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Interaction of amyotrophic lateral sclerosis/frontotemporal lobar degeneration–associated fused-in-sarcoma with proteins involved in metabolic and protein degradation pathways

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ABSTRACT

Fused-in-sarcoma (FUS) is a nuclear protein linked to amyotrophic lateral sclerosis and frontotemporal dementia. Under pathologic conditions, FUS frequently is accumulated in cytosoplasm, but how this altered distribution affects the protein interaction pattern of FUS is unclear. Using dual-tag affinity purification and mass spectrometry, we compared the interactome of the wild-type FUS and the P525 L mutant, which causes juvenile amyotrophic lateral sclerosis with the most severe phenotypes. The mutant FUS retained the ability to bind proteins involved in RNA metabolism. We found significant increased binding of P525 L to many metabolic enzymes. Furthermore, we identified and confirmed some novel interactions between FUS and proteins involved in neurodegenerative diseases and/or ubiquitin proteasome pathway, such as VCP/p97, PSF, UBA 1, and 26S proteosome non-ATPase regulatory subunit 12 (PSMD12/Rpn5). Accordingly, we have observed significantly reduced ATP levels and increased accumulation of poly-ubiquitinated proteins in cells with FUS accumulation. Therefore, our study suggested new mechanisms whereby FUS accumulation leads to defective energy metabolism and protein degradation by directly interacting with key regulators in these pathways.

1. Introduction

Fused-in-sarcoma (FUS) or translocated-in-sarcoma is an RNA/DNA-binding protein originally identified as an oncoprotein (Crozat et al., 1993). It participates in a variety of nuclear functions, which include transcription, RNA splicing, and DNA repair (Dichmann and Harland, 2012; King et al., 2012; Kwon et al., 2013; Mackenzie and Neumann, 2012; Qiu et al., 2014; Wang et al., 2008, 2013; Yamazaki et al., 2012). Together with 2 other structurally and functionally similar proteins, Ewing's sarcoma protein and TATA-box binding protein–associated factor 15, they form the FET family of proteins (King et al., 2012). Recently, mutations in FET proteins have been directly associated with amyotrophic lateral sclerosis (ALS) and/or frontotemporal dementia (FTD), 2 neurodegenerative diseases sharing many pathologic, clinical, and genetic features (Couthouis et al., 2011, 2012; Gitler and Shorter, 2011; Kwiatkowski et al., 2009; Vance et al., 2009).

Pathogenic mutations frequently affect the nuclear localization signal of FUS, leading to increased cytosolic distribution of the protein (Dormann et al., 2010; Mackenzie et al., 2010). The likely consequences of this change are the loss of normal nuclear activities of FUS and the potential gain of functions in the cytosol. Recent studies have identified a large number of target RNAs or genes regulated by the wild-type FUS, suggesting the potential deleterious effects caused by the mutation-induced mislocalization (Hoell et al., 2011; Lagier-Tourenne et al., 2012; Tan et al., 2012). Besides mutation-caused mislocation, wild-type FUS is found to be a component of cytoplasmic inclusions observed in the brains of some patients with ALS or FTD (Mackenzie and Neumann, 2012). In transgenic rodent or drosophila models, FUS accumulation results in neurodegeneration or even lethality (Chen et al., 2011; Huang et al., 2011); however, the underlying molecular and cellular mechanisms are still not clear.

Protein–protein interaction analysis can help to generate insight into the functions of a protein. In a proteomic study using nuclear extract, Yamazaki et al. (2012) indicated that FUS interacts with many proteins involved in RNA metabolism and transcription and shed light on the mutation-caused loss of nuclear functions of FUS. Given the cytoplasmic accumulation of FUS under disease conditions, however, it will be important to unveil the FUS interactome in

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the whole-cell level and identify the changes caused by pathogenic mutations. The missense mutation P525 L in FUS causes juvenile-onset ALS with the most severe clinical manifestation among all known FUS mutation (Kwiatkowski et al., 2009). Therefore, in this study we used a stringent 2-tag affinity purification followed by proteomic analysis to compare the interactome of the wild-type FUS with that of the P525 L FUS using total cell lysate. In addition to confirming many of the previously described interactions and functions of FUS, we found that FUS, especially the mutant, interacts with key proteins in energy metabolism and protein degradation pathways. Furthermore, FUS accumulation results in decreased cellular ATP level and increased poly-ubiquitinated proteins.

2. Methods

2.1. Materials

Expression plasmids for human FUS, valocin-containing protein (VCP), UBA1, and Rpn-5 were generated by reverse transcription polymerase chain reaction via the use of human cDNA library and subcloned into pcDNA3.1-based vector with indicated tags (Invitrogen). Flag-tagged PSF was described previously (Zhong et al., 2006). The P525 L mutation and various truncation constructs of FUS were generated by polymerase chain reaction–directed mutagenesis. The primary antibodies and the dilution used are: mouse anti-PSF antibody (P2860; Sigma, 1:2000); rabbit anti-FUS antibody (ab23439; Abcam, 1:800); rabbit anti-VCP antibody (2985-1; Epitomics, 1:3000); Mouse anti-tubulin antibody (M30109; Abmart, 1:2000); mouse anti-FUS antibody (sc-47711; Santa Cruz Biotechnology, 1:800); mouse anti-Flag tag (DYKDDDDK-Tag) antibody (M20008; Abmart, 1:3000, IP 1:100); rabbit anti-Flag tag (DYKDDDDK-Tag) antibody (2368; Cell Signaling Technology, 1:1000); mouse anti-His tag antibody (M30111; Abmart, 1:5000); mouse anti-actin antibody (M20010; Abmart, 1:2000); mouse antihemagglutinin tag antibody (M20003; Abmart, 1:5000); rabbit antihemagglutinin antibody (H6908; Sigma, 1:2000); mouse anti-Ub antibody (sc-8017; Santa Cruz Biotechnology, 1:1000); rabbit anti-K48 antibody (4289; Cell Signaling Technology, 1:1000). Unless indicated, the dilutions listed are for immunoblotting. The secondary antibodies used in immunoblots were from either Abmart or Jackson ImmunoResearch.

2.2. Cell culture and transfection

HEK293 cells were cultured at 37 °C in 5% CO_2 in Dulbecco's modified Eagle's medium (Invitrogen), supplemented with 10% fetal bovine serum (Invitrogen) and antibiotics. Cells were transfected using Lipofectamine 2000 reagent (Invitrogen). The total amount of plasmid DNA was adjusted to 4 μg per 35-mm dish or 20 μg per 10-cm dish with an empty vector or green fluorescent protein expression plasmid. Cells were harvested at 36–48 hours' posttransfection for Western blotting.

2.3. FUS affinity purification and mass spectrometry

Four plates of HEK 293T cells grown in 10-cm dishes were transiently transfected with 30 μg/plate of Flag-His double tagged WT or P525 L FUS. Cells were harvested and lysed in NP-40 buffer with protease inhibitors 48 hours posttransfection. Then, 5 mg of lysates were first subjected to Ni-NTA agarose beads pulldown over night and then washed 4 times, and finally eluted in NP40 buffer containing 250 mM imidazole. The eluted proteins were then incubated with 30 μL of anti-Flag antibody-conjugated agarose beads (Abmart) for 2 hours. After washing 3 times with NP40 buffer, FUS conjugates were eluted with FLAG peptide (Sigma-Aldrich) and resolved by 4%–20% Tris–glycine gradient gel (Bio-Rad). Coomassie-stained protein bands were excised and processed for in-gel digestion with trypsin and subsequent mass spectrometry by EttanTM MDLC system (GE healthcare) at Applied Protein Tech, Shanghai, China. The complete list of proteins identified was included in Supplementary Table 4, and the raw data sets were included in the Supplementary Materials (FUS raw data). For data analysis, tandem mass spectrometry spectra were automatically searched against the International Protein Index Human sequence database v3.53 from the European Bioinformatics Institute (http://www.ebi.ac.uk/) using the Bioworks Browser (Thermo Electron, San Jose, CA). Protein identification results were extracted from SEQUEST out files with Build Summary. The relative abundance of detected protein was determined by normalizing the peptide counts of each protein with those of affinity-purified FUS protein. For proteins bound to both the wild-type and P525 L FUS (common proteins), the ratio of relative abundance was calculated to assess the change in affinity, and was listed in Tables 1–3.

2.4. Immunoblotting and immunoprecipitation

The procedures for Western blotting and immunoprecipitation were described previously (Zhong et al., 2006). For coimmunoprecipitation, a 10-cm plate of transfected 293T cells or a cortex hemisphere of adult mouse was lysed in nondenaturing NP-40 buffer (50 mM Tris, pH 8.0; 150 mM NaCl; 1% NP-40) with protease inhibitors cocktail (Roche). Then, 1-mg extracts of cell lysates or 20 mg of brain lysates were incubated with primary antibodies or control IgG for 16 hours at 4 °C with gentle inversion mixing. Subsequently, protein A/G sepharose beads (A10001; Abmart) were added and incubated for 2 hours. The beads were collected and washed with lysis buffer, and the immunoprecipitated proteins were eluted either by 1× sodium dodecyl sulfate sample buffer or by peptides followed by sodium dodecyl sulfate polyacrylamide gel electrophoresis analysis.

2.5. ATP level determination

HEK293 T cells seeded in 6-well plates were transfected with WT or P525 L FUS. Then, 48 hours later, cells were collected and harvested

Table 1
FUS-interacting proteins associated with neurodegenerative diseases

Protein name	Gene symbol	Related diseases	P525 L/WT[a]
Isoform 1 of ataxin-2-like protein	ATXN2L	Spinocerebellar ataxia	P525 L unique
Transitional endoplasmic reticulum ATPase	VCP	FTD, ALS	3
Isoform long of splicing factor, proline- and glutamine-rich	SFPQ	FTD, AD, PD	2
14-3-3 protein zeta/delta	YWHAZ	ALS	1.7
14-3-3 protein theta	YWHAQ	ALS, AD, FTD	1
Profilin-1	PFN1	ALS	1

Key: AD, Alzheimer disease; ALS, amyotrophic lateral sclerosis; FTD, frontotemporal dementia; FUS, fused-in sarcoma; PD, Parkinson disease; SFPQ, Splicing factor, proline- and glutamine-rich; VCP, valocin-containing protein; WT, wild type.
[a] The numbers in the column of *P525 L/WT* represent the ratio of relative abundance of the protein (as determined by normalized peptide counts) in P525 L and WT FUS interactome.