Human Molecular Genetics, 2012, Vol. 21, No. 22 ***4845–4856***
doi:10.1093/hmg/dds321
Advance Access published on August 7, 2012

High-content RNAi screening identifies the Type 1 inositol triphosphate receptor as a modifier of TDP-43 localization and neurotoxicity

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Received April 28, 2012; Revised July 3, 2012; Accepted July 31, 2012

Cytosolic aggregation of the nuclear RNA-binding protein (RBP) TDP-43 (43 kDa TAR DNA-binding domain protein) is a suspected direct or indirect cause of motor neuron deterioration in amyotrophic lateral sclerosis (ALS). In this study, we implemented a high-content, genome-wide RNAi screen to identify pathways controlling TDP-43 nucleocytoplasmic shuttling. We identified ~60 genes whose silencing increased the cytosolic localization of TDP-43, including nuclear pore complex components and regulators of G2/M cell cycle transition. In addition, we identified the type 1 inositol-1,4,5-trisphosphate (IP3) receptor (ITPR1), an IP3-gated, endoplasmic reticulum (ER)-resident Ca^{2+} channel, as a strong modulator of TDP-43 nucleocytoplasmic shuttling. Knockdown or chemical inhibition of ITPR1 induced TDP-43 nuclear export in immortalized cells and primary neurons and strongly potentiated the recruitment of TDP-43 to Ubiquilin-positive autophagosomes, suggesting that diminished ITPR1 function leads to autophagosomal clearance of TDP-43. The functional significance of the TDP-43-ITPR1 genetic interaction was tested in *Drosophila*, where mutant alleles of ITPR1 were found to significantly extended lifespan and mobility of flies expressing TDP-43 under a motor neuron driver. These combined findings implicate IP3-gated Ca^{2+} as a key regulator of TDP-43 nucleoplasmic shuttling and proteostasis and suggest pharmacologic inhibition of ITPR1 as a strategy to combat TDP-43-induced neurodegeneration *in vivo*.

INTRODUCTION

Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disorder of middle age that preferentially targets motor neurons controlling muscle movement. There is no effective treatment for ALS, which is usually fatal (1). More than 90% of ALS cases occur sporadically (sALS), without clear family history, whereas 10% of cases have a clear genetic link and are familially inherited (fALS). Dominant mutations in SOD1 were the first identified genetic cause of fALS and rodent SOD1 models have been used to elucidate mechanisms of ALS neuropathogenesis (2,3). More recently, Neumann *et al*. identified TDP-43 as a major component of cytoplasmic inclusions in affected neurons of patients with ALS or the pathologically related condition frontotemporal lobar dementia (FTLD) (4). TDP-43 is an essential nuclear RNA-binding protein (RBP) that participates in transcriptional repression, inhibition of exon splicing and messenger RNA (mRNA) stabilization (5–8). Soon after TDP-43 aggregates were described in sALS, dominant mutations in the gene encoding TDP-43, *TARDBP*, were found to cause a subset of inherited FTLD-U and ALS cases (9–12), which strongly supports a direct role for TDP-43 cytoplasmic aggregation in ALS pathogenesis. It was subsequently found that dominant mutations in a second RBP, fused in sarcoma (FUS), also cause inherited forms of ALS/FTLD (13,14). Like TDP-43, mutant FUS forms insoluble cytosolic aggregates in affected neurons, though these are typically distinct from TDP-43 aggregates (13).

ALS-associated mutations in TDP-43 cluster in a Gly-rich domain with prion-like characteristics (15). Insoluble C-terminal fragments of TDP-43 containing the Gly-rich domain are frequently detected in postmortem brain samples of ALS–frontotemporal dementia patients, and the Gly-rich domain is intrinsically aggregation-prone *in vitro* and in cell culture (16–18). FUS also contains a prion-like domain and is extremely

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aggregation prone (15). ALS-associated mutations in FUS cluster in a noncanonical PY-type nuclear localization sequence that is recognized by Transportin (19). These findings strongly imply that cytosolic accumulation of FUS is essential for its neurotoxicity.

Transgenic expression of TDP-43 in rodents, zebrafish, *Drosophila* and *Caenorhabditis elegans* elicits age- and dose-dependent neurodegeneration and motor defects, indicating that TDP-43 misexpression is severely neurotoxic (20–25). Although ALS-associated mutations are reported in some studies to be more toxic than wild-type TDP-43, mutations are not absolutely required for TDP-43-dependent neurodegeneration. Interestingly, the extent of TDP-43 cytosolic aggregation reported in these models has been variable; some studies have reported TDP-43 aggregation that correlates with neurodegeneration (22,23,26), whereas others have observed neurodegeneration in the absence of gross TDP-43 aggregation (20,27).

Degenerating neurons with TDP-43 cytoplasmic pathology frequently exhibit a near complete loss of nuclear TDP-43, a phenomenon referred to as 'nuclear clearing' (4). Interestingly, several laboratories discovered that TDP-43 negatively regulates its own mRNA by promoting the exclusion of an alternatively spliced exon that is required for message stability (28–30). An overexpression of TDP-43 transgenes *in vivo* led to the depletion of endogenous nuclear TDP-43 (29), and it has been proposed that cytosolic aggregation of TDP-43 compromises TDP-43 autoregulation, leading to feed-forward aggregation and nuclear clearing of TDP-43 (31).

Nucleocytoplasmic shuttling of TDP-43 and FUS is dynamically regulated in response to various cellular insults, including axon resection, hypoxia, heat shock, Ca^{2+} perturbation and oxidative stress (32–35). These agents promote the reversible accumulation of TDP-43 and FUS in cytosolic stress granules (SGs), which are sites of paused translation and/or mRNA storage (32,35–39). Although SG formation is part of a cytoprotective mechanism, it is conceivable that these structures nucleate pathologic TDP-43 and FUS aggregation under conditions of chronic stress (40). Consistent with this possibility, mutations in SG-related genes were found to modulate FUS toxicity in yeast (36). Finally, age-dependent reductions in nuclear import (41) may also promote cytosolic accumulation and aggregation of TDP-43 and FUS, as suggested by Dormann and Haass (40). Clearly, it is important to comprehensively identify pathways controlling nucleocytoplasmic shuttling of TDP-43 and FUS *in vivo*.

In this study, we used high content RNAi (HC-RNAi) screening to identify inositol-1,4,5-trisphosphate receptor type 1 (ITPR1) as a novel regulator of TDP-43 nuclocytoplasmic shuttling. ITPR1 is an endoplasmic reticulum (ER)-resident calcium channel that mediates Ca^{2+} efflux in response to extracellular stimuli that raise inositol-1,4,5-trisphosphate (IP3) levels (42–45). We show that ITPR1 silencing promoted cytosolic accumulation of TDP-43, which correlated with diminished TDP-43 neurotoxicity *in vivo*. These findings indicate that TDP-43 expression and localization are under genetic control by Ca^{2+} and further suggest diminishing ITPR1-gated Ca^{2+} fluxes as a strategy for reducing TDP-43 proteinopathy *in vivo*.

RESULTS

HC-RNAi screen for genetic modifiers of TDP-43 localization

We screened a SMARTpool siRNA library targeting 18 230 human genes for modifiers of TDP-43 and FUS localization in HeLa cells. Gene-specific pools of siRNAs were reverse-transfected into HeLa cells in 96-well plates and the localization of endogenous TDP-43 and FUS assessed 48 h later by immunostaining (Fig. 1A and B, see the Materials and Methods section). Among the 18 230 genes screened, we identified ~60 leads whose silencing enhanced the cytoplasmic/nuclear ratio of TDP-43 to a ratio $\geq$0.4 (Fig. 1C and Supplementary Material, Table S1). Remarkably, no single knockdown caused nucleocytoplasmic repartitioning of FUS using this criterion. Nuclear transport-related genes including NupL1, Nup54 and Importin β/KPNB1 were identified as hits in our TDP-43 screen, which is consistent with the results of Nishimura *et al*., who identified these genes in a focused RNAi screen of nuclear import factors regulating TDP-43 nuclear localization (Fig. 1D and Supplementary Material, Table S1) (46). In addition, RAN (Ras-related nuclear protein), MRPL35 (mitochondrial ribosomal protein 35), NNAT (neuronatin) and the proteasome subunit PSMD1 were identified and validated as modulators of TDP-43 localization (Supplementary Material, Fig. S1 and Table S1). The potential links between these factors and TDP-43 are discussed in more detail below.

ITPR1 is a novel regulator of TDP-43 nucleocytoplasmic shuttling

ITPR1, which encodes the major ER-resident IP3 receptor, was identified as a strong modifier of TDP-43 localization from the RNAi screen (Fig. 1D). ITPR1 is one of three IP3 receptors in the human proteome that possess different biophysical properties and nonidentical expression patterns (47). ITPR1 plays a key role in intracellular calcium homeostasis and cellular energetics, and monoallelic inactivating mutations in *ITPR1* cause spinal cerebellar ataxia-type 15 (SCA15), a slowly progressive cerebellar ataxia that damages Purkinje neurons (43). Given its abundant expression in the brain and links to neurodegenerative disease, we chose to study the relationship between ITPR1 and TDP-43 in further detail.

We first validated that distinct ITPR1 siRNAs caused cytosolic localization of TDP-43 in HeLa cells, which was confirmed using several TDP-43-specific antibodies (Supplementary Material, Fig. S2A–C). Importantly, ITPR1 knockdown did not significantly affect the localization of other nuclear proteins, including MCM4 or cAMP response element-binding-binding protein (Supplementary Material, Fig. S2C). Doxycycline (Dox)-inducible ITPR1 shRNA vectors also induced TDP-43 cytoplasmic localization in HeLa cells (Supplementary Material, Fig. S3), and fractionation experiments confirmed cytoplasmic enrichment of TDP-43 in ITPR1 knockdown cells (Fig. 2A and B). ITPR1 knockdown also increased TDP-43 cytosolic localization in immortalized SH-SY5Y neuroblastoma cells (Fig. 2C) or primary rat cortical neurons (Fig. 2D and E). To test whether ALS-associated mutations in TDP-43 impacted its regulation by ITPR1, we performed

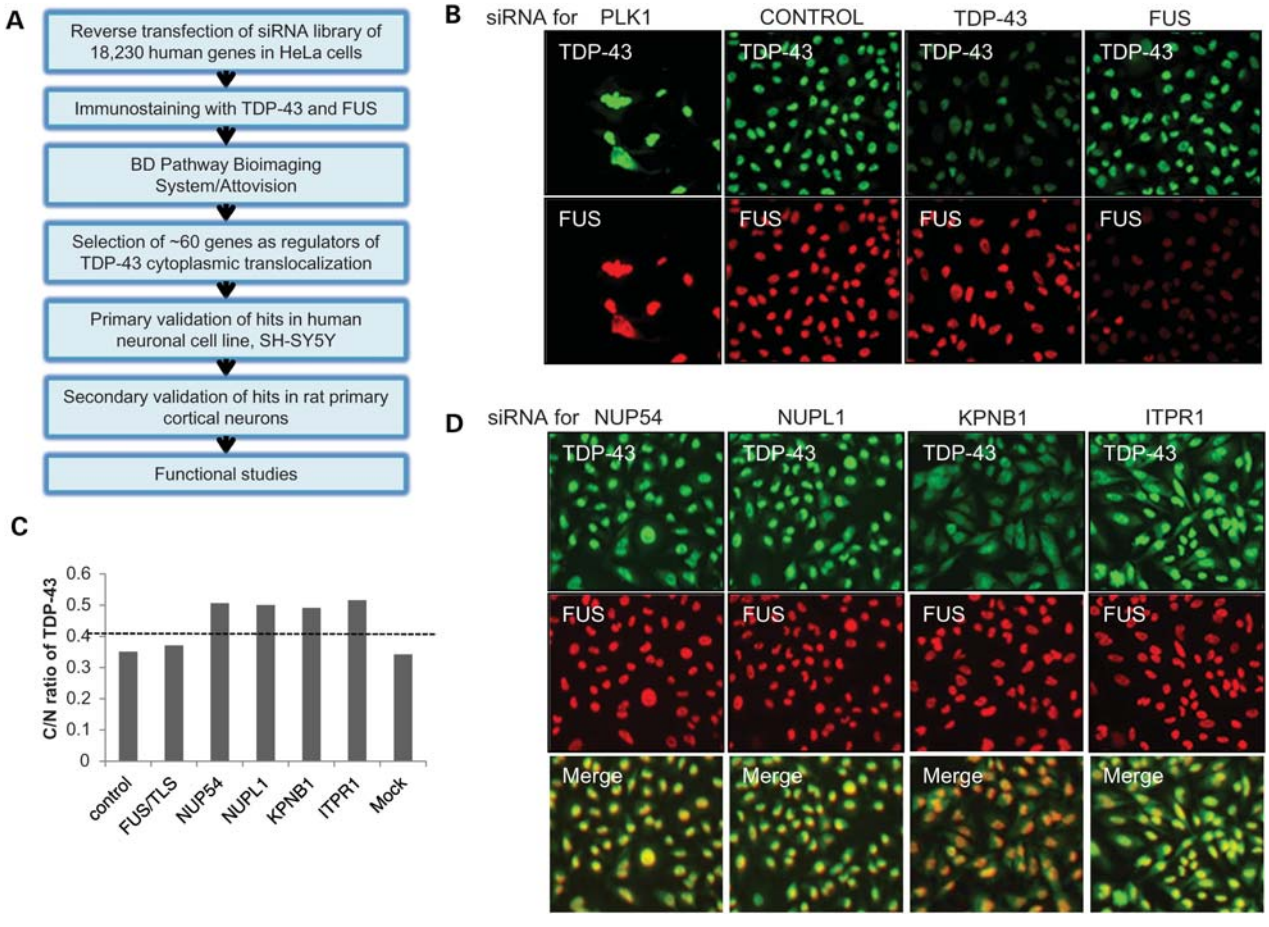

Figure 1. HC-RNAi screen for modifiers of TDP-43 localization. (**A**) Flow chart of HC-RNAi screen. (**B**) Controls of RNAi screen. HeLa cells were transfected with siRNA library of human genome and immunostained with α-TDP-43 and α-FUS antibodies. The immunostained cells were analyzed using BD Pathway Bioimaging Systems (20×). The knockdown of PLK1 (polo-like kinase 1), TDP-43 and FUS were positive controls for the RNAi screen, showing near 100% transfection efficiency. (**C**) Hits from the RNAi screen. After analysis using BD Pathway Bioimaging Systems, the average intensity of TDP-43 staining in the cytoplasm and nucleus was measured and cytoplasm/nucleus intensity (C/N ratio) was calculated. (**D**) Validation of several primary hits from the TDP-43 HC-RNAi. Knockdown of NUP54 (nuclear pore protein 54), NUPL1 (nuclear pore protein like 1), KPNB1 (Importin β1) and ITPR1 increased the TDP-43 C/N ratio in HeLa cells.

ITPR1 knockdowns in isogenic HeLa cell lines expressing Dox-inducible wild-type, Q331K or M337V mutants of TDP-43 (48). Although ectopically expressed TDP-43 also accumulated in the cytoplasm of ITPR1 knockdown cells, point mutations in TDP-43 did not affect the extent of TDP-43 mislocalization. Similar results (48) were obtained following knockdown of KPNB1 (Supplementary Material, Fig. S4). Finally, we found that the ITPR1 channel inhibitor 2-aminoethoxydiphenyl borate (2-APB) (49) induced cytosolic TDP-43 in HeLa and SH-SY5Y cells (Fig. 3A and B), as did the Ca^{2+} chelator, bis-N,N,N′,N′-tetraacetic acid-AM (BAPTA-AM). The combined results suggest that reduced ITPR1-dependent Ca^{2+} fluxes lead to cytosolic accumulation of TDP-43.

Knockdown of ITPR1 enhanced TDP-43 nuclear export

ITPR1 silencing could increase the cytosolic content of TDP-43 by inhibiting its nuclear import or enhancing its nuclear export. To distinguish between these possibilities, we treated ITPR1 knockdown cells with the CRM1 inhibitor, leptomycin (LMB). LMB antagonized TDP-43 cytoplasmic accumulation in ITPR1 knockdown cells, suggesting that cytoplasmic TDP-43 originated from the nuclear pool (Fig. 3C and Supplementary Material, Fig. S5). In addition, cycloheximide (CHX) treatment did not prevent cytoplasmic localization of TDP-43 following ITPR1 knockdown (Fig. 3D). These results suggest that ITPR1 downregulation induces export of nuclear TDP-43 rather than preventing nuclear import of newly synthesized TDP-43.

ITPR1 knockdown increases TDP-43 targeting to UBQLN-positive autophagosomes

Previous studies from our laboratory revealed that TDP-43 is recruited to autophagosomes through interaction with the cochaperone ubiquilin 1 (UBQLN1), which binds to TDP-43 via a C-terminal ubiquitin-associated domain (20,50). It is also established that chemical or genetic silencing of ITPR1 potently induces autophagy through derepression of the essential autophagy regulator Beclin 1 and/or by suppressing mitochondrial bioenergetics (42,51–55). Given these facts, we tested whether

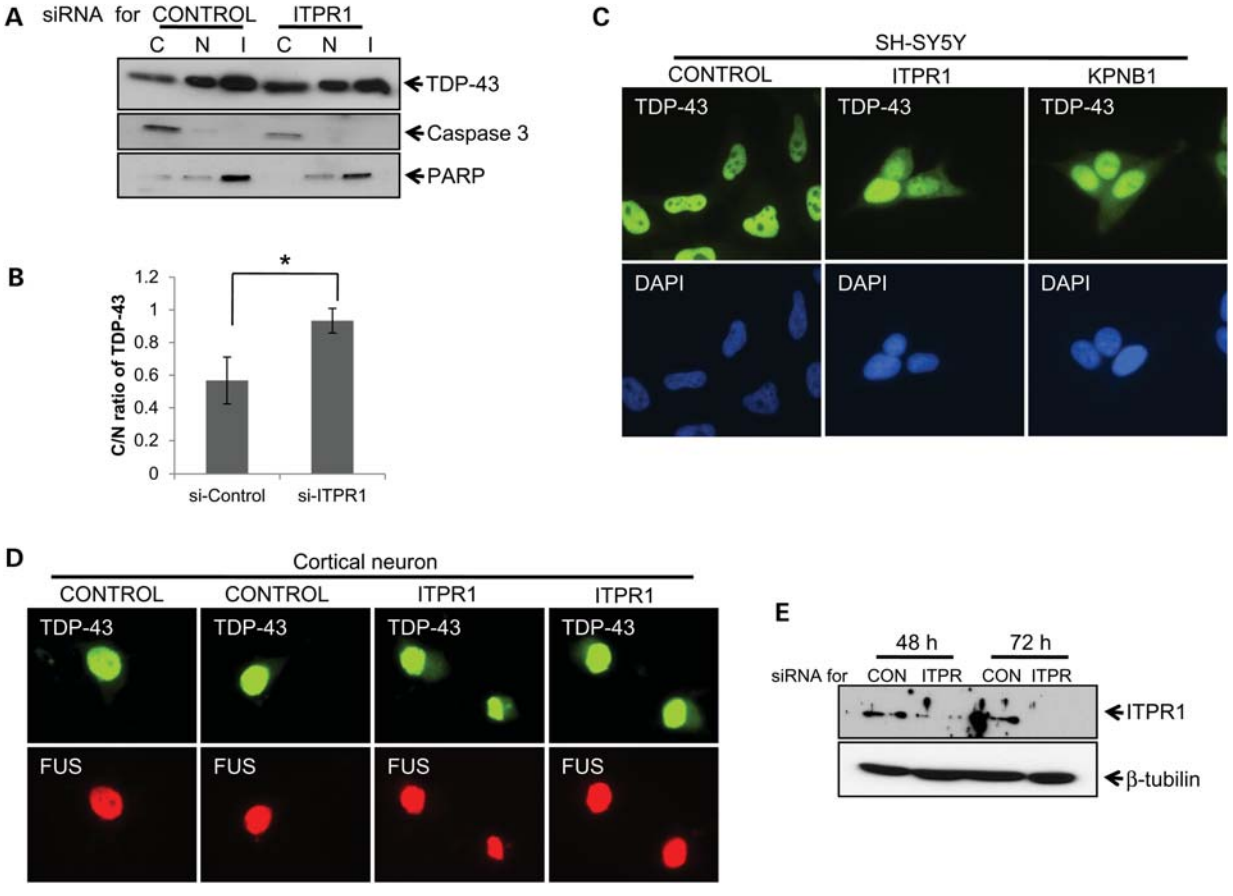

Figure 2. Knockdown of ITPR1 induces nuclear TDP-43 cytoplasmic accumulation in neurons. (**A**) HeLa cells were transfected with control siRNA or ITPR1 siRNA for 3 days, and fractionated into cytoplasm (C), nucleus (N) and insoluble/pellet (I) fractions. The fractions were immunoblotted with α-TDP-43, α-PARP (nuclear control) and α-caspase-3 (cytoplasmic control) antibodies. (**B**) Quantification of band intensity from western blot analysis of the cytoplasmic and nuclear TDP-43 measured with ImageJ. The cytoplasmic/nuclear TDP-43 ratio (C/N ratio) from the western blot analysis was normalized to total soluble TDP-43 ($n = 3$, $^{*}P < 0.005$). (**C**) SH-SY5Y cells were transfected with siRNAs for both control and ITPR1 and immunostained with α-TDP-43 antibodies and 4′,6-diamidino-2-phenylindole (DAPI). The immunostained cells were analyzed with a fluorescence microscope (40×). (**D**) Rat primary cortical neurons were transfected with siRNA for rat ITPR1 and immunostained with α-TDP-43 and α-FUS antibodies. The immunostained cells were analyzed with a fluorescence microscope (40×). (**E**) Rat primary cortical neurons were transfected with siRNA for rat ITPR1 or control siRNA using DharmaFECT3 for 48 and 72 h, and the transfected cells were lyzed with Hi-salt buffer. The cell extracts were immunoblotted with α-ITPR1 and α-β-tubulin antibodies.

ITPR1 silencing enhanced recruitment of TDP-43 to autophagosomes and/or enhanced autophagy-dependent TDP-43 degradation. Consistent with the previous studies, we found that ITPR1 knockdown promoted degradation of cleaved LC3 (LC3-II) in a bafilomycin A1-sensitive manner, indicating that ITPR1 silencing induced autophagy in our system (Fig. 4A). Additionally, ITPR1 knockdown induced cytoplasmic colocalization of UBQLN and TDP-43 following treatment with the proteasome inhibitor, MG-132 (Fig. 4B and C). These TDP-43 aggregates colocalized with the autophagosomal markers LC3 and p62 and, as expected, UBQLN aggregates colocalized with LC3 (Fig. 4D). Although knockdown of KPNB1 also induced cytosolic TDP-43 aggregation; these punctae were uniformly UBQLN negative (Fig. 4C), instead staining positive for TIAR1, a marker of SGs (Supplementary Material, Fig. S6). These findings indicate that downregulation of ITPR1 facilitated TDP-43 targeting to UBQLN-positive autophagosomes in response to proteotoxic stress.

The finding that ITPR1 knockdown caused endogenous TDP-43 to target to autophagosomes suggested that ITPR1 silencing might be used as a strategy to reduce TDP-43 expression and/or aggregation. To explore this hypothesis further, we overexpressed hemagglutinin-tagged TDP-43 (HA-TDP-43) in HeLa cells and split the transfected cells into two dishes, which were then transfected with control or ITPR1 siRNA and harvested 24 h or 48 h later. We found that the level of overexpressed TDP-43 was reproducibly decreased in ITPR1-silenced cells versus controls (Fig. 5A and Supplementary Material, Fig. S7), which is consistent with the idea that loss of ITPR1 facilitates turnover of TDP-43.

Effect of ITPR1 mutation on TDP-43 toxicity in *Drosophila*

We next sought to determine impacts of ITPR1 deficiency on TDP-43-associated neurotoxicity. Others and we have shown that overexpression of wild-type or ALS mutants of human TDP-43 in *Drosophila* leads to age- and dose-dependent neurodegeneration, paralysis and lethality (20,56–61). Motor neuron-directed expression of TDP-43 in flies (D42>TDP-43 flies) leads to a rapid-onset paralysis, with ∼90% of flies

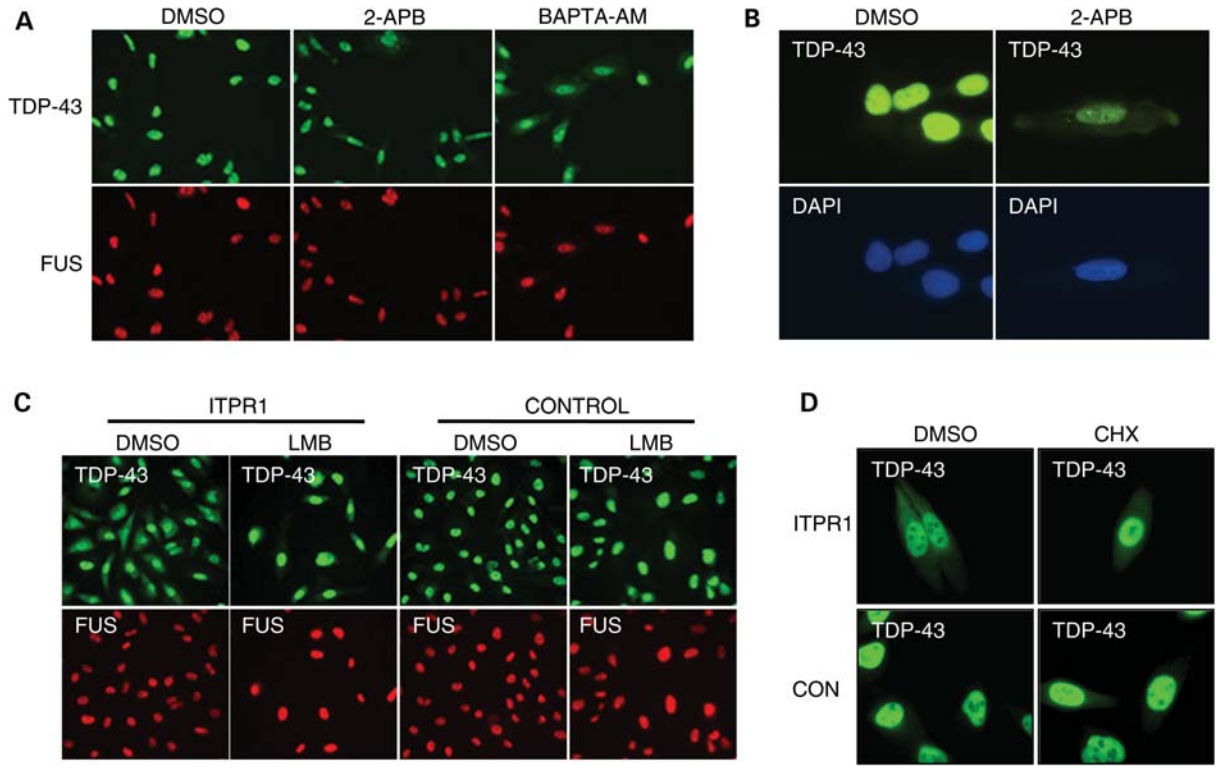

Figure 3. Inhibition of ITPR1 enhanced TDP-43 nuclear export. (**A**) HeLa cells were treated with the ITPR inhibitor 2-APB, or the Ca^{2+} chelator BAPTA-AM for 1 h. The cells were immunostained with α-TDP-43 and α-FUS antibodies and analyzed using a BD Pathway Bioimaging System (20×). (**B**) SH-SY5Y cells were treated with 2-APB for 1 h, and immunostained with α-TDP-43 antibodies. The immunostained cells were analyzed with a fluorescence microscope (40×). (**C**) HeLa cells were transfected with siRNA for ITPR1 for 24 h and the cells were treated with 10 nM LMB, an inhibitor of the nuclear export factor CRM1, for 24 h. The cells were immunostained with α-TDP-43 and α-FUS antibodies and analyzed using the BD Pathway Bioimaging System (20×). (**D**) HeLa cells were transfected with ITPR1 siRNA for 24 h and then treated with CHX for 24 h. The cells were immunostained with α-TDP-43 antibodies and analyzed with a fluorescence microscope (40×).

succumbing between 3 and 4 weeks post-eclosion (20). We crossed D42>TDP-43 flies to flies harboring mutations in *Itp-r83A*, the sole *Drosophila ITPR* ortholog. We found that three different mutant alleles of *Itp-r83A (Itp-r83A^{ug3}*, Itp-*r83A^{sv35}* and *Itp-r83A^{90B})* partially rescued the climbing defect of 15-day-old D42>TDP-43 flies (Fig. 5B). We also tested *Itp-r83A^{ug3}* and Itp-*r83A^{sv35}* for impacts on longevity and found that the mutant alleles extended the median survival of D42>TDP-43 flies by 6.5 and 5.5 days, respectively [$P < 0.0001$, (Gehan–Breslow–Wilcoxon test] Fig. 5C)]. Neither *Itp-r83A* allele extended the lifespan of D42>Gal4 control flies (Supplementary Material, Fig. S8).

The above findings imply that reducing of Itp-r83A-mediated Ca^{2+} fluxes confers protection against TDP-43-mediated neuronal damage, perhaps by promoting cytosolic accumulation and/or degradation of TDP-43. To explore this, we crossed flies expressing TDP-43 under the pan-neuronal Elav driver to the *Itp-r83A^{ug3}* and Itp-*r83A^{sv35}* strains and immunostained larval brain sections with α-TDP-43 antibodies (Fig. 5D). We detected a subtle qualitative increase in the cytosolic signal of TDP-43 in neurons of Elav-TDP-43 flies on the *Itp-r83A^{ug3}* and Itp-*r83A^{sv35}* genetic backgrounds (Fig. 5D). Although such findings must be interpreted conservatively, they are compatible with the notion that reduced *Itp-r83A* gene dosage leads to cytosolic accumulation of TDP-43 in *Drosophila*, much as it does in mammalian cells.

TDP-43 regulates ITPR1 mRNA expression

ITPR1 was recently identified as a candidate TDP-43 target in genome-wide studies of TDP-43 occupancy in neurons (28). Given that ITPR1 regulates TDP-43 localization and potentially toxicity, we wished to explore this link further. Consistent with a role in ITPR1 regulation, we found that knockdown of TDP-43 caused an ∼40% reduction in ITPR1 mRNA in HEK 293T and HeLa cells (Fig. 6A and Supplementary Material, Fig. S9). Next, we tested for TDP-43 occupancy of the ITPR1 transcript. ITPR1 contains a large intron (intron 4) that harbors UG repeats, which serve as preferred binding sites for TDP-43 (Fig. 6B) (28,62). Thus, we performed RNA immunoreprecipitation (RIP)-qPCR experiments using primer pairs spanning *ITPR1* intron 4 as well as upstream introns. This revealed TDP-43 binding at or near the 3′ end of intron 4, which contains a 12X UG repeat (Fig. 6C). These findings demonstrate that in addition to being a regulator of TDP-43, ITPR1 requires TDP-43 for its maximal expression. The possibility that TDP-43 and ITPR1 comprise a mutually codependent regulatory loop is discussed below.

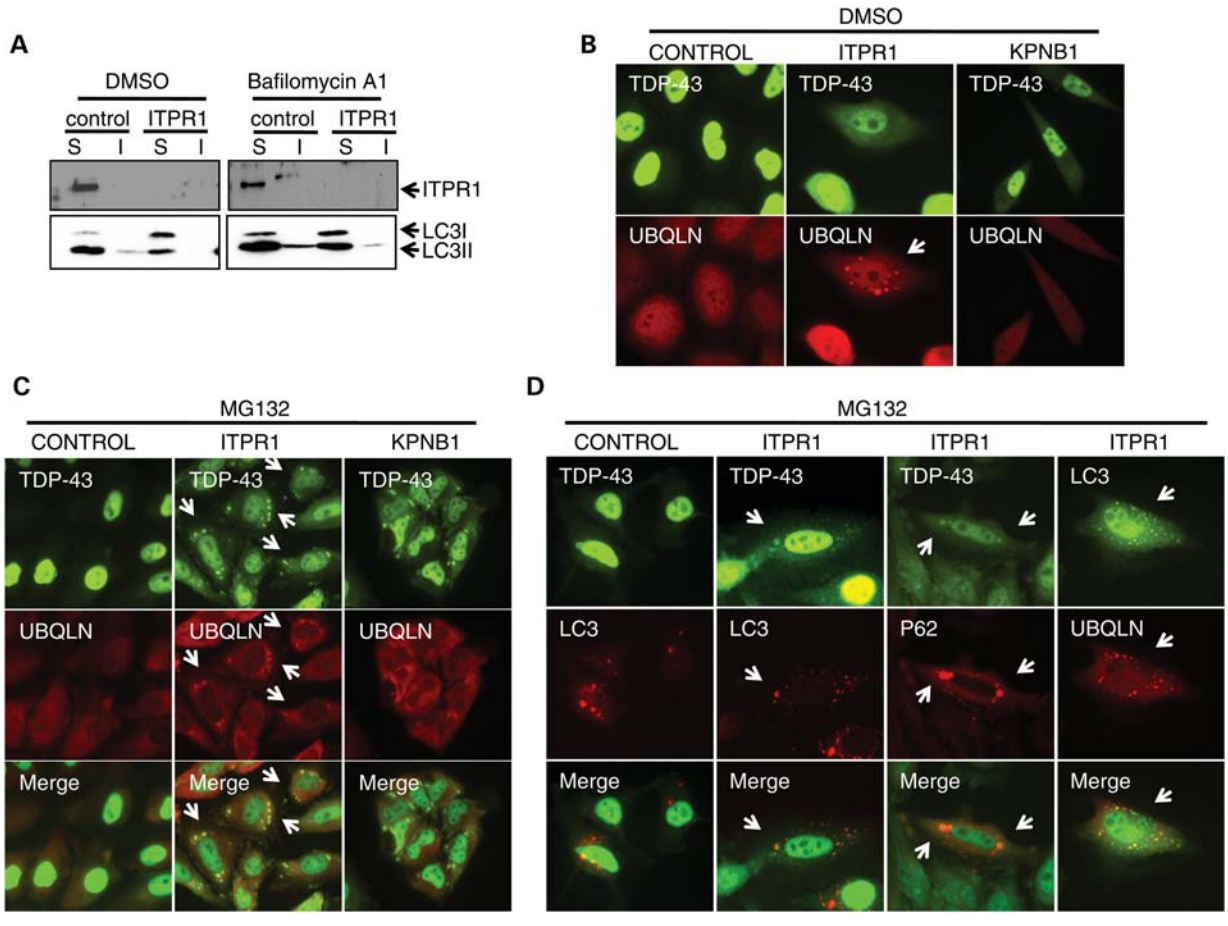

Figure 4. ITPR1 deficiency, but not nuclear import defects, causes selective recruitment of TDP-43 to autophagosomes. (**A**) ITPR1 knockdown induces autophagy. HeLa cells were transfected with control siRNA or ITPR1 siRNA and treated with DMSO or 100 nM bafilomycin A1 for 2 h. Detergent soluble (S) and insoluble (I) fractions were analyzed using western blotting with α-ITPR1 and α-LC3 antibodies. (**B** and **C**) HeLa cells were transfected with control siRNA, ITPR1 siRNA or KPNB1 siRNA for 46 h and treated with DMSO (B) or 10 μM MG-132 (C) for 2 h. The cells were immunostained with α-TDP-43 and α-UBQLN antibodies and analyzed with a fluorescence microscope (100×). (**D**) ITPR1 silencing reduced steady-state TDP-43. HeLa cells were transfected with control siRNA or ITPR1 siRNA for 46 h and treated with MG-132 for 2 h. The cells were immunostained with α-TDP-43 and α-LC3 antibodies, α-TDP-43 and α-p62 antibodies and α-LC3 and α-UBQLN antibodies. The immunostained cells were analyzed with a fluorescence microscope (100×). Arrows point to protein aggregates.

DISCUSSION

In a recent study, Nishimura *et al*. recently reported the results from a focused RNAi screen of 82 nuclear transport proteins for impacts on TDP-43 localization. They found that knockdown of NUP62, NUP54, NUPL1 and KPNB1 enhanced TDP-43 cytoplasmic localization (46). Each of these genes was also identified in our genome-wide HC-RNAi screen (Supplementary Material, Table S1). A fifth nuclear import regulator identified by Nishimura *et al*., CAS, was not identified in our screen for unknown reasons. Remarkably, not a single siRNA knockdown caused cytosolic redistribution of FUS, which utilizes a noncanonical PY-type nuclear import sequence (19). Nevertheless, defective nuclear import of FUS is strongly implicated in the pathogenesis of ALS-associated FUS mutants (37).

In addition to nuclear import factors, which are expected hits in our screen, we identified ∼60 other genes whose silencing increased the TDP-43 C/N ratio (Supplementary Material, Table S1). These genes were functionally diverse; however, the list was enriched for mitosis regulators (DOCK11, CEP192, CDC25B and CDC25C) and small guanosine-5′-triphosphate-binding proteins (GIMAP5, RASGRP3, GNAT1 and RAN). The basis for enhanced cytosolic localization of TDP-43 following knockdown of G2/M regulators (e.g. CEP192) is not clear. However, in many instances, we observed that such cells were in the process of completing cytokinesis (Supplementary Material, Fig. S1). We suspect that enhanced cytosolic TDP-43 under these conditions is a manifestation of its relatively slow nuclear import following nuclear envelope reassembly. Interestingly, FUS retained a strong nuclear localization in CEP192 knockdown cells, suggesting that its post-mitotic nuclear import is more efficient than that of TDP-43.

Other genes of interest identified in our screen include the mitochondrial protein MRPL35 and the calcium-binding protein NNAT. MRPL35 is a component of mitochondrial ribosomes and may impact cellular energetics. NNAT encodes a proteolipid protein family membrane protein that his highly expressed in the developing brain (63). NNAT is localized to the ER in neurons and is implicated in Ca^{2+} mobilization in

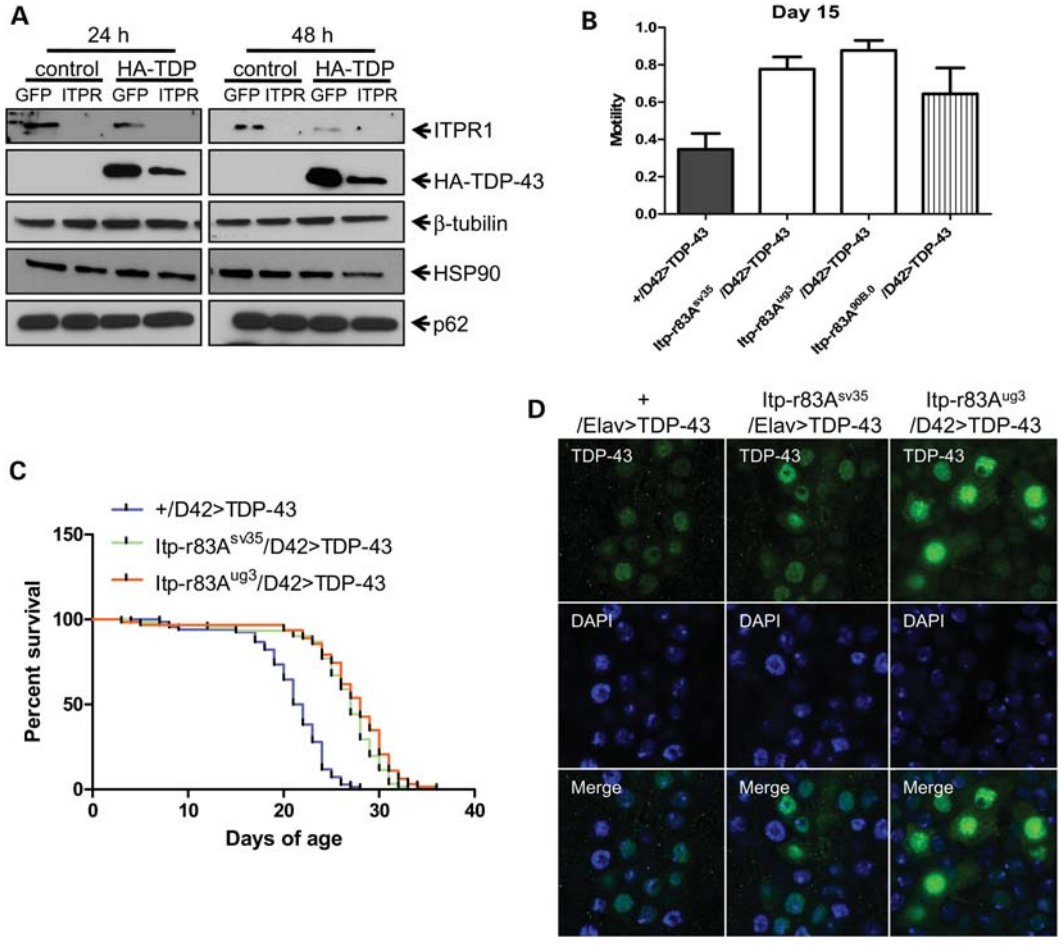

Figure 5. ITPR mutants rescued TDP-43 motor neuron phenotypes in *Drosophila*. (**A**) ITPR1 knockdown impacted TDP-43 levels in HeLa cells. These cells were transfected with HA vector or HA-TDP-43 for 24 h. The transfected cells were split into four plates and transfected with GFP or ITPR1 siRNA for either 24 or 48 h. Cell extracts were immunoblotted with the indicated antibodies. (**B**) Day 15 climbing performance of D42>TDP-43 flies crossed to wild-type or mutant *Itp-r83A* genetic backgrounds ($n > 30$). (**C**) Survival curve of control +/D42>TDP-43, Itp-r83A^{sv35}/D42>TDP-43, and Itp-r83A^{ug3}/D42>TDP-43 flies ($n = 100$). (**D**) ITPR mutants promote TDP-43 cytoplasmic localization in Larval VNCs. TDP-43 was expressed under pan-neuronal elav driver in ITPR mutant backgrounds. VNCs from third instar wandering larvae were stained with antibody against TDP-43 and DAPI.

dendrites (64). It may, therefore, impact TDP-43 localization via a mechanism shared with ITPR1.

Although pharmacologic manipulation of Ca^{2+} is known to cause cytosolic accumulation of TDP-43 (35), this study is the first to implicate a genetic regulator of Ca^{2+} homeostasis, ITPR1, in TDP-43 nucleocytoplasmic shuttling. Silencing of ITPR1 may enhance TDP-43 cytosolic accumulation through one of several mechanisms. Higo *et al*. reported that genetic silencing of ITPR1 caused ER stress and sensitized neurons to ER-stress-induced cell death (65). Thus, cytosolic accumulation of TDP-43 in ITPR1-deficient cells may occur in response to ER stress, arising from defects in ER Ca^{2+} discharge. However, we did not observe induction of ER stress markers (GRP78) following ITPR1 knockdown in HeLa cells (Supplementary Material, Fig. S10), suggesting that ER stress is unlikely to be the major cause of TDP-43 nuclear export. Alternatively, TDP-43 nuclear export may occur in response to altered cellular energetics arising in the absence of ITPR1 function. Chicken DT40 B cells harboring deletions in all the three ITPR genes (*ITPR1*, *ITPR2* and *ITPR3*) exhibited oxidative phosphorylation defects and compromised ATP production as a consequence of reduced ER-to-mitochondria Ca^{2+} transfer (42). The diminished ATP production of ITPR-deficient cells led to AMP-activated protein kinase activation and induction of macroautophagy through an mTOR-independent mechanism. However, it has also been proposed that ITPR1 inhibition derepresses Beclin 1/Atg6, a key upstream autophagy regulator (52). We hypothesize that TDP-43 exits the nucleus of ITPR1-deficient cells as part of a specific or nonspecific response to an altered cellular metabolic state and is subsequently degraded by autophagy, which is intrinsically elevated in ITPR1-deficient cells. Although we were unable to demonstrate changes in steady-state levels of endogenous TDP-43 in ITPR1 knockdown cells (data not shown), we found that a subset of MG-132-induced TDP-43 aggregates in ITPR1 knockdown cells colocalized with UBQLN, LC3 and p62, regulators of autophagy (Fig. 4C and D) (66). In addition, overexpressed TDP-43 was decreased in ITPR1 knockdown cells compared with the control knockdown cells (Fig. 5A). Downregulation of ITPR1 recruits

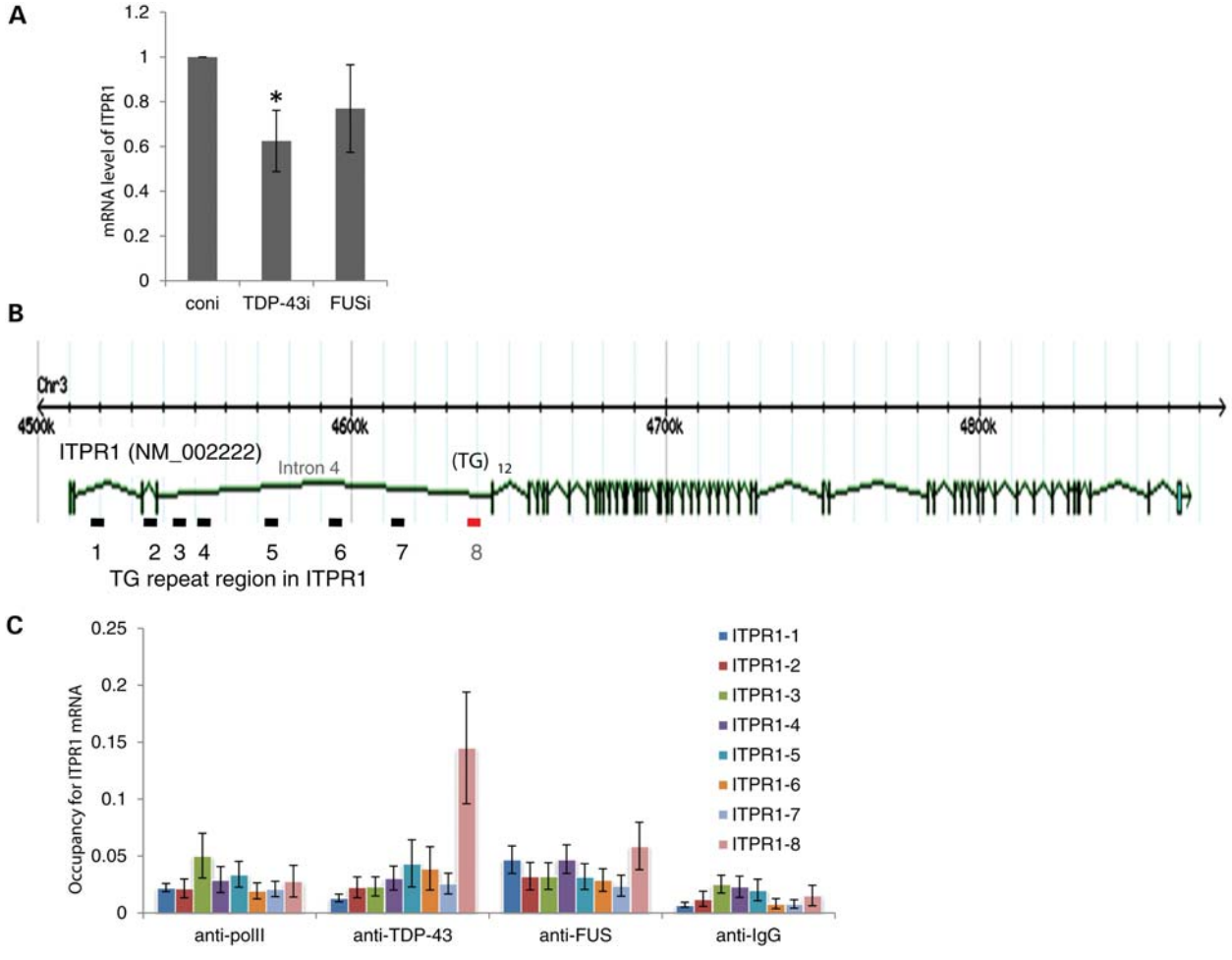

Figure 6. TDP-43 regulates ITPR1 mRNA expression. (**A**) TDP-43 is required for maximal ITPR1 expression. HEK 293T cells were transfected with siRNA for control, TDP-43 or FUS for 48 h. Total RNA was prepared from the transfected cells and cDNA was analyzed using qRT-PCR using a primer set against ITPR1 and normalized to glyceraldehyde-3-phosphate dehydrogenase. $^{*}P < 0.005$. (**B**) Gene structure of human ITPR1. The UG repeat regions are indicated with black bars. (**C**) TDP-43 interacts with ITPR1 RNA. HEK 293T extracts were immunoprecipitated with α-RNA polII, α-TDP-43, α-FUS and α-rabbit IgG antibodies. The co-immunoprecipitated RNA was analyzed with qRT-PCR using primer sets flanking the eight intronic UG repeats shown in B ($n = 3$).

TDP-43 to the cytoplasm and may enhance autophagosomal degradation of mislocalized TDP-43. Future studies will address whether ITPR silencing enhances autophagic clearance of aggregation prone fragments of TDP-43 *in vivo*.

Mutations in the sole *Drosophila* ITPR ortholog, *Itp-r83A*, increased lifespan and attenuated climbing defects of D42>TDP-43 flies (Fig. 5B and C). These findings suggest that ITPR-mediated Ca^{2+} signaling contributes to TDP-43-induced neurotoxicity and are consistent with studies showing that inhibition of ITPR1 is neuroprotective in experimental models of HD and spinocerebellar ataxia type 2 (SCA2) in which the expression of polyQ-expanded Htt and Ataxin 2, respectively, was shown to hyperactivate ITPR-mediated Ca^{2+} mobilization (67–69). Although the mechanism of neuroprotection conferred by *Itp-r83A* mutation in D42>TDP-43 transgenic *Drosophila* is not known, we observed a subtle increase in the cytosolic localization of TDP-43 in these flies (Fig. 5D). Thus, we speculate that *Itp-r83A* mutations mitigate TDP-43 toxicity by reducing its nuclear dosage. Nonexclusively, *Itp-r83A* mutation may induce neuroprotective autophagy, and reduce Ca^{2+}-mediated excitotoxicity; or work through a combination of all three mechanisms.

Finally, it is interesting to note that PolyQ repeat expansions in Ataxin 2 are also a risk factor for ALS and PolyQ-expanded Ataxin-2 exacerbated TDP-43-dependent neurodegeneration in *Drosophila* (60). It is, therefore, conceivable that PolyQ-Ataxin 2 worsened TDP-43-dependent neurodegeneration, in part, through activation of ITPR-mediated Ca^{2+} fluxes. Altogether, the combined studies suggest targeting of ITPR1 as a therapeutic opportunity in ALS and other neurodegenerative proteinopathies.

Recent CLIP-Seq studies have shown that RNAs encoding many key regulators of Ca^{2+} homeostasis, including ITPR1, are regulated by TDP-43 (28,62). Polymenidou *et al*. showed that TDP-43 depletion by antisense oligonucleotides in mouse brain decreased ITPR1 mRNA expression (28), which is consistent with our finding that TDP-43 knockdown reduced ITPR1 message ∼40% in HEK 293T and HeLa cells (Fig. 6A and Supplementary Material, Fig. S9). RIP-qPCR experiments implied that TDP-43 binds proximally to an UG repeat in

intron 4 of the ITPR1 message. This implies that TDP-43 controls Ca^{2+} homeostasis and ultimately its own nucleocytoplasmic shuttling and autophagic degradation through regulation of ITPR1 and other Ca^{2+} modulators. Pathologic TDP-43 aggregation may disrupt Ca^{2+}-dependent TDP-43 shuttling, leading to feed-forward rounds of TDP-43 nuclear export which, combined with defects in TDP-43 mRNA autoregulation, may contribute to nuclear clearing of TDP-43 that is observed in end-stage ALS.

MATERIALS AND METHODS

Cell culture and transfections

HeLa and SH-SY5Y cells were maintained in Dulbecco's Modified Eagle medium containing 10% FBS. Primary cortical neuronal cells were grown in Neurobasal medium containing 10% B27. siGENOME siRNAs SMARTpool (Dharmacon) was used for knockdown of TDP-43 (M-012394-01), FUS/TLS (L-009397-00), KPNB1 (M-017523-01), human ITPR1 (M-006207-01) and rat ITPR1 (MQ-095360-00). HeLa cells were transfected with siRNAs using DharmaFECT1 by a reverse transfection method and SH-SY5Y cells and rat cortical neurons were transfected with siRNA using DharmaFECT3.

RNAi screen

A SMART pool siRNA library targeting ~18 230 open reading frames in the human genome (Dharmacon) was accessed through The UW Small Molecule Screening & Synthesis Facility (SMSSF). siRNAs were transferred to black with clear bottomed 96-well plates (BP Falcon). Ten thousand HeLa cells per well were reverse-transfected with 20 nM of each siRNA using DharmaFECT1 (Dharmacon) for 48 h. The transfected cells were fixed with paraformaldehyde, and permeabilized with 0.1% Triton X-100 and processed for immunostaining with rabbit α-TDP-43 (ProteinTech, 10782-2-AP), and mouse α-FUS (Santa Cruz, SC47711) antibodies and 4′,6-diamidino-2-phenylindole. The primary antibodies were used at a concentration of 0.2 μg/ml. A-rabbit-Alex-488 and α-mouse-Alexa-594-conjugated secondary antibodies were used at a dilution of 0.2 μg/ml. The immunostained cells were analyzed using a BD Pathway Bioimaging System (20X magnification). Cytoplasmic/nuclear (C/N) intensity of TDP-43 and FUS were analyzed using the BD pathway system and Attovision software, after which the FlowJo software program was used to define intensity ratios for each well. Those with a C/N ratio ≥0.4 were considered positive for validation studies (Fig. 1C).

Protein analysis

SH-SY5Y and HeLa cell extracts were prepared using Hi-salt buffer as described (50) and cytoplasm/nuclear fractionation carried out as described (46).

Immunofluorescence microscopy

HeLa, SH-SY5Y and primary cortical neuronal cells were grown on glass coverslips coated with Poly-L-lysine, and siRNAs were transfected using DharmaFECT1 or DharmaFECT3. Following transfections, the cells were immunostained as described above with the indicated antibodies. Fluorescent images were generated using a Nikon Fluorescence microscope at the UW Paul P. Carbone Comprehensive Cancer Center.

Quantitative RT-PCR and RNA immunoprecipitation (RIP)

RNA analysis was carried out as described (70). The gene-specific primers were used: 5′-TGTTACAAGCCCCATTC ACA-3′ (ITPR1-F) and 5′-TTCTGCATGAAGCCAAA CTG-3′ (ITPR1-R) for mRNA of ITPR1. For RIP, isolated RNA from RIP was analyzed by qRT-PCR. The results were expressed as the relative-fold occupancy of the target precipitation when compared with the input. The ITPR1 primers for RIP 5′- AGTTTGCTTTGCAGCCAGTT-3′ (ITPR1-F1), 5′-GAAGAACTTTCCCTGCCTGA-3′ (ITPR1-R1), 5′-ACCTC CAAATGGCTTCATCT-3′ (ITPR1-F2), 5′-GCTGGGATTAC AGGCGTTAG-3′ (ITPR1-R2) 5′-TGGTGGCTTTCCTGG AGTAG-3′ (ITPR1-F3), 5′-AAAAGCCAACCCAATCTT GA-3′ (ITPR1-R3), 5′-TTCAACATCGAACACACTTGC-3′ (ITPR1-F4), 5′-GATGTGCAGCCAACCTTTTC-3′ (ITPR1-R4), 5′-CCCCTCCTTTAAGTTTCATCG -3′ (ITPR1-F5), 5′-AGAACCACTTGAACCCAGGA-3′ (ITPR1-R5), 5′-TCGGT TTGACCTGATGGATT-3′ (ITPR1-F6), 5′-TGGCTGTGGG ACATTATGAG-3′ (ITPR1-R6), 5′-TTGCTGGGGATACTG TGGAT-3′ (ITPR1-F7), 5′-TGACAAATGCCAGGAAAA TG-3′ (ITPR1-R7), 5′-TGGCTTCAGTCCAGGAGTTC-3′ (ITPR1-F8) and 5′-CACACACACACACACACACAGA-3′ (ITPR1-R8) were used for qPCR.

Experiments with *Drosophila*

The W1118 strain was used as a wild-type control in our study. The upstream activating sequences-human TDP-43 transgenic fly was generated through p-element insertion as previously described (20). The following stocks are obtained from the Bloomington Stock Center: D42-Gal4 (71), Itp-r83A^{ug3} (72) and Itp-r83A^{sv35} (73). For longevity assays, flies were maintained at 25°C with no >20 flies per vial. For each genotype, a total of >70 flies were used. Fresh food was replaced every 3–5 days. Death events were recorded on a daily basis as irresponsiveness to tapping on the vial. The longevity curve was plotted by Kaplan–Meier analysis (GraphPad Prism version 5.01 for Windows, GraphPad Software, La Jolla, CA, USA, www.graphpad.com). The climbing assay was adapted as previously described (74). A total of forty 15-day-old male flies were used for each genotype. For each test run, 10–15 flies were placed in a standard 15 ml conical tube and tapped to the bottom of the tube. Motility was scored as percentage of flies reached the 10-ml mark after 5 s. The result was captured by using an iPhone4 camera and the Photo Timer application V4.3 (Developed by CodeGoo). The final motility performance is plotted based on the average score of five sequential trials. For confocal microscopy, the third instar larvae brain was dissected and immunostained as previously described (75). The ventral nerve cord (VNC) was stained with primary antibody against TDP-43 for 48 h at 4°C (1:50; Proteintech #10782-2-AP). Alexa 488-conjugated goat anti-rabbit secondary antibody

was used at 1:100 for 24 h at 4°C. Samples were mounted in Vectorshield medium (H-1000). Confocal images were collected using a scanning laser microscope (Nikon Digital Eclipse C1 plus) at UW-Madison Waisman Center CMN Core. Images were analyzed using ImageJ.

AUTHORS CONTRIBUTION

S.H.K. performed experiments, including the HC-RNAi screen, and drafted the manuscript. K.A.H. and L.Z. assisted with *Drosophila* experiments. R.S.T. supervised the experiments and edited the manuscript.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at *HMG* online.

ACKNOWLEDGEMENTS

The authors would like to thank Huey Yee Lim for assistance with the HC-RNAi screen. The authors thank Dr Don Cleveland (Ludwig Institute for Cancer Research and Department of Cellular and Molecular Medicine, University of California at San Diego) for a gift of isogenic HeLa cell lines expressing Dox-inducible wild-type, Q331K, or M337V mutants of TDP-43.

Conflict of Interest statement. None declared.

FUNDING

This work was supported by a grant from the NIH (1R21NS067572 to R.S.T.) and a Shaw Scientist Award to R.S.T. from the Milwaukee Foundation.

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