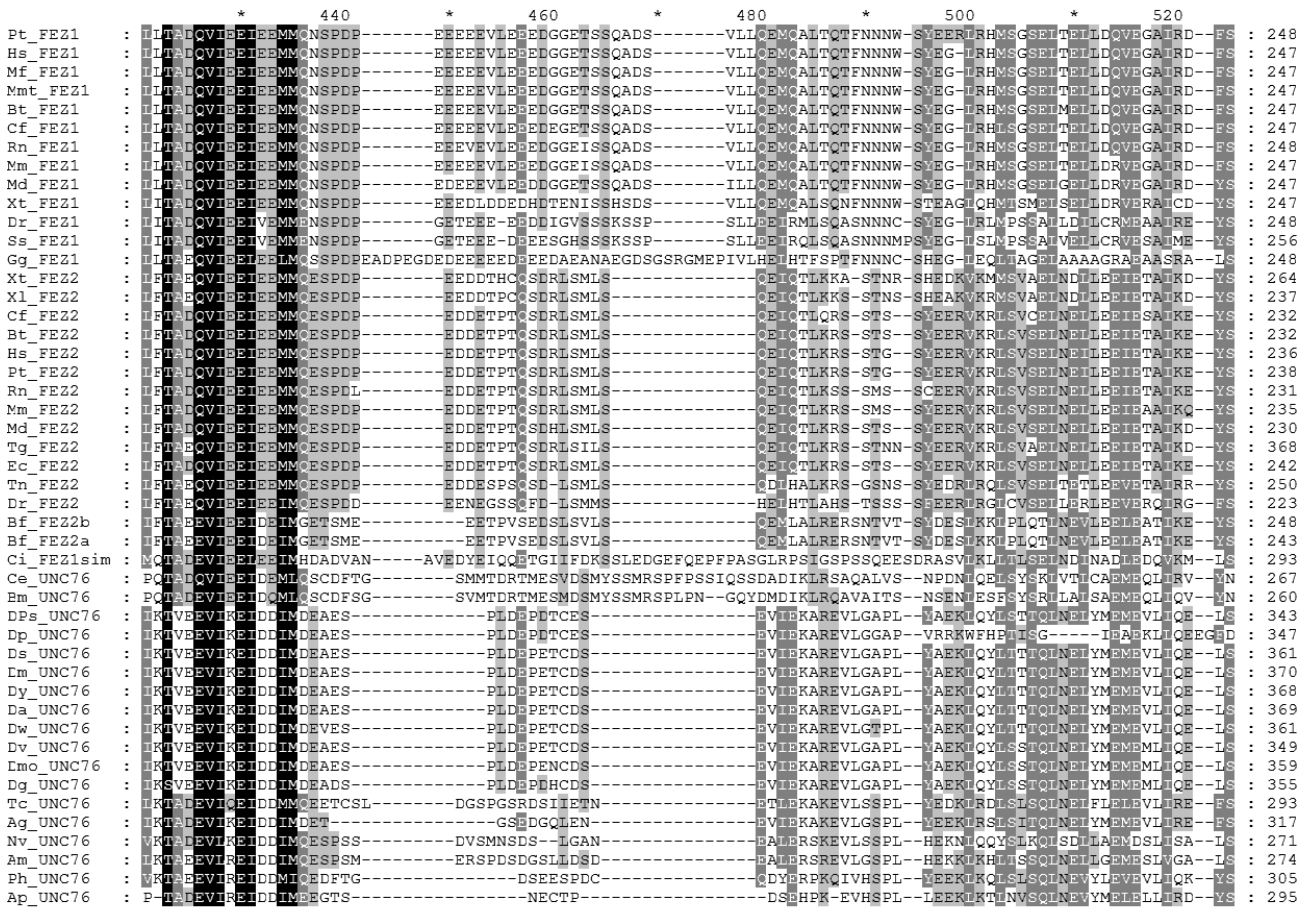
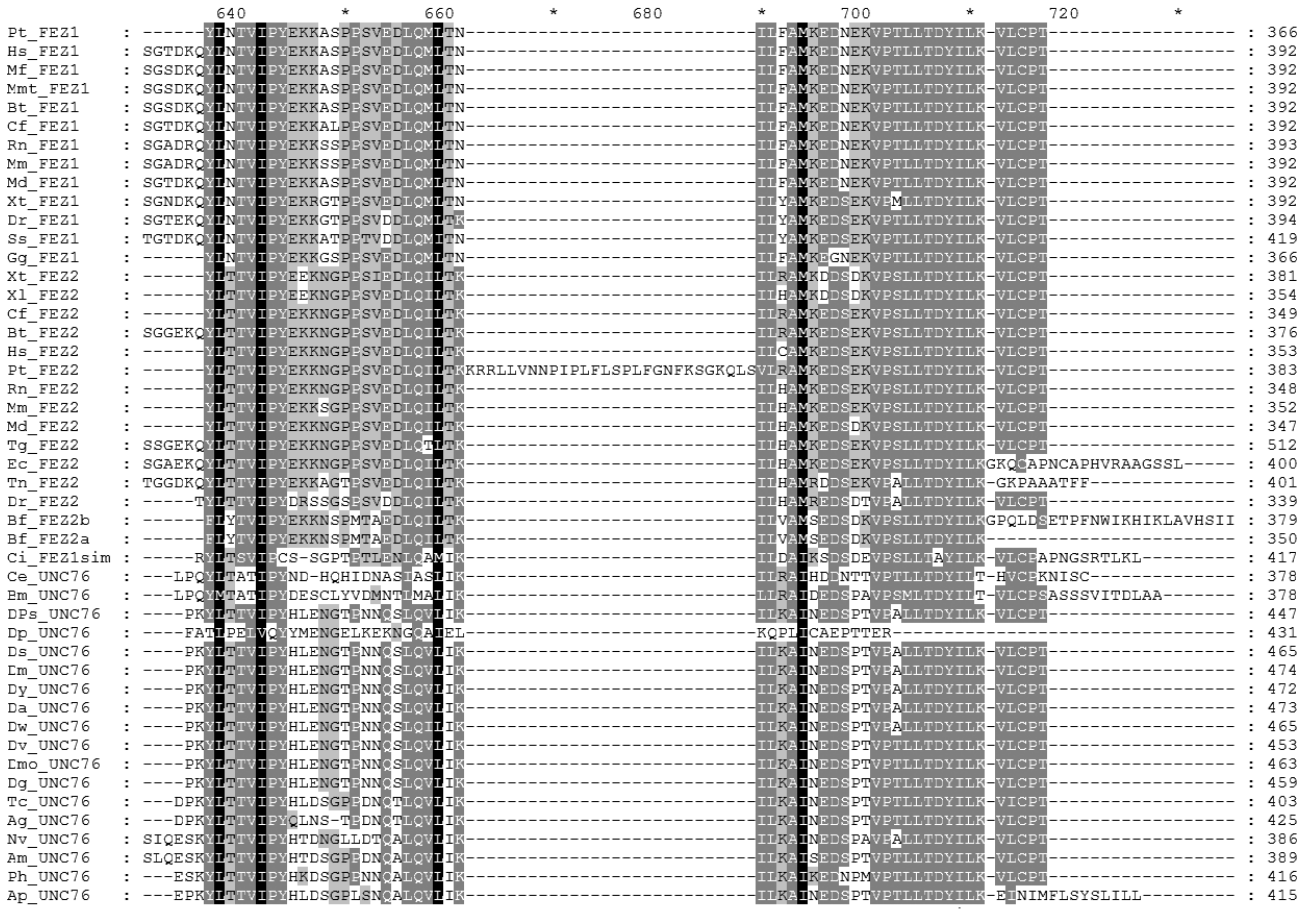
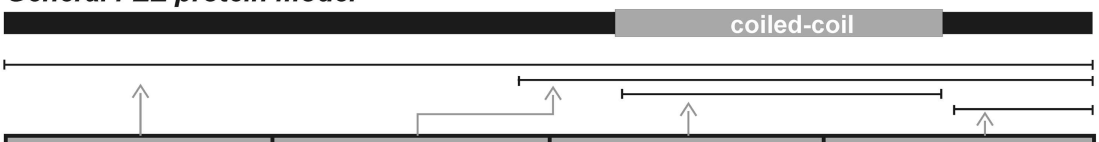






Supplementary Fig. S1. Amino acid sequence alignment of the members of the FEZ family. Names of the sequences are given in the Supplemental Table 1. The residues in the alignment are shaded light grey, grey, or black to indicate shared identity in 40%, 70% or 100% of the analyzed sequences, respectively. The bars indicate regions predicted to form coiled-coil.

General FEZ protein model



Full Length			C-terminal			Coiled-coil			Extreme c-terminal		
FEZ1 (1-392) FEZ2 (1-353) UNC-76 (1-378)			FEZ1 (221-392) FEZ2 (207-353) UNC-76 (242-378)			FEZ1 (224-306) FEZ2 (207-353) UNC-76 (244-317)			FEZ1 (340-392) FEZ2 (301-353) UNC-76 (323-378)		
FEZ1 x UNC-76:	I	25.94%	FEZ1 x UNC-76:	I	33.52%	FEZ1 x UNC-76:	I	39.76%	FEZ1 x UNC-76:	I	42.11%
	S	18.63%		S	19.89%		S	27.71%		S	15.79%
	W	13.68%		W	8.52%		W	6.02%		W	15.79%
	D	41.75%		D	38.07%		D	26.51%		D	26.32%
FEZ2 x UNC-76:	I	27.04%	FEZ2 x UNC-76:	I	39.07%	FEZ2 x UNC-76:	I	39.76%	FEZ2 x UNC-76:	I	42.11%
	S	20.92%		S	23.84%		S	26.51%		S	17.54%
	W	10.46%		W	9.93%		W	7.23%		W	15.79%
	D	41.58%		D	27.15%		D	26.51%		D	24.56%
FEZ1 x FEZ2:	I	41.63%	FEZ1 x FEZ2:	I	57.71%	FEZ1 x FEZ2:	I	62.65%	FEZ1 x FEZ2:	I	83.02%
	S	16.75%		S	13.14%		S	21.69%		S	5.66%
	W	9.61%		W	4.00%		W	4.82%		W	5.66%
	D	32.02%		D	25.14%		D	12.84%		D	5.66%

Supplementary Fig. S2. Identities and similarities between human proteins FEZ1 and FEZ2 and *C. elegans* UNC-76. A general scheme of the FEZ family proteins is shown at the top. The identity and similarity comparisons were made of two-by-two proteins both for the complete protein alignment as well as for local alignment of FEZ fragments by NPS@ (http://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page=npsa_clustalw.html). I = identity, S = strongly similar, W = weakly similar, D= different.

3.3. Artigo III

Human FEZ1 Protein Forms a Disulfide Bond Mediated Dimer: Implications for Cargo Transport

Alborghetti MR, Furlan AS, Silva JC, Paes Leme AF, Torriani ICL, Kobarg J

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Human FEZ1 Protein Forms a Disulfide Bond Mediated Dimer: Implications for Cargo Transport

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Received April 6, 2010

The human proteins FEZ1 (fasciculation and elongation protein zeta 1) and FEZ2 are orthologs of the protein UNC-76 from *C. elegans*, involved in the growth and fasciculation of the worms axon. Pull down assays showed that the protein FEZ1 interacts with other proteins (e.g., the protein SCOCO, short coiled-coil protein), mitochondria, and vesicles. These components may therefore represent cargoes to be transported along the microtubule, and the transport may be mediated through FEZ1 reported binding to kinesins (KIF3A). We previously showed that FEZ1 dimerizes in its N-terminal region and interacts with other proteins, including the candidate cargoe proteins, through its C-terminus. Here, we studied the fragment FEZ1(92−194) as well as full-length 6xHis-FEZ1 (1−392) *in vitro* and endogenous FEZ1 isolated from HEK 293 cells and were able to demonstrate the formation of an intermolecular disulfide bond through FEZ1 Cys-133, which appears to be essential for dimerization. This disulfide bond may be important for the FEZ1 role as a dimeric and bivalent transport adaptor molecule, since it establishes a strong link between the monomers, which could be a prerequisite for the simultaneous binding of two cargoes.

Keywords: neuron • homodimer • disulfide bond • cargo transport • microtubules • kinesin • SAXS • FEZ1 • flower-like phenotype

Introduction

Many fundamental eukaryotic trafficking mechanisms are conserved in neurons.[1] Among the molecular motors, three large superfamilies have been identified: kinesins, dyneins and myosins. The requirement for kinesin-based transport is especially acute in neurons, in which long-distance transport from the cell body to the synapse occurs.[2] In some cases, the transport machinery requires an adaptor protein that directly associates with the motor protein and is essential for the transport and association of components such as cargo complexes or vesicles.[3]

In 2003, Gindhart and colleagues showed that the UNC-76 is a kinesin-cargo adaptor and their interaction is essential for intracellular transport. The UNC-76 function, as well as that of its human orthologues FEZ1 (fasciculation and elongation protein zeta 1) and -2, is important and necessary for normal axonal outgrowth and fasciculation. The mutant unc-76 discovered in a locomotion defective worm showed failure to fully extend axon bundles of the dorsal and ventral nerve cords, as well as a defective neuronal fasciculation.[4,5]

FEZ1 has several features of natively unfolded proteins with coiled-coil predictions.[6] Recent studies showed the importance of the Carboxy-terminus of UNC-76/FEZ1 in mediating protein−protein interactions that mainly involve coiled-coil pairing.[7−10] Interestingly, several proteins related to neuronal development have been described to interact with UNC-76 or FEZ1 through its C-terminal region: DISC1, PKCzeta, PTPS, synaptotagmin, and SCOCO (short coiled-coil protein), among others,[4,10−13] all of which may represent cargo proteins. The dimeric configuration of a construct representing only its N-terminal region (aa 1−227) in FEZ1 suggested that it could act as a bivalent transport adaptor protein and that the dimerization is mediated by the N-terminal.[6] The two C-terminal regions of the full-length dimer are pointing outward and may serve as docking domains for cargo proteins or may interact indirectly with the microtubules via CLASP2, KIF3A or other connecting proteins yet to be identified.

The N-terminal halves of FEZ1 and FEZ2 show less conservation in their amino acid sequence than their C-terminal halves (Figure 1A), although both the N and C terminals show substantial sequence similarity to UNC-76 of *C. elegans*, as well

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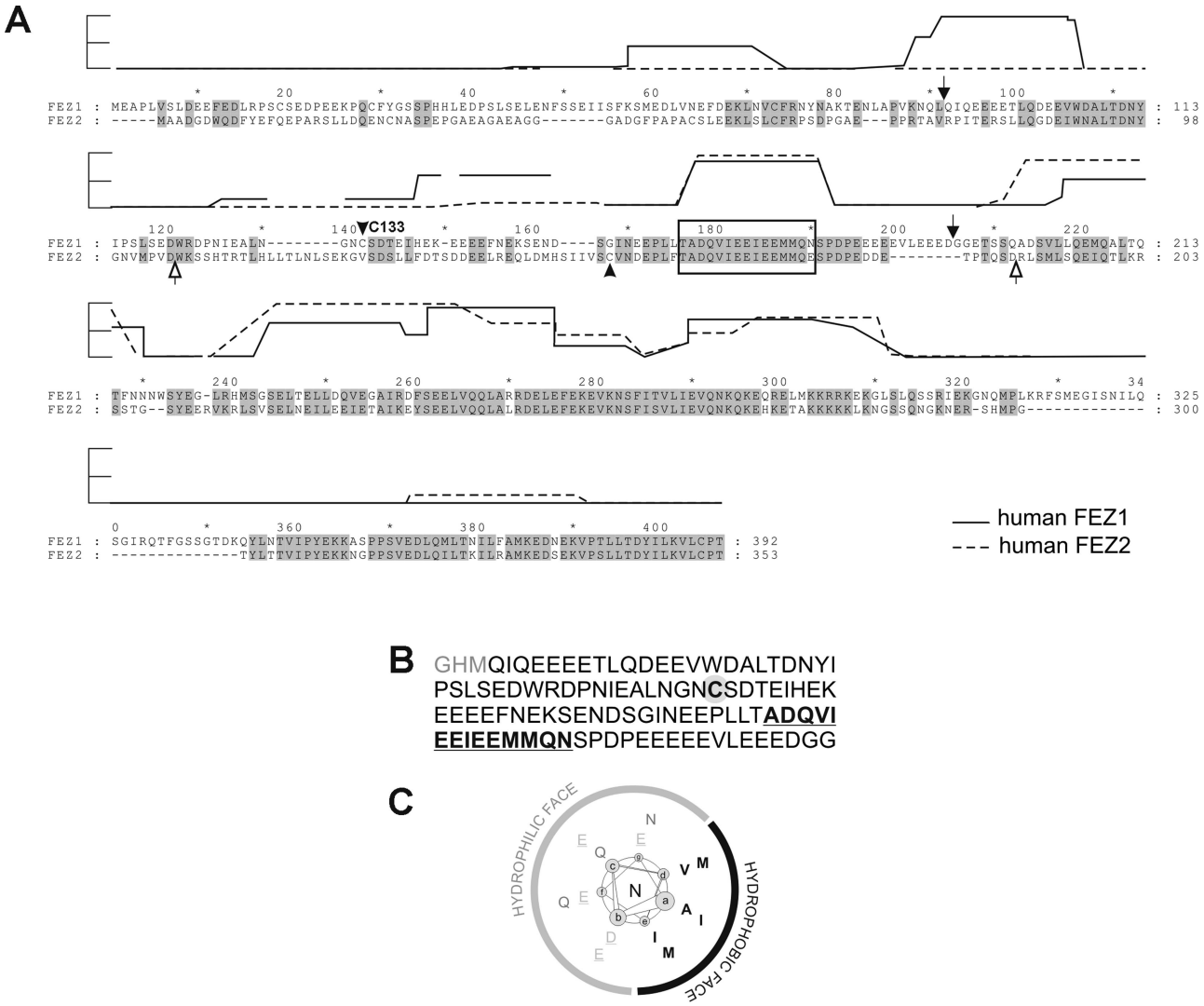

Figure 1. Sequence analysis of FEZ1 and FEZ2 protein. (A) Probability of coiled-coil formation of the full-length FEZ1 and FEZ2 protein was predicted by the program COILS (Lupas et al. 1999); gaps in the prediction were inserted according to alignment. The conserved residues in the alignment are shaded in gray. The rectangle shows the conserved region with propensity to form coiled-coils. The black arrows delimitate the expressed fragment FEZ1 (92−194). The black arrowheads show the cysteine residue present in that fragment (FEZ1) or the probably corresponding residue in FEZ2. The white arrows indicate the start and stop positions of the constructs FEZ2 (106−189). (B) Amino acid sequence of FEZ1 (92−194). A motif with a high probability for coiled-coil formation as determined by the program COILS (window 14) is underlined. A single cysteine (C133) is bolded and highlighted by a gray circle. (C) Helical wheel representation of a coiled coil based on the predicted heptad repeats (residues 164−177, underlined in B). The amino acids in black are hydrophobic and in gray hydrophilic. The view is from the N down to the C terminus. The predicted heptad repeats are denoted as "abcdefg".

as a conserved subcellular localization of the protein.[4] Most interestingly, the amino acids 1−197 of UNC-76 have been shown to target a fused green fluorescent protein or β-galactosidase to axons in *C. elegans*.[14] It is worthwhile to compare the axon targeting characteristic of this protein region of UNC-76 to the dendrite targeting activity of the mouse Glutamate Receptor-Interacting Protein (GRIP1), which binds to KHC homologues KIF5a/b/c and targets them to the dendrite.[14,15] This could suggest that the amino terminus of UNC-76 may have an analogous role in axon targeting of kinesins or other motor proteins.[2]

Here, we characterized a fragment from the N-terminus of FEZ1 (amino acid residues 92−194), that has a highly conserved region predicted to contain a coiled-coil motif just as in FEZ2 (Figure 1B and C) and worm UNC-76 proteins, and obtained its solution structure model. We performed native acryl amide gel electrophoresis under reducing or nonreducing conditions and analyzed the samples by Small Angle X-ray Scattering (SAXS), where we confirmed that the molecular mass of the nonreduced sample (dimer) was exactly twice the mass of the TCEP-reduced sample (monomer). Finally, a comparative mass spectrometry analysis of reduced and nonreduced protein samples showed that the cysteine 133 is indeed oxidized in the full-length FEZ1 dimer. In summary, our results suggest that the disulfide bond at the FEZ1 N-terminus region is important for the protein dimerization. The disulfide bond may be a key feature for FEZ1 role as a bivalent adaptor molecule, since it establishes a strong link between the monomers. Our model of the full length FEZ1[6] further suggests that the FEZ1 C-terminal may be divided in two regions: one may interact with microtubule associated proteins (tubulin, KIF3A, CLASP2 are FEZ1 interactors) while the other one may interact with

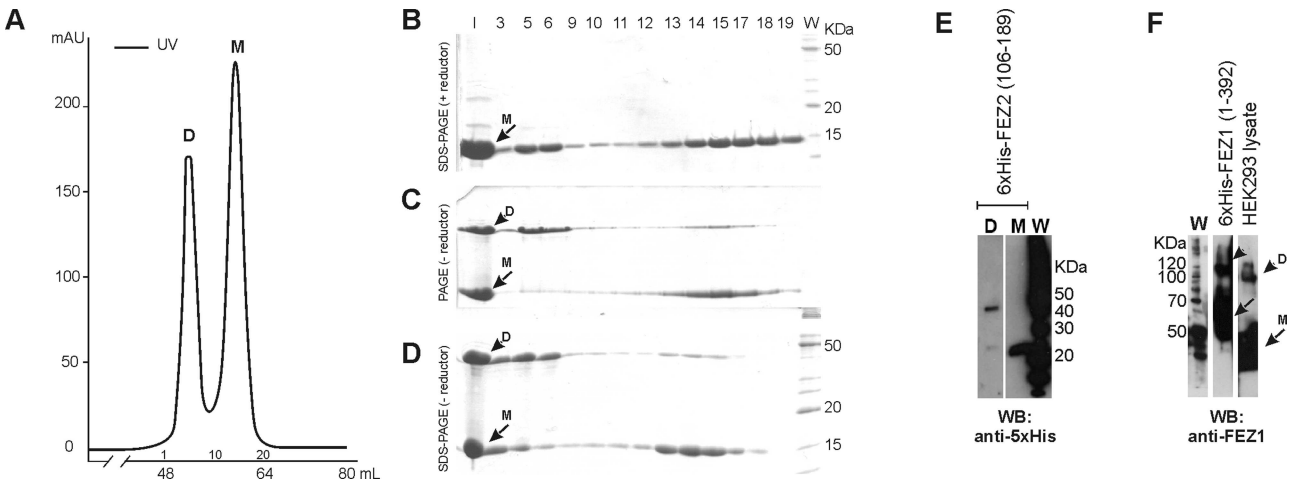

Figure 2. Dimerization of FEZ1 and FEZ2. (A) Absorbance curve of FEZ1 (92−194) purified by gel filtration on a HiLoad 16/60 Superdex 75 prep grade column. (B) SDS-PAGE (15% AA) of eluted fractions (number of fractions according to absorbance curve in A) under denaturing (reduced) conditions. (C) PAGE 15% of eluted fractions under nondenaturing (and nonreduced) conditions. (D) SDS-PAGE (15% AA) of eluted fractions under nonreduced conditions. The gels were colored with Coomassie Blue. The arrows and arrow heads indicate the monomer and dimer, respectively. (E) 6xHis-FEZ2 (106−189) SDS-PAGE was performed under non- (*D* = dimer) and reduction (*M* = monomer) conditions and analyzed by Western blot. (F) Western blot of purified 6xHis-FEZ1 (1−392) protein from *E. coli* and HEK 293 cell lysate after SDS-PAGE under mildly reducing conditions. Blot was analyzed with an anti-FEZ1 antibody. *W* = molecular weight marker proteins.

candidate cargo proteins (e.g., the protein SCOCO), vesicles and mitochondria.

Results

Conserved N-Terminal Regions in FEZ1 and FEZ2 Proteins. Based on primary sequence analysis, FEZ1 and FEZ2 proteins show conserved regions in both its C- and N-terminal regions. The C-terminus of FEZ1/2 is related to FEZ-cargo protein−protein interactions mediated by coiled-coil formation. The N-terminal halves are less conserved but still show substantial amino acid sequence similarity,[4] mediating FEZ1/2 homodimerization.[6] There are some weak predictions for short coiled-coils formation in this region when using the program COILS (see Materials and Methods) for both FEZ1 and FEZ2, and this prediction matches with highly conserved amino acid sequence regions: the amino acids 164−177 in FEZ1 and 162−175 in FEZ2 (Figure 1). FEZ1 and FEZ2 have cysteine residues near this coiled-coil region (Figure 1A, black arrowheads) that could contribute to the dimerization process and dimer stability (Figure 1). A coiled-coil domain at the N-terminus could be also important to mediate protein−protein interactions that are required for the proteins correct localization in axons. The hydrophobic interface formed by amino acid residues in the positions "a, d and e" of the helical wheel could be essential for these interactions, while the opposing hydrophilic surface could be exposed to the solvent (Figure 1B and C).

Intermolecular Disulfide Bond in FEZ1 (92−194) and in FEZ2 (106−189) Dimers. Previous studies showed that FEZ1 dimerizes through its N-terminal region.[6] Here, we selected the smaller FEZ1 N-terminal region (92−194) (Figure 1A, black arrows) to test the formation of a probable disulfide bond by the unique cysteine in the position Cys133 of this fragment, which may be involved in protein dimerization. The recombinant protein was expressed in *E. coli* (BL21 DE3 strain) and purified by nickel affinity chromatography. The 6xHis tag was cleaved using TEV protease and repurified by anionic exchange purification. Size exclusion chromatography was performed and two populations of FEZ1(92−194) were obtained (Figure 2A). The eluted samples were analyzed by PAGE with and without SDS, under both reducing and nonreducing conditions (Figure 2B−D). SDS-PAGE under reducing condition (Figure 2B) demonstrated that there is a single protein band with a molecular weight around 13 kDa, as expected for monomeric FEZ1(92−194). However, two protein bands (13 and 40 kDa) were evidenced when the same samples were analyzed by either PAGE or SDS-PAGE under nonreducing condition, in accordance with the absorbance curve of size exclusion purification (Figure 2A). These results demonstrate that FEZ1 (92−194) dimerizes through its N-terminus, as shown previously for the full length protein by SAXS and other experiments.[6] The dependency of the migratory behavior of FEZ1 (92−194) on the reducing or oxidizing conditions suggests also that the dimerization involves a disulfide bond formation. The 40 kDa observed mass would predict a trimeric FEZ1 (92−194), but abnormal migration is frequently observed in PAGE experiments with FEZ1 protein, due to the presence of many charged amino acids, especially at its N-terminus.[6] From the SAXS and mass spectrometry experiments to be described below we can be certain that FEZ1(92−194) forms only dimers and not trimers.

Furthermore, we performed the cloning, expression and affinity purification of 6xHis-FEZ2 (106−189) (Figure 1A, white arrows). The recombinant protein was expressed at low concentration and because of that the oligomerization state was only analyzed by SDS-PAGE under reducing and nonreducing conditions followed by Western blott analysis (WB). The expected monomeric band was observed upon reduction (20 kDa), while a probable dimer of apparently 40 kDa appears under nonreducing condition (Figure 2E). Both FEZ1 (92−194) and FEZ2 (106−189) present only one cysteine residue (Cys133 and Cys153, for FEZ1 and 2, respectively).

We wondered if the formation of the disulfide bridge in the dimerization of FEZ1 could also occur in vivo in the reducing cytosolic cell environment. Therefore we analyzed the cell lysate of human HEK293 cells under mildly reducing conditions in

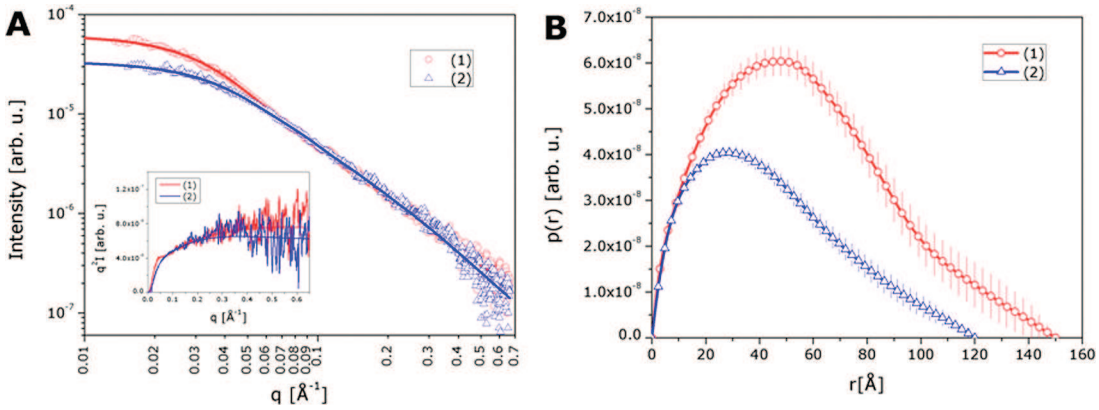

Figure 3. SAXS (small angle X-ray scattering) experiments of FEZ1 (92−194). (A) Scattering intensity curves of FEZ1 (92−194) under native conditions [red, (1)] and after 4 mM TCEP reduction [blue, (2)] (Inset) Respective Kratky plots. (B) Pair distance distribution functions ($p(r)$ functions).

order to allow good protein separation in the SDS Gel (Figure 2F). The experiment showed clearly that albeit the slightly reducing conditions dimerization of endogenous FEZ1 protein was also observed in HEK293 cell lysate. Although FEZ1 protein is under the extraction conditions predominantly presented in the monomeric form (arrow), we could clearly observe a significant fraction of dimeric FEZ1 (arrowhead), which seemed to be very stable and resistant to the mild reducing conditions applied (3% beta-mercaptoethanol). In this case, the molecular weight of the dimer band showed twice of the molecular weight of the monomer band. Both purified recombinant proteins purified from *Escherichia coli* lysate as well as endogenous protein from HEK293 lysate detected by the anti-FEZ1 antibody showed the same two bands, representing monomer and dimer. This demonstrated the specificity of the antibody. The bands of the recombinant protein are slightly higher, since this construct contains 32 additional amino acids, including the 6xHis tag and poly linker encoded amino acids, at its N-terminal.

FEZ1 (92−194) Dimerizes Involving Formation of a Disulfide Bond. Coiled-coil proteins are usually dimers, but some may also form trimers, tetramers, or pentamers.[16] To investigate the oligomeric state of FEZ1 (92−194), we used SAXS (small angle X-ray scattering). SAXS is a very useful technique for the study of flexible and low compactness proteins, providing important parameters like the overall size, shape and oligomerization status of the macromolecules in solution.[17−21] We performed SAXS analyses with samples predicted to be both monomeric or dimeric in accordance with the corresponding fractions obtained from the size exclusion chromatography (Figure 2A), as well as with a reduced sample that is predicted to be monomeric (see Materials and Methods). The SAXS data indicate that FEZ1(92−194) is a dimer in solution without a disulfide bond reducing agent but becomes a monomer after the addition of 4 mM of reductor TCEP. The scattering intensity curves of the dimeric oxidized sample and the monomeric reduced form of FEZ1 (92−194) are displayed in Figure 3A. The corresponding Kratky plots are shown in the inset. The Kratky plots present a plateau for $q > 0.2$ Å^{-1}, indicating that both proteins have a flexible conformation, which is a typical characteristic of intrinsically unstructured proteins. A discrete shoulder around $q = 0.05$ Å^{-1} may indicated a small content of structured chains in the dimer, probably located in the dimerization region. The corresponding pair distance distribution functions (the $p(r)$ functions) are displayed in Figure 3B.

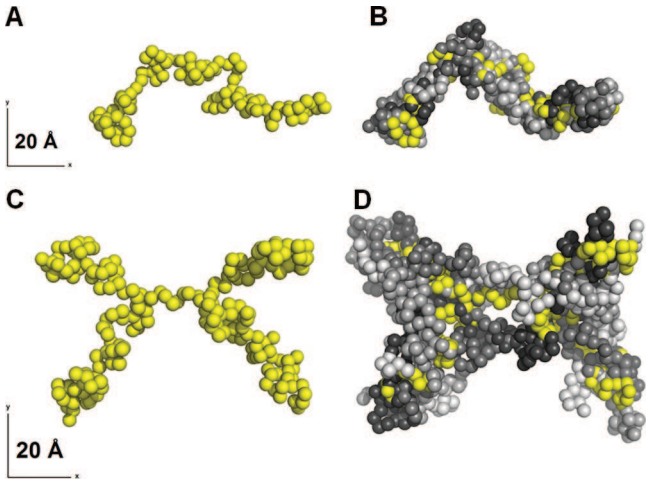

Figure 4. Low-resolution ab initio models for reduced and nonreduced FEZ1(92−194) derived from SAXS data. (A and C) Representative *dummy-residue* single models for the monomer (A) and dimer (C). (B and D). Four additional representative *dummy-residue* models (in gray scales) are superimposed on the yellow model from A and C for the monomer (B) or the dimer (D), respectively.

The $p(r)$ function shapes indicate that the proteins have an elongated conformation in both cases, with a maximum dimension of ∼120 Å for the monomer and ∼150 Å for the dimer. The radii of gyration R_g were estimated as (36 ± 1) Å and (45 ± 1) Å, respectively. The molecular masses were estimated as ∼11 and ∼22 kDa for the monomeric and dimeric species, respectively. These molecular masses are in good agreement with the value calculated using the ProtParam tool for the amino acid sequence of monomeric and dimeric FEZ1 (92−194), that is, 12.2 and 24.4 kDa, respectively.

Conformations of the Oxidized and Reduced FEZ1(92−194) in Solution. The low resolution models restored from the SAXS data for the oxidized (dimeric) and the reduced (monomeric) FEZ1 (92−194) in solution are presented in Figure 4. The NSD values for the set of 10 models range from 2.25 to 2.69 for the dimeric sample and from 1.27 to 1.67 for the monomeric samples. These values are considered reasonably good for unstructured proteins.[6,20] The low resolution of the models does not allow the unambiguous determination of the spatial positions of the secondary structure elements but provides a tridimensional view of the overall shape of the most frequent

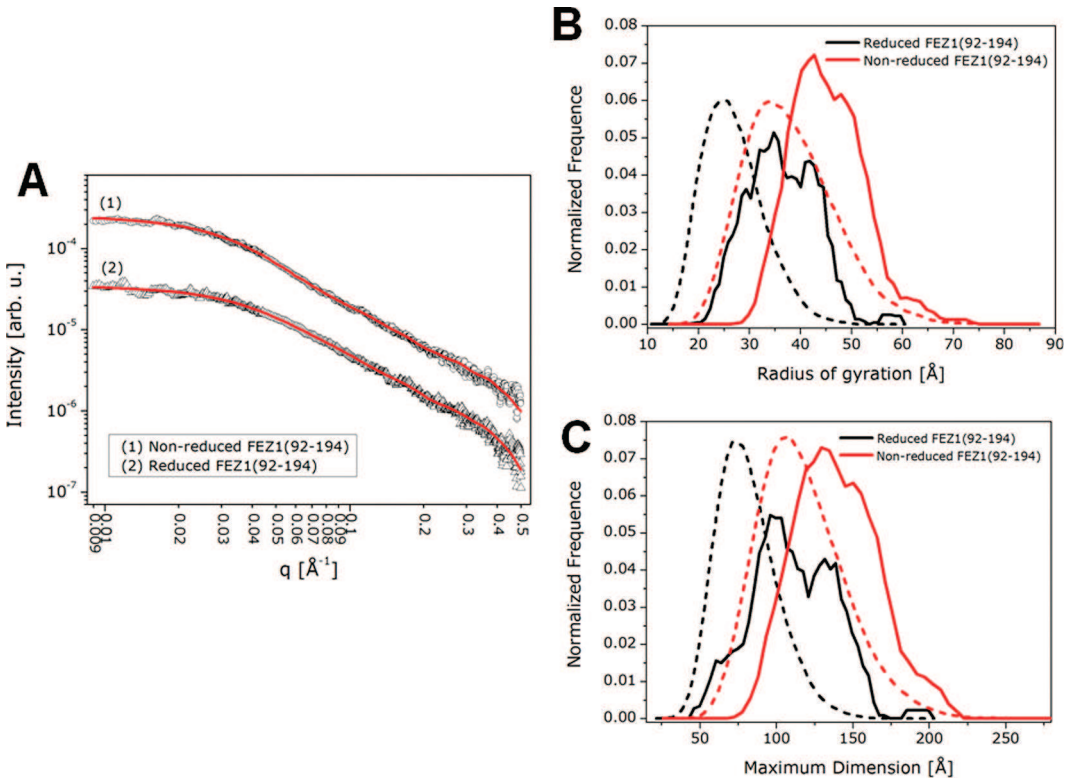

Figure 5. Ensemble optimization method results for the reduced and nonreduced FEZ1(92−194). (A) Experimental intensity curves of FEZ1 (92−194) under native conditions [(2), ○] and after 4 mM TCEP reduction [(1), △]. The curves have been shifted for better visualization. The solid lines represent the fit of the calculated intensity averaged over the individual curves obtained for each conformer of the selected ensemble. (B) R_g distribution for the sample in native condition (red) and reduced condition (black). (C) D_{max} distribution for the sample in native condition (red) and reduced condition (black). The dashed line represents the initial randomized distribution of conformers and the solid one represents the optimized distribution obtained.

conformations adopted by the protein samples in solution. Although the conformation of the monomer is expected to be less compact than that of the dimer (see Kratky Plot in the inset of Figure 3A), the model reconstructions of the monomer seem to be more stable than those of the dimer. Representative models of reduced and nonreduced FEZ1(92−194) are shown in Figure 4A and C, and the result of the superimposition of five average models for each protein after aligning their inertial axes are shown in Figure 4B and D.[22] These results indicated that both proteins explore a large conformational space, as expected from an elongated particle.

Analyzing the ensemble of conformations instead of one average conformation space, we obtained the R_g and D_{max} distributions presented in Figure 5B and C, respectively. The average R_g and D_{max} values obtained from those distributions were, respectively, 37.5 Å and 114.2 Å for the monomer and 46.5 and 142.0 Å for the dimer. These average values are in close agreement with those values previously obtained using the $p(r)$ functions and the approximations for small values of q (Figure 3). Comparing the distributions calculated for the nonreduced and the reduced molecule, we noticed that the whole ensemble of conformations changed after the addition of the reductor agent. We clearly observed that the R_g and D_{max} distributions shifted to smaller values when the disulfide bond is reduced, which confirms the monomerization of the whole set of molecules. The width of each distribution for the monomer becomes larger because the monomer is expected to be more unstructured than the dimer as observed by the Kratky Plot (inset of Figure 3).

Mass Spectrometry Confirms the Disulfide Bond in Full Length 6xHis-FEZ1. By analyzing the recombinant human 6xHis-FEZ1 (1−392) produced in *E. coli* BL21[7] by ESI Q-TOF mass spectrometry, we were able to identify and assign the peptides that resulted from enzymatic digestion with trypsin in both the oxidized and reduced forms. According to the results of Mascot search, the corresponding peptide region 123−141 showed no coverage for the oxidized protein. However, this sequence could be identified in the analysis of the reduced protein sample. The theoretical and experimental monoisotopic masses of the dimeric peptide in different charged states are shown in Figure 6. These data confirm the presence of the dimeric peptide formed by a Cys133-Cys133 disulfide bond, showing that the two different charged states were found in the spectra of oxidized FEZ1 with m/z of 1049.51 [M + 4H] and 839.83 [M + 5H], as predicted by the theoretical calculations. All other possibilities of intra- and intermolecular disulfide bonds in the molecule were analyzed in the oxidized protein, but no mass corresponding to the C-terminal VLCPT, or other cysteines, was identified (data not shown). In conclusion, the disulfide bridge between the two Cys133 was the only one that could be directly established, suggesting that this Cys residue is responsible for maintaining the dimerization of human FEZ1.

SEQUENCE	THEORETICAL MASSES					
(-)DPNIEALNGN**C**SDTEIHEK(-) | (-)DPNIEALNGN**C**SDTEIHEK(-)	Theor(Bo)	[M+H]	[M+2H]	[M3+H]	[M+4H]	[M+5H]
	4193.84	4194.85	2097.93	1398.95	1049.47	839.78

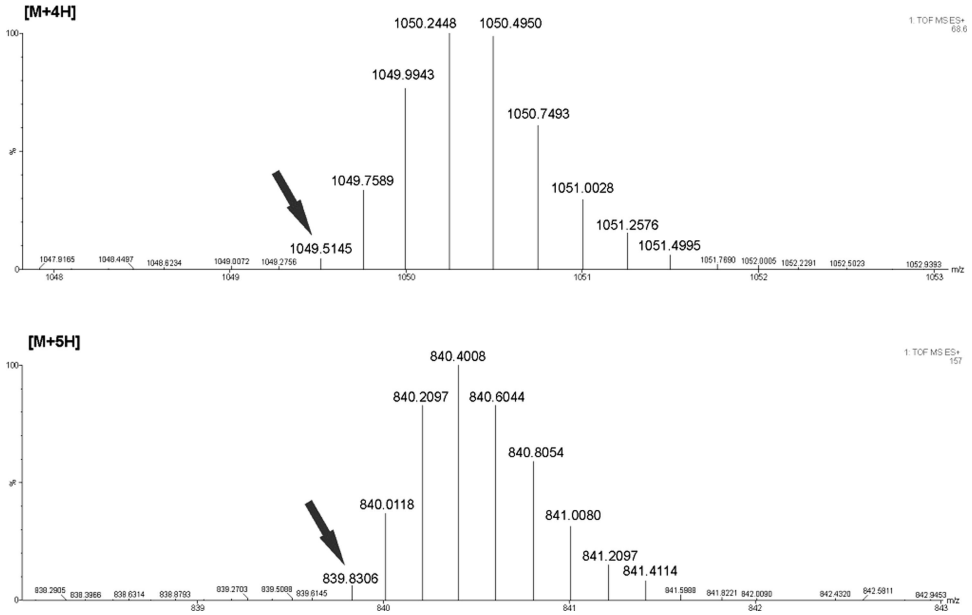

Figure 6. Cys133 disulfide bond in oxidized 6His-FEZ1 (123−141). The spectra show the isotope distribution of peptides cross-linked by one disulfide bond. The theoretical masses of the dimeric peptide are shown in the box. The arrows highlight the monoisotopic peaks of dimeric peptide with charge states of +4 and +5.

Discussion

The human FEZ1 protein has several features of natively unfolded proteins and dimerizes in solution through its N-terminal region.[6] The C-terminus region is able to interact with other proteins, mainly through predicted coiled-coils regions.

FEZ1 involvement in axon outgrowth is related to the anterograd microtubular transport.[2,9,23] In Drosophila, the FEZ1 orthologue UNC-76 associates with a kinesin motor protein for the axonal transport of vesicles and organelles[2,24] and *Unc-76* mutations cause neuromuscular phenotypes similar to those observed when axonal microtubule motor function is disrupted.[2] Moreover, Blasius and colleagues, in 2007, showed that FEZ1 is the first protein identified that binds to the inhibitory globular tail of Kinesin Heavy Chain (KHC), indicating that FEZ1 is not likely to be strictly a cargo (or cargo adaptor) of Kinesin-1. Perhaps FEZ1 relieves the inhibitory folded conformation of KHC and activates the kinesin-1 together with c-Jun N-terminal kinase-interacting protein 1 (JIP1).[5]

In 2008, Lanza and co-workers showed by SAXS analysis that FEZ1 is able to assume a dimer conformation mediated through its N-terminus region and discussed that the dimerization may be important for FEZ1 to act as a bivalent transport adaptor protein involved in microtubules directed neurit outgrowth.[6] Here we were able to get some evidence about FEZ1 protein dimerization in HEK293 cells and our *in vitro* analyses, describes details about the FEZ1 dimerization through its N-terminus. Here, the cysteine 133 of the fragment FEZ1 (92−194) was found to form a disulfide bond between two FEZ1 monomers. PAGE assays confirm the oligomeric state of FEZ1 (92−194) in nonreducing buffer (Figure 2C). SAXS analyses of the sample showed definitively that oxidized FEZ1(92−194) is dimeric but monomeric upon reduction (Figure 3−5). This demonstrates that FEZ1 (92−194) is capable of forming a disulfide bond. Since there is only one cysteine residue in this FEZ1 fragment, we could propose that cysteine 133 is involved in the disulfide bond formation. This assumption could be confirmed by mass spectrometry, which showed that this disulfide bond is the only one found in the full length protein (Figure 6). Moreover, we speculate that a stretch of 14 amino acid residues predicted to form coiled-coils (Figure 1), and which is situated in close proximity to Cys133, may drive the dimerization process and the disulfide bridge formation.

The disulfide bond-mediated dimer may be a requirement for FEZ1 biological function by creating a strong link between the FEZ1 monomers, exposing the C-termini of FEZ1 to interact at the same time with cytoskeleton elements (microtubules, kinesin) and cargoes (SCOCO, DISC1, PKCzeta, mitochondria, vesicles). Moreover, partitioning-defective protein 3 (PAR3), a component of the polarity complex PAR3-PAR6-atypical Protein Kinase C, was recently shown to interact with KIF3A, which indicates that the polarity complex might be transported by KIF3A.[3,25] Interestingly, FEZ1 protein is crucial to cell polarity, and also interacts with KIF3A, the atypical PCKzeta[10] and mitochondria.[23] As a bivalent adaptor protein, FEZ1 may be involved in promoting or stabilizing this protein complex involved in cell polarity by directing it through the axon. This has been previously shown for mouse GRIP1, which targets kinesin to dendrites.[2,14]

In 2008, we described that GFP-FEZ1 (1−392) overexpression led to a pronounced formation of so-called “flower-like”

multilobulated nuclei in over 40% of transfected HEK293 cells. Nocodazole treatment completely abolished the multilobulated nucleus formation, indicating the necessity of intact microtubules for the formation of the flower-like nuclei. The overexpression of GFP-FEZ1 (1−392) may promote an artificial cross-linking between different microtubules, which provokes a "lasso"-formation that leads to the observed constriction of the nucleus.[26] For such an artificial "cross-linking" of microtubules crucial features may be that the overexpressed FEZ1 is both dimeric and bivalent, thereby acting as a bridging element. Moreover, it may be essential that the dimer be stabilized by a covalent cysteine disulfide bond, as described here, to support the mechanical forces involved in the nuclear constriction. However, additional experiments need to be performed to show the importance of FEZ1 dimerization *in vivo*.

In summary, our molecular description of FEZ1 dimerization opens new possibilities to address the involvement of FEZ1 in the flower-like phenotype, which is frequently described in human acute lymphocytic leukemia cell lines resistant to Vincristine.[27]

Materials and Methods

Bioinformatics. Coiled-coil propensities were estimated using the program COILS (http://www.ch.embnet.org/software/COILS_form.html)[28] using a window width of 14. Amino acids alignment was performed by ClustalW2 (http://www.ebi.ac.uk/Tools/clustalw2/index.html) and the amino acid conserved percent colored by GeneDoc Multiple Sequence Alignment Editor & Shading Utility Version 2.6.002.

Cloning. The DNA fragment encoding human FEZ1 N-terminus (residues 460−660) was amplified by PCR using FEZ1 (1−392)[7] as a template and a pair of specific primers: Forward primer 5′-CATATGCAGAT CCAAGAGGAGGAG-3′, Reverse primer 5′-GTCGACTTATCCTCCATCCTCTTCTTC-3′. *Nde* I and *Sal* I cleavage sites were included at the ends of the forward and the reverse primers, respectively. After PCR, the amplified DNA product was purified from the mixture using the QIAquick gel extraction kit (Qiagen), cloned into the pGEM-T easy vector (Promega) and digested by the two restriction enzymes simultaneously. The digested DNA was then recovered from a 1% agarose gel using the QIAquick gel extraction kit (Qiagen) and cloned in fusion with a 6xHis tag in expression vector pET28a (Novagen/EMD Biosciences, San Diego, CA), followed by a TEV cleavage site. The correct nucleotide sequence of FEZ1 (92−194) was confirmed by DNA sequencing. The DNA fragment encoding FEZ2 N-terminus (residues 106−189) was amplified by PCR using a human fetal brain cDNA library (Clontech) as a template. Other cloning procedures are the same as previously described for FEZ1(1−227), with specific primers.[6] Forward primer 5′-gc catatg TGGAAGTCATCGCATACT-3′, Reverse primer 5′-gtcgac CTA ATCTGACTGTGTAGGGGT-3′

Expression and Purification. 6xHistidine-tagged recombinant proteins were expressed in *E. coli* BL21 (DE3), transformed with plasmids pET28aTEV_FEZ1 (92−194) or pET28aTEV_FEZ2 (106−189). *E. coli* cells were grown in LB medium containing kanamycin (30 μg/mL) at 37 °C up to an OD_{600} of ∼1.0, when the temperature was shifted to 30 °C and the cultures were induced with 0.2 mM IPTG for 4 h. Following induction, the cells were harvested by centrifugation (5000× *g*) for 10 min at 4 °C and suspended in buffer A (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.4). The cells were lysed by lysozyme treatment (300 μg/mL for 1 h on ice), followed by sonication and the extract isolated by centrifugation (23.000× *g*) for 30 min at 4 °C. Purification of the recombinant proteins was carried out by affinity chromatography on a 5 mL HiTrap Chelating HP column (GE Healthcare) pre-equilibrated in buffer A and eluted with a 100 mL gradient of 0−100% buffer B (buffer A + 250 mM imidazole). The chromatography was performed on an ÄKTA-FPLC system (GE Healthcare), with a flow rate of 1 mL/min and fractions of 1 mL were collected. 6xHis-FEZ1 (92−194) protein fractions were dialyzed against TEV buffer (100 mM Tris.HCl, 1 mM DTT, 1 mM EDTA, pH 8.0) and the 6xHis tag was cleaved by TEV protease (TEV: 6xHis-FEZ1 ratio 1:14 mg) for 16 h at 30 °C. The protein was purified further by ion exchange chromatography on a 1 mL HiTrap Q-Sepharose HP column equilibrated with buffer A and eluted with a 0−100% gradient of buffer containing buffer A and 1 M NaCl in 30 mL. The chromatography was performed at a flow rate of 1 mL/min and fractions of 1 mL were collected. The samples were dialyzed against buffer A and 6 mg of purified FEZ1 (92−194) were loaded on a SuperdexTM 75 16/60 Prep grade column (GE Healthcare) that had been equilibrated previously with buffer A. The final protein was tested by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) or PAGE under non- or reducing conditions.

Small Angle X-Ray Scattering. The SAXS experiments were performed at theD02A-SAXS2 beamline of the Laboratório Nacional de Luz Síncrotron (LNLS, Campinas, Brazil). The measurements were performed with a monochromatic X-ray beam with a wavelength of $\lambda = 1.488$ Å and the X-ray patterns were recorded using a two-dimensional MARCCD detector. The sample-to-detector distances were set at 500.895 mm and 1508.512 mm, resulting in a scattering vector range of 0.01 Å$< q <$ 0.65 Å^{-1}, where q is the magnitude of the q-vector defined by $q = (4\pi/\lambda) \sin \theta$ (2θ is the scattering angle). Before the SAXS experiments, the protein samples were submitted to DLS (dynamic light scattering) analysis. The DLS data of two samples (FEZ1(92−194) in native and reduced condition) showed narrow, single peaks in a monodisperse solution. For the SAXS measurements, the protein samples (0.97 mg/mL for predicted dimers) were placed in a 1-mm path length cell with mica windows, temperature-controlled (20 °C) via water circulation. The samples were dialyzed in the following buffer: 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.4. Three successive frames of 300 s each were recorded for each sample and one 300s-frame more was also recorded for the corresponding buffer scattering. Thereupon, the scattering curves were individually corrected for the detector response and scaled by the incident beam intensity and the samples absorption. Then, the buffer scattering was subtracted from the corresponding sample scattering and the resulting curve was carefully inspected to check for possible radiation-induced damage, but no such effects were observed. The molecular masses of each FEZ1 (92−194) samples (reduced and nonreduced) were estimated from the SAXS data using the extrapolated values of the intensity at the origin I(0).[29]

The SAXS data analyses were carried out following similar steps to those described in Bressan et al. (2008) and Lanza et al. (2009). Briefly, the radius of gyration (R_g) of both samples was evaluated using the Guinier approximation.[30−33] Since this approximation holds true on a very restricted q range of the scattering curve from unstructured proteins, we also use the Debye's approximation[30−33] to obtain a more accurate determination of the R_g values and confirmed the values obtained by the first approximation. Moreover, the R_g values were also obtained from the pair distance distribution function $p(r)$ which

was calculated using the indirect Fourier transform package GNOM.[34] The $p(r)$ function represents the histogram of all distances within a molecule and provides the maximum dimension D_{max} of the molecule.[35,36] In order to analyze the flexibility of the conformation of non- and reduced FEZ1 (92−194) chains, we used the so-called Kratky Plot ($q^2I(q)$ versus q). For compact and structured proteins, the Kratky representation presents a bell-shaped plot with a well-defined maximum, because the scattering function satisfies Porod's law for large q values (i.e., $I(q)$ is proportional to $1/q^4$). Conversely, the Kratky representation of an ideal Gaussian chain,[30] (equivalent to a random coil in the case of an infinitely thin chain) presents a plateau at high q-values because $I(q)$ is proportional to q^2.

Low Resolution Modeling of the Reduced and Nonreduced FEZ1 (92−194) and Their Ensembles of Conformations. Although the proteins under study present an unstructured conformation, whose shape is hard to define, we tried to restore the low resolution average conformations of the reduced and nonreduced samples, based on the experimental SAXS curves. We applied an *ab initio* approach where the protein is represented as a chain of dummy residues (DRs). The number of DRs is usually known *a priori* from the protein amino acid sequence. Starting from a randomly distributed gas-like ensemble of DRs inside a spherical volume of diameter D_{max}, a simulated annealing protocol is employed to find a chain-compatible spatial distribution of DRs which fits the experimental scattering pattern. This algorithm is implemented in the program GASBOR.[37] The resulting low resolution models were used in this work to gain further insight into the possible three-dimensional arrangement of the protein samples. The use of this modeling approach has been previously used by Moncoq et al.[18] and Bressan et al.[20] in the study of unstructured proteins. We would like to stress that no unique solution can be obtained from these calculations. For this reason, 10 independent calculations were run for each sample data. Analyzing the normalized spatial discrepancy (NSD, which indicated the dissimilarity between pairs of models[22]) of the several calculations, the models with common features (less dissimilarities) led to the selection of a representative, low resolution average conformation for both FEZ1 (92−194) (reduced and nonreduced) proteins. A pairwise comparison and NSD evaluation were performed using the DAMAVER program suite[38] complemented by the SUPCOMB routine.[22]

To analyze not only an average conformation, but also a set of different conformations in solution, we applied the ensemble optimization method to the SAXS data of both FEZ1 (92−194) samples. This method is described in Benardó et al.[19] Using this method, we can obtain a distribution of conformers in one ensemble whose average intensity fits to the SAXS intensity curve. The idea is to analyze the changes in this ensemble upon reduction of the oxidized sample, monitoring the changes in the statistical distribution (normalized frequency) of the parameters R_g and D_{max} calculated from the selected conformers.

Disulfide Bond Analysis by Mass Spectrometry. Oxidized and reduced 6xHis-FEZ1 (1−392) protein bands were excised and in-gel trypsin digestion was performed according to Hanna et al. (2000).[39] An aliquot (4.5 μL) of the peptide mixture was separated by C18 (100 μm × 100 mm) RP-UPLC (nanoAcquity UPLC, Waters) coupled with nanoelectrospray tandem mass spectrometry on a Q-Tof Ultima API mass spectrometer (MicroMass/Waters) at a flow rate of 600 nL/min. The gradient was 0−80% acetonitrile in 0.1% formic acid over 45 min. The instrument was operated in the "top three" mode, in which one MS spectrum is acquired followed by MS/MS of the top three most-intense peaks detected. The resulting spectra were processed using Mascot Distiller 2.2.1.0, 2008, Matrix Science (MassLynx V4.1) and a human nonredundant protein database (NCBI, Release of 31-July-2009) was searched, using Mascot, with or without carbamidomethylation as a fixed modification, oxidation of methionine as a variable modification, one trypsin missed cleavage and a tolerance of 0.1 Da for both precursor and fragment ions. Furthermore, the fragment corresponding to the residues 123−141 of 6xHis-FEZ1 cross-linked by one disulfide bond was theoretical calculated by Peptide Sequencing (BioLynx) supplied with Masslynx 4.1 software package and this fragment was manually validated in the spectra.

Cell Culture and Antibodies. Adherent HEK293 cells were cultivated in bottles containing DMEM medium, 10% FCS and penicillin and streptomycin at 37 °C and 5% CO_2 atmosphere. Cells were harvested and washed twice in ice-cold phosphate buffer (PBS), (137 mM NaCl, 0.3 mM KCl, 8.1 mM Na_2HPO_4, 6.7 mM KH_2PO_4, pH 7.2) resuspended and lysed for 1 h in ice-cold lysis buffer (20 mM Tris, 150 mM NaCl, 5 mM EDTA, 1% NP40, pH 7.45). The soluble fraction was subjected to previous denaturation under mildly reducing conditions to allow a good separation of lysate proteins in the SDS PAGE (buffer: 45 mM Tris pH 6.8, 0.13% SDS, 7% glycerol, 3% beta-mercaptoethanol). After SDS-PAGE (10% gel), Western blot was performed by using a primary rabbit polyclonal antibody anti-FEZ1 (1:500, ProSci) and secondary goat antirabbit antibody (1:5000, Santa Cruz Biotechnology) conjugated with HRP.

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3.4. Manuscrito I

Structural studies by SAXS and NMR of the FEZ1 dimer and the tetramer FEZ1 / SCOCO

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Alborghetti MR, Furlan AS, Silva JC, Miguelleti DL, Sforça ML, Neves JL, Zeri AC, Torriani ICL, Kobarg J

Abstract

The *C. elegans* protein UNC-69 (orthologous to human SCOCO – short coiled-coil protein) interacts physically with UNC-76 (orthologous to human FEZ1 – fasciculation and elongation protein zeta 1) and both act in the same pathway to control axon extension. Exploiting the feature of FEZ1 protein as adapter of the transport mediated by kinesins and FEZ1 protein interaction with SCOCO, we investigated the structural aspects of this interaction by SAXS (Small Angle X-ray Scattering) studies. The data showed that a FEZ1 homodimer is able to interact with two molecules of SCOCO, according to the proposition that FEZ1 act as a transport bivalent adaptor molecule that can interact with both cytoskeletal elements by one of the monomers and with cargo elements with the other monomer. The topology of homodimerization was accessed through NMR (Nuclear Magnetic Resonance) studies of the region involved in this process, corresponding to FEZ1 (92-194). Through these studies involving the protein in its monomeric configuration (reduced) and dimeric, we conclude that homodimerization occurs with FEZ1 chains oriented in parallel topology. These data allowed concluding that FEZ1 can act as a bivalent adaptor of transport mediated by kinesins and, probable, in the transcription machinery.

Introduction

Neuronal differentiation *in vitro* and *in vivo* involves coordinated changes at various levels, including in the gene expression, cytoskeleton rearrangements and trafficking processes [1]. During the neuronal differentiation, the growth of neurite processes from the cell body involves a massive increase in cell surface area [2]. The membrane increment occurs in a very dynamic axon structure called growth cone. For growth cones to extend, vesicles derived from the Golgi apparatus fuse with the plasma membrane by a process of regulated exocytosis [3]. These vesicles are not only transported but are also differentially sorted into dendrites or axons [4,5]. Localization of dendritic, axonal and pre-synaptic proteins is dependent of kinesin mediated transport [6]. Knockdown of the kinesin motors using antisense oligonucleotides not only disrupts dendritic or axonal localization of these proteins, but also suppresses neurite outgrowth, presumably by blocking kinesin-dependent vesicle transport [7,8].

The vesicular transport during neuronal development has been extensively studied using the nematode model *Caernohabditis elegans*. Through genetic screenings using this mutant worm, many genes related to axonal growth were also identified to cause defects in locomotion. These genes were termed as *unc* (uncoordinated). Through this approach some kinesins have been identified as UNC104/KIF1A, UNC-116/kinesin heavy chain (KHC) and kinesin light chain KLC-2, and other proteins. In *unc-69* (the orthologue to human SCOCO – short coiled coil protein) mutants (like *e587* mutant) several outgrowth defects are observed, including premature termination of axonal processes, ectopic extension of branches, and de-fasciculation of axon bundles [9]. This spectrum of phenotypes resembles the disruption of UNC-76 (the orthologue to human FEZ1 – fasciculation and elongation protein zeta 1) in *C. elegans* (like *e11* mutants) [10,11] (figure 1), a protein that binds to the carboxyl terminus of the kinesin heavy chain (KHC) [12]. In 2006, SU et al., showed that UNC-69 interacts physically with UNC-76 and both act in the same pathway to control axon extension. Moreover, UNC-116/KHC is required for proper subcellular distribution of both UNC-76 and UNC-69. In unc116 mutants, UNC-76 failed to co-localize with UNC-69, and the normal puncta distribution of both protein was changed to a more diffuse distribution.

UNC69 interacts with the protein Arl1 (ADP-ribosylation factor like protein). The overall function of Arl1 is, like other Arfs and Rabs, presumably to recruit specific effectors to a specific membrane in the cell, in this case the trans-Golgi [13]. Studies in mammalian cells have also indicated that Arl1 acts on the Golgi to facilitate the arrival of membrane traffic from the endosomal system. The protein is present on the membranes of the trans side of the Golgi, and it recruits by direct binding GRIP-domain coiled-coil proteins, and possibly recruits or activates other effectors [13]. UNC-69 could act as

an effector of Arl1 protein. In fact, mutations in UNC-69 preferentially disrupt membrane traffic within axons.

It has been suggested a role of bivalent adapter transport mediated by kinesins for FEZ1 (which dimerizes via disulfide bridge), and probably for FEZ2 [14]. FEZ family proteins have the ability to interact with a wide spectrum of proteins (FEZ1 interacts with 80 proteins and FEZ2 proteins with these same 80 plus 19 more specific interactions). This high amount of protein-protein interaction classifies FEZ proteins as interaction hubs. The interaction partners identified could, among other functions, be cargoes to be transported by adaptor protein FEZ1 bound to transporter kinesins. Many identified proteins are membrane components of mitochondria and vesicles, and these organelles are likely to be transported by the same system. It has been demonstrated that inhibition of FEZ1 protein expression by iRNA interference changes the location for these organelles in the cell [12].

Moreover, FEZ1 protein is able to interact with a plenty of nuclear proteins, including proteins involved in transcription regulation (DRAP1, GTFIRD2, MCM7, MED7, MLF1IP, RARA, SAP30, SAP30L, SFRS8, SLTM) that could be involved in the regulation of expression of *sox2* gene. Thereby the pattern of function of the proteins that interact with FEZ1 at large resembles that of proteins interacting with SCOCO.

To date, the majority of proteins interacted with the C-terminal of the FEZ family proteins. This region has a strong prediction to form coiled-coil structures, which are responsible for protein-protein interaction. The UNC-69 protein, which also has a strong prediction for coiled-coil structures in almost its entire length, interacts trough this region, and according to SU and colleagues, the integrity of the coiled-coil in UNC-76 is required for interaction with UNC-69. There are no other "structured" regions in FEZ proteins [15], and these proteins are classified as intrinsically disordered protein. Intrinsically disordered proteins are now recognized as crucial in areas such as transcriptional regulation, translation and cellular signal transduction as a consequence of the use of new paradigms in biochemical methodology. Many eukaryotic proteins are modular — that is, they contain independently folded globular domains that are separated by flexible linker regions. The protein FEZ1, however, shows no globular domain, not showing the modular character mentioned above (figure 1).

Here, we confirmed and characterized the interaction between humans 6xHis-FEZ1 (1-392) and GST-SCOCO (42-122) by SAXS (Small Angle X-ray Scattering) and performed NMR (Nuclear Magnetic Resonance) study about FEZ1 N-terminal homodimerization and model refinement of 6xHis-FEZ1/GST-SCOCO protein complex. We concluded that FEZ1 interacts itself in a dimeric topology and interacts with two molecules of SCOCO simultaneously. These data are in accordance with the model of FEZ1 protein acting as a bivalent adaptor of transport mediated by kinesin as so a bivalent function in transcription modulation. Moreover, this is the first low-resolution model, to our knowledge, of a

complex with structured elements (such as GST fused to SCOCO), but with a predominance of intrinsically unstructured regions modeled through a strategy that uses a mixed approach to development models of rigid bodies combined with ab initio modeling of unknown regions.

Results

FEZ1 dimer interacts with two molecules of SCOCO

To act as a bivalent adaptor, FEZ1 protein should be able to interact with two proteins simultaneously. To verify this condition we performed co-purification assays with 6xHis-FEZ1 (1-392) and GST-SCOCO (42-122) (figure 2A) followed by SAXS data acquisition and analyses (figure 2B). We chose to fuse the protein SCOCO with GST protein to get more distinction of SCOCO molecule in the complex, since its small size (9.2 kDa, according to ProtParam) would be a drawback for a SAXS analysis of this protein within a larger complex. Our results showed a homodimer of FEZ1 protein interacting with two molecules of SCOCO. According to ProtParam predictions the 6xHis-FEZ1 (1-392) protein has an expected size of 48.8 kDa protein and GST-SCOCO (42-122) an expected size of 36.8kDa. The mass calculated from the SAXS data was approximately 172kDa, which agrees with the state of the heterotetramer complex (predicted to be 171.2 kDa). The maximum diameter obtained for the complex was 320Å and Rg 104Å (+/- 2). The pattern of the *p(r)* curve suggests as elongated region in the complex as globular regions, which corroborates with the fact of FEZ1 be an intrinsically unstructured protein (elongated) and with the fact of SCOCO be fused to GST (which is globular).

Complex model agrees with C-terminal interaction in FEZ1 by SCOCO

As described before by Su and co-workers (2006) and Assmann and co-workers (2006), *C. elegans* UNC-69 interacts with the C-terminal region of *C. elegans* UNC-76 and human SCOCO interacts with the C-terminal region of FEZ1, respectively. The theoretical curve scattering from the low resolution model (figure 3) of 6xHis-FEZ1 interacting with GST-SCOCO generated by SAXS data and *ab initio* modeling was the best with SCOCO interacting in the C-terminal of FEZ1 (data not show).

FEZ1 dimerization involves few amino acids

We verified the topology of homodimerization of FEZ1 by NMR spectroscopy. The possible assignments of amide resonances (1HN and 15N) are represented in the 2D 15N-HSQC spectrum of the protein in Figure 4. Two FEZ1 (92-194) 2D 15N-HSQC spectrums are showed in figure 4: the protein in monomeric conformation (black) and dimeric conformation (red). The monomeric and dimeric protein was completely disordered, but some distinct chemical shifts were observed among monomers and dimers. Relaxation data (figure 5) are consistent with the presence of residues maintaining contacts each other between the chains of the protein FEZ1. Through NOE analysis (data not shown) of the assigned residues that maintaining these contacts, we can suggest that the homodimerization occur in an anti-parallel topology. However, due to the high proportion of identical residues both intra and inter-chains, the results are still preliminary and the parallel topology cannot be discarted (figure 6).

Discussion

Transport of vesicles and organelles often occurs over long distances. For example, membrane receptors destined for synapses in neuronal cells need to be transported from the cell body down axons that can reach a meter in length. Diffusion would be prohibitively slow and cells have therefore evolved molecular motors that transport vesicle cargoes along microtubule tracks. Kinesins are among these motors and use the energy of ATP to move towards the plus ends of microtubules [4–6]. A general picture is emerging whereby kinesin family members use adaptor/scaffolding proteins to link to their cargoes, although examples of direct interactions with transmembrane proteins exist. Importantly, FEZ1 (and FEZ2) is the first protein identified that binds to the inhibitory globular tail of KHC, and together with JIP1 (that binds to KLC), release the kinesin-1 from the auto inhibitory sate. These data indicate that FEZ1 is not likely to be strictly a cargo of Kinesin-1.

Recent findings purpose that FEZ1 could act as a bivalent adaptor of transport mediated by kinesin. Through its function as adaptor protein, FEZ1 would be able to interact with two other protein molecules simultaneously. The dimeric conformation of FEZ1 by the N-terminal described by Lanza and colleagues (2008) [15] is in accordance with this requirement, since, once dimer, the protein have two C-terminal regions (involved in protein-protein interaction). This paper support and bring more structural details to the hypothesis. SAXS experiments confirmed the composition of heterotetramer of two molecules of 6xHis-FEZ1 (1-392) and 2 molecules of GST-SCOCO (421-122), a partner of the

interaction of FEZ1 identified in two-hybrid screenings in yeast, corroborating the hypothesis of bivalent adapter. NMR data of FEZ1 protein (92-194) further demonstrate that FEZ1 dimerizes in parallel topology. In 2010, we demonstrated by native gel, SAXS and mass spectrometry that FEZ1 dimerizes by disulfide trough the cysteines 133 of each monomer [14]. *In vivo*, this dimeric state covalently bound may be important for transport of proteins mediated by kinesins along microtubules, providing stability to the dimer during the transport process. The protein SCOCO interacts with proteins involved in trans-Golgi network, as Arl1, present on the membranes of the trans side of the Golgi, probably involved to recruit specific effectors to a specific membrane in the cell. Our data suggest that FEZ1 act as a link, as an adapter, of the machinery transport mediated by kinesins and proteins from trans-Golgi network destined to vesicle transport, for instance.

In fact, mutations in both FEZ1 and SCOCO cause similar phenotypes and lead to axonal growth deficiencies, mistakes of synaptic vesicles transport, among others. Has not been confirmed that the interaction between FEZ1 and SCOCO is interrupted in these phenotypes. Certainly in the mutant *e11* the interaction with FEZ1/UNC-76 and SCOCO/UNC-69 is disrupted. The mutation promotes the formation of a premature stop codon before the minimal interaction region of SCOCO in FEZ1. The mutant *e587* also has a mutation that causes a premature stop codon, but in SCOCO/UNC-69. However, the truncated region, apparently, does not lose a segment of its coiled-coil (structure involved in protein-protein interaction). The deleted region is predicted to be a coil (figure 1). Unfortunately, it is unclear whether FEZ1 interacts with this region. If interacts, then probably the interaction FEZ1-SCOCO is essential for normal neuritogenesis. Otherwise, besides the interaction is important, this segment of SCOCO probably interacts with proteins essential for neuritogenesis, relating to both transport mechanisms and the mechanisms involved in regulating the expression of genes involved in the process.

The fact that FEZ1 is intrinsically unstructured, dimer and promiscuous in relation to its large number of interactions with its C-terminal regions and interacts with cytoskeletal elements (kinesin, tubulin, CLASP2) can be crucial to explain the formation of bridges between microtubules and constriction of the nucleus, resulting in turn in the flower-like phenotype when FEZ1 is over-expressed [18]. The covalent dimerization can also be important for stabilization during operation of traction to the constriction of the nucleus.

In summary, our data show that FEZ family proteins can act as bivalent adaptor proteins, as in the transport as in the transcriptional machineries. We demonstrate that FEZ1 protein dimerizes through disulfide bond, parallel and can interact in their dimeric form, with two other proteins at the same time. As an intrinsically disordered protein, FEZ1 is essential to neuronal function, as SCOCO is.

Material and Methods

SAXS sample preparation

The nucleotide sequence of human SCOCO (42-122) in the library vector pACT2, which are inserted between restriction sites *Eco* RI and *Xho* I and code for the interacting protein identified in the yeast two-hybrid system screen [19], was 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. The cells were induced for 4 h to protein expression at 37 °C using 0.5 mM isopropyl 1-thio-β-D-galactopyranoside. The protein 6xHis-FEZ1 (1-392) expression was performed as described in Assmann (2006). Cells from 2L of expression of 6xHis-FEZ1 (1-392) and 1L of GST-SCOCO (42-122) were harvested together by centrifugation at 4,500 X *g* for 10 min, and the cell pellet was ressuspended and incubated for 30 min with 10 volumes of lysis buffer (137mMNaCl, 2.7mMKCl, 10mMNa_2HPO_4, 1.8mM KH_2PO_4, 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 X *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 Na_2HPO_4, 1.8 mM KH_2PO_4, 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 6xHis-FEZ1 (1-392) and GST-SCOCO (42-122) followed by a new step of purification in a GST-Trap column (GE Healthcare). Again, aliquots of each eluted fraction obtained were analyzed by SDS-PAGE and 1 mL aliquot was dialyzed with buffer (137 mM NaCl, 2.7 mM KCl, 10mM Na_2HPO_4, 1.8 mM KH_2PO_4) and analyzed by DLS (Dynamic Light Scattering). The aliquots that showed the most narrow, single peaks and predicted masses and percentage of poly-dispersivity indicative of a monodisperse solution were selected to SAXS data acquisition.

SAXS experiments

The SAXS experiments were performed at the D02A-SAXS2 beamline of the Laboratório Nacional de Luz Síncrotron (LNLS, Campinas, Brazil). Measurements were performed with a monochromatic X-ray beam with a wavelength of $\lambda = 1.488$ Å. The X-ray patterns were recorded using a two-dimensional position-sensitive MARCCD detector. The sample-to-detector distances were set at 1374.4 mm and 1788.8 mm, resulting in a scattering vector range of $0.009Å^{-1} < q < 0.25Å^{-1}$, where q is the magnitude of the q-vector defined by $q = (4\pi/\lambda)\sin\theta$ (2θ is the scattering angle). For SAXS

measurements protein samples were placed in a 1mm path length cell with mica windows, temperature-controlled (T = 20°C) via water circulation[15].Three successive frames of 300s each were recorded for each sample. The buffer scattering data were recorded before and after the sample scattering data for 300 s each and thereafter averaged. The scattering curves were individually corrected for the detector response and scaled by the incident beam intensity and the samples absorption. The average buffer scattering was subtracted from the corresponding sample scattering. The resulting curve was carefully inspected to check for possible radiation-induced damage, but no such effects were observed. The scattering patterns were recorded at concentration of 1.16 mg/mL. The molecular mass of each 6xHis-FEZ1 complexed with GST-SCOCO (42-122) sample was inferred from the ratio of the extrapolated value of the intensity at the origin *I(0)* [20].

SAXS data analysis

The radius of gyration was first evaluated using the Guinier approximation[21,22,23]. It is worth noting that in the case of an unstructured protein, the Guinier approximation holds true on a very restricted q range corresponding to $q < 1/Rg$. Such a very narrow range contains a small number of experimental points, thereby limiting the accuracy of the Rg determination. In that case, a more accurate determination of the radius of gyration can be obtained by Debye's equation[15,24], which adequately describes the scattering in the domain $q < 1.4/R_g$ by:

$$\frac{I(q)}{I(0)} = \frac{2}{x^2}(x - 1 + e^{-x})$$

where $x = q^2R_g^2$.

Moreover, R_g was also evaluated from the pair distance distribution function *p(r)* which was calculated using the indirect transform package GNOM[25]. The *p(r)* function represents the histogram of all distances within a molecule and provides the maximum dimension D_{max} of the molecule where its value reaches zero [15].To analyze the natively unfolded conformation of 6xHis-FEZ1(1-392) chains, a particularly useful representation of the SAXS intensity was used: the so-called Kratky Plot ($q^2I(q)$ vs. q). This plot is usually applied to study unstructured proteins because it provides information about the compactness of a molecule. For compact and structured proteins, the Kratky representation presents a bell-shaped plot with a well-defined maximum, because the scattering function satisfies Porod's law for large q values (i.e. *I(q)* is proportional to $1/q^4$). Conversely, the Kratky representation of an ideal Gaussian chain [15], (equivalent to a random coil in the case of an infinitely thin chain) presents a plateau at high q-

values because I(q) is proportional to $1/q^2$. Finally, in the case of a persistence length chain model, where short-range interactions between adjacent chain segments produce stiffness of the chain, the Kratky plot also displays a plateau over a specific q-range followed by a monotonic increase at still higher *q*-values [24,26].

Ab initio modeling

A combined approach to modeling rigid body and flexible structure was used to generate the low-resolution model of 6xHis-FEZ1 (1-392) complexed with GST-SCOCO (42-122). Although the ‘‘shape’’ of naturally unfolded proteins is per se hard to define, we restored the low resolution average conformations of 6xHis-FEZ1(1-392) complexed with GST-SCOCO (42-122) from the experimental SAXS curves by the following ab initio approach. To obtain an overall view from the conformational space occupied by the proteins, the program DAMMIN [25], was used. In this approach, each protein was represented as an assembly of densely packed spherical beads (dummy atoms) of radius $r_0 << D_{max}$ inside a sphere of diameter D_{max}, which was directly determined from the scattering data using the GNOM routine. Using simulated annealing, the program DAMMIN starts from a random configuration of beads and searches for a configuration that fits the experimental scattering pattern. Ten calculations were performed. The normalized spatial discrepancies (NSD) were evaluated using the DAMAVER suite [27], and the most typical model (with lowest NSD value) were considered as close descriptions of the possible conformations of the molecule.

NMR sample preparation

The cloning of FEZ1 (92-194) is described in Alborghetti et al. (2010) [14]. pET-FEZ1 (92-194) plasmid was transformed into BL21(DE3) *Escherichia coli* expression cells and grown at 37°C in M9 minimal medium supplemented with 30 µg/mL kanamycin, 4 g/L [13C]glucose and 1 g/L [15N]ammonium chloride. Expression was induced at an OD600 of 0.8-1.0 by the addition of 0.2 mM IPTG for 24 h. The cells were harvested by centrifugation at 5000 g for 10 min and the purification methodology is described Alborghetti et al. (2010) [14]. Fractions containing pure 6xHis-FEZ1 (92-194) were pooled and concentrated to 0.3 mM in buffer containing 20mM phosphate, 50mM NaCl, pH 6.2. The sample was divided in two aliquots and one was reduced with 4mM DTT and both were analyzed by NMR spectroscopy.

NMR Spectroscopy

NMR experiments for structure determination were performed at 293 K using a Varian Inova 600 MHz spectrometer equipped with a cryogenic probe. The following experiments were recorded: 15N-HSQC; 15N-edited NOESY (80 ms mixing time, chosen after a build up curve with other mixing time values); 15N-edited TOCSY; HNCA; HN(CO)CA; HNCACB; CBCA(CO)NH; HNCO; HN(CA)CO [28].

Relaxation Measurements

For the backbone amide relaxation measurements, 15N T1, 15N T2 and heteronuclear NOE experiments were recorded at a 15N frequency of 60.78 MHz on a Varian Inova 600 MHz spectrometer equipped with a cryogenic probe. T1 relaxation delays were set to 10, 210, 410, 610, 810, 1010, 1210, 1410, 1710, 2010, and 2410ms. T2 relaxation delays were set to 10, 30, 50, 70, 90, 110, 130, 150, 170, 190 and 210ms. In all the experiments a relaxation delay of 3s was used. For the heteronuclear NOE measurements, a pair of spectra was recorded with and without proton saturation. Spectra recorded with proton saturation utilized a 5s recycle delay followed by a 15s period of saturation, while spectra recorded in the absence of saturation employed a recycle delay of 15s. Peak volumes were fitted to a single exponential decay function using the program NMRView. The two-dimensional experiments were acquired with 1024 x 128 complex points [28].

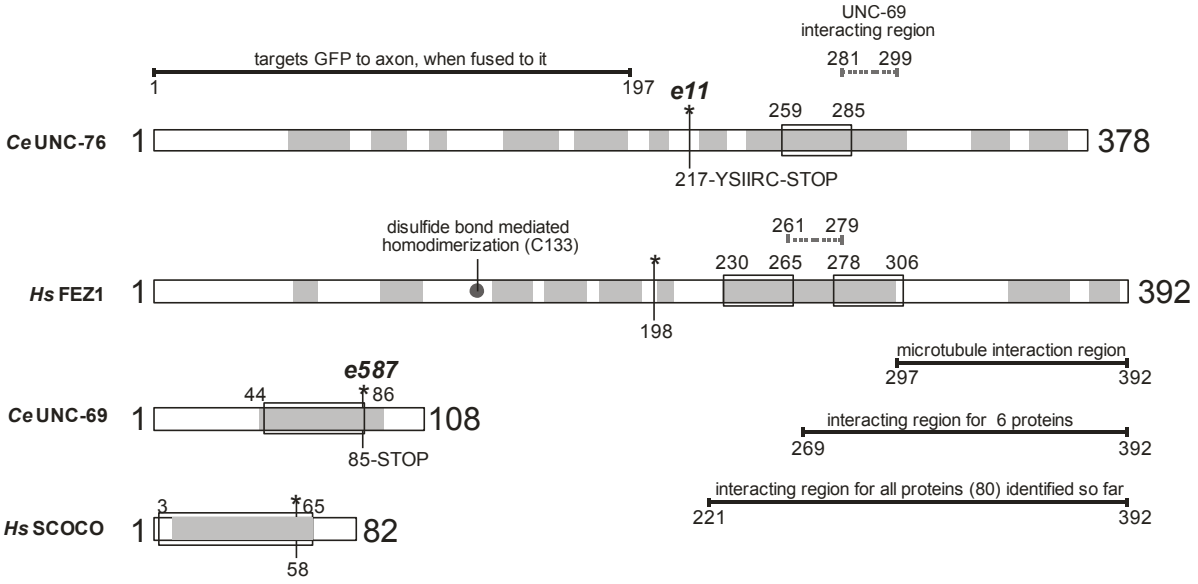

Figure 1 – General scheme of UNC-76/FEZ1 (*C. elegans/H. sapiens*) and UNC-69/SCOCO (*C. elegans/H. sapiens*) proteins. Mutations observed *in C. elegans*, and the corresponding region in the human protein, are indicated by asterisks. Coiled-coils: box, alpha-helix prediction: gray

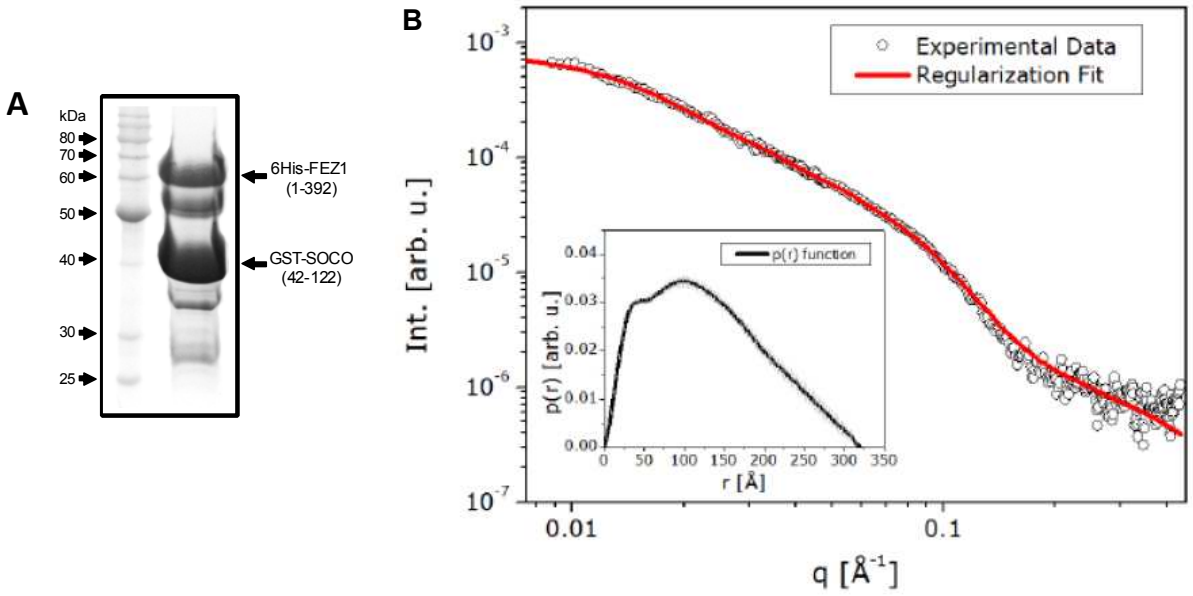

Figure 2. A) SDS-PAGE 10% of the 6xHis-FEZ1 (1-392) and GST-SCOCO (42-122) protein complex. The complex was analyzed by SAXS at 1.16 mg/mL in PBS buffer. The complex polydispersivity was 28,0% according to DLS assay. B) SAXS (Small Angle X-ray Scattering) experiments of 6xHis-FEZ1 (1-392) interacting with GST-SCOCO (42-122). Scattering intensity curve of FEZ1 (1-392) Inset: Pair Distance Distribution Functions (p(r) functions).

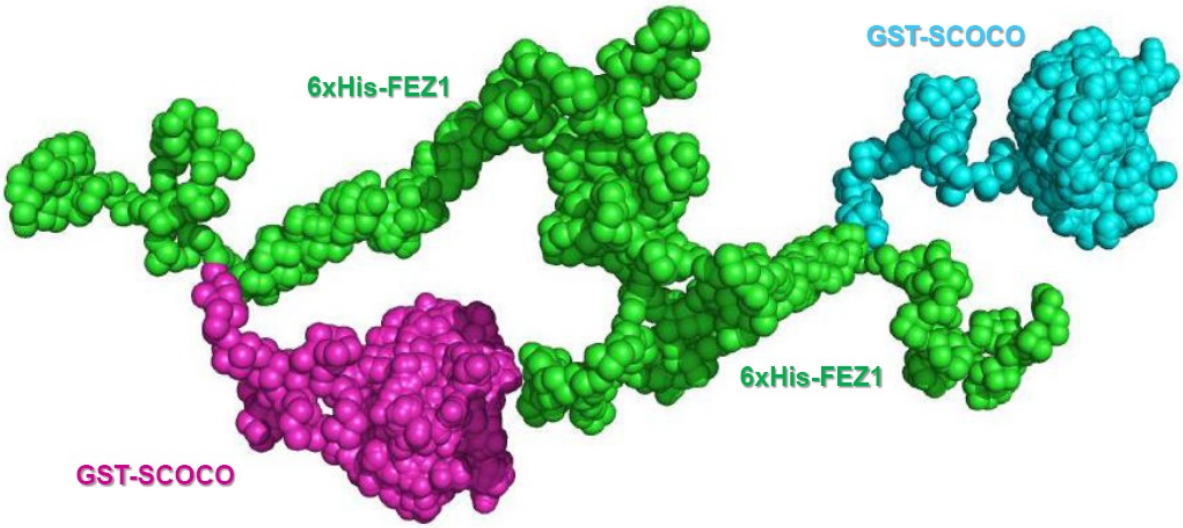

Figure 3. Low resolution *ab initio* model for 6xHis-FEZ1 (1-392) with GST-SCOCO (42-122) derived from SAXS data. Representative dummy-residue single model. A heterotetrameric state is observed, which consists of two GST-SCOCO molecules attached to two FEZ1 molecules.

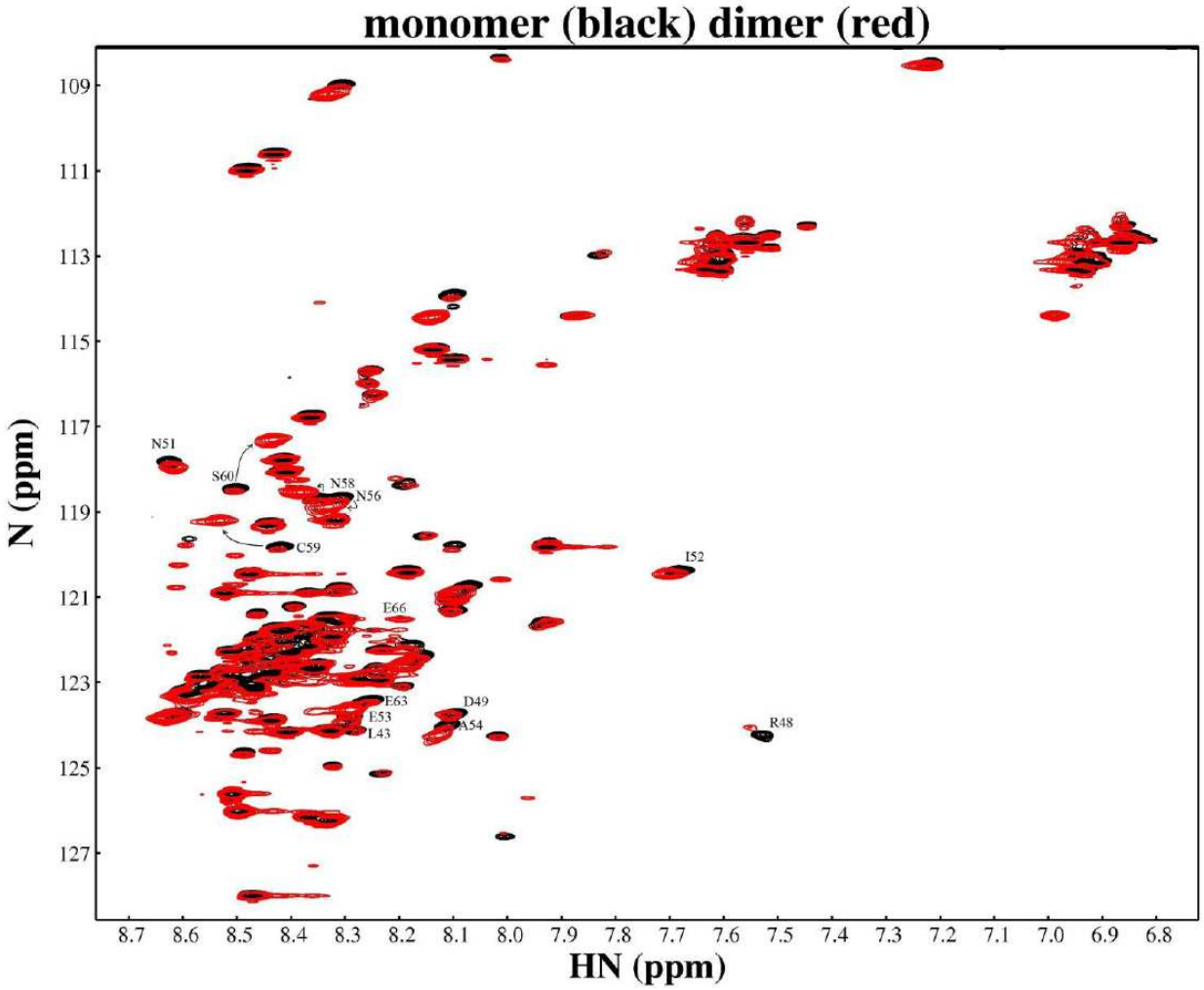

Figure 4. 15N-HSQC FEZ1 (92-194) Nuclear Magnetic Resonance (NMR) spectra. HSQC shows chemical shifts in reduced monomeric protein (black) and non-reduced dimeric protein (red). The spectrum was obtained in spectrometer 600 MHz in NMR Laboratory of LNBio/CNPEM. For the series of experiments, isotope 15N was introduced in minimal medium for growth of bacteria and induction of protein expression.

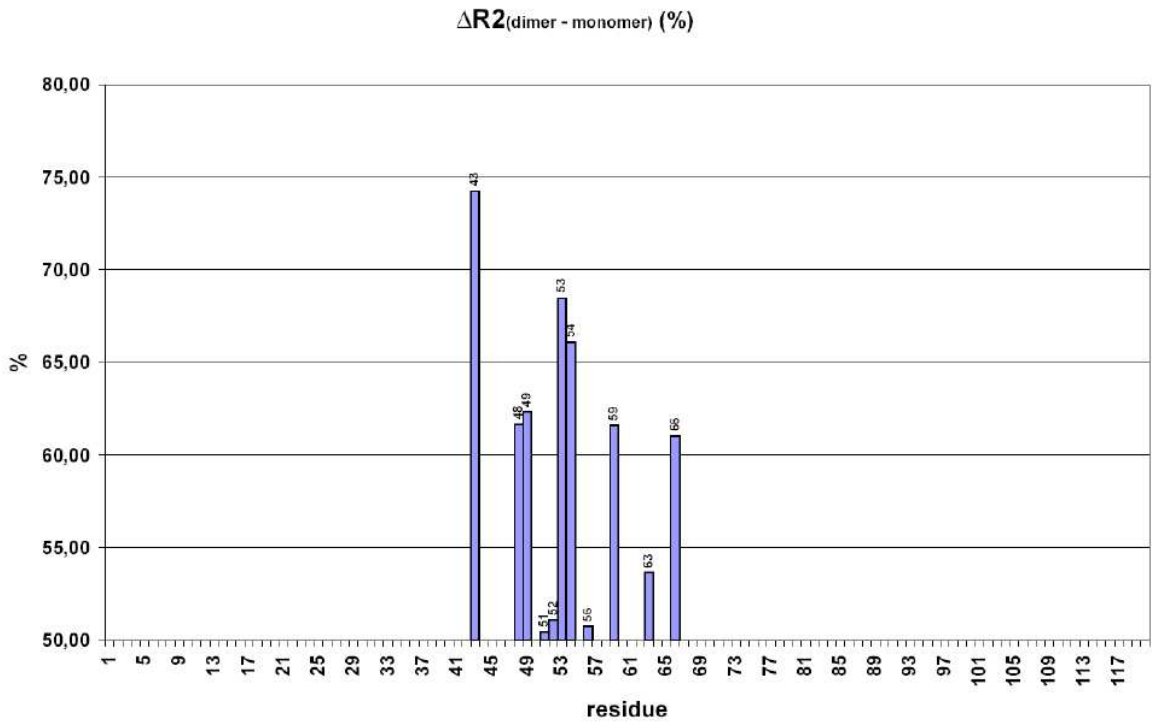

Figure 5. Relaxation assay by spin-spin interaction (T2), where R2is the inverse of T2, the results refer to the ratio of dimer and monomer.

Figure 6. Amino acid sequence of 6xHis-FEZ1 (92-194) studied by nuclear magnetic resonance labeled with the isotope 13C. Cysteine is marked in red and dotted lines indicate the possible contacts between monomers obtained from NOESY spectra.

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4.1. Análise global da duplicação gênica de *unc-76* e do interactoma das proteínas homólogas

→ 4.1.1. Duplicação gênica de *unc-76* originando *FEZ1* e *FEZ2*

A proteína UNC-76 de *C. elegans* e suas ortólogas humanas FEZ1 e FEZ2 constituem uma nova família de proteínas por não apresentarem nenhuma similaridade com outras. Mutações em *unc-76* acarretam em falhas na extensão e na fasciculação neuronal, comprometendo a locomoção do verme, sendo o gene humano *FEZ1* capaz de complementar estas mutações. Isso demonstra que a função dos genes da família FEZ está conservada desde vermes até humanos e que esta família de proteínas e as proteínas com as quais elas interagem são importantes componentes da rede de moléculas que regulam a morfologia celular[1]. Estudos em outros modelos animais (*Mus musculus* e *Drosophila melanogaster*) confirmaram o envolvimento desta família de proteínas (mais especificamente estudando as proteínas UNC-76 e FEZ1) no desenvolvimento neuronal[39]. Contudo, uma análise sistemática da presença de genes da família FEZ nas mais diversas espécies ainda não havia sido realizada.

Um banco de dados com 47 sequências que julgamos serem membros da família de proteínas FEZ foi compilado. Sequências protéicas da família FEZ foram encontradas em uma variedade de espécies, desde nematóides, artrópodes até mamíferos, mas não em plantas, fungos ou protistas. De um modo geral, a presença de genes da família FEZ correlaciona-se com organismos dotados de células nervosas. A partir do alinhamento das sequências obtidas, construímos uma árvore filogenética, o que permitiu estimar o período em que ocorreu a duplicação gênica como sendo após a divergência do ramo do anfioxo, o que é concomitante com a origem dos cordados. De acordo com análises de sintenia entre anfioxos e humanos, duas rodadas de duplicação genômica ocorreram no ramo dos cordados, após a divergência dos cefalocordados mas antes da divisão entre teleósteos e tetrápodes. Acreditamos que a duplicação gênica de *unc-76* tenha ocorrido durante esses eventos de duplicação genômica.

→ 4.1.2. Aspectos evolutivos: padrão de interação entre os homólogos

Os dados apresentados nos artigos "*FEZ1 Dimerization and Interaction with Transcription Regulatory Proteins Involves Its Coiled-coil Region*" e "*FEZ2 Has Acquired Additional Protein Interaction Partners Relative to FEZ1: Functional and Evolutionary Implications*" permitem classificar toda a família de proteínas FEZ como uma família de proteínas *hubs*. Proteínas *hubs* são aquelas que interagem com um grande número de proteínas. As redes de PPI (*protein-protein interaction* – interação proteína-proteína) possuem poucos nós (proteínas) altamente conectados e muitos nós pobremente conectados. Estudos demonstram que a deleção de uma proteína *hub* é mais letal do que a de uma

proteína não-*hub*, um fenômeno conhecido como regra da centralidade-letalidade. E como esperado, PPIs de proteínas essenciais são evolutivamente mais conservadas do que PPIs de proteínas não essenciais[35]. O que se observa no interactoma das proteínas da família FEZ é justamente esta tendência da conservação entre as PPIs entre proteínas essenciais. UNC-76 e FEZ1 conservam praticamente todas as interações, ao passo que FEZ2 também conserva e agrega novas. Este resultado, associado ao fato de que o gene *unc-76* duplicou-se no ramo dos vertebrados, explica os fenótipos de mutação, *knockout* e complementação observados em *C. elegans*, *D. melanogaster* e *M. musculus*[1,3,9]. Defeitos no desenvolvimento axonal foram observados quando da mutação deste gene nos citados invertebrados e em *C. elegans* estes defeitos não se mantiveram quando da complementação por FEZ1 humana. Já camundongos *knockouts* para *FEZ1* não apresentaram nenhum traço de comprometimento no desenvolvimento axonal, sendo tão somente mais responsivos à administração de metanfetamina, apresentando níveis elevados de dopamina no *núcleo accumbens*. A não observação do comprometimento do desenvolvimento axonal poder ser explicada pela possível complementação de função pelo gene *FEZ2* (cuja proteína interage com as mesmas proteínas que FEZ1) no genoma do camundongo, ao passo que invertebrados não apresentam o gene duplicado.

Entender as vantagens evolutivas obtidas pela manutenção da duplicação do gene de *unc-76* no ramo dos vertebrados é uma das premissas para a compreensão da função dos genes da família FEZ. Makino e colegas[36] sugerem que a taxa de evolução de uma proteína que possui mais parceiros de PPI é muito menor do que a daquela que possui poucos parceiros de PPI. Três vias têm sido propostas para diferenciação funcional para genes duplicados[36]. Primeiro, uma das cópias pode ser silenciada por acumulação de mutações deletérias e eventualmente tornar-se indistinguível de regiões genômicas vizinhas não-codificantes, enquanto que a outra cópia retém a função original. Segundo, enquanto que uma cópia mantém a função original, a outra adquire uma nova função possivelmente por mutações vantajosas e que são selecionadas positivamente. Terceiro, ambas as cópias acumulam mutações que alteram a função original, mas a função original é mantida e compensada cooperativamente. Nossos resultados indicam que, provavelmente, a segunda via é a que ocorre no processo de evolução através dos homólogos aqui estudados (*FEZ1, FEZ2* e *unc-76*). Embora as novas interações adquiridas por FEZ2 não indiquem uma nova função de forma clara, elas corroboram com sua função em outros tecidos, visto de sua expressão tecidual ubíqua. Como tem sido proposto, a proteína FEZ1 atuaria como uma proteína adaptora do transporte mediado por kinesinas. Considerando FEZ2 como uma proteína adaptadora do transporte mediado por kinesinas, as novas interações adquiridas por FEZ2 observadas pelo ensaio de duplo híbrido aumentariam o repertório de cargas da proteína motora e podem estar implicadas em mecanismos envolvidos na diferenciação celular e/ou manutenção do estado diferenciado das células/tecidos onde é expressa.

Salienta-se que estas interações, muito provavelmente, podem não estar ocorrendo simultaneamente na célula. Vias de sinalização que levem à modificações pós-traducionais nas proteínas da família FEZ podem regular o aporte destas cargas. Muitos são os sítios preditos para fosforilação em FEZ1[34]. Um padrão de fosforilação resultante de diversas vias de sinalização poderia funcionar como um "código de barras" regulando o aporte de cargas específicas em determinada fase do ciclo celular.

→ 4.1.3. Padrão diferencial das interações entre FEZ1 e FEZ2 – tendências

A estratégia adotada para se obter indícios que justifiquem a manutenção do gene duplicado no genoma foi estudar as propriedades dos produtos gênicos (proteínas) de todos os parceiros de interação para FEZ2 que foram identificados na triagem por duplo-híbrido em levedura com FEZ2 como isca. Consultamos o banco de dados *Gene Ontology Annotation* (UniProtKB-GOA), através do NCBI (*National Center for Biotechnology Information*), e compilamos manualmente um banco de dados organizado de acordo com os três principais domínios abrangidos pela ontologia: componente celular (*cellular component*), que é a região definida intra- ou extracelularmente; função molecular (*molecular function*), que compreende as atividades elementares de um produto gênico no nível molecular, como ligação ou catálise; e processo biológico (*biological process*), abrangendo as operações ou conjuntos de eventos moleculares com início e fim definidos, pertinentes às unidades funcionais integradas: células, tecidos, órgãos e organismo[37]. Definidas essas propriedades gênicas dos parceiros de interação, procuramos avaliar o perfil de todos aqueles que interagiram com FEZ2, daqueles que eram exclusivos à FEZ2 (representando possível ganho de função) e por fim daqueles cuja interação era compartilhada por FEZ1 (representando possível conservação de função).

Dentre os processos biológicos com maior representação entre os parceiros de interação comuns à FEZ1 e FEZ2 (e com tendência à conservação), podemos destacar (figura 5):

- função mitocondrial (com 3 presas relacionadas, 0 específica para FEZ2),
- transcrição (com 8 presas relacionadas, 1 específica para FEZ2),
- desenvolvimento do sistema nervoso (com 4 presas relacionadas, 1 específica de FEZ2),
- transdução de sinal (com 5 presas relacionadas, 1 específica para FEZ2),
- ciclo celular (com 3 presas relacionadas, 0 específica para FEZ2).

Dentre os processos biológicos com maior representação entre os parceiros de interação específicos de FEZ2 (e provável ganho de função), podemos destacar (figura 5):

- tradução (com 6 presas relacionadas, sendo 4 específicas de FEZ2),
- transporte e citoesqueleto (com 7 presas relacionadas, sendo 4 específicas de FEZ2)

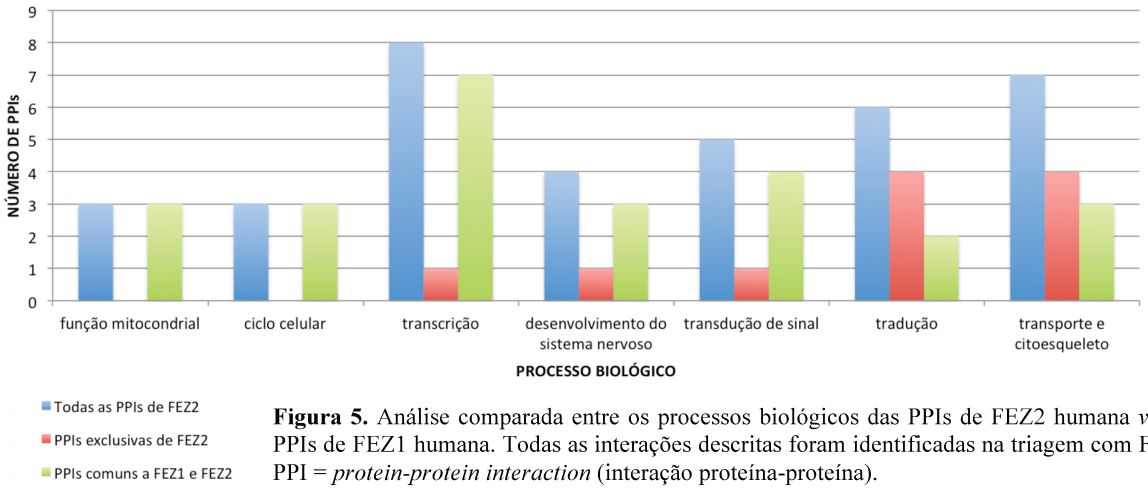

Figura 5. Análise comparada entre os processos biológicos das PPIs de FEZ2 humana *versus* PPIs de FEZ1 humana. Todas as interações descritas foram identificadas na triagem com FEZ2. PPI = *protein-protein interaction* (interação proteína-proteína).

Os dados referentes à função são congruentes aos do processo biológico: com tradução e ligação a nucleotídeos predominando entre os parceiros de interação específicos de FEZ2 e com transcrição, como processo que se destaca dentre os parceiros comuns à FEZ1 e FEZ2. Contudo, a anotação da função no banco de dados é menos comum, e muitas proteínas – embora com o processo biológico anotado – podem não ter a função anotada. Desta forma, embora congruentes, as diferenças foram menos expressivas.

Em relação aos componentes celulares (figura 6) com maior representação entre os parceiros de interação comuns à FEZ1 e FEZ2 (e com tendência à conservação), podemos destacar a localização nuclear (com 18 proteínas identificadas, 3 específicas de FEZ2). Já em relação aos componentes celulares com maior representação entre os parceiros de interação específicos de FEZ2 (e provável ganho de função), podemos destacar a localização citoplasmática (com 38 proteínas identificadas, sendo 18 específicas de FEZ2).

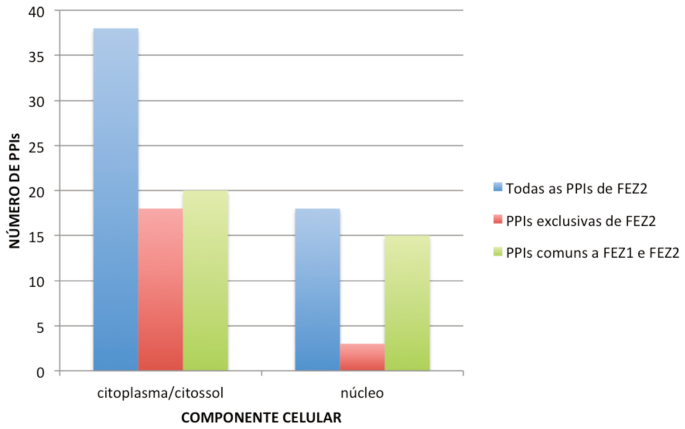

Figura 6. Análise comparada entre os componentes celulares onde localizam-se as PPIs de FEZ2 humana *versus* PPIs de FEZ1 humana. Todas as interações descritas foram identificadas na triagem com FEZ2. PPI = *protein-protein interaction* (interação proteína-proteína).

De modo resumido, eventos relacionados à transcrição gênica e desenvolvimento do sistema nervoso, principalmente, apresentam-se com tendência à conservação. Por outro lado, eventos relacionados à tradução se apresentam como "novidades evolutivas" dentre as interações específicas para FEZ2. De forma congruente, torna-se relevante a quantidade de proteínas presentes no núcleo como interagindo com as homólogas (18 de 56 proteínas identificadas, 32,14%). Dentre as 18 proteínas nucleares identificadas na triagem com FEZ2, 15 mantiveram sua interação com FEZ1 (83,33%) – dentro da expectativa da transcrição gênica como processo biológico conservado. Fato este muito relevante, pois ainda não foi caracterizada a atividade funcional de FEZ1 no núcleo, embora experimentos de fracionamento celular já tenham identificado a proteína neste compartimento subcelular[32]. Ainda, das 38 proteínas citoplasmáticas identificadas, 18 interagem especificamente com FEZ2 (56,25%) – dentro da expectativa da tradução sendo uma "novidade evolutiva" dentro dos processos biológicos dos parceiros de interação específicos de FEZ2.

→ 4.1.4. Hipótese para a interação diferencial entre as homólogas

Os ensaios para detecção de interação proteína-proteína foram realizados com a região C-terminal das proteínas homólogas (UNC-76, FEZ1 e FEZ2) como isca, a qual apresenta forte predição para a estruturação em *coiled-coil* mediante interações. Contudo, diferenças em predições estruturais (estrutura secundária, *coiled-coil*, composição de aminoácidos) não evidenciaram nenhuma caracterísitca exclusiva à FEZ1 e UNC-76 em relação à FEZ2, nem vice-versa, que pudesse explicar o padrão diferencial de interação entre as homólogas. Como já foi mencionado, e será novamente abordado a seguir, proteínas da família FEZ podem ser fosforiladas (por UNC-51, PKCζ, dentre outras cinases não identificadas) de modo a regular interação proteína-proteína e conseqüentemente o conjunto de cargas a serem transportadas. Como, provavelmente, as vias de sinalização afetam este transporte mediado por proteínas da família FEZ, no âmbito das interações mediante seu estado de fosforilação, averiguamos o conteúdo de resíduos de aminoácidos que podem ser fosforilados (serina, treonina e tirosina) na região C-terminal das homólogas.

Os resíduos fosforiláveis conservados entre FEZ1 e FEZ2 foram descartados por não constituirem diferenças entre as parálogas (Figura 7A). Assim, os resíduos diferentes foram comparados com UNC-76. Verificamos se existia conservação destes entre UNC-76 e FEZ1, mas nenhum foi conservado, indicando que FEZ1 e UNC-76 não apresentam resíduos fosforiláveis a mais que FEZ2 (figura 7B). Comparando-se os resíduos presentes em FEZ2 e que não eram conservados em FEZ1 com UNC-76, observamos que 4 resíduos não foram conservados (figura 7C). Destes, apenas 1 estava conservado em todas as espécies portadoras do gene *FEZ2* e não estava conservado em nenhuma seqüência de FEZ1 ou UNC-76. Predição para fosforilação utilizando-se a seqüência completa de FEZ2 foi realizada utilizando-se o programa GPS

2.1 (*Group-based Prediction System version* 2.1)[38]. Este programa prediz também quais cinases poderiam estar realizando a fosforilação. No caso da treonina 230 específica em FEZ2, as seguintes cinases foram preditas como candidatas para fosforilarem FEZ2 neste resíduo: CAMK (*score* 2,675 e *cutoff* 2,059), NIK (*score* 6,000 e *cutoff* 5,500), NEK9 (*score* 6,000 e *cutoff* 6,000), TLK e MAP3K11 (*score* 5,000 e *cutoff* 3,667). Desta forma, a presença da treonina 230 em FEZ2 humana pode estar relacionada com este padrão diferencial de interação entre as homólogas, observado no duplo híbrido, além de constituir um ponto extra para a regulação para o aporte de cargas.

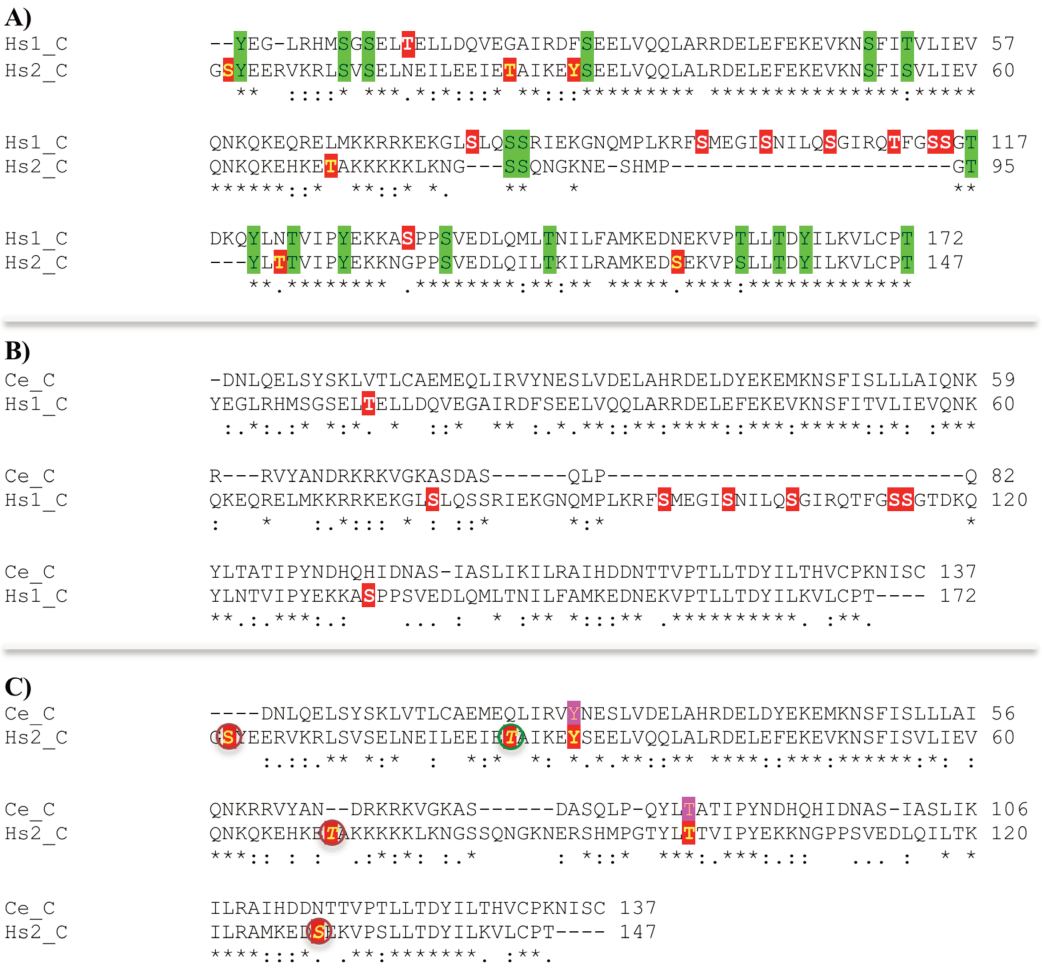

Figura 7. Comparação entre as sequências C-terminais de FEZ1, FEZ2 e UNC-76 com enfoque em aminoácidos que possam ser fosforilados: serina, treonina e tirosina. **A, B e C)** Aminoácidos fosforiláveis idênticos ou semelhantes (como a troca de S→T) conservados entre as homólogas estão sombreados em verde, os não conservados estão sombreados em vermelho, destes, os presentes somente em FEZ1 estão com a letra em branco e os presentes somente em FEZ2 estão com a letra em amarelo. **A)** Comparação entre FEZ1 (221-392) humana (Hs1_C) e FEZ2 (207-353) humana (Hs2_C). **B)** Comparação entre os aminoácidos fosforiláveis exclusivos de FEZ1 (sombreados em vermelho com letra branca) com os de UNC-76 (242-378) de *C. elegans* (Ce_C) – nenhum aminoácido fosforilável demonstrou-se conservado. **C)** Aminoácidos fosforiláveis em UNC-76 correspondentes e conservados em FEZ2 que não apresentaram conservação entre FEZ1 e FEZ2, estão sombreados em rosa. Aminoácidos fosforiláveis exclusivos de FEZ2 estão destacados por um círculo, sendo o vermelho os exclusivos de FEZ2 não conservados entre as espécies onde FEZ2 está presente, e o círculo verde correspondendo ao aminoácido fosforilável exclusivo de FEZ2 e que está conservado em todas as espécies.

4.2. Análise Pontual do Interactoma

Os dados referentes à análise pontual do interactoma provém de um levantamento que difere dos dados levantados na análise global (oriundas diretamente do *Gene Ontology*). Neste caso, realizou-se uma pesquisa manual e individual na literatura para cada parceiro de interação identificado na triagem com FEZ2 no PubMed (http://www.ncbi.nlm.nih.gov/pubmed/), tendo como base os itens relacionados nos próximos tópicos (de 4.2.1 à 4.2.4).

→ 4.2.1. Parceiras de interação envolvidas com esquizofrenia

A esquizofrenia, um dos principais distúrbios mentais, é devastadora e relativamente comum, acomentendo cerca de 1% da população em geral, sendo sua etiologia, assim com a de outras doenças psiquiátricas, amplamente desconhecida[39]. Um dos motivos para este desconhecimento é o fato de que biologia da esquizofrenia é complexa e com muitas hipóteses que suportam a base da doença. Dentre estas, está a regulação da função sináptica, acreditada estar entre as principais etiologias de desordens psiquiátricas. A liberação normal de neurotransmissores depende de um complexo grupo de proteínas pré-sinápticas que regulam a ancoragem vesicular, fusão e fissão de membranas, incluindo *synaptophysin*, *syntaxin*, *synaptosomal-associated protein-25* (SNAP-25), *vesicle associated membrane protein* (VAMP), α-*synuclein* e *dynamin* I (*dynamin I-like* foi identificada como parceira de interação de FEZ1 e FEZ2 neste trabalho). Além disso, proteínas estruturais e de sinalização, como NCAM (*neural cell adhesion molecule*), mantêm a integridade sináptica[40]. Muitas vias também apontam o fator de risco *DISC1* (*disrupted in schizophrenia 1*) como sendo capaz de explicar hipóteses díspares[41]. DISC1 tem sido identificada como um possível regulador positivo da via Wnt canônica, via inibição da GSK3*β* (*glycogen synthase kinase 3 beta)* e conseqüente aumento dos níveis de *β*-catenina, regulando a proliferação dos progenitores neurais. O interactoma de DISC1 e sua localização subcelular enquadram esta proteína em funções relacionadas à sinapse e ao centrossomo[41], funções em comum com proteínas FEZ. DISC1 também está envolvida na formação/manutenção do cílio primário, uma estrutura celular onde concentram-se, dentre outros, diversos receptores dopaminérgicos relacionados à esquizofrenia. A formação e a manutenção do cílio primário está intimamente relacionada com o centrossomo e o material pericentriolar[21]. Estudos recentes demonstram também que a cinase NEK1 – que interage com FEZ1 – regula a ciliogênese[42].

O gene *DISC1* apresenta uma translocação t(1;11)(q42.1;q14.3) que segrega com esquizofrenia e outros transtornos mentais associados à esta em uma família escocesa. A proteína FEZ1 relaciona-se à esquizofrenia devido sua interação com a proteína DISC1. A interação entre FEZ1 e DISC1 é interrompida quando da translocação de DISC1, indicando que FEZ1 poderia atuar na mesma via que

DISC1. Embora alguns estudos populacionais relacionem SNPs em FEZ1 à esquizofrenia outros não encontram tal correlação[43].

Considerando-se o possível envolvimento de FEZ1 com esquizofrenia, verificamos, dentre os parceiros de interação de FEZ1 e FEZ2, quais aqueles relacionados à esta patologia. Das 59 proteínas interagindo com FEZ2, 11 (18,64%) apresentaram relação com a esquizofrenia, de acordo com dados provenientes, de um modo geral, de levantamentos de proteômica e genômica. Destas, 4 interações foram específicas para FEZ2. Proteínas relacionadas à esquizofrenia e que também apresentaram envolvimento com sinapse somaram um total de 4, sendo que destas, todas interagiram tanto com FEZ2 quanto FEZ1 (4/7 = 57,14%), ou seja, das proteínas que interagiram tanto com FEZ2 quanto com FEZ1 e que estão relacionadas com esquizofrenia, 57,14% estão envolvidas com sinapse (tabela 1).

Esta correlação corrobora com a hipótese de que alterações de base sináptica estão entre as possíveis causas da esquizofrenia. Os dados também são consoantes às alterações comportamentais observadas em camundongos *knockout* para *FEZ1*. Embora este modelo animal não apresente prejuízos de locomoção nem alterações morfológicas em neurônios (como observado em *C. elegans* mutados em *unc-76*), as alterações comportamentais observadas neste camundongo *knockout* são condizentes à esquizofrenia. Embora a complementação por *FEZ2* possa evitar tais danos severos para o desenvolvimento do animal, alterações na base sináptica podem ser mais sensíveis às possíveis diferenças entre FEZ1 e FEZ2 quando da complementação por *FEZ2*, em termos, por exemplo, das interações específicas à esta assim como o nível de expressão da paráloga.

Tabela 1. Proteínas identificadas no interactoma com FEZ2 como isca e que apresentam correlação com esquizofrenia. SP = sinapse. As referências para as evidências estão relacionadas no rodapé da tabela.

	Gene	**SP**	**Evidência**
FEZ1 e FEZ2	RARA	X	super-expressão (proteína) em células granulares do giro dentado
	PTN	X	redução (RNA) da expressão
	WWC1		genotipagem – SNP
	FEZ1	X	genotipagem - SNP e associação com DISC1
	TSNAX		splicing intergênico com DISC1 - fusão de DISC1 e TSNX
	GPRASP2		genotipagem – SNP
	CAP1	X	superexpressão (proteína) no tálamo mediodorsal
FEZ2	NQO2		inserções e deleções no promotor
	SHMT1		polimorfismos no promotor e níveis de expressão gênica aumentados
	GNAS		polimorfismo
	HSPA1A		polimorfismo

RARA[44], PTN[45], WWC1[46], FEZ1[43], TSNAX[47], GPRASP2[48], CAP1[49], NQO2[50], SHMT1[51], GNAS[52], HSPA1A[53]

→ 4.2.2. Parceiras de interação envolvidas no desenvolvimento do sistema nervoso

Neurônios são células altamente polarizadas que utilizam vários mecanismos para iniciar, manter e regular a sua função biológica. Os neurônios possuem até 10000 vezes mais área de superfície que qualquer outro tipo celular, de modo que o crescimento, a remodelagem e a manutenção dos processos axonais e dendríticos dependem fortemente do tráfego de membrana, exigindo uma extensa rede de tráfego pós-Golgi para a entrega das membranas e receptores em destinos específicos. A coordenação deve ocorrer entre os processos de classificação (*sorting*) de proteínas, brotamento de vesículas, transporte ao longo do citoesqueleto e fusão das vesículas[26]. Assim, o processo de extensão do neurito, transporte de receptores transmembrana e sinapse é altamente acoplado, de modo que o transporte axonal mediado por microtúbulos é de vital importância para a função e viabilidade neuronal. Muitos estudos focam a maquinaria motora neste processo, contudo os processos regulatórios para a montagem do complexo motor-carga ainda não são muito bem compreendidos. Em 2008, Hirofumi Toda e colaboradores demonstraram que a cinase UNC-51/ATG1 pode regular a interação entre vesículas sinápticas e complexos motores durante o transporte em *D. melanogaster*. UNC-51 interage com UNC-76 (desde já classificada como uma proteína adaptadora da cadeia pesada de kinesina – *Kinesin Heavy Chain*, KHC). Tanto defeitos em *unc-51* quanto em *unc-76* levam à segregação de vesículas sinápticas dos complexos motores, que se acumulam ao longo dos axônios. UNC-51 fosforila UNC-76 na serina 143, UNC-76 fosforilada interage com *Synaptotagmin*-1, uma proteína de vesícula sináptica, sugerindo que as interações motor-carga são reguladas de maneira dependente de fosforilação. Além disso, os defeitos no transporte axonal em mutantes de *unc-76* só foram recuperados por UNC-76 fosfo-mimética na Ser143 e não pela fosfo-deficiente, demonstrando mais uma vez que o transporte axonal depende da fosforilação de proteínas adaptadoras. Outas cinases podem fosforilar FEZ1/UNC-76, como a PKCζ e influenciar na interação proteína-proteína, como descrito por Lanza e colegas[34]. Neste caso, a fosforilação em FEZ1 por PKCζ reduziu a interação entre o C-terminal de FEZ1 e CLASP2, *in vitro*. O sítio de fosforilação ainda não foi identificado. Ainda, outras cinases poderiam fosforilar FEZ1 e influenciar no aporte de cargas para o complexo motor, em outras regiões, que influenciariam todo o metabolismo celular e de maneira retro-alimentada à função e ao estado de fosforilação de FEZ1.

Dentro desta lógica, é importante destacar que, muitas das proteínas identificadas interagindo com FEZ1 e com FEZ2 neste interactoma e relacionadas como desenvolvimento do sistema nervoso (tabela 2), estão relacionadas à eventos de sinalização, como SHMT1 (*serine hydroxymethyltransferase 1*), PTN (*pleiotrophin*), RARA *(retinoic acid receptor, alpha*) e TSNAX (*translin-associated factor X*). As mais notórias são RARA e PTN. O ácido retinóico aumenta os níveis de expressão de PTN, que é secretada e atua em receptores com atividade de tirosina fosfatase (RPTP, *receptor protein tyrosine phosphatase* – que também interagem com FEZ1), acarretando sua homodimerização e inibição de sua

atividade. O resultado final é um aumento global de tirosinas fosforiladas, regulando a atividade de inúmeras proteínas que, dentre outros processos, leva à eventos como o crescimento de neuritos. Destaca-se também que FEZ1 é essencial para a função do NGF, uma vez que o silenciamento de *FEZ1* acarretou em prejuízos para a extensão de neuritos em células estimuladas com este fator. Alterações no transporte destes elementos (PTN, RARA, RPTP, por exemplo), acarretariam em prejuízos para o normal desenvolvimento neuronal e/ou sináptico.

Outras proteínas que estão envolvidas na neurogênese e que foram identificadas nesta triagem são DNM1L (*dynamin-1 like*) e SCOCO (*short coiled-coil*). As dinaminas são expressas em células de mamíferos e estão envolvidas nos processos de tráfego de vesículas. DNM1L é conhecida por constringir e tubular membranas e dividir mitocôndrias e peroxissomos. Outras funções no complexo de Golgi vem sendo atribuídas à DNM1L, relacionadas à maquinaria de *sorting* na TGN (*Trans-Golgi Network*)[54]. A proteína SCOCO também está envolvida diretamente no transporte de vesículas sinápticas, aparentemente em cooperação com a proteína FEZ1 e associada também ao complexo de Golgi. Ambas proteínas (DNM1L e SCOCO), que estão diretamente envolvidas com o tráfego de vesículas, não estão associadas à esquizofrenia de acordo com a literatura, ao passo que a maioria das proteínas de sinalização descritas anteriormente estão relacionadas à esquizofrenia e à processos de sinapse. Isto sugere que, se FEZ1 está realmente envolvida na esquizofrenia, este envolvimento se dá no transporte de proteínas de sinalização específicas e que também estão envolvidas com sinapse e não em mecanismos mais basais que envolvem a formação de vesículas (que poderia ser mais deletério para a célula).

Tabela 2. Proteínas identificadas no interactoma com FEZ2 como isca e que apresentam correlação com o desenvolvimento do sistema nervoso. EF = esquizofrenia e SP = sinapse. As referências para as evidências estão relacionadas no rodapé.

	Gene	**EF**	**EF e SP**	**Evidência**
FEZ1 e FEZ2	RARA	X	X	baixos níveis de expressão levam à morte neuronal
	PTN	X	X	extensão de neuritos
	FEZ1	X	X	essencial para a correta fasciculação e extensão do neurito
	DNM1L			essencial para o desenvolvimento do embrião e do cérebro de camundongos. Deleção acarreta em divisão anormal das mitocôndrias
	SCOCO			deleção do C-terminal acarreta em prejuízos para a extensão do neurito
	TSNAX	X		interage e regula os níveis de expressão de GAP43 (*growth associated protein 43*) durante a fase regenerativa do neurônio
	CAP1	X	X	superexpressão aumentou o tamanho do neurito, localiza-sem em cones de crescimento
FEZ2	SERPINF1			extra-celular e neurotrófico
	SHMT1			envolvida no metabolismo do folato, associado à deficiências no tubo neural

RARA[55], PTN[56], FEZ1[1], DNM1L[54], SCOCO[57], TSNAX[58], CAP1[59], SERPINF1[60], SHMT1[61]

→ 4.2.3. Parceiras de interação envolvidas com infecção viral

Neurônios estão entre os poucos tipos celulares que não suportam a replicação do HIV tipo 1 (HIV-1). Este tipo celular naturalmente expressa altos níveis de FEZ1 quando comparado aos astrócitos ou células de microglia e são, correspondentemente, menos susceptíveis à infecção por HIV-1[30]. Como já introduzido, propõe-se que FEZ1 teria a habilidade de bloquear o transporte do DNA viral para o núcleo[29]. Quando FEZ1 é super-expressa – no caso do vírus JC, que possui DNA dupla-fita circular – prejudica a função da agnoproteína no transporte dos virions, provavelmente por favorecer o transporte de elementos neuronais ao invés de elementos virais pela agnoproteína. Embora diferentes, os mecanismos de atuação ante o bloqueio da infecção viral por retro-vírus ou DNA-vírus convergem para a maquinaria de transporte intracelular.

Das proteínas identificadas no interactoma, 12 (20,34%) apresentam dados de envolvimento com infecção viral, sendo 4 interações específicas para FEZ2 (tabela 3). Em termos qualitativos, diversas são as evidências para o envolvimento destas proteínas no ciclo de replicação viral e elas não parecem convergir para uma um único mecanismo, como o de transporte, por exemplo. Tampouco foi encontrada correlação entre as proteínas relacionadas com infecção viral e esquizofrenia. Neste caso, os mecanismos que levariam à resistência por infecção viral quando da super-expressão de FEZ1 parecem diferir dos mecanismos que posicionam esta proteína em vias afetadas pela esquizofrenia.

Tabela 3. Proteínas identificadas no interactoma com FEZ2 como isca e que apresentam correlação com infecção viral. EF = esquizofrenia e SP = sinapse. As referências para as evidências estão relacionadas no rodapé.

	Gene	**EF**	**EF e SP**	**Evidência**
FEZ1 E FEZ2	PDCD7			redução da expressão em cultura de células transfectadas com vírus de pacientes aidéticos e com demência
	DEFA3			aumento da expressão reduz a progressão da doença
	SAP30			PBF (*Papillomavirus binding factor*) se liga a SAP30 e inibe a transcrição do HPV
	SMC3			parte do interactoma da proteína Tat do HIV-1
	PTN	X	X	induz a replicação do HIV-1
	FEZ1	X	X	linhagens resistentes à infecção viral apresentaram super-expressão de FEZ1
	MCM7			citomegalovírus humano reprime a replicação por inibir o recrutamento do complexo MCM
	AATF			envolvido na manutenção da imunidade adaptativa contra a invasão pelo HIV-1 ao nível de ácido nucléico
FEZ2	HSP90B1			chaperona de RE essencial para a infecção por virus stomatitis
	RPS25			RPS25 é essencial para o início da tradução via IRESs (*internal ribosome entry site*) pelos vírus *Dicistroviridae* e da hepatite C
	DNAJB11			interage com uma glicoproteína transmembrana do KSHV chamada K1, que é capaz de transformar fibroblastos e inibir a apoptose. Inibir DNAJ inibe expressão de K1
	TUBA1B			a proteína viral Tat, diretamente aumenta a polimerização da tubulina

PDCD7[62], DEFA3[63], SAP30[64], SMC3[65], PTN[66], FEZ1[29], MCM7[67], AATF[68], HSP90B1[69], RPS25[70], DNAJB11[71], TUBA1B[72]

→ 4.2.4. Parceiras de interação envolvidas com a maquinaria de transporte

A proteína FEZ1 pode participar na formação dos microtúbulos em neurônios em diferenciação, provavelmente através de um complexo formado por FEZ1, PKCζ e α-/β-tubulina. O complexo pode estabilizar e estender a rede de microtúbulos em paralelo com o transporte de cargas via kinesinas[43]. Corroborando com isso, a inibição da expressão do mRNA de FEZ1 em células hipocampais de camundongo acarretou em defeitos na morfologia e na ocupância de mitocôndrias, o que foi correlato aos defeitos na polarização neuronal. Sugere-se ainda que a mitocôndria seria uma carga transportada via FEZ1, além de vesículas pré-sinápticas.

Das proteínas identificadas pela triagem com FEZ2, 8 (13,6%) foram relacionadas diretamente à maquinaria de transporte. De um modo geral estão envolvidas com formação e manutenção do centrossomo, fuso mitótico e coesão das cromátides irmãs (C14orf94 e SMC3), *sorting* de proteínas (WWC1, LOC1), proteínas adaptadoras (FEZ1, TSNAX, CAP1), citoesqueleto (TUBA1B). Destas 8 interações, 2 foram específicas para FEZ2 e não foram relacionadas à esquizofrenia. Das 6 proteínas que também interagiram também com FEZ1, 4 (66,67%) apresentavam associação com esta patologia sendo que destas, apenas 2 também foram associadas à sinapse (50%). Considerando-se que uma das hipóteses é a de que esquizofrenia é uma doença com base em defeitos na sinapse e que mecanismos de transporte podem influenciar o padrão de distribuição de receptores de membrana assim como a entrega de vesículas sinápticas pode-se inferir que nossos resultados demonstram que proteínas da família FEZ poderiam atuar tanto em um quanto em outro processo, contudo deve ser considerado o número pequeno de proteínas analisadas nesta seção do interactoma.

Tabela 4. Proteínas identificadas no interactoma com FEZ2 como isca e que apresentam correlação com a maquinaria de transporte. EF = esquizofrenia e SP = sinapse. As referências para as evidências estão relacionadas no rodapé.

	Gene	EF	EF e SP	Evidência
1 e 2	C14orf94			regula a integridade do centrossomo e do fuso mitótico
	SMC3			envolvido na coesão das cromátides irmãs
	WWC1	x		associa-se com a cadeia leve da dineína. Relacionada com *sorting* de receptores pela TGN (*Trans-Golgi Network*)
	FEZ1	x	x	associa-se com microtúbulos e kinesinas
	TSNAX	x		interage com KIF2Abeta e pode funcionar como adaptadora para o transporte do complexo TB-RBP/*translin*
	CAP1	x	x	CAP1 fornece um link direto do citoesqueleto de actina à mitocôndria
2	TUBA1B			interage com ARL7 (*ADP-ribosylation factor like 7*) e ARL8 e está envolvida no transporte da TFN (*transferrin*) via TGN
	TLOC1			envolvida no transporte co-traducional

C14ORF94[73], SMC3[74], WWC1[75], FEZ1[9], TSNAX[76], CAP1[77], TUBA1B[78], TLOC1[79]

4.3. Análise estrutural da homodimerização e da formação de heterotetrâmeros com implicações na função

Mediante sua função como proteína adaptadora, FEZ1 teria de ser capaz de interagir com outras duas moléculas protéicas ao mesmo tempo. A conformação dimérica de FEZ1 pelo N-terminal descrita por Lanza e colegas (2008)[34] vai de encontro à este requisito, visto que, uma vez dimérica, o homodímero possuiria duas regiões C-terminais (envolvidas na interação proteína-proteína). O manuscrito e o artigo "*Structural studies by SAXS and NMR of the FEZ1 dimer and the tetramer FEZ1 / SCOCO*" e "*Human FEZ1 Protein Forms a Disulfide Bond Mediated Dimer: Implications for Cargo Transport*", respectivamente, também corroboram e trazem maior detalhamento estrutural para a hipótese.

→ 4.3.1. FEZ1 como molécula adaptadora bivalente do transporte mediado por kinesinas

Su e colaboradores demonstraram em 2006 que o complexo UNC-76 e UNC-69 (ortóloga de SCOCO humana em *C. elegans*) é essencial para o desenvolvimento neuronal e de modo paralelo nosso grupo demonstro que as homólogas humanas, FEZ1 e SCOCO, interagem entre si. Neste estudo de Su e colaboradores, demonstrou-se também que estas proteínas, em *C. elegans*, atuam na mesma via, uma vez que a inibição da expressão de ambas ao mesmo tempo não causou defeitos mais severos do que a inibição de cada uma isoladamente. Em homólogos de leveduras e mamíferos, Slop1p e SCOCO, interagem, respectivamente, com Arl3p e ARL1 – associadas ao Golgi, *GTP-binding ADP-ribosylation fator (ARF)-like proteins*. ARL1 de mamífero está envolvida no transporte pós-Golgi. Arl3p de levedura direciona Arl1p ao Golgi, ocorrendo a montagem de vesículas oriundas do endossomo ao Golgi[57,80]. Ainda neste trabalho propôs-se que UNC-76 e UNC-69 participaria de um complexo localizado em certos compartimentos subcelulares que controlariam o transporte de vesículas entre o Golgi e a membrana plasmática, sendo este complexo recrutado e direcionado pela kinesina-1 a seus destinos finais nos axônios.

Os resultados provenientes dos experimentos de SAXS (*Small Angle X-ray Scattering*) iniciaram uma abordagem mecanística de como o complexo poderia atuar junto à maquinaria de transporte. Demonstramos a composição heterotetramérica de 2 moléculas de 6xHis-FEZ1 (1-392) e 2 moléculas de GST-SCOCO (421-122) humanas no complexo, corroborando com a hipótese de adaptador bivalente, uma vez que FEZ1 dimeriza pela região N-terminal. O modelo heteroteramérico foi gerado com base no fato de que SCOCO, como presa, foi identicada nas triagens por duplo híbrido em levedura utilizando a região C-terminal de FEZ1 ou FEZ2 como isca – uma vez que a proteína com a sequência completa de aminoácidos auto-ativa os genes repórteres do sistema, dificultando sua utilização para triagens e mapeamentos de regiões mínimas de interação. O modelo em si não evidencia se a homodimerização de

FEZ1 é paralela ou anti-paralela (não há resolução suficiente para tal afirmação a partir dos dados de SAXS). Contudo, os contatos entre cadeias laterais dos aminoácidos presentes em cada monômero de FEZ1 (92-194), identificados através de dados de RMN (Ressonância Magnética Nuclear) da proteína FEZ1 (92-194) marcada com ^{15}N e ^{13}C em amostras reduzidas e não reduzidas, demonstraram que FEZ1 dimeriza de forma paralela.

→ 4.3.2. Bases moleculares da homodimerização de FEZ1 e fenótipo *flower-like*

Embora dados anteriores sugerissem a dimerização de FEZ1 pela região N-terminal e o modelo de FEZ1 complexada com SCOCO sugerisse o mesmo, ainda não se sabia quais eram as bases bioquímicas desta homodimerizacão. Por gel nativo, SAXS e espectrometria de massas verificamos que FEZ1 dimeriza por ponte dissulfeto, através das cisteinas 133 de cada monômero. *In vivo*, este estado dimérico de forma covalente pode ser importante para transporte mediado por kinesinas de proteínas ao longo dos microtúbulos, provendo estabilidade ao dímero durante o processo de transporte. Embora observamos a interação do C-terminal de FEZ1 com o seu próprio C-terminal, ainda não compreendemos o papel desta interação, que poderia estar relacionada com uma função regulatória para o aporte de cargas.

O fato de que FEZ1 é intrinsecamente desestruturada, dímero e promíscua em relação ao seu grande número de interações, com suas regiões C-terminais interagindo com elementos do citoesqueleto (tubulina, CLASP2) pode ser crucial para explicar a formação de pontes entre os microtúbulos e a constrição do núcleo, resultado por sua vez no fenótipo *flower-like*. A dimerização covalente também pode ser importante para estabilização durante o trabalho de tração para a constrição do núcleo pelos laços formados pelos microtúbulos, acarretando o referido fenótipo.

Integração dos dados e proposição de um modelo de atuação para as proteínas FEZ

De uma forma em geral, nossos dados demonstram que proteínas da família FEZ podem atuar como proteínas adaptadoras bivalentes. Demonstramos que a proteína FEZ1 dimeriza através de ponte dissulfeto, de forma paralela e é capaz de interagir, em seu formato dimérico, com outras duas proteínas ao mesmo tempo. Classificamos todas as proteínas da família FEZ como *hubs*, propondo a duplicação do gene no ramo dos vertebrados, com a conservação do padrão de PPI por FEZ1 e agregação de novas PPIs por FEZ2. A partir disto e dos dados presentes na literatura, propomos que as proteínas da família FEZ são adaptadoras diméricas bivalentes do transporte mediado por kinesinas e que as forças evolutivas direcionam o gene *FEZ2* para a manutenção e agregação de proteínas parceiras de interação (figura 8), que podem estar relacionadas com sua ainda incompreendida função nos diferentes tecido onde é expressa. Este trabalho salienta a importância de FEZ2 em novos estudos de desenvolvimento neuronal e de outros tecidos. O desenvolvimento de animais *knockout* para *FEZ2*, assim como uma caracterização mais detalhada das interações descritas em conjunto com uma análise de localização subcelular de proteínas da família FEZ de acordo com os estados de modificações pós-traducional, tem essencial importância para tais estudos e proverão dados para a compreensão dos mecanismos de atuação de DISC1 na etiologia da esquizofrenia, dentre outros aspectos relacionados à resistência à infecção viral.

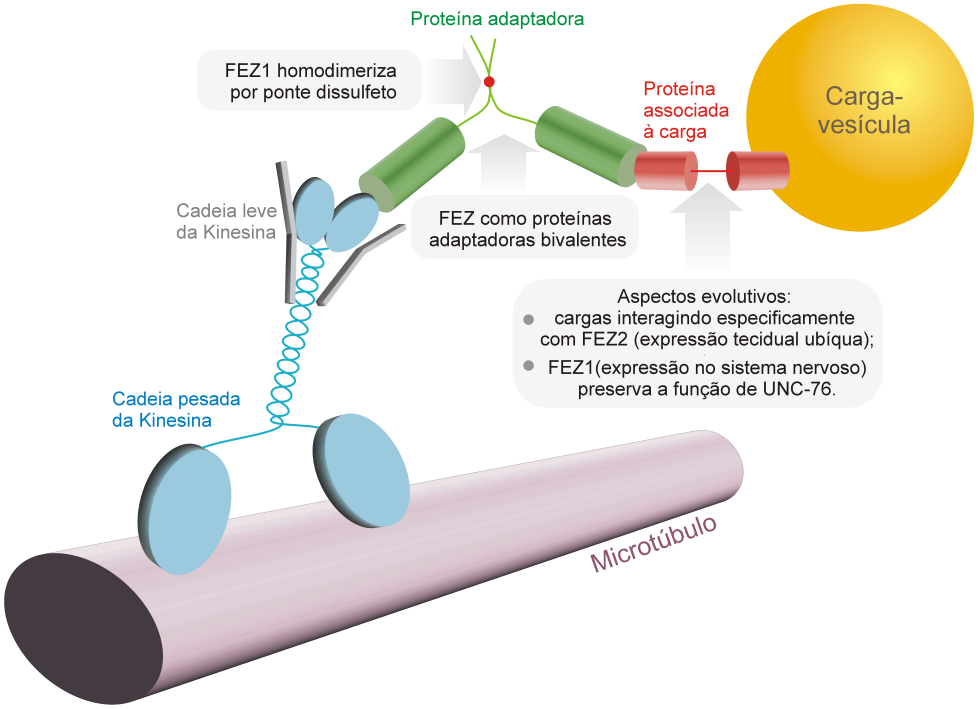

Figura 8. Proteínas da família FEZ como adaptadoras do transporte mediado por kinesinas: estrutura, função e evolução. As principais conclusões deste trabalho estão destacadas em caixas cinzas.

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DECLARAÇÃO

Declaro para os devidos fins que o conteúdo de minha dissertação de Mestrado/tese de Doutorado intitulada Proteínas da família FEZ (*Fasciculation and Elongation protein Zeta*) como adaptadoras bivalentes do transporte: aspectos funcionais, estruturais e evolutivos:

( ) não se enquadra no § 3º do Artigo 1º da Informação CCPG 01/08, referente a bioética e biossegurança.

Tem autorização da(s) seguinte(s) Comissão(ões):

(X) CIBio – Comissão Interna de Biossegurança , projeto No. JK06.02, Instituição: Associação Brasileira de Tecnologia de Luz Síncrotron.

( ) CEUA – Comissão de Ética no Uso de Animais , projeto No. _____, Instituição: _____.

( ) CEP - Comissão de Ética em Pesquisa, protocolo No. _____, Instituição: _____

** Caso a Comissão seja externa ao IB/UNICAMP, anexar o comprovante de autorização dada ao trabalho. Se a autorização não tiver sido dada diretamente ao trabalho de tese ou dissertação, deverá ser anexado também um comprovante do vínculo do trabalho do aluno com o que constar no documento de autorização apresentado.*

Aluno: (Marcos Rodrigo Alborghetti)

Orientador: (Jörg Kobarg)

Para uso da Comissão ou Comitê pertinente:
(X) Deferido ( ) Indeferido

Carimbo e assinatura

Prof. Dr. MARCELO LANCELLOTTI
Presidente da Comissão Interna de Biossegurança
Instituto de Biologia - UNICAMP

Para uso da Comissão ou Comitê pertinente:
( ) Deferido ( ) Indeferido

Carimbo e assinatura

Uso exclusivo da CIBio:

Número de projeto / processo: JK06.02

Formulário de encaminhamento de projetos de pesquisa para análise pela CIBio - Comissão Interna de Biossegurança da ABTLuS – Associação Brasileira de Tecnologia de Luz Síncrotron

Título do projeto: Estudos estruturais e funcionais de proteínas que interagem com a proteína reguladora humana FEZ1

Pesquisador responsável: Jörg Kobarg

Experimentador: Marcos Rodrigo Alborghetti

Nível do treinamento do experimentador: [ ]-Iniciação científica, [ ]-mestrado, [ ]-doutorado, [x]-doutorado direto, [ ]-pós-doutorado, [ ]-nível técnico, [ ]-outro, especifique:_____

Resumo do projeto:

A proteína humana FEZ1 (***fasciculation and elongation protein ζ 1***) é ortóloga da proteína UNC-76, de *Caenorhabditis elegans,* a qual é necessária para o crescimento axonal neste nematóide. Estudos anteriores têm mostrado que a proteína FEZ1 interage com a PKC ζ (protein kinase C ζ), DISC1 (disrupted-in-schizophenia 1) e agnoproteína do poliomavirus humano JC. Nosso grupo descreveu anteriormente que FEZ1 e seu parálogo FEZ2 são proteínas que interagem com NEK1 (NIMA related kinase 1), uma proteína quinase envolvida na doença renal policística e em mecanismos de reparo na fase G2/M do ciclo celular. Um ensaio de duplo-híbrido, com biblioteca de cérebro fetal humano (Clontech) utilizando a proteína FEZ1 (aminoácidos 221-396) como isca identificou 16 proteínas interagindo com a última. As 13 proteínas com função conhecida participam de processos como: regulação da transcrição gênica e remodelagem da cromatina (7 proteínas), regulação do desenvolvimento de neurônios (2 proteínas), mecanismos de transporte celular (3 proteínas) ou participam da apoptose (2 proteínas). Neste projeto pretende-se estudar a possível função de regulação da transcrição gênica exercida pela proteína FEZ1, através de metologias que envolvem sua associação com as proteínas BAF60a, KIBRA e SAP30L, envolvidas em processos de regulação da transcrição e identificadas nos experimentos de duplo híbrido. Estudos estruturais (cromatografia de exclusão molecular, dicroísmo circular, cristalografia e footprinting oxidativo) e funcionais (co-localização *in vivo* e microarranjo de DNA) serão realizados com o intuito de fornecer pistas acerca desta possível função da proteína FEZ1.

A CIBio analisou este projeto em reunião realizada no dia: 16.4.2007 .

Parecer final: [X]-projeto aprovado, [ ]-projeto recusado, [ ]-projeto com deficiências, favor comentários abaixo:

_____

_____

_____

Jörg Kobarg

Presidente de CIBio - ABTLuS
Prof. Dr. Jörg Kobarg

Celso Benedetti

Membro da CIBio - ABTLuS
Prof. Dr. Celso Eduardo Benedetti

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