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Genome wide array analysis indicates that an amyotrophic lateral sclerosis mutation of FUS causes an early increase of CAMK2N2 *in vitro*

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ABSTRACT

Mutations in the RNA binding protein FUS (fused in sarcoma) have been linked to a subset of familial amyotrophic lateral sclerosis (ALS) cases. The mutations are clustered in the C-terminal nuclear localization sequence (NLS). Various FUS mutants accumulate in the cytoplasm whereas wild-type (WT) FUS is mainly nuclear. Here we investigate the effect of one ALS causing mutant (FUS-ΔNLS, also known as R495X) on pre-mRNA splicing and RNA expression using genome wide exon-junction arrays. Using a non-neuronal stable cell line with inducible FUS expression, we detected early changes in RNA composition. In particular, mutant FUS-ΔNLS increased calcium/calmodulin-dependent protein kinase II inhibitor 2 (CAMK2N2) at both mRNA and protein levels, whereas WT-FUS had no effect. Chromatin immunoprecipitation experiments showed that FUS-ΔNLS accumulated at the CAMK2N2 promoter region, whereas promoter occupation by WT-FUS remained constant. Given the loss of FUS-ΔNLS in the nucleus through the mutation-induced translocation, this increase of promoter occupancy is surprising. It indicates that, despite the obvious cytoplasmic accumulation, FUS-ΔNLS can act through a nuclear gain of function mechanism.

1. Introduction

Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disease characterized by the progressive loss of motor neurons in the central nervous system. Recent studies found that mutations in the RNA binding protein FUS (fused in sarcoma) can cause a subset of hereditary forms of ALS in a dominant fashion [1,2]. It has been largely unknown how FUS mutations cause ALS.

FUS, also known as TLS (translocated in sarcoma) and as hnRNP P2, is a nucleic acid binding protein that is mainly nuclear, but can shuttle between the cytosol and the nucleus. FUS has been implicated in multiple steps of mRNA metabolism, including transcriptional regulation, regulation of RNA processing, and RNA export [3–7]. FUS contains several domains that can bind to nucleic acids including an RNA recognition motif that binds both to DNA and RNA [8], which partially explains the multiple roles in gene expression.

Genomic translocation affecting FUS can result in cancer, especially liposarcomas [9,10]. In these genomic rearrangements, the promoter and N-terminal part of FUS is translocated to the C-terminal domain of various DNA-binding transcription factors, resulting in a fusion protein with a strong transcriptional activation domain (reviewed in [11]). This indicates a role of FUS in gene transcription.

The familial ALS related mutations are clustered in the C-terminal region, which was later determined as a nuclear localization sequence (NLS) [12–14]. Mutations in the NLS decrease the nuclear import and cause cytoplasmic accumulation of the mutant FUS protein. In particular, the FUS-ΔNLS mutation lacks the NLS and causes a relatively aggressive ALS clinical phenotype with an early onset [14]. It has been proposed that the loss of a nuclear function or the gain of a cytosolic function or both can be the possible cause for the disease [15–17].

Recent studies have reported thousands of RNA targets that can bind to FUS [18–20]. The structure of the RNA recognition motif of FUS shows a non-canonical nucleic acid binding site and supports the relatively low specificity binding of RNAs to FUS [8]. To gain insight into how FUS mutations may interfere with mRNA transcription and processing, we performed genome wide exon junction array studies using stably transfected HEK293 cells with inducible FUS expression. We specifically concentrated on early changes in gene expression. Among more than 100 genes showing small changes after 24 h of FUS expression, calcium/calmodulin-dependent protein kinase II inhibitor 2 (CAMK2N2) showed the most striking difference between wild type and mutant FUS. The change of CAMK2N2 was

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validated at both RNA and protein levels. Furthermore, chromatin immunoprecipitation experiments showed that FUS-ΔNLS accumulated at the CAMK2N2 promoter region, whereas promoter occupation by WT-FUS remained constant. The results from this study suggest that a nuclear gain of function could also be an underlying mechanism contributing to FUS mutation induced alterations.

2. Material and methods

2.1. Cell culture

HEK293 stable cells with inducible WT GFP-FUS and FUS-ΔNLS expression have been described before. They were created by FRT-mediated recombination into a single genomic locus. After antibiotic selection, the cells remained pooled to avoid clonal bias [14]. The cells were cultured in DMEM with 10% TET-tested fetal bovine serum (Atlanta Biologicals, s10350H), 2 mM L-glutamine (Gibco, 25030), 15 μg/ml blasticidin (Invitrogen, R210-01), 150 μg/ml hygromycin B (Invitrogen, 10687-010), and 1% penicillin and streptomycin solution. Doxycyline (Clontech, 1 μg/ml) was added to induce the expression of GFP-FUS 6, 24, or 48 h before harvest.

2.2. Array analysis

RNA was isolated using Qiagen kits. Its quality was determined by RNA integrity (RIN) number analysis and samples with a RIN > 9.5 were used following the Affymetrix labeling procedure.

For the analysis, the signal from Affymetrix human junction arrays (HJAY) was normalized using the "Probe scaling" method. The background was corrected with ProbeEffect from GeneBase [21]. The gene expression index was computed from probes that were selected using ProbeSelect from GeneBase [21]. The gene expression signals were computed using these probes. Genes were considered expressed if the mean intensity was ≥500. Genes were considered regulated if 1) they were expressed in at least one condition (*i.e.*, VPA and/or control); 2) the fold-change was greater or equal than 1.5 and; 3) the unpaired *t*-test p-value between gene intensities was ≤0.05. For each probe, a splicing-index was computed. Unpaired t-tests were performed to determine the difference in probe expression between the two samples as described previously [22]. Probe p-values in each probeset were then summarized using Fisher's method. Using annotation files, splicing patterns (cassette exons, 5′/3′ alternative splice sites and mutually exclusive exons) were tested for a difference between isoforms, selecting the ones with a minimum number of regulated probesets (with a p-value ≤ 0.01) in each competing isoform (at least one third of "exclusion" probesets have to be significant; at least one third of "inclusion" probesets have to be significant and show an opposite regulation for the splicing-index compared to the "exclusion" probesets). For example, for a single cassette exon, the exclusion junction and at least one of the three inclusion probesets (one exon probeset and two inclusion junction probesets) have to be significant and have to show an opposite regulation for the splicing-index.

2.3. RT-PCR

Total RNA from HEK293 cells was extracted using the GenElute Mammalian Total RNA Miniprep Kit (Sigma) according to the manufacturer's instructions. cDNAs were synthesized from 1 μg of each RNA using SuperScript® III Reverse Transcriptase (Life Technologies). Reverse transcriptase-polymerase chain reaction (RT-PCR) experiments were carried out using 1 μl of each cDNA as template and specific primers. Products were visualized on gel electrophoresis after ethidium bromide staining. PCR primers were as follows:

β-ACTIN_FOR: AGAGCTACGAGCTGCCTGAC; β-ACTIN_REV: GGA TGTCCACGTCACACTTC; F_CAMK2N2: ATGTCCGAGATCCTGCCCTA: R_CAMK2N2: ACGTCGTCTATCCGGTCATC; CAMK2N2_cFOR2: ACA CAGCGAAAGAGATACGG; CAMK2N2_cREV: AGCAGGAGGGCAAAA GCT

2.4. Chromatin IP

HEK293 cells were lysed and sheared by sonication in a 0.1% NP-40/PBS (Sigma) lysis buffer to generate cellular chromatin fragments of 400–500 bp. The chromatin was immunoprecipitated for 14–16 h at 4 °C using antibodies against GFP coupled to sepharose (GFP-Trap_A, Chromotek). After the incubation, chromatin immunoprecipitates were purified, then 2 μl of each sample were analyzed by real-time PCR. The real-time PCR was carried out in the Stratagene Mx3005P (Agilent Technologies), using SYBR green reagent (Life Technologies). The relative expression was estimated as follows: $2^{C_t(\text{reference}) - C_t(\text{sample})}$, where C_t (reference) and C_t (sample) were input DNA and specific histone modification chromatin, respectively. For each experiment, at least three immunoprecipitations were analyzed.

2.5. Antisera

Mouse monoclonal anti-FUS antibody (sc-47711) and goat polyclonal anti-β-actin (sc-459) were purchased from Santa Cruz Biotechnology. The CAMK2N2 antibody (N-term) was a peptide affinity purified rabbit polyclonal antibody (Pab) (AP11140a) from Abgent.

2.6. Transfection

HEK293 cells were seeded in 6 well plates, and were transfected with pEGFP-C3 expression plasmids containing the wild type or ΔNLS FUS cDNA using lipofectamine (Invitrogen) according to the manufacturer's instructions [13].

2.7. Quantification and statistical analysis

Quantification and statistical analysis were performed using Quantity One® 1-D Analysis Software (Biorad). Results are presented as means ± S.D. Differences between means in two groups were compared using the Student's *t* test. A 5% or 1% significance level and 95% or 99%, respectively, confidence interval were used in the statistical analysis.

3. Results

3.1. Characterization of stable cell lines with inducible expression of FUS

To determine the molecular role of WT-FUS as well as FUS-ΔNLS, we chose stable HEK293 cell lines with Tet-On inducible expression of either human WT-FUS or FUS-ΔNLS (Fig. 1A). Both proteins were GFP-tagged to allow discrimination from endogenous FUS that is expressed in all cells [14]. After 6 h of induction, the expression of both WT and FUS-ΔNLS started becoming evident (Fig. 1B). The exogenous GFP-FUS expression was comparable to the endogenous FUS expression in the cells.

The FUS-ΔNLS variant was detected predominantly in the cytoplasm in familial ALS patient tissues. This feature is reflected by the cytosolic accumulation of GFP tagged FUS variants in our cell culture system (Fig. 1C). In contrast, WT-FUS is mainly nuclear. We noted that GFP tagged FUS-ΔNLS still showed detectable nuclear staining when it was expressed at the relatively low levels in these cells. This inducible cell culture system offers comparable WT and mutant FUS expression levels, which likely mimics the situation in human ALS patients.

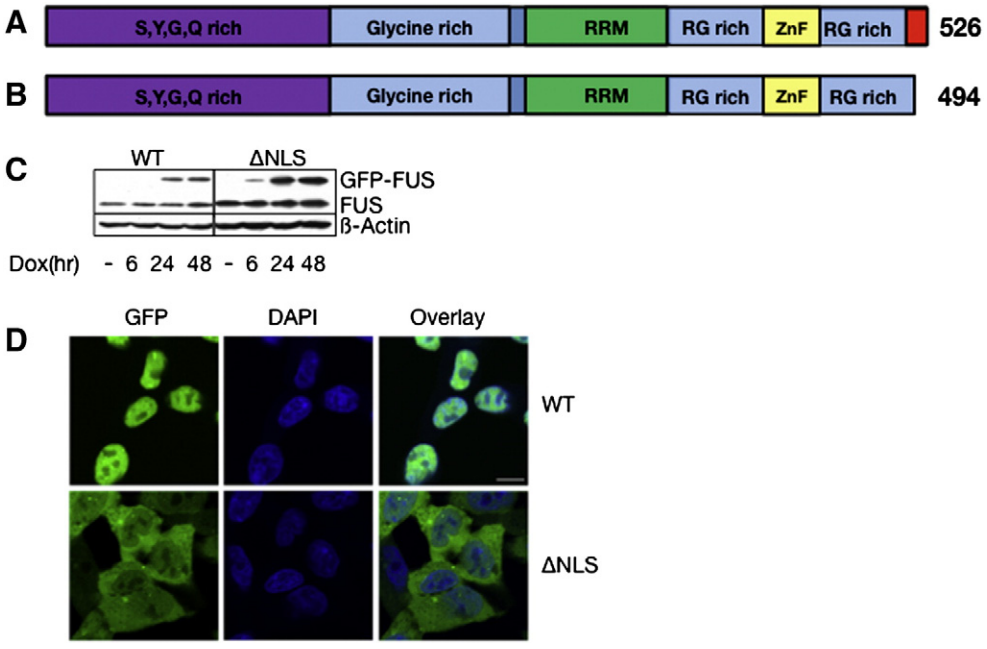

Fig. 1. Expression of GFP-FUS and GFP FUS-ΔNLS in stable cell lines. A. Domain structure of FUS and FUS-ΔNLS. B. Protein expression after FUS and FUS-ΔNLS induction. HEK293 cells were stably transfected with GFP-FUS and GFP-FUS-ΔNLS expression constructs. The cells were induced with doxycycline for the times indicated. Western blots were performed with an anti FUS antiserum and an anti-actin serum as a loading control. C. Intracellular localization of the FUS variants. The detection of the overexpressed proteins was through their GFP tag. The cells were counterstained with DAPI. The size bar is 10 μm.

3.2. Genome-wide exon junction array analysis

FUS is part of the hnRNP complex and can bind both to single and double stranded DNAs, as well as to RNA [11]. Since a significant amount of FUS-ΔNLS leaves the nucleus and accumulates in the cytosol, we hypothesized that the mutation will cause a change in gene expression. We therefore analyzed changes in mRNA expression using Affymetrix genome wide exon junction arrays. In addition to detecting changes in alternative splicing, exon junction arrays are a robust tool to monitor overall gene expression, as each mRNA is detected by multiple probes located in all mRNA regions. To identify only early changes, we compared 24 h of induction, when both WT-FUS and FUS-ΔNLS started to express. We concentrated on early changes to avoid detection of secondary effects that are not directly caused by FUS-ΔNLS overexpression. To minimize effects caused by different growth conditions, all cells were seeded at the same sub-confluent density prior to doxycyclin induction.

Three datasets were generated for 24 h of induction. We measured gene expression in cells expressing GFP, WT-FUS and FUS-ΔNLS. These expression levels where then compared and changes larger than 1.5 fold with a p-value smaller than 0.05 were compared (Fig. 2). Comparing GFP overexpression with wild type indicated five changes (Supplementary Table 1). This low number reflects that a modest increase of WT-FUS expression (approximately two fold since the GFP-FUS is comparable to the endogenous FUS, Fig. 1B) has no strong effect at the timeframe of the experiment. Comparing FUS-ΔNLS with GFP indicated 156 changes (Supplementary Table 3), which suggested that FUS-ΔNLS had a slightly stronger influence on gene expression. Finally, comparing FUS-ΔNLS with FUS-WT showed 43 changes (Supplementary Table 2). This comparison resembles most strongly the situation in humans, where the mutant is expressed together with WT-FUS. There are 23 changes common to the datasets when overexpression of FUS-ΔNLS is compared to WT-FUS and uninduced cells, respectively (Fig. 2).

An analysis of KEGG pathways did not indicate pathways that are changed in a statistically significant pathway ($p < 10^{-3}$). The arrays indicated only one change in alternative splicing with high confidence in the comparison of FUS-ΔNLS with GFP, which could however not be validated by RT-PCR (Supplementary Table 3, Pathway analysis tab).

Together, these data suggest that induction of FUS-ΔNLS has a stronger effect on gene expression than expressing WT-FUS. The low number of changes suggests that we monitor only early, direct changes in FUS-ΔNLS dependent modification in gene expression.

3.3. Differential expression of CAMK2N2

We next validated the effects of WT-FUS and FUS-ΔNLS overexpression on gene expression using RT-PCR with primers indicated in Fig. 3D. We concentrated on the gene with the most pronounced consistent differences during RT-PCR validations, which was CAMK2N2 (calcium/calmodulin-dependent protein kinase II inhibitor 2). CAMK2N2 inhibits calcium/calmodulin-dependent protein kinase II, a major signaling molecule in the brain. It also interacts with the ARHGAP32 protein, also called RICS for Rho GTPase activating protein 32 [23]. RICS promotes GTP hydrolysis on RHOA, CDC42 and RAC1 small GTPases. As shown in Fig. 3, WT-FUS down-regulated CAMK2N2 whereas FUS-ΔNLS up-regulated the CAMK2N2 mRNA levels after 6, 24 and 48 h of FUS expression. These differences between WT and mutant FUS expressing cells were statistically significant (Fig. 3C), when normalized to the actin signal that was not influenced by FUS expression.

CAMK2N2 changes were highly reproducible in more than four different experiments. Other changes shown in Supplementary Table 1 varied between independent experiments and we did not observe statistically significant differences. These variations between experiments likely reflect the early time points of the analysis that were chosen to detect the early changes caused by the mutation. We therefore identified a change in a mRNA encoding a regulatory protein as a response to the expression of an ALS-causing variant of FUS.

3.4. FUS-ΔNLS upregulates CAMK2N2 protein

To determine whether the changes on the RNA level are reflected on the protein level, we analyzed CAMK2N2 protein levels after

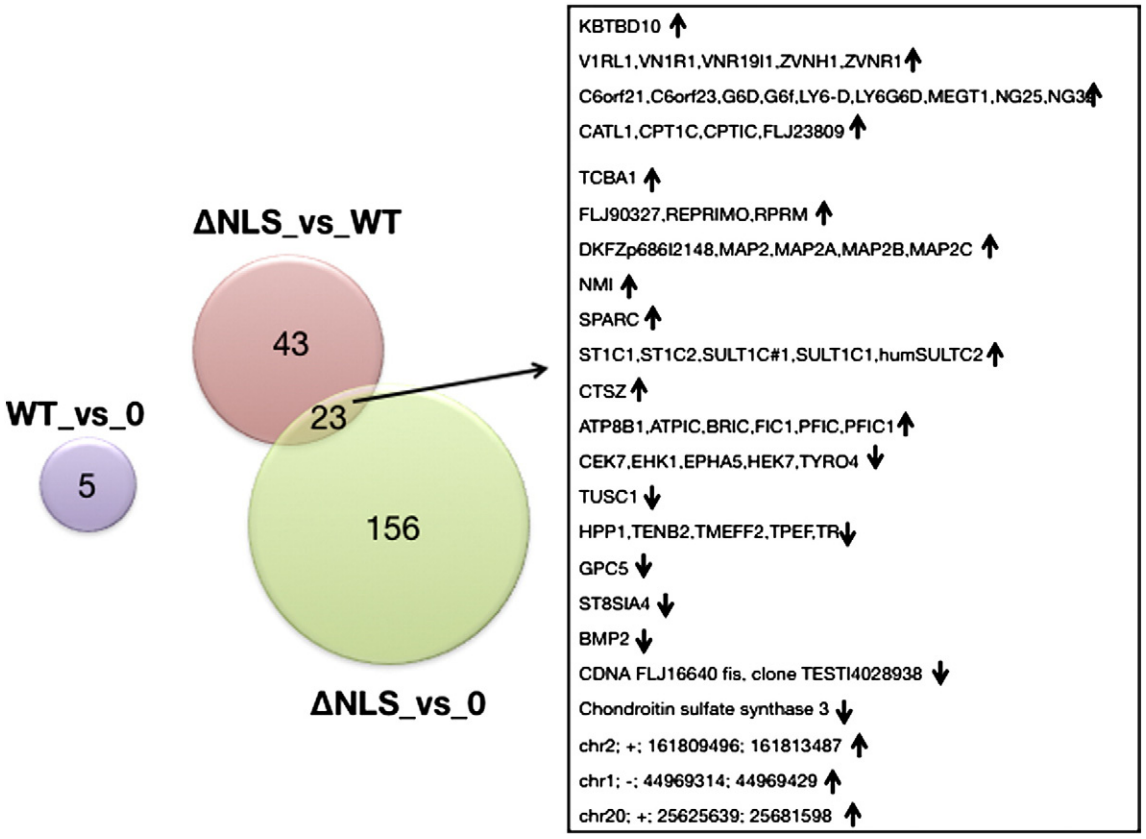

Fig. 2. Summary of the array analysis. The Venn diagram shows pathways most affected by 24 h WT-FUS and FUS-ΔNLS overexpression. The changes of the arrays are shown in Supplementary Tables 1–3.

WT-FUS and FUS-ΔNLS inductions. As shown in Fig. 4, after induction of WT-FUS, CAMK2N2 levels show no statistically significant changes. In contrast, when FUS-ΔNLS is overexpressed, we observe an about 2.5 fold induction on the protein level that reflects the increase on the mRNA level.

3.5. FUS-ΔNLS upregulates CAMK2N2 in transient transfections

To rule out the selection of specific clones when making stable cell lines, we tested the effect for FUS-ΔNLS on CAMK2N2 in transient transfection assays. As shown in Fig. 5A, similar to the situation in stable cell lines, WT-FUS had no effect on CAMK2N2 mRNA levels. In contrast, we observed a steady increase of CAMK2N2 after transfecting FUS-ΔNLS (Fig. 5B). After 48 h, CAMK2N2 is upregulated about 2.5 fold, similar to the effect observed in stable cell lines.

We next determined the effect of FUS variants on the protein level in transient transfections and observed an about two fold increase of CAMK2N2 with FUS-ΔNLS, but no statistically significant increase with the wild type (Fig. 6A–C). The data suggest that FUS-ΔNLS increases both mRNA and protein levels of CAMK2N2, both in stable and transient transfections.

3.6. FUS-ΔNLS binds stronger to the CAMK2N2 promoter than wild-type FUS

FUS has been shown to bind to DNA and to activate transcription [3–5]. We therefore measured the binding of WT-FUS and FUS-ΔNLS to the CAMK2N2 promoter. To compare WT and mutant FUS proteins, we immunoprecipitated them using the GFP tag from the stable cell lines. The cells were analyzed at 6 and 48 h, to capture the early and strongest changes on the mRNA and protein levels. The levels of CAMK2N2 promoter DNA bound to the immunoprecipitates were determined using real-time PCR. As shown in Fig. 7, a 3.5 fold increase of FUS-ΔNLS binding to the CAMK2N2 promoter was observed at 48 h as compared to WT-FUS. Interestingly, there is a slight decrease after 6 h that precedes the strong increase, suggesting rearrangements at the promoter. In contrast, WT-FUS bound to the promoter remains constant. The data suggest that FUS-ΔNLS accumulates on the CAMK2N2 promoter, which likely causes its induction.

4. Discussion

FUS-ΔNLS has been identified as a rare mutation that causes ALS. To gain insight into the molecular mechanism of this mutation, we identified early changes in gene expression caused by FUS-ΔNLS expression. This resembles the disease situation, where FUS-ΔNLS is expressed in addition to WT-FUS. However, in the human system one WT allele is substituted by a mutated allele. Our overexpression system differs from other studies where WT-FUS was knocked out, which resulted in multiple changes in pre-mRNA processing and gene expression [19,20].

We detected only a few changes in gene expression that are small. This is expected, as the disease has an onset of several years and we concentrated only on early changes in gene expression to identify directly regulated genes.

We did not find any changes in pre-mRNA splicing, despite the known involvement of FUS in this process [18]. This is likely due to the early time point of analysis and the fact that we overexpressed FUS, which did not drastically change the total amount of WT-FUS.

We found that the FUS-ΔNLS mutant causes an about two-fold increase in CAMK2N2 mRNA and about 3.5 fold increase in protein levels in non-neuronal cells. This was the only change that could be validated in several independent RT-PCR experiments. It therefore validated CAMK2N2 as this first direct target of FUS-ΔNLS.

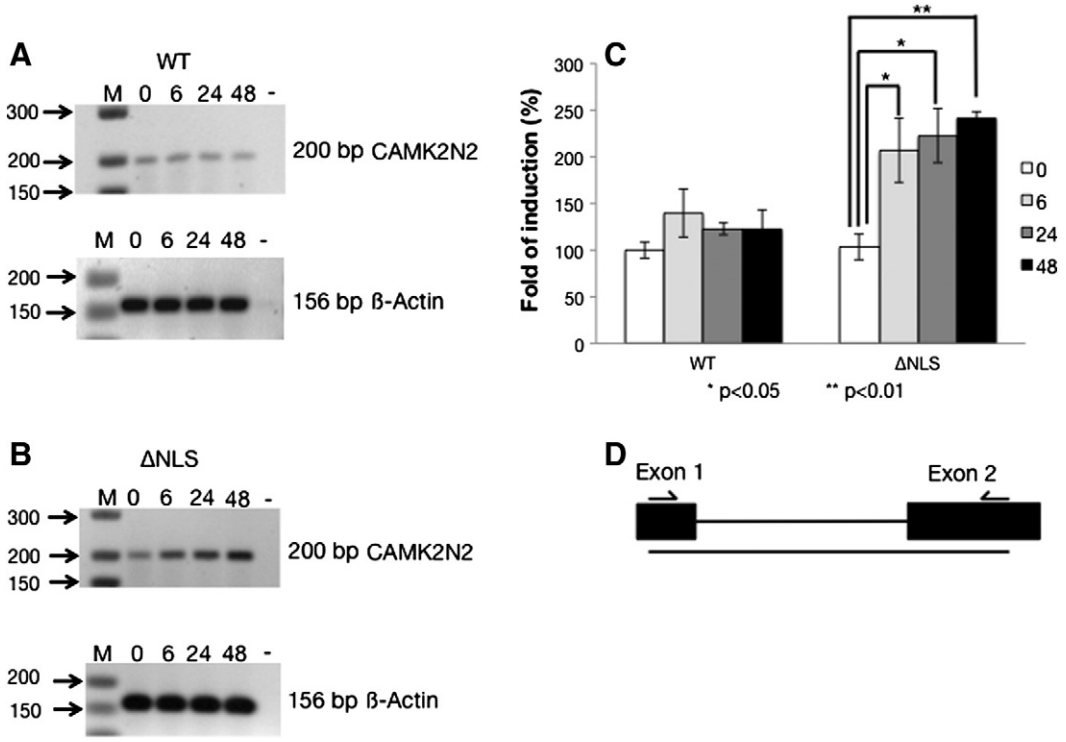

Fig. 3. The CAMK2N2 gene is changed after FUS-ΔNLS expression. A. RT-PCR detecting CAMK2N2 expression after WT-FUS was induced at the times indicated. B. RT-PCR detecting CAMK2N2 expression after FUS-ΔNLS was induced at the times indicated. C. Quantification of the CAMK2N2 signal after normalization to beta actin. D. Localization of the primer for detection. Primers were F_CAMK2N2 and R_CAMK2N2.

A deregulation of CAMK2N2 will have consequences for the cell. CAMK2N2 is an inhibitor of calmodulin dependent kinase 2 (CaMKII) that arrests calmodulin on the kinase, which inhibits it. CAMK2N2 also physically interacts with the Rho GTPase activating protein 32 (RICS), a brain specific regulator for Rho-GAP signaling [23]. Both CAMK2N2 and RICS are involved in signal transduction in the brain. One mode of action for the FUS-ΔNLS variant is the disruption of signaling networks, which could contribute to the disease *in vivo*. CaMKII is known to phosphorylate AMPA-type glutamate receptors, which are implicated in ALS [24,25]. It is therefore possible that CaMKII-dependent changes in the phosphorylation of AMPA receptors contribute to ALS.

Mechanistically, the change in CAMK2N2 expression is caused by the binding of FUS-ΔNLS protein to the CAMK2N2 promoter. Since WT-FUS binds to the promoter, it is likely that the mutant substitutes endogenous WT-FUS and accumulates over time, which changes the transcription. The molecular reason for this accumulation is unclear, but it could involve an increase of binding to single stranded DNA in

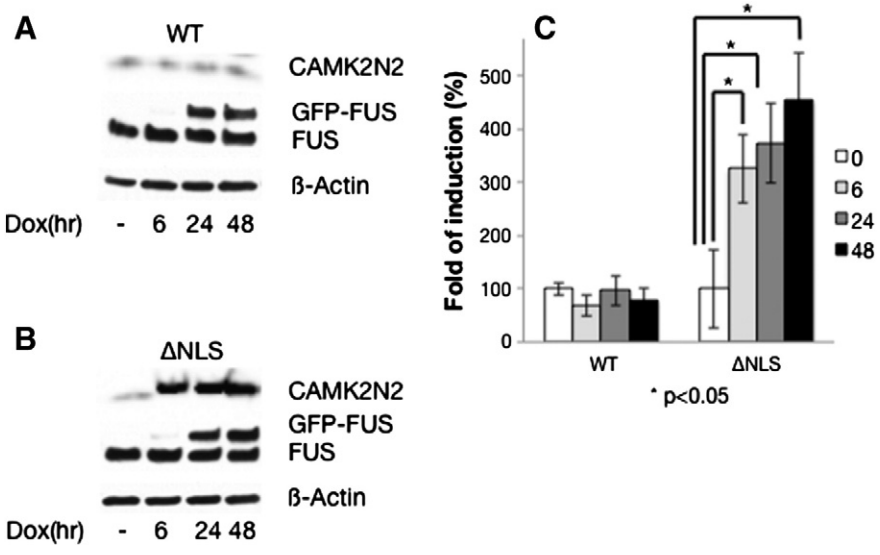

Fig. 4. FUS-ΔNLS upregulates CAMK2N2 protein levels. A. After WT-FUS induction, CAMK2N2 expression was detected by Western blot in doxycycline induced cell lines for the times indicated. B. After FUS-ΔNLS induction, CAMK2N2 expression was detected by Western blot in doxycycline induced cell lines for the times indicated. C. The quantification of the data is shown on the right.

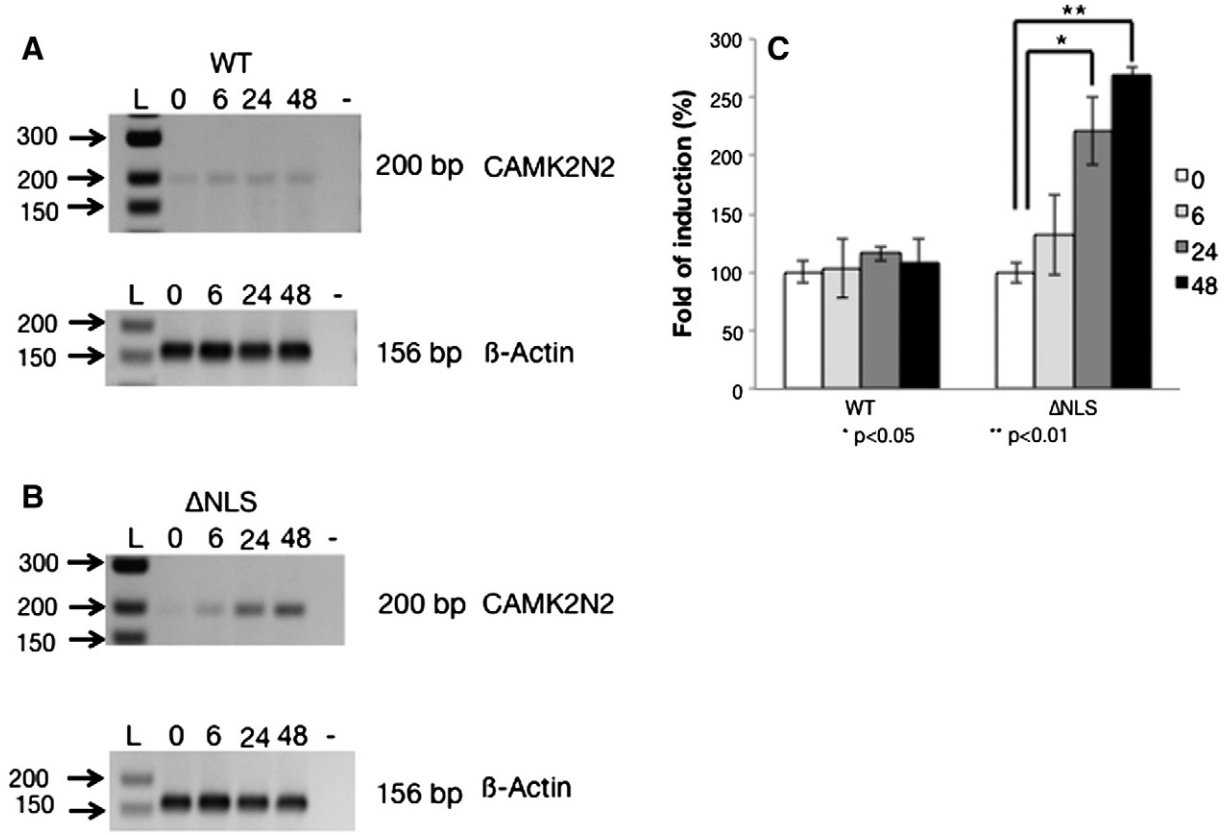

Fig. 5. The CAMK2N2 gene is changed after FUS-ΔNLS transient expression. A. RT-PCR detecting CAMK2N2 expression after WT-FUS was transfected at the times indicated. 2 μg of expression plasmid per 300,000 cells were used. B. RT-PCR detecting CAMK2N2 expression after FUS-ΔNLS was transfected at the times indicated. 2 μg of expression plasmid per 300,000 cells were used. C. Quantification of the CAMK2N2 signal after normalization to beta actin.

the open promoter complex that is aided by proteins that bind to the FUS-ΔNLS mutant stronger than the wild type. Therefore, despite the cytosolic accumulation of FUS-ΔNLS, the protein could act through a nuclear gain of function mechanism.

Together the data indicate that mutant FUS could change the expression of a regulatory protein CAMK2N2 by deregulation of its promoter and that such changes could be involved in the etiology of ALS.

Array analysis of HEK293 cells stably transfected with constructs expressing GFP, WT-FUS type and FUS-ΔNLS. Supplementary data related to this article can be found online at http://dx.doi.org/10.1016/j.bbadis.2013.03.015

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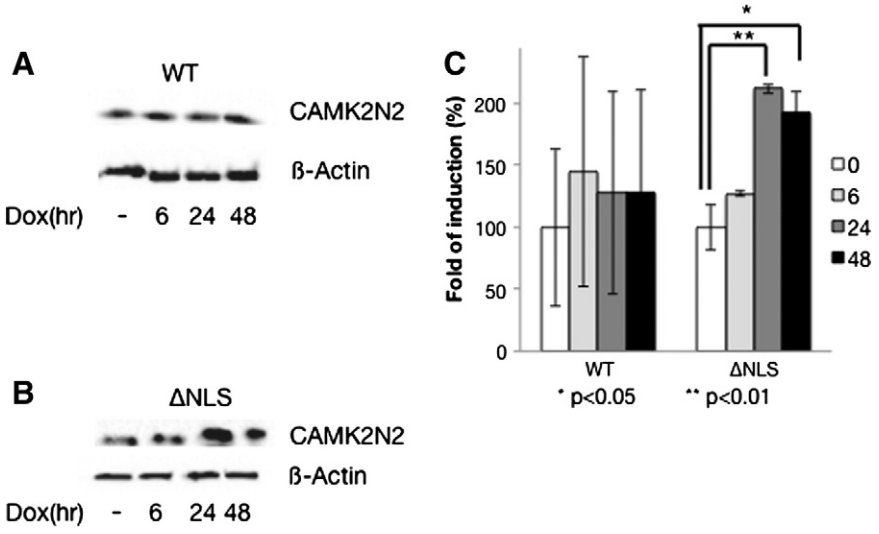

Fig. 6. FUS-ΔNLS upregulates CAMK2N2 protein levels after transient transfections. A. After WT-FUS transfection, CAMK2N2 expression was detected by Western blot at the times indicated. B. After FUS-ΔNLS transfection, CAMK2N2 expression was detected by Western blot at the times indicated. C. The quantification of the data is shown on the right.

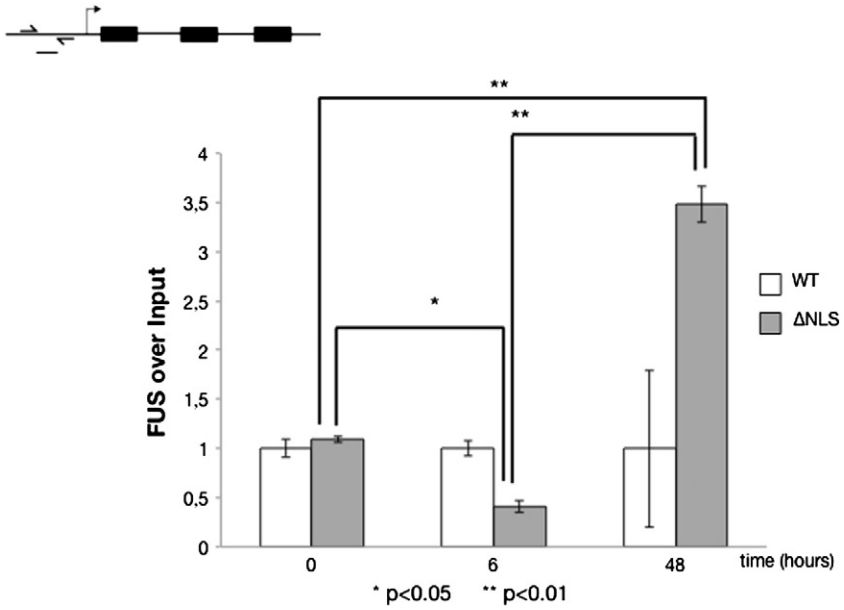

Fig. 7. Chromatin immunoprecipitations of CAMK2N2 DNA with FUS and FUS-ΔNLS. The stable cell lines were induced for 6 and 48 h and GFP-tagged FUS-wild type and FUS-ΔNLS were immunoprecipitated using anti-GFP. Chromatin bound to the immunoprecipitates was detected by PCR using the primers located in the promoter regions, as indicated in the cartoon on top. The graph represents three independent experiments. The expression level at time zero was set to one. The differences for FUS-ΔNLS are significant, $p < 0.05$ (*) and $p < 0.01$ (**), as indicated.

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