Association of UBQLN1 mutation with Brown–Vialetto–Van Laere syndrome but not typical ALS[☆],[☆☆]

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ABSTRACT

Genetic variants in UBQLN1 gene have been linked to neurodegeneration and mutations in UBQLN2 have recently been identified as a rare cause of amyotrophic lateral sclerosis (ALS).

Objective: To test if genetic variants in UBQLN1 are involved in ALS.

Methods: 102 and 94 unrelated patients with familial and sporadic forms of ALS were screened for UBQLN1 gene mutations. Single nucleotide variants were further screened in a larger set of sporadic ALS (SALS) patients and unrelated control subjects using high-throughput Taqman genotyping; variants were further assessed for novelty using the 1000Genomes and NHLBI databases. *In vitro* studies tested the effect of UBQLN1 variants on the ubiquitin–proteasome system (UPS).

Results: Only two UBQLN1 coding variants were detected in the familial and sporadic ALS DNA set; one, the missense mutation p.E54D, was identified in a single patient with atypical motor neuron disease consistent with Brown–Vialetto–Van Laere syndrome (BVVLS), for whom c20orf54 mutations had been excluded. Functional studies revealed that UBQLN1[E54D] protein forms cytosolic aggregates that contain mislocalized TDP-43 and impairs degradation of ubiquitinated proteins through the proteasome.

Conclusions: Genetic variants in UBQLN1 are not commonly associated with ALS. A novel UBQLN1 mutation (E45D) detected in a patient with BVVLS altered nuclear TDP-43 localization *in vitro*, suggesting that UPS dysfunction may also underlie the pathogenesis of this condition.

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Introduction

Amyotrophic lateral sclerosis (ALS) is the most common adult-onset degenerative motor neuron disease (MND). The incidence of ALS is 1–2 per 100,000, the prevalence is 4–6 per 100,000 and the lifetime ALS risk is approximately 1/600 (McGuire et al., 1996; Mitsumoto et al., 1998). While most ALS cases are sporadic (SALS), approximately 10% are familial (FALS). The most common genetic cause in FALS is the recently defined expansion of a hexanucleotide repeat in the non-coding region of C9orf72 and mutations in the gene encoding cytosolic superoxide dismutase 1 (SOD1), which together may account for at least 50% of FALS cases (Dejesús-Hernández et al., 2011; Renton et al., 2011; Rosen et al., 1993). Mutations in TAR DNA binding protein (TARDBP) and fused-in-sarcoma/translated-in-liposarcoma (FUS/TLS) genes each account for about 5% of FALS cases (Kabashi et al., 2008; Kwiatkowski et al., 2009; Sreedharan et al., 2008; Vance et al., 2009). Less frequently, mutations in genes encoding vesicle-associated membrane protein-associated protein B (VAPB), senataxin (SETX), dynactin (DCTN1), alsin (ALS2), angionenin (ANG), optineurin (OPTN), valosin-containing protein (VCP) are predicted to cause FALS (Chen et al., 2004; Greenway et al., 2006; Johnson et al., 2010a; Maruyama et al., 2010; Münch et al., 2005; Nishimura et al., 2004; Yang et al., 2001). Recently, some cases of X-linked ALS have been ascribed to mutations in UBQLN2 (Deng et al., 2011).

Genetic variants in ubiquilin 1 (UBQLN1) have been linked to neurodegeneration. Two family-based cohorts showed a positive association

between an intronic variant (UBQ-8i) and Alzheimer disease, although this association was not consistently confirmed (Bensemain et al., 2006; Bertram et al., 2005, 2007; Brouwers et al., 2006; Slifer et al., 2005; Smemo et al., 2006). The plausibility of this association was reinforced by the observation that UBQLN1 interacts with polyubiquitinated TDP-43 protein through its ubiquitin-associated (UBA) domain and regulates the transport of TDP-43 to both the proteasome and the autophagy systems through a ubiquitin-like (UBL) domain (Heir et al., 2006; Kim et al., 2009; Ko et al., 2004). During our study, mutations in UBQLN2 were identified in 2% of dominant X-linked ALS families and ubiquilin pathology has been observed on autopsy-confirmed ALS without mutations in this gene (Brettschneider et al., 2012; Deng et al., 2011), which reinforces our interest in UBQLN1 as a candidate ALS gene. Here, we report our mutational analysis of the UBQLN1 gene in ALS.

Subjects and methods

The study was approved by the local ethics committee; participants provided written informed consent. The diagnosis of ALS was based on the revised El Escorial criteria including patients affected with possible, probable or definite ALS (Brooks et al., 2000). SOD1, TARDBP and FUS/TLS mutations were ruled out in all cases; hexanucleotide expansion in the c9orf72 gene was ruled out in ALS patients identified as carriers of UBQLN1 variants.

Genotyping analysis

Three single nucleotide polymorphisms (SNPs) in the UBQLN1 gene (rs2780995, rs 2781002, rs12344615 [UBQ-8i]) were tested for association with risk for ALS in more than 900 SALS cases and healthy controls.

Sequence analysis

102 and 94 unrelated patients with FALS and SALS, respectively, were screened for UBQLN1 gene mutations. Whole genome amplification was performed on blood DNA (Illustra Genomiphi V2 DNA Amplification kit; GE HealthCare cat. #25-6600-31). UBQLN1 exons 1 through 11, and exon–intron junctions, were amplified by PCR (primers available on request). AmpliTaq Gold PCR Master Mix 2500U (Applied Biosystems cat. #4327059) was used for touchdown PCR. The high GC content of exon1 (70%) required addition of 3 μl of a 10X Combinatorial Enhancer Solution (10X CES) with betaine 0.54 M betaine, 1.34 mM DTT, 1.34% DMSO, and 11 μg/mL BSA in 30 μl PCR reaction volume.

DNA samples were resolved by capillary electrophoresis on an ABI 3730XL DNA Analyzer.

High-throughput SNP genotyping was performed using TaqMan assay for each novel variant in a larger set of SALS patients and unrelated control subjects.

Analysis of alternative splicing

Total lymphoblast RNA was extracted and reverse transcribed to complementary DNA (cDNA) using oligodT primers, according to the manufacturer's instructions (Invitrogen cat #74104, 18080–051). cDNA amplification was carried out with two previously reported specific PCR primers that annealed to exon 7 and exon 9 (Bertram et al., 2005). Two transcripts were extracted from agarose gel (Qiagen cat #28706) and directly sequenced to confirm their specificity for UBQLN1 sequence.

Cell culture and transfection

HEK293T, Hela-CCL2 and FUS-GFP stable cell lines were cultured in Dulbecco's Modified Eagle Medium (DMEM; Sigma, Poole, UK) supplemented with 10% fetal calf serum (Sigma) at 5% CO_2, 37 °C (Bosco et al., 2010). The cDNA of human UBQLN1 was cloned into the PCR-Blunt vector (Invitrogen K2700-20). Site-directed mutagenesis was performed to create the mutant constructs (Stratagene cat #200522-5). Subsequently the wild-type (wt) and mutant UBQLN1 cDNAs were cloned into MoPrP. Xho was inserted into the pBluescript vector (Stratagene cat #212205) at the Xho site (gift from Dr. David Borchelt). Flag-tagged UBQLN1 mutant constructs were derived from pCS2-Flag-UBQLN1 (Addgene, Plasmid 8663). Various plasmids were transiently transfected into HEK293T or Hela-CCL2 cells using Lipofectamin2000 (Invitrogen) following manufacturer's instructions.

Fly strains

Flies were raised on standard food medium and kept at 25 °C. To generate UAS-hUBQLN1, UAS-hUBQLN1-S503C, UAS-hUBQLN1-E54D, UAS-HA-hUBQLN1, UAS-HA-hUBQLN1-S503C and UAS-HA-hUBQLN1-E54D constructs, corresponding DNA fragments were synthesized and cloned into the pUAST vector (GenScript, Inc). These constructs were confirmed by DNA sequencing and microinjected into wildtype (w^{1118}) Drosophila embryos to generate transgenic lines (Rainbow Transgenic Flies, Inc.). Individual transgenic lines were crossed with GMR-Gal4 or other Gal4 drivers for expression and phenotypic analyses.

Western blots and immunoprecipitation

Levels of wildtype (wt) and mutant UBQLN1 and TDP-43 proteins in transfected HEK293T or HeLa-CCL2 cells were determined using western immunoblotting according to standard methods (Lu et al., 2009a). For fly protein expression analysis, adult flies were frozen with dry ice and vortexed to remove their heads. Approximately 20 heads from each genotype were homogenized in cold lysis buffer (50 mM Tris–HCl, pH 7.5, 150 Mm sodium chloride, 1% Nonidet P40, 0.5% sodium deoxycholate, 1 tablet complete Mini protein inhibitor cocktail/10 mL). Homogenates were centrifuged at 4 °C for 20 min at 13,000 rpm. Protein concentrations were determined using Bradford Assay (Bio-Rad).

Supernatants containing 5 μg of protein were mixed with 4X SDS sample buffer (1 M Tris–HCl, pH 6.8, 8% SDS, 40% glycerol, 0.1% bromophenol blue) and boiled for 5 min. These samples were then separated on a 10% polyacrylamide SDS gel and transferred to a PVDF membrane (Bio-Rad) in a wet transfer system at 4 °C for 60 min at 100 V. The membrane was incubated in the blocking solution TBST (25 mM Tris–HCl, 137 mM NaCl, 3 mM KCl, pH 7.4, and 0.1% Tween-20) containing 5% milk at room temperature for 1 hr. The membrane was then incubated with anti-dTDP-43 antibody (1:1000 in blocking solution) at room temperature for 3 hr or at 4 °C overnight, and finally with anti-rabbit HRP-conjugated secondary antibody (Jackson ImmunoResearch; 1:10,000) for 1 hr (Lu et al., 2009b). The signal was visualized with chemiluminescent substrate (Supersignal West Pico, Pierce). For other western blot analyses, the primary antibodies were anti-hTDP-43 antibody (1:2000; Proteintech, 10782-2-AP), anti-ubiquilin antibody (1:2000; Abcam, ab3341), mouse monoclonal anti-actin antibody (1:8,000, Sigma, A2228) and β-actin antibody (1:2000; Cell Signaling, 4967).

Immunostaining

Anti-UBQLN1 antibody (Abcam, ab3341 and ab96870), anti-EGFP antibody (Clontech, 632375) and anti-TDP-43 antibody (Proteintech, 10782-2-AP and 60019-2-Ig) were used as primary antibodies. Goat anti-rabbit Alexa Fluor 568 and goat anti-mouse Alexa Fluor 488 (Invitrogen) were used as secondary antibodies. Cells were washed three times in PBS and mounted with Vectashield (Vector Laboratories Ltd, Peterborough, UK). Immunostaining signals were examined with the IX-70 microscope or a Leica TCS-SP inverted scanning confocal microscope.

UPS reporter assay

HEK293T cell lines were grown in 6-well plates and co-transfected with an UPS-reporter vector encoding Ub^{G76V}-GFP (Addgene, 11941), and an expression vector encoding either human *UBQLN1*^{wt} or *UBQLN1*$^{E54D/S503C}$. Cells were harvested 36 hr after transfection and resuspended in PBS. The fluorescence intensities were measured by flow cytometry. All flow-cytometry data were collected and analysed using a MoFlo cell sorter and BD FACSCaliburs. Argon-ion (488 nm) laser was used for excitation. The GFP signals were collected using 530/540-nm band pass filter. In all experiments, data were gated on GFP labeled cells. At least 50,000 such events were recorded in each experiment. Data were collected from three independent experiments.

Statistic analyses

Pearson's chi-squared test, Fisher's exact test and two-tailed unpaired Student's *t*-test were performed considering $p < 0.05$ as significant value. Association analyses were based on allele-based comparisons of ALS cases vs. healthy controls using a chi-square statistic and computing allelic odds ratios and 95% confidence intervals.

Results

Genetic studies

No significant association was detected for any of the three *UBQLN1* SNPs previously associated with risk for Alzheimer disease (Supplementary Table 1). To further evaluate a role for *UBQLN1* in ALS, we sequenced all coding exons of *UBQLN1* in 102 FALS and 92 SALS cases. We identified four variants by direct sequencing: two missense SNPs in exon 1 (c.162 G>T, p.E54D) and exon 10 (c.1508 C>G, p.S503C), a silent SNP in exon 8 (c.1685 C>T, p.L442L), and a non-coding change located 24 bp upstream of exon 10 (i10:-24 A>T).

For three of these variants (E54D, S503C, L442L), we next performed a TaqMan assay in 1,474 SALS patients and 1,378 unrelated control subjects. The non-coding change (i10:-24 A>T) was detected by TaqMan analysis in one of the first 380 controls and was thus considered to be a benign polymorphism. Neither of the two missense variants was detected in our set of 1378 controls. The p.S503C variant was identified in an additional patient with definite SALS; it was not list in the 1000Genomes database (http://www.1000genomes.org/home) but was present in dbSNP (rs151070080) and in the NHLBI exome database (http://evs.gs.washington.edu/EVS/); we therefore conclude that this is a rare polymorphism. The p.E54D variant was not detected in the dbSNP, 1000Genomes or NHLBI databases and thus is potentially disease-specific (Table 1).

The E54D mutation was present in a heterozygous form in an Italian female patient diagnosed with an early-onset motor neuron disease characterized by bulbar palsy. There were no affected relatives or consanguinity in the family. After ruling out other disease processes that might explain the signs of motor neuron degeneration, this patient was diagnosed with *clinically possible ALS* according to El Escorial criteria (Brooks et al., 2000). Subsequent clinical examinations revealed the presence of sensorineuronal hearing loss that, together with the early onset of the motor neuron symptoms, suggested the diagnosis of Brown–Vialetto–Van Laere syndrome (BVVLS) (Mégarbané et al., 2000). We therefore excluded mutations in all five exons of the *c20orf54* gene, which reportedly cause some (but not all) cases of BVVLS (Green et al., 2010; Johnson et al., 2010b). These data suggest that the UBQLN1 E54D mutation is causal. Because the glutamate residue at position 54 is highly conserved, one suspects that its mutation to aspartate is functionally significant, although PMut (http://mmb2.pcb.ub.es:8080/PMut/), PolyPhen (http://genetics.bwh.harvard.edu/pph/) and SIFT (http://sift.jcvi.org) predicted that this change is not likely to be functionally deleterious.

Lastly, L442L was identified in a male patient diagnosed with definite SALS. This C-to-T nucleotide change, which is located 7 bp upstream of the 3′ end of exon 8, was well preserved across species. Statistically, this variant is under-represented in ALS cases (Table 1B); however, its biological significance is unclear. We note that there normally are two spliced variants of *UBQLN1*: TV1 (entire coding region) and TV2 (excludes exon 8). We did not detect an impact of this variant on the alternative splicing of exon 8 (see Methods, data not shown).

Functional studies

We next studied the functional significance of E54D variant since it was novel, evolutionarily conserved, and located within UBL domain, and interestingly was identified in a BVVLS patient of unknown genetic cause. S503C was also tested in parallel although its presence in controls predicted a wild type response. We first investigated the effect of wt and mutant *hUBQLN1* on the TDP-43 distribution (Fig. 1). When co-expressed with Flag-h*UBQLN1*^{E54D}, hTDP-43-EGFP was mislocalized from the nucleus to the cytoplasm in 30% of the co-transfected cells, whereas this phenotype was observed in only 9.5% and 12% of cells when co-expressing Flag-h*UBQLN1*^{WT} and Flag-h*UBQLN1*^{S503C}, respectively. Furthermore, TDP-43 and UBQLN1 protein were clearly identified as co-stained cytoplasmic aggregates when co-expressed with Flag-h*UBQLN1*^{E54D} but not when co-expressed with UBQLN1^{WT} (Fig. 1-l). Similar cellular phenotypes were obtained when testing the effect of tagged and untagged *UBQLN1*^{WT}, *UBQLN1*^{E54D}, and *UBQLN1*^{S503C} constructs on endogenous TDP-43 (Fig. 1 m-t, Supplementary Figs. 1 and 2). Although some cytosolic distribution of endogenous TDP-43 was observed when expressing *UBQLN1*^{WT} and *UBQLN1*^{S503C}, no aggregates were identified in any of these cases. It thus might be argued that the finding of cytosolic TDP-43 aggregation induced by UBQLN1^{E54D} is a consequence of high levels of UBQLN1 expression rather than the missense mutation. However, we note that western immunoblotting showed approximately equivalent expression of *hUBQLN1* or Flag-h*UBQLN1* in wt and mutant forms (Fig. 2). These data strongly support the view that the mislocalization and aggregation effects of TDP-43 in cells transfected with *UBQLN1*^{E54D} is a consequence of the E54D mutation and not due to high levels of UBQLN1 expression.

As a further approach, we quantified the endogenous levels of the soluble fraction of TDP 43 protein when over-expressing untagged and tagged *UBQLN1* constructs in both HEK293T cells and *Drosophila melanogaster* (Fig. 2). In these experiments, forced expression of Flag-h*UBQLN1*^{E54D} in HEK293T cells significantly reduces soluble TDP-43 levels (Figs. 2A and B). To exclude the possibility that this effect is due to over-expression of the mutant protein or adverse effects of the Flag tag, we also expressed non-tagged h*UBQLN1*^{WT}, h*UBQLN1*^{S503C}, or h*UBQLN1*^{E54D} whose expression is driven by the weaker PrP promoter. A reduction of endogenous TDP-43 was also observed in HEK293T cells expressing h*UBQLN1*^{E54D} (Figs. 2C and D). To determine if this in vitro effect of h*UBQLN1*^{E54D} is evident in vivo, we generated multiple UAS-transgenic fly lines that express h*UBQLN1*^{WT}, h*UBQLN1*^{S503C}, or h*UBQLN1*^{E54D} under specific Gal4 drivers, such as eye-specific GMR-Gal4. We first selected transgenic lines that express these proteins at comparable levels, such that their biological effects can be compared (Fig. 2E). We found that expression of h*UBQLN1*^{E54D} in the eye of *D. melanogaster* also led to a reduction in the level of endogenous dTDP-43 (Figs. 2E and F) although no obvious morphological phenotype was observed. By contrast, no significant differences were found when h*UBQLN1*^{S503C} was over-expressed.

In a related functional study, we also assessed the impact of *UBQLN1* mutants on the degradation of ubiquitinated proteins through the proteasome using a GFP-tagged, mutant ubiquitin (Ub^{G76V}-GFP) as a reporter, as previously reported (Dantuma et al., 2000). Expression of *UBQLN1*^{E54D} but not *UBQLN1*^{S503C} slightly enhanced accumulation of

Table 1
Results of *UBQLN1* mutational screening.

Exon	Variant		Sequence		TaqMan assay		Total cases	Total alleles	Control allele	Chi-square test (2)		
	SNP	AA	FALS	SALS	SALS	control	FALS/SALS	FALS/SALS	Frequency (1)	p	OR	95% CI
1	c.162 G>T	E54D	1/102	0/92	0/1474	0/1378	1/102	1/204	0/2,756[a]	0.0002	40.64	1.649–1001
8	c.1685 C>T	L442L	0/102	1/92	0/1474	0/1378	1/1566	1/3,132	21/4,940[a,b]	0.001	0.07	0.010–0.557
10	c.1508 C>G	S503C	0/102	1/92	1/1474	0/1378	2/1566	2/3,132	6/8398[a,c]	0.8905	0.89	0.180–4.432
intro	i10:-24 A>T	no	0/102	1/92	no	1/380	1/92	1/184	1/760[a]	0.2756	4.15	0.258–66.67

(1) Control frequency includes data from
  a. Taqman assay
  b. 1,000 Genome database
  c. NHBLI exome server, European-American population.
(2) Total alleles in FALS or SALS cases compared to control allele frequency.

Ub^{G76V}-GFP, as compared to *UBQLN1*^{wt} (Supplementary Fig. 3). This change was statistically significant but modest and thus is of uncertain biological significance.

Finally, because TDP-43 and FUS/TLS have similar domain structures, are involved in RNA binding and transport, and are both implicated in FALS and SALS, we tested the possibility that co-expression of *UBQLN1*^{wt}, *UBQLN1*^{S503C} and UBQLN1^{E54D} with GFP-WT-FUS would cause cytosolic retention of FUS/TLS, as it did with TDP-43 (Ito and Suzuki, 2011). This was not observed when *UBQLN1*^{E54D} was co-expressed with GFP-WT-FUS (Fig. 3).
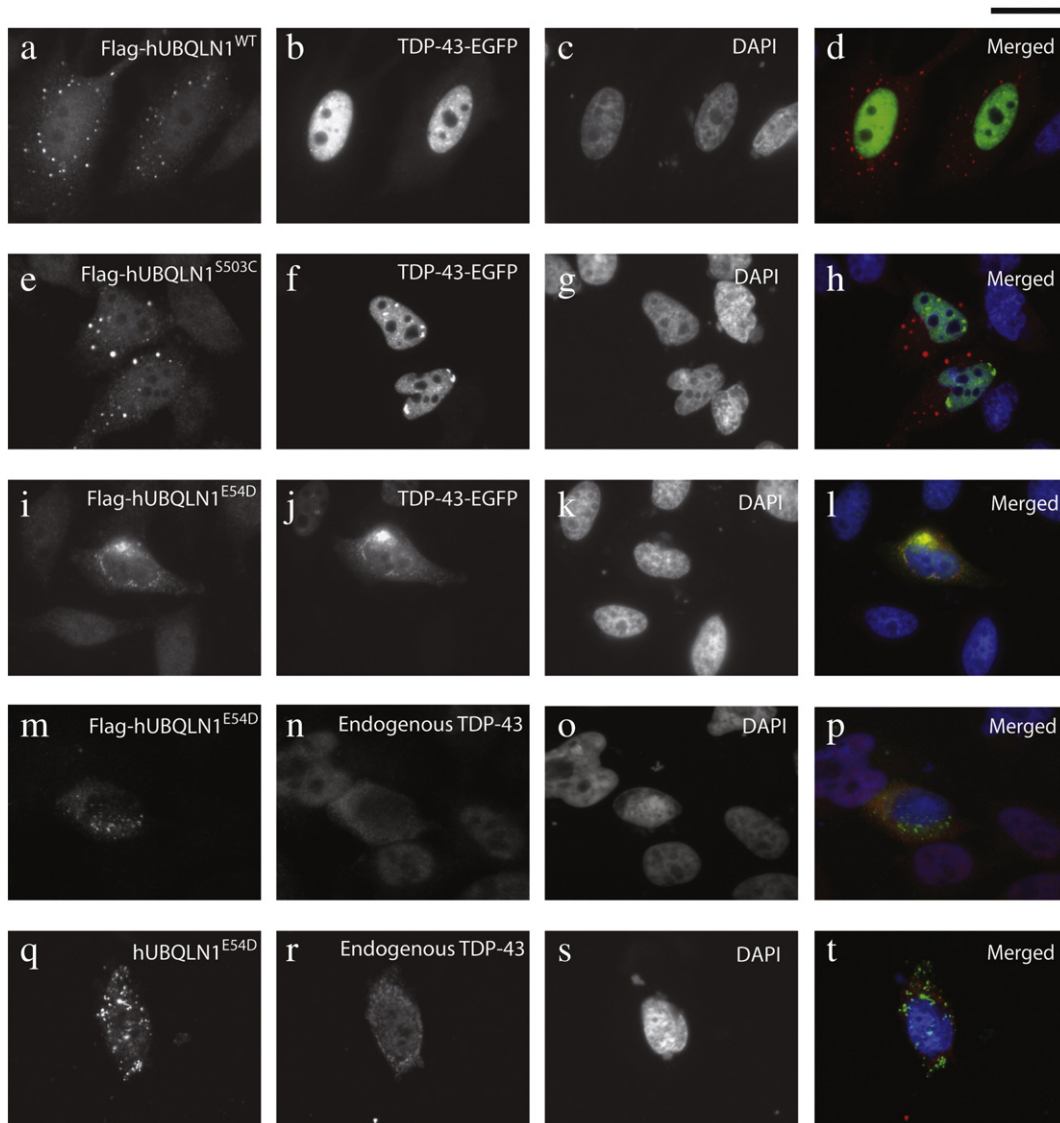

Fig. 1. h*UBQLN1*^{E54D} perturbs the distribution of TDP-43 protein in HEK293 cells. Co-expression of Flag-h*UBQLN1*^{E54D} and TDP-43-EGFP redistributes TDP-43 to the cytoplasm of HEK293 cells, where TDP-43 co-localizes with mutant *UBQLN1*^{E54D}, primarily in aggregate forms (i–l). This phenotype was not observed with *UBQLN1*^{WT} (a-d) or *UBQLN1*^{S503C} (e–h), although a loss of nuclear TDP-43 was observed in the latter case. Flag-h*UBQLN1*^{E54D} and h*UBQLN1*^{E54D} reproduced the results seen with *UBQLN1*^{E54D} (m–t).
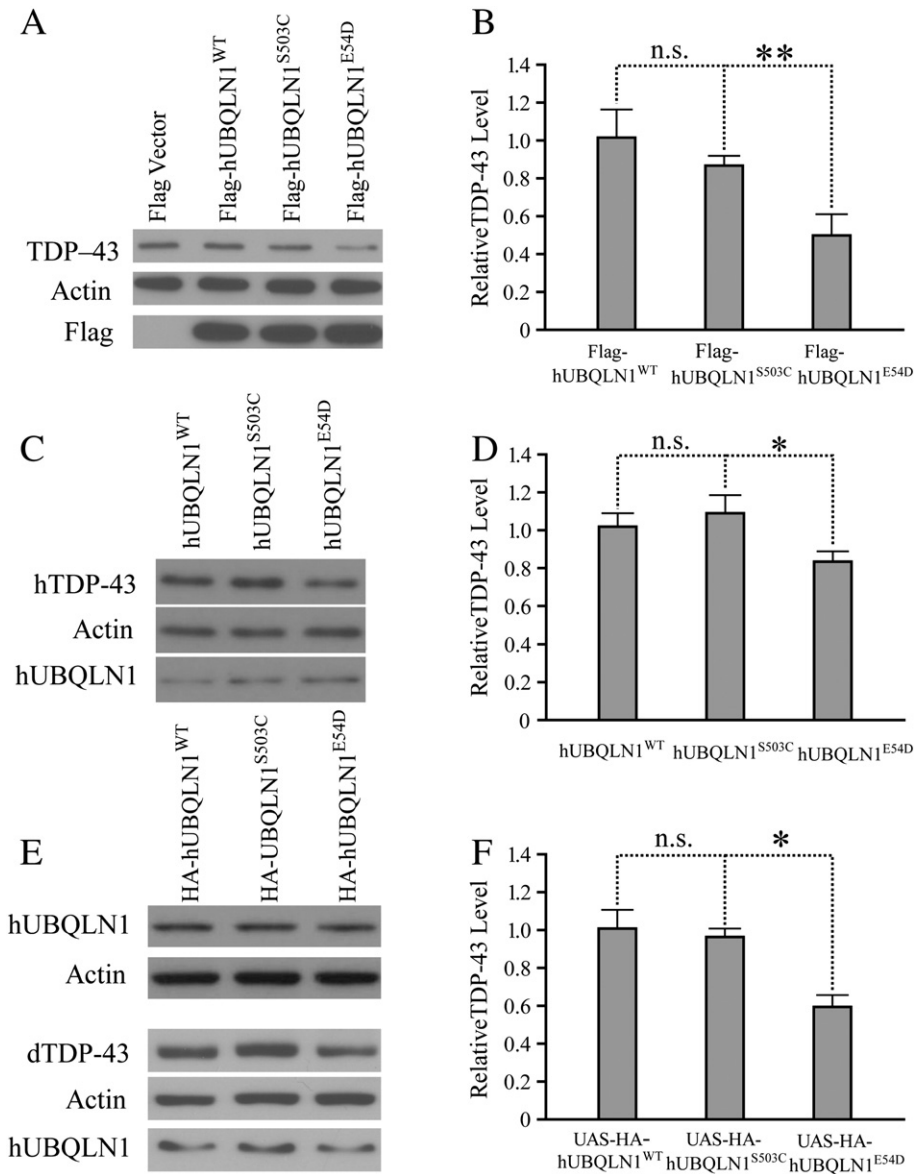
Fig. 2. h*UBQLN1*[E54D] reduces the level of endogenous TDP-43 in both HEK293 cells and *D. melanogaster*. HEK293T cells were transfected with tagged (A and B) and untagged (C and D) human *UBQLN1*[WT], *UBQLN1*[S503C] and *UBQLN*[E54D] constructs. Endogenous TDP-43 levels were significantly reduced in cells transfected with PrP-*UBQLN1*[E54D] and Flag-*UBQLN1*[E54D] when compared to h*UBQLN1*[WT]; transfection with h*UBQLN1*[S503C] did not produce this effect. The same effect was also evident after over-expression of UAS-HA-h*UBQLN1*[WT], UAS-HA-h*UBQLN1*[S503C] and UAS-HA-h*UBQLN1*[E54D] in the eye of *D. melanogaster* (E and F).

Discussion

Despite the absence of association between any of the three *UBQLN1* SNPs and ALS, and despite the fact that UBQLN1 lacks the PXX domain that is mutated in UBQLN2 in ALS, we screened for *UBQLN1* mutations in an ALS cohort for two reasons. UBQLN1 interacts directly with polyubiquitinated TDP-43 (Kim et al., 2009; Ko et al., 2004), the main component of cytoplasmic inclusions in ALS (Neumann et al., 2006). And, there is distinct ubiquilin pathology in autopsy-confirmed ALS cases without mutations in *UBQLN2* (Deng et al., 2011), raising the possibility that there may be mutations in other ubiquilin genes.

By direct sequencing of the *UBQLN1* gene, we have not detected novel *UBQLN1* gene variants in ALS. However, we have identified one novel coding variant (E54D) in a juvenile-onset form of MND (BVVLS). This variant was not detected in 1378 unrelated control subjects or in three large databases of DNA variants. We conclude that polymorphisms in *UBQLN1* (a PXX domain-lacking ubiquilin) do not commonly contribute to risk for ALS, while rare variants may contribute to the pathogenesis of atypical motor neuron disease.

We recognize that the E54D variant may be a rare polymorphism. Indeed, it is striking that apparently normal individuals typically harbor ~200 novel SNPs per exome (30 Mb) (http://useast.ensembl.org/index.html). Given that the coding sequence for the major UBQLN1 protein is 589 amino acids, encoded by a cDNA domain of 1,767 bp (Ensemble entry ENST00000376395), one would estimate that the number of novel variants in *UBQLN1* in each individual is $200 \times 1,767/30 \times 10^6$, or 1.2×10^{-2}. Since we performed full mutational screening on 196 individuals, we would therefore expect $196 \times 1.2 \times 10^{-2} = 2.3$ (i.e. 2 or 3) novel SNPs from the analysis.

These considerations prompt caution in interpreting the E54D variant as causal in BVVL. We therefore undertook studies to define potential adverse functional effects of this variant. The ubiquilin proteins (1–4) function as adaptor proteins that chaperone cargos to proteasomes and autophagosomes. Chaperone partners include TDP-43, presenilin-1, huntingtin and amyloid precursor protein (Hiltunen et al., 2006; Kim et al., 2009; Stieren, 2011; Thomas et al., 2006; Wang and Monteiro, 2007). Essential for the linking of adaptor protein–partner complexes to the proteosome is the ubiquitin-like domain (UBL) in the N-terminus of
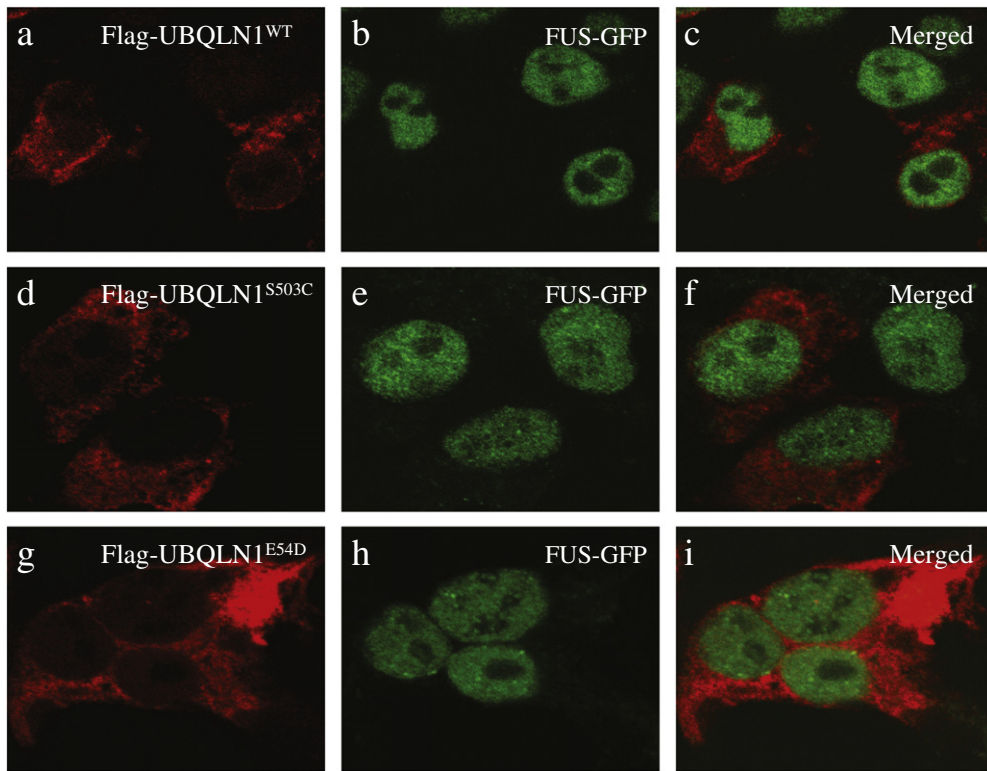
Fig. 3. *UBQLN1* mutants did not influence FUS distribution in HEK293 cells. Nuclear distribution of FUS protein was not affected when co-expressing Flag-h*UBQLN1*[E54D/S503C/WT] and FUS-GFP.

UBQLN1. It is therefore interesting that the predicted glutamate-to-aspartate amino acid change at position 54 involves a residue that is highly conserved across species within this UBL domain. We were intrigued to find that UBQLN1[E45D] induces cytosolic mislocalization of TDP-43, primarily in aggregate forms that co-localize with the UBQLN1 protein. While it is possible that cytosolic retention of TDP-43 is a non-specific reaction to stress induced by UBQLN1[E45D], we note that there is clear documentation that UBQLN1 can directly interact with TDP-43 both in yeast and *in vitro* (Kim et al., 2009).

Our data suggest that the UBQLN1[E45D] variant may weakly, but significantly, inhibit ubiquitin degradation, as assayed using a Ub[G76V]-GFP reporter. The weakness of effect could explain the absence of phenotype
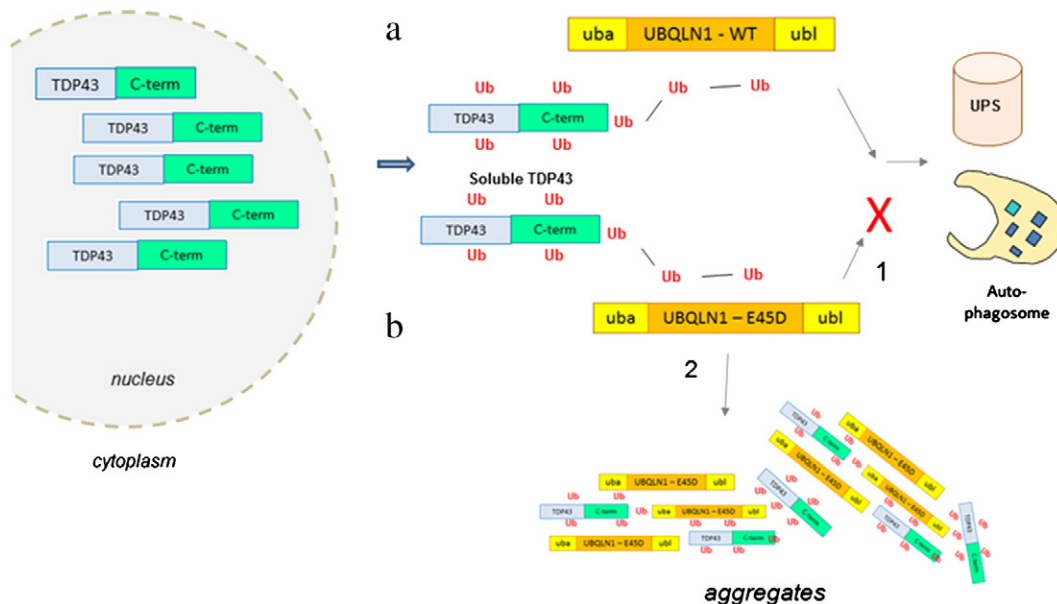

Fig. 4. Speculative model of the effect of *UBQLN1*[E54D] on TDP-43. The E54D mutation in *UBQLN1* is presumed to prevent the UBL domain from interacting with the proteasome or autophagosomes. Consequently, polyubiquitinated proteins that bind UBQL1, like TDP-43, are not degraded and accumulate in cytoplasm.

in transgenic flies when h*UBQLN1*[E54D] is expressed in the eye and neurons of *D. melanogaster*, whereas the ubiquitous overexpression of both mutant and wt forms prevented flies from developing into the adult stage (data not shown). We hypothesize that one of two pathogenic events arises: (1) this UBQLN1[E54D] mutant in the UBL domain prevents normal interactions with proteasome and autophagosomes, leading to secondary aggregation with binding partners such as TDP-43. (2) Alternatively, the mutations may enhance aggregation of UBQLN1 with TDP-43, secondarily impairing trafficking to proteasomes and autophagosomes (Fig. 4).

The presence of ubiquilin protein in aggregates in autopsies from ALS patients is consistent with the view that perturbations in protein turnover are important elements in ALS pathogenesis and with the finding that *UBQLN2* mutations are infrequently detected in ALS cases (Deng et al., 2011; Millecamps et al., 2012), even though, as we report here, genetic variations in *UBQLN1* are not commonly associated with ALS. The concept that protein aggregation and distressed protein turnover is critical in neurodegeneration is firmly supported by an immense body of experimental data both in humans and in animal and cellular models. Potentially of particular importance is the interplay between the ubiquilins and RNA-binding proteins like TDP-43, given recent discoveries of causative ALS genes that implicate disruptions of RNA metabolism as the fundamental agent in ALS pathogenesis, at least in a subset of cases.

Contributors

Paloma González-Pérez: genotyping, mutational analysis, analysis and interpretation of the data, drafting manuscript.

Yubing Lu: functional studies of wildtype and mutant UBQLN1, analysis and interpretation of the data, manuscript revision.

Ru-Ju Chian: cloning of WT and mutant UBQLN1, manuscript revision.

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Rudolph E. Tanzi: conceptualization of the study, manuscript revision.

Lars Bertram: conceptualization of the study, statistical analyses, manuscript revision.

Fen-Biao Gao: design of experiments in *Drosophila* and cell cultures, interpretation of the data, drafting and revision of the manuscript.

Robert H. Brown: design, conceptualization of the study, interpretation of the data, drafting and manuscript revision.

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