Identification of ter94, Drosophila VCP, as a strong modulator of motor neuron degeneration induced by knockdown of Caz, Drosophila FUS

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In humans, mutations in the fused in sarcoma (FUS) gene have been identified in sporadic and familial forms of amyotrophic lateral sclerosis (ALS). Cabeza (Caz) is the Drosophila ortholog of human FUS. Previously, we established Drosophila models of ALS harboring Caz-knockdown. These flies develop locomotive deficits and anatomical defects in motoneurons (MNs) at neuromuscular junctions; these phenotypes indicate that loss of physiological FUS functions in the nucleus can cause MN degeneration similar to that seen in FUS-related ALS. Here, we aimed to explore molecules that affect these ALS-like phenotypes of our Drosophila models with eye-specific and neuron-specific Caz-knockdown. We examined several previously reported ALS-related genes and found genetic links between Caz and ter94, the Drosophila ortholog of human Valosin-containing protein (VCP). Genetic crossing the strongest loss-of-function allele of ter94 with Caz-knockdown strongly enhanced the rough-eye phenotype and the MN-degeneration phenotype caused by Caz-knockdown. Conversely, the overexpression of wild-type ter94 in the background of Caz-knockdown remarkably suppressed those phenotypes. Our data demonstrated that expression levels of Drosophila VCP ortholog dramatically modified the phenotypes caused by Caz-knockdown in either direction, exacerbation or remission. Our results indicate that therapeutic agents that up-regulate the function of human VCP could modify the pathogenic processes that lead to the degeneration of MNs in ALS.

INTRODUCTION

Amyotrophic lateral sclerosis (ALS) is a devastating neurodegenerative disease that is characterized by degeneration of motoneurons (MNs); this degeneration leads to progressive muscle weakness and eventually fatal paralysis typically within 1–5 years after disease onset (1). Frontotemporal lobar degeneration (FTLD) is a dementia syndrome with clinically diverse phenotypes that include behavioral changes, semantic dementia and progressive non-fluent aphasia (2). It is well established that ALS and FTLD form a clinical disease continuum (3, 4). Up to 15% of ALS patients meet the clinical criteria of FTLD and 30–50% has subtle cognitive deficits (5); likewise, up to 15% of FTLD patients meet the clinical criteria for ALS and up to one-third have at least minor MN dysfunction (5).
A substantial number of proteins linked to ALS are directly or indirectly involved in RNA processing (6). Mutations in genes encoding two such RNA-binding proteins—transactive response DNA binding protein 43 kDa (TDP-43, gene TARDBP) and fused in sarcoma (FUS, gene FUS)—have been identified as major genetic causes in both familial and sporadic ALS (2,7–15). TDP-43 and FUS are RNA-binding proteins implicated in multiple aspects of RNA metabolism including transcriptional regulation, mRNA splicing and shuttling of mRNAs between the nucleus and the cytoplasm (16,17).

There is a single Drosophila ortholog each for human FUS and TDP-43 named Cabeza (Caz) and TBPH, respectively. Reportedly, Drosophila lacking TBPH presents deficient locomotive behaviors, reduced life span and anatomical defects at neuromuscular junctions (NMJs); these phenotypes indicate that a loss of TDP-43 nuclear functions could be a causative factor for the neurodegeneration observed in patients with ALS/FTLD (19). The RNAi of the fly lines we generated (responder DNA binding protein 43 kDa (TDP-43, gene TARDBP) and fused in sarcoma (FUS, gene FUS) also causes MN degeneration that can be rescued by the respective wild-type but not mutant FUS (20,21). These findings suggest that FUS is important for the survival of MNs and that a “loss-of-function” mechanism could be the fundamental pathogenic mechanism causing ALS/FTLD.

Using our established fly model of FUS-ALS induced by Caz-knockdown, we aimed to explore molecules that affect phenotypes presented by the model. Specifically, we intended to elucidate the molecular mechanisms leading to neuronal dysfunction in FUS-related ALS/FTLD (19). The RNAi of the fly lines we generated (responder DNA binding protein 43 kDa (TDP-43, gene TARDBP) and fused in sarcoma (FUS, gene FUS) ortholog each for human FUS and Drosophila, respectively. Phenotypes associated with the resultant genotypes are summarized. Each Caz-knockdown severely enhanced the Caz-knockdown phenotypes in flies; it severely exacerbated locomotive disabilities and the degeneration of MNs induced by neuron-specific Caz-knockdown. Conversely, the overexpression of ter94 rescued those phenotypes.

### RESULTS

**Knockdown of Caz in eye imaginal discs induces morphologically aberrant rough eyes**

To investigate the molecular mechanisms of FUS-related neurodegeneration, we have already generated Caz-knockdown fly models of ALS by using the highly versatile GAL4/UAS-targeted expression system (19). To eliminate the possibility of off-target effects, we generated 11 independent transgenic fly lines and obtained one fly line from the Vienna Drosophila RNAi center (VDRC; Table 1); Caz double-stranded RNA (dsRNA; inverted repeats, IRs) targeted to the different region of the Caz mRNA is expressed in those fly lines as described in our previous study (19). The RNAsi of the fly lines we generated (responder controls) was targeted to the region corresponding to residues 1–167 (four lines, UAS-Caz-IR1–167) and 180–346 (seven lines, UAS-Caz-IR180–346) and 333–399 (seven lines, UAS-Caz-IR333–399) of Caz (22–24).

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<th>Transgene strain</th>
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We used two independent Caz-RNAi constructs, UAS-Caz-IR1–167 and UAS-Caz-IR180–346, to generate 11 independent transgenic fly lines. UAS-Caz-IR333–399 was obtained from VDRC. To drive the expression of Caz dsRNA in the whole body of the flies, or specifically in the eye imaginal discs or neuronal tissues, we cross UAS-Caz-IR flies with Act5C-GAL4, GMR-GAL4 or elav-GAL4 flies, respectively. Phenotypes associated with the resultant genotypes are summarized. Each transgenic strain shows a consistent phenotype. NE, no effect; ND, not determined; LD, locomotive dysfunction.
dsRNA is expressed, with the transgenic flies carrying a UAS-typical characterization of two P-element alleles of was described previously (28). According to this report, female germ-line clones of a strong loss-of-function allele of ter94K15502 affect the phenotypes caused by central nervous system (CNS), and Caz protein is present in eye imaginal discs (27). To efficiently screen for genes that affect the phenotypes caused by Caz-knockdown, we generated model flies with eye-specific Caz-knockdown. Specific knockdown of Caz in eye imaginal discs was achieved by crossing the transgenic flies carrying a UAS-Caz, in which Caz dsRNA is expressed, with GMR-GAL4 driver lines (GMR-GAL4; UAS-Caz-IR/+; +). Phenotypes of those model flies are easily characterized by abnormal rough eye morphology; scanning electron microscope (SEM) images showed fusion of ommatidia and loss of mechanosensory bristles (Fig. 1). Phenotypes of those fly lines carrying each UAS-Caz-IR crossed with the GMR-GAL4 driver strain are summarized in Table 1. Flies carrying GMR-GAL4; +; + alone exhibited apparently normal eye morphology (Fig. 1A; GMR). Flies carrying GMR-GAL4, UAS-Caz-IR1–167/+; + (GMR > UAS-Caz-IR1–167) and those carrying GMR-GAL4; UAS-Caz-IR363–399/+; + (GMR > UAS-Caz-IR363–399) showed essentially the same rough-eye phenotype (Fig. 1B and C). These results demonstrated that the rough-eye phenotype observed in Caz-knockdown flies was not due to a possible insertionional mutation or off-target effect, but rather to reduced Caz protein levels. Throughout the following studies, we used fly strains carrying UAS-Caz-IR363–399, and UAS-Caz-IR hereafter refers to this fly strain.

Loss-of-function mutations and overexpression of ter94 conversely modified the compound eye degeneration induced by Caz-knockdown

To examine a genetic interaction between Caz and ALS-causing genes, we first crossed eye-specific Caz-knockdown flies with several fly lines carrying different mutations in various ALS-causing genes, and their progeny were screened for eye phenotypes. From these screens, we detected a genetic interaction between Caz and ter94, the Drosophila ortholog of human VCP. We crossed Caz-knockdown flies with ethyl methanesulfonate-induced ter94 mutations, ter94K15502, and ter94 derived from an adaptor library. The phenotypic characterization of two P-element alleles of ter94 mutations was described previously (28). According to this report, female germ-line clones of a strong loss-of-function allele of ter94, ter94K15502, do not produce germlia or egg chambers, and female germ-line clones of another slightly less strong loss-of-function allele of ter94, ter94K3775, formed germlia which give rise to stage 6 or 7 egg chambers before degeneration occurs (28). From these findings, we used the ter94K15502 mutation as a strongest loss-of-function allele and the ter94K3775 mutation as a strong loss-of-function allele. The strongest (ter94K15502) and strong (ter94K3775) loss-of-function mutations in the heterozygous states remarkably enhanced the rough-eye phenotype induced by eye-specific Caz-knockdown GMR > UAS-Caz-IR363–399/ter94K15502 (Fig. 1D) and GMR > UAS-Caz-IR363–399/ter94K3775 (Fig. 1E), respectively. The progeny of eye-specific Caz-knockdown flies became lethal at the pupal stage when crossed with ter94-knockdown or a chromosomal deficiency line: Df (2R) X1 lacking the genomic region 46C2–47A01 that contains ter94. These results indicate that loss-of-function mutations of ter94 act as dominant enhancers of the Caz-knockdown-induced rough-eye phenotype. Conversely, the overexpression of wild-type ter94 (GMR > UAS-Caz-IR363–399/UAS-ter94) obviously suppressed the rough-eye phenotype induced by eye-specific Caz-knockdown (Fig. 1G) based on a comparison with the responder control of UAS-ter94 flies (GMR > UAS-Caz-IR363–399/UAS-GFP) (Fig. 1F).

Loss-of-function mutation and overexpression of ter94 had opposite effects on nuclear Caz signals in the larval CNS

We demonstrated in our previous paper that Drosophila Caz was strongly expressed in the CNS of third instar larvae and localized in the nucleus (19). Therefore, we investigated whether neuron-specific Caz-knockdown changed the expression or subcellular localization of Caz or both; specifically, we crossed fly lines carrying UAS-Caz-IR with elav-GAL4 driver lines. To monitor Caz expression and localization, we immunostained brain-ventral ganglia complexes (BVGCs) of third instar larvae with anti-Caz antibody, which was developed previously (19), and quantified the immunofluorescent signals. The BVGCs of the control larvae, which carried w; +; elav-GAL4/+ (elav/+), exhibited ubiquitous signals of endogenous Caz (Fig. 2, a driver control, A1), but the signal intensity of endogenous Caz in the BVGCs was remarkably reduced in neuron-specific Caz-knockdown larvae carrying UAS-Caz-IR/+; elav-GAL4/+ (Fig. 2, elav > UAS-Caz-IR, C1). In driver control larvae, anti-Caz immunoreactivity was evident in the nucleus of the neuronal cells, but it did not colocalize with actin filaments stained with phalloidin (Fig. 2B, B1, B2, B1 + B2) or with chromosomes stained with diamino-2-phenylidole (DAPI; Fig. 2, B1, B3, B1 + B3). These findings indicated that Caz must localize in the nucleolasm. The intensity of nuclear Caz signals was significantly reduced in the BVGCs of neuron-specific Caz-knockdown larvae carrying elav > UAS-Caz-IR [intensity units = 8.89 (arbitrary units), measured in Fig. 2, D1] compared with that of driver control larvae (intensity units = 31.5, measured in Fig. 2B, P < 0.001, Fig. 2G).

Next, we examined the effects of ter94 on neuron-specific Caz-knockdown with regard to Caz levels and localization. First, we examined Caz expression and localization in larvae carrying a heterozygous loss-of-function ter94 mutation and neuron-specific Caz-knockdown constructs. Larvae carrying the strongest loss-of-function allele of ter94 and neuron-specific Caz-knockdown, UAS-Caz-IR/ter94K15502; elav-GAL4/+ (Fig. 2, elav > UAS-Caz-IR/ter94K15502, E1) exhibited remarkably reduced Caz signals in the BVGCs when compared with UAS-Caz-IR/++; elav-GAL4/+ larvae. The intensity of nuclear Caz signal was significantly reduced in these larvae carrying elav > UAS-Caz-IR/ter94K15502 (intensity units = 6.68, measured in Fig. 2, F1), even compared with that of the larvae carrying elav > UAS-Caz-IR (Fig. 2, D1) (P < 0.05, Fig. 2G). These results indicated that neuron-specific Caz-knockdown significantly reduced nuclear Caz expression, and this reduction was enhanced by genetic crossing with the strongest loss-of-function allele of ter94.

Conversely, compared with the Caz signals in BVGCs of larvae carrying UAS-Caz-IR/UAS-GFP; elav-GAL4/+ (Fig. 3, elav > UAS-Caz-IR/UAS-GFP, A1), Caz signals in
Figure 1. The rough-eye phenotype induced by Caz-knockdown is modified by genetic changes in ter94. Each panel shows a scanning electron micrograph of the compound eye of a 3-day-old adult fly. Each lower panel is a higher magnification image of the corresponding upper panel. Specific knockdown of Caz in eye imaginal discs was achieved by crossing the transgenic flies that carried UAS-Caz-IR with the GMR-GAL4 driver. (A) The eyes of a control fly carrying GMR-GAL4; +/+ (GMR) exhibit apparently normal eye morphology having an organized ommatidial architecture. (B and C) Adult eyes from two independent fly lines with eye-specific Caz-knockdown. Flies from line with UAS-Caz-IR1–167 (strain 4, Table 1) or from the line with UAS-Caz-IR363–399 were crossed with flies from the GMR-GAL4 driver strain. Resultant flies carrying GMR > UAS-Caz-IR1–167 (B) or GMR > UAS-Caz-IR363–399 (C) have essentially the same rough-eye phenotype and exhibit ommatidial degeneration. (D and E) Adult eyes from two independent fly lines; each line has eye-specific Caz-knockdown and a distinct loss-of-function mutation in ter94. The eye-specific Caz-knockdown fly line (GMR > UAS-Caz-IR363–399/UAS-Caz-IR363–399) was crossed with flies carrying the strongest (ter94K15502) or a strong (ter94K12875) loss-of-function ter94 mutation. The resultant flies carrying GMR > UAS-Caz-IR363–399/ter94K15502 (D) or GMR > UAS-Caz-IR363–399/ter94K12875 (E) show rough-eye phenotypes that is enhanced relative to that observed in flies with GMR > UAS-Caz-IR363–399 alone (C). Adult eyes from fly lines resulting from crosses of eye-specific Caz-knockdown flies with UAS-GFP (GMR > UAS-Caz-IR363–399/UAS-GFP, a responder control, F) or UAS-ter94 (GMR > UAS-Caz-IR363–399/UAS-ter94, G). The rough-eye phenotype induced by eye-specific Caz-knockdown is obviously less severe in the presence of UAS-ter94 (G) than in the presence of UAS-GFP (F). Posterior is to the right, and dorsal is to the top. The flies were developed at 28°C. Scale bars indicate 50 μm.
Effects of a loss-of-function mutation and of overexpression of ter94 on the mobility defects caused by neuron-specific Caz-knockdown

Because ALS is an age-related motor neuron disease, we examined the effect of neuron-specific Caz-knockdown on the locomotory function of adult flies of different ages by using a well-established climbing assay (29). We also examined the effects of loss-of-function or overexpression of ter94 on changes in climbing ability caused by neuron-specific Caz-knockdown. All the fly strains showed an age-dependent decline in the climbing ability (Fig. 4). Neuron-specific Caz-knockdown flies carrying elav>UAS-Caz-IR showed a significantly decreased climbing ability at the following days of age: day 7, −10.4%; and day 21, −16.8%, P < 0.001; day 14, −10.6%, P < 0.01; day 28, −14.7%, P < 0.05 (Fig. 4A, gray columns). Flies carrying the strongest loss-of-function allele of ter94 and neuron-specific Caz-knockdown (elav>UAS-Caz-IR/ter94^K15502^) had significantly worse locomotive ability than did flies with neuron-specific Caz-knockdown alone (elav>UAS-Caz-IR) for every age examined (day 3, −14.0%; day 7, −19.5%; day 14, −35.2%; day 21, −49.7%; day 28, −63%; P < 0.001, Fig. 4A, black columns). Conversely, flies that overexpressed wild-type ter94 in the background of neuron-specific Caz-knockdown (elav>UAS-Caz-IR/UAS-ter94) had significantly better climbing ability than did control flies with neuron-specific Caz-knockdown (elav>UAS-Caz-IR/UAS-GFP) until day 14 (day 3, +24.8%; day 14, +21.8%, P < 0.001; day 7, +15.5%, P < 0.01; Fig. 4B, black columns), but not after that (day 21, not significant, P = 0.98; day 28, reduced climbing ability, −37.4%, P < 0.01; Fig. 4B, black columns). There were no significant differences in climbing abilities among elav+/ (a driver control), UAS-Caz-IR+/ (a responder control) and ter94^K15502^/+ flies in each day after exclusion that was monitored until 14 days (Supplementary Material, Fig. S2). Flies carrying elav>UAS-Caz-IR/UAS-GFP exhibited a significantly decreased climbing ability for every age examined (day 3, −32.8%; day 7, −23.8%; day 14, −26.1%; day 21, −42.4%; day 28, −47.9%, P < 0.001; Fig. 4B, gray columns) compared with those carrying elav+/ (Fig. 4B, white columns). Until day 3, the climbing ability of flies carrying elav>UAS-Caz-IR/UAS-ter94 was recovered almost as well as that of flies carrying elav+/. However, that of flies carrying elav>UAS-Caz-IR/UAS-ter94 was not fully recovered, and significantly less than that of those carrying elav+/ after day 3 at the following days of age: day 7, −12.8%; day 21, −42.4%; day 28, −27.8%, P < 0.001; day 14, −10.0%; P < 0.01 (Fig. 4B, black columns) compared with those carrying elav+/ (Fig. 4B, white columns). Together, our data demonstrate that neuron-specific Caz-knockdown leads to a severe progressive locomotive defect in adult flies, and that overexpression of wild-type ter94 could rescue the locomotive defect until certain fly ages, whereas loss of ter94 function significantly exacerbated this Caz-knockdown defect throughout the adult life span of these flies. We next examined the fly life span of the fly models with neuron-specific Caz-knockdown and genetically modified ter94. There were no significant differences in life spans among the control flies carrying elav+/ (the average life span = 50.9 days, n = 151), neuron-specific Caz-knockdown flies carrying elav>UAS-Caz-IR (48.3 days, n = 123), and flies carrying the strongest loss-of-function allele of ter94 and neuron-specific Caz-knockdown (elav>UAS-Caz-IR/ter94^K15502^, 47.5 days, n = 120) (Supplementary Material, Fig. S3A). Similarly, there were no significant differences in life spans between neuron-specific Caz-knockdown flies carrying elav>UAS-Caz-IR/UAS-GFP (responder control, the average life span = 44.8 days, n = 140) and those carrying ter94 overexpression in the background of neuron-specific Caz-knockdown, elav>UAS-Caz-IR/UAS-ter94 (41.1 days, n = 140) (Supplementary Material, Fig. S3B). In our Caz-knockdown fly models, the expression of Caz protein was decreased to 40–60% in the CNS, but their life spans were not reduced (19). These results suggest that the substantial expression of Caz in neuronal tissues, even though it is not fully expressed, could sufficiently keep their life spans within normal range.

The effects of loss-of-function mutation and overexpression of ter94 on the morphology of MN presynaptic terminals in the NMJs of neuron-specific Caz-knockdown flies

Based on the finding that our Caz-knockdown flies showed motor deficits in the climbing assay (19) and the fact that FUS, the human ortholog of Caz, is involved in ALS that impairs motor neurons, we analyzed the morphology of MN presynaptic
Neuron-specific Caz-knockdown reduces Caz signal in nuclei within larval CNS, and this reduction is significantly exacerbated by a loss-of-function ter94 mutation. (A)–(F) are representative images of corresponding genotypes. (A1)–(A3) are immunofluorescent images of larval CNS, which comprises BVGC, taken from a driver control larva carrying elav+/+. (C1)–(C3) are BVGCs of a Caz-knockdown larva carrying UAS-Caz-IR. (E1)–(E3) are the BVGCs of a larva co-expressed with ter94K15502 in the background of Caz-knockdown carrying elav>UAS-Caz-IR/ter94K15502. (B1)–(B3), (D1)–(D3) and (F1)–(F3) are higher magnification images of the boxed area in (A1), (C1) and (E1), respectively. (B1+B2), (B1+B3), (D1+D2), (D1+D3), (F1+F2) and (F1+F3) are merged images. The indirect immunofluorescence in A1, B1, C1, D1, E1 and F1 is signal from the polyclonal anti-Caz antibody. The fluorescence in A2, B2, C2, D2, E2 and F2 is from phalloidin, which labels actin; the fluorescence in A3, B3, C3, D3, E3 and F3 is from DAPI, which labels DNA. The BVGC of driver control larvae carrying elav/+ show ubiquitous signals from endogenous Caz (A1), but the signal intensity from endogenous Caz in the BVGC is remarkably reduced in larvae carrying elav>UAS-Caz-IR (C1). Anti-Caz antibody immunoreactivity is evident in the nuclei of neuronal cells (B1) and does not colocalize with phalloidin-stained actin filaments (B2, B1+B2). Caz does not colocalize with DAPI (B3, B1+B3). The intensity of nuclear Caz signal is significantly reduced in the BVGCs of larvae carrying...
elav>UAS-Caz-IR/UAS-GFP (Caz-IR x responder control) compared with that of driver control larvae (B1) \((P < 0.001, G)\). The larvae carrying the strongest loss-of-function allele of \(ter94\) and neuron-specific Caz-knockdown (elav>UAS-Caz-IR/ter94\(^{K15502}\)) (E1) also show remarkably reduced Caz signals in the BVGCs. The intensity of the nuclear Caz signal is significantly reduced in these larvae with the strongest loss-of-function allele of \(ter94\) and Caz-knockdown (F1), even compared with that of the Caz-knockdown larvae (D1) \((P < 0.05, G)\). The scale bars indicate 100 \(\mu m\) (A1–A3 and C1–C3) and 5 \(\mu m\) (B1–B3 and D1–D3). (G) This graph plots the mean (± SE) of the intensity of the nuclear Caz signal from third instar larvae as fluorescence emission in arbitrary units. Columns and horizontal bars show the mean and SE of 15 nuclei, respectively. **\(P < 0.001\), \(P < 0.05\).
significantly less than that of flies carrying elav+/+ (94.4 ± 8.0 μm, Fig. 5A), which was significantly decreased in neuron-specific Caz-knockdown larvae carrying elav>UAS-Caz-IR (53.8 ± 6.0 μm, Fig. 5B; P < 0.001, Fig. 5D). Furthermore, this decreased branch length caused by neuron-specific Caz-knockdown was significantly enhanced by genetic crossing with the strongest loss-of-function allele of ter94 (elav>UAS-Caz-IR/ter94R13502, 39.5 ± 1.7 μm, Fig. 5C; P = 0.035, Fig. 5D). The average number of synaptic boutons per MN was also significantly smaller in neuron-specific Caz-knockdown larvae (9.7 ± 0.5, Fig. 5B) than in control larvae (14.7 ± 1.0, Fig. 5A; P < 0.001, Fig. 5E). This decrease in the number of synaptic boutons in the neuron-specific Caz-knockdown larvae was significantly enhanced by genetic crossing with the strongest loss-of-function allele of ter94 (6.5 ± 0.5, Fig. 5C; P < 0.001, Fig. 5E). However, there were no significant differences in the size of synaptic boutons among these genotypes (Fig. 5F).

Conversely, the total branch length was significantly longer in the larvae with ter94 overexpression in the background of neuron-specific Caz-knockdown (elav>UAS-Caz-IR/ter94+; Fig. 5H and I) than in responder control larvae (elav>UAS-Caz-IR/ter94+; Fig. 5G; 110.7 ± 12.0 versus 54.7 ± 2.5 μm, P < 0.001, Fig. 5J). The total branch length in the larvae carrying elav>UAS-Caz-IR/ter94+ was also significantly increased compared with those carrying elav+/+ (94.4 ± 8.0 versus 54.7 ± 2.5 μm, P < 0.001, Fig. 5J). However, there were no significant differences about the total branch length between the larvae carrying elav+/+ and elav>UAS-Caz-IR/ter94+ (Fig. 5J). These results indicated that a loss-of-function ter94 mutation and wild-type ter94 overexpression have opposite effects on the synaptic terminal growth and morphogenesis that is impaired by Caz-knockdown. Notably, in the larvae with ter94 overexpression in the background of neuron-specific Caz-knockdown (elav>UAS-Caz-IR/ter94+), the extent of increase in the total branch length showed considerable variability (Fig. 5H and I). Of the larvae with ter94 overexpression in the background of neuron-specific Caz-knockdown, 28% had branch lengths (Fig. 5I) that were 2-fold or more elongated relative to the responder controls (Fig. 5G). The larvae carrying elav>UAS-Caz-IR/ter94+ (Fig. 5H and I) also showed significantly increased number of synaptic boutons of MN terminals (25.6 ± 3.5) compared with those carrying elav>UAS-Caz-IR/ter94+ (8.7 ± 0.5; Fig. 5G; P < 0.001, Fig. 5K). The number of synaptic boutons in the larvae carrying elav>UAS-Caz-IR/ter94+ was also significantly decreased compared with that of those carrying elav+/+ (8.7 ± 0.5 versus 14.7 ± 1.0, P < 0.01, Fig. 5K). The number of synaptic boutons in the larvae carrying elav>UAS-Caz-IR/ter94+ was significantly increased compared with that of those carrying elav+/+ (14.7 ± 1.0 versus 25.6 ± 3.5, P < 0.05, Fig. 5K).

Figure 4. A loss-of-function ter94 mutation and wild-type ter94 overexpression have opposite effects on the climbing ability of neuron-specific Caz-knockdown flies. (A) The locomotive ability of driver control flies, which carrying elav+/+ (n = 366, white columns), is significantly better than that of neuron-specific Caz-knockdown flies, which carrying elav>UAS-Caz-IR (n = 296, gray columns) for every age examined other than day 3. On each day after eclosion that was monitored, adult flies carrying elav>UAS-Caz-IR/ter94R13502 (n = 210, black columns) exhibited significantly worse climbing ability than flies carrying neuron-specific Caz-knockdown alone. (B) Conversely, adult flies carrying elav>UAS-Caz-IR/ter94R13502 (n = 215, black columns) have significantly better climbing ability than flies carrying elav>UAS-Caz-IR/ter94R13502 (n = 210, black columns) on days 3, 7 and 14, but not after day 14. The climbing ability of flies carrying elav+/+ (n = 366, white columns) is significantly better than those carrying elav>UAS-Caz-IR/ter94R13502 for every age examined, same as those in (A). Until day 3, the climbing ability of flies carrying elav>UAS-Caz-IR/ter94R13502 is recovered almost as well as that of flies carrying elav+/+. However, that of the flies carrying elav>UAS-Caz-IR/ter94R13502 is significantly less than that of flies carrying elav+/+ after day 3. Columns and horizontal bars show the mean and SE of the measurements, respectively. **P < 0.001, ***P < 0.001 and *P < 0.05.
the larvae carrying elav > UAS-Caz-IR/UAS-ter94 due to the growth of synaptic terminals. There were no significant differences in the size of synaptic boutons among these genotypes (Fig. 5L). These results indicated that Caz is required for growth of MN terminals and formation of synaptic boutons at the NMJ, and these functions of Caz at the MN terminals are affected by the levels of ter94 protein.

**DISCUSSION**

Here, we demonstrated that eye-specific and neuron-specific Caz-knockdown induced a rough-eye phenotype and locomotive dysfunction, respectively; moreover, the locomotive dysfunction was due to the degeneration of MNs. The strongest loss-of-function allele of ter94 (ter94^{k15502}) enhanced such rough-eye and locomotive-dysfunction phenotypes induced by Caz-knockdown. Conversely, the overexpression of wild-type ter94 significantly suppressed the phenotypes induced by Caz-knockdown such as rough-eye phenotype, locomotive disabilities and degeneration of MNs. Moreover, neuron-specific Caz-knockdown decreased Caz levels in nuclei, and overexpression of wild-type ter94 significantly suppressed the effects on nuclear Caz-expression levels induced by Caz-knockdown.

VCP is a member of the AAA protein family; these proteins are involved in diverse cellular functions and in a variety of physiological processes such as cell cycle regulation, membrane fusion, ER-associated degradation, ubiquitin-mediated protein degradation and nucleocytoplasmic shuttling (22–24). VCP is implicated in various neurodegenerative disorders. Mutations in the human VCP gene have been reported to cause frontotemporal dementia associated with IBMPFD or familial ALS, and VCP is consequently now considered as a causative gene for FTLD/ALS (25,26). Additionally, previous studies demonstrated that VCP is a binding partner of polyglutamine (polyQ) disease proteins with expanded polyQ tracts (huntingtin, ataxin-1, ataxin-3, ataxin-7 and androgen receptor) (32,33). Previously, the Drosophila ortholog of VCP, ter94, was identified in a screen for genetic modifiers of the eye degeneration phenotypes induced by eye-specific expression of an expanded polyQ tract (34). Moreover, VCP may be involved in diseases that are caused by changes in protein conformation; notably, VCP has been shown to colocalize with pathological protein aggregates in cases of Parkinson’s disease, dementia with Lewy bodies, superoxide dismutase 1-associated ALS and Alzheimer’s disease (32,35–37).

Our results demonstrate, for the first time, a genetic link between Caz and ter94, the Drosophila orthologs of FUS and VCP, respectively. Although it would be necessary to confirm whether that is Drosophila-specific or not, our results suggest genetic interaction between FUS and VCP in human. Genetic interaction between TDP-43 and VCP in Drosophila was demonstrated previously; IBMPFD-causing mutations in ter94 lead to redistribution of TDP-43, from the nucleus to the cytoplasm, and redistribution of TDP-43 is sufficient to induce morphologically aberrant rough eyes (24). This previous report suggests that VCP can balance the amount of TDP-43, which is a constituent of larger heteronuclear ribonucleoprotein (hnRNP) complexes, between nucleus and cytoplasm by acting as a nucleocytoplasmic shuttling molecule (Fig. 6).

In this schema, VCP functions to remove TDP-43 from RNP complexes, import TDP-43 into nuclei and degrade TDP-43 via autophagy (24,38,39). VCP might have similar functions with respect to FUS because FUS and TDP-43 have significant structural and functional similarities and are implicated in similar molecular processes (40–42). For example, TDP-43 and FUS act in the context of larger hnRNP complexes. FUS also continuously moves between the nucleus and the cytoplasm (16,17,43,44); therefore, FUS not only regulates gene expression in the nucleus, but also has important functions in the cytoplasm (5). Here, we showed that the decreased level of Caz in the nucleus and the resultant motor disturbance induced by neuron-specific Caz-knockdown could be rescued by overexpressed wild-type ter94 despite lacking any change of Caz protein in the CNS (Supplementary Material, Fig. S1A and B). If VCP has a shuttling function as shown in Figure 6, wild-type ter94 overexpression could translocate Caz from cytoplasm to nucleus because nuclear importing function of ter94 would be dominantly induced in the situation with the deficiency of Caz in the nucleus. Conversely, the loss-of-function allele of ter94 (ter94^{k15502}) exacerbated the depletion of Caz from the nucleus probably because ter94-mediated nuclear import of Caz was compromised.

It has been demonstrated that a polyQ tract can interact with VCP in Drosophila (34); specifically, either the strongest (ter94^{k15502}) or strong (ter94^{d1772}) loss-of-function allele of ter94 suppressed the eye degeneration induced by an expanded polyQ tract, whereas the overexpression of wild-type ter94 in the background of Caz-knockdown enhanced this phenotype. Additionally, a chromosomal deletion of 46C3–46E02, the genomic region that contains ter94, acted as a dominant suppressor of the polyQ-induced phenotype (34). Our present study and these previous reports together indicate that gain and loss of ter94 function rescued and exacerbated Caz-knockdown phenotypes, respectively, and that they had the converse effects on polyQ-induced phenotypes. These converse effects could be explained by the difference in disease pathogenesis; in polyQ-induced disease models, polyQ-containing pathogenic aggregates exist in nuclei of affected neurons; in contrast, Caz expression in nuclei is deficient in Caz-knockdown disease models. Overexpression of wild-type ter94, which functions in nuclear import of polyQ or Caz, would exacerbate nuclear polyQ aggregation, but could alleviate the nuclear deficiency of Caz protein.

Neuron-specific Caz-knockdown flies showed an age-dependent decline in climbing ability that was significantly worse than driver control flies for every age examined after day 7. Overexpression of wild-type ter94 significantly rescued the declined locomotor ability caused by Caz-knockdown up to day 14, but it did not rescue the phenotype at later stages. Regarding the age-dependent ability to rescue locomotive deficits, we considered the two following possible explanations. First, the elongation of the branch length of MN terminals at NMJs caused by overexpression of wild-type ter94 in neuron-specific Caz-knockdown flies may have alleviated the locomotive defects caused by neuron-specific Caz-knockdown. However, the extent of this elongation was highly variable. A previous report showed that larvae with NMJ overgrowth phenotypes exhibited mobility defects; this finding indicates that the elongation of nerve terminal branches beyond some adequate
Figure 5. A loss-of-function \textit{ter94} mutation and wild-type \textit{ter94} overexpression change the morphology of MN presynaptic terminals in the NMJ of MN4 in neuron-specific \textit{Caz}-knockdown larvae in opposite ways. A representative image of anti-horseradish peroxidase staining of muscle 4 synapses in third instar larvae with elav/+ (A; a driver control), neuron-specific \textit{Caz}-knockdown (B, elav>UAS-Caz-IR), neuron-specific \textit{Caz}-knockdown crossed with the strongest loss-of-function mutation of \textit{ter94} (C; elav>UAS-Caz-IR/ter94K15502), neuron-specific \textit{Caz}-knockdown crossed with UAS-GFP (G, elav>UAS-Caz-IR/UAS-GFP; a responder control) or neuron-specific \textit{Caz}-knockdown crossed with UAS-\textit{ter94} (H and I; different larvae with the same genotype, elav>UAS-Caz-IR/UAS-\textit{ter94}). (D and J) Total branch length of the NMJ from muscle 4 for each of the indicated genotypes. Compared with the total length of synaptic branches of MNs in driver control larvae (A), that in neuron-specific \textit{Caz}-knockdown larvae (B) is significantly decreased ($P < 0.001$, $n = 10$, D). This decrease in branch length observed in the neuron-specific \textit{Caz}-knockdown larvae (B) is significantly worsened in larvae carrying the strongest loss-of-function allele of \textit{ter94} and neuron-specific \textit{Caz}-knockdown (C) ($P < 0.05$, $n = 10$, D). Conversely, the total branch length in larvae that overexpressed wild-type \textit{ter94} in the background of neuron-specific \textit{Caz}-knockdown (H and I) is significantly longer than that in larvae carrying elav>UAS-Caz-IR/UAS-GFP (G) ($P < 0.001$, $n = 14$, J). The extent of increase in the total branch length of elav>UAS-Caz-IR/UAS-\textit{ter94} was highly variable among individual flies (H and I). The total branch length of synaptic branches of MNs in the larvae carrying elav>UAS-Caz-IR/UAS-GFP is significantly decreased compared with that of larvae carrying elav/+ ($P < 0.001$, $n = 12$, J). (E and K) The number of synaptic boutons for each of the indicated genotypes. The number of synaptic boutons of MNs in neuron-specific \textit{Caz}-knockdown larvae (B) is also significantly decreased compared with driver control larvae (A) ($P < 0.001$, $n = 10$, E). This decrease in the number of synaptic boutons in the neuron-specific \textit{Caz}-knockdown larvae is significantly worsened in larvae carrying the strongest loss-of-function allele of \textit{ter94} and neuron-specific \textit{Caz}-knockdown (C) ($P < 0.001$, $n = 10$, E). Conversely, the number of synaptic boutons in the larvae carrying wild-type \textit{ter94} overexpression in the background of neuron-specific \textit{Caz}-knockdown (H and I) is significantly higher than that in responder control larvae (G) ($P < 0.001$, $n = 10$, K). Compared with the larvae carrying elav/+, the number of synaptic...
length could cause disturbances in locomotive ability (45). Therefore, synaptic MN terminals may have to be within some optimal range of lengths. Second, the age-dependence of ability to rescue the locomotive defects might be due to the age-dependent difference in the expression levels of ter94. Tissue expression data from FlyBase (http://flybase.org) show that mRNA expression levels of ter94 are very high in the CNS of third instar larvae, but they are relatively low in the head, eye or brain of adults. Age-dependent changes in ter94 expression levels might determine the period within which wild-type ter94 overexpression can rescue locomotive deficits caused by Caz-knockdown. Age-dependent effects of ter94 are also evident in fly models of poly-Q-induced neurodegeneration. Between the third instar larval stage and the late pupal stage, levels of ter94 were elevated, and elevated levels of ter94 induced severe apoptotic cell death in those pupae (34).

In some IBMPFD-associated VCP mutants, it was previously reported that pathogenic VCPs could bind to cofactors, such as Npl4, Ufd1 or p47, more efficiently than wild-type VCP (46, 47). However, little is known about which of the VCP cofactors relate to FUS-nuclear translocation or how the conformational change of VCP affects the interactions of VCP cofactors with other proteins.

In conclusion, we found a genetic interaction between Caz and ter94. Our data indicate that chemicals that up-regulate the function of VCP or facilitate nuclear import of FUS may suppress the pathogenic processes that lead to the degeneration of MNs in FUS-associated ALS/FTLD. This might be the first step to develop candidate drugs for the disease-modifying therapy of human ALS.

**MATERIALS AND METHODS**

**Fly stocks**

Fly stocks were maintained at 25°C on standard food containing 0.7% agar, 5% glucose and 7% dry yeast. Canton S was used as the wild-type strain. The strain: w1118, P[w[+mC]=GAL4;elav.L] 3 (Bloomington BL8760) (elav-GAL4), y[w+]; P[+[+mC]=GAL4;elav.L] 3 (Bloomington BL8760) (elav-GAL4), y[w+]; P[[+mC]= Act5C-GALA4] 17bFO1/TM6B, Tb(b) (BL3954) (Act5C-GALA4), y[w+]; P[w[+mC]= lacW] ter94k15502/CyO (BL10454) (ter94k15502) and en2 P[ry[+17.2]= PZ] ter9403773/CyO; ry306 (BL11349) (ter9403773) were obtained from the Bloomington Drosophila stock center in Indiana. Establishment of the lines carrying GMR-GAL4 was as described previously (48). We crossed transgenic UAS-Caz-IR flies with Act5C-GALA4, GMR-GAL4 or elav-GAL4 flies to drive expression of Caz dsRNA throughout the whole body of flies, specifically in eye imaginal discs or specifically in neuronal tissues, respectively. We generated eye-specific Caz-knockdown flies (GMR-GAL4; UAS-Caz-IR/+); +) (GMR>UAS-Caz-IR) and neuron-specific Caz-knockdown flies (w; UAS-Caz-IR+/; elav-GAL4+/) (elav>UAS-Caz-IR). Each transgenic strain showed a consistent phenotype (Table 1).

Dr Kakizuka kindly provided UAS-ter94 flies. The UAS-ter94-IR strain: w1118, P[GD9777] v24354 (VDRC strain v24354) (ter94-knockdown) was obtained from the VDRC. VDRC reports that the ter94-RNAi construct is inserted into chromosome 2 and has no off-target effects. The lines generated in this study are as follows: GMR-GAL4; +; (GMR), GMR-GAL4; UAS-Caz-IR363–399; +; (GMR>UAS-Caz-IR), GMR-GAL4; UAS-Caz-IR363–399/UAS-Caz-IR363–399; + (GMR>UAS-Caz-IR/UAS-Caz-IR), GMR-GAL4; UAS-Caz-IR363–399; + (GMR>UAS-Caz-IR/ter94k15502), GMR-GAL4; UAS-Caz-IR363–399; + (GMR>UAS-Caz-IR/ter94k15502), GMR-GAL4; UAS-Caz-IR363–399; + (GMR>UAS-Caz-IR/UAS-Caz-IR), GMR-GAL4; UAS-Caz-IR363–399; + (GMR>UAS-Caz-IR/ter94k15502), GMR-GAL4; UAS-Caz-IR363–399; + (GMR>UAS-Caz-IR/UAS-Caz-IR), GMR-GAL4; UAS-Caz-IR363–399; + (GMR>UAS-Caz-IR/UAS-Caz-IR), GMR-GAL4; UAS-Caz-IR363–399; + (GMR>UAS-Caz-IR/UAS-Caz-IR), GMR-GAL4; UAS-Caz-IR363–399; + (GMR>UAS-Caz-IR/UAS-Caz-IR), GMR-GAL4; UAS-Caz-IR363–399; w; +; elav-GAL4+/ (elav+; a driver control), UAS-Caz-IR363–399; + (UAS-Caz-IR; a responder control), ter94k15502; w; UAS-Caz-IR363–399; + (elav>UAS-Caz-IR), w; UAS-Caz-IR363–399/UAS-Caz-IR363–399;
Rabbit anti-Caz antibodies were raised against amino acid residues 29–45 and 383–399 of Caz and were produced previously (19). For immunohistochemical analysis, CNS tissues were dissected from third instar larvae and fixed in 4% paraformaldehyde/phosphate buffered saline (PBS) for 15 min at 25°C. These tissue samples were washed with PBS containing 0.3% Triton X-100; fixed samples were then incubated with Alexa 488-conjugated phalloidin (1 unit/200 µl) in PBS containing 0.3% Triton X-100 for 20 min at 25°C. The samples were then blocked with blocking buffer (PBS containing 0.15% Triton X-100 and 10% normal goat serum) for 30 min at 25°C, and then incubated with 1:1000 diluted rabbit anti-Caz antibody in the blocking buffer for 20 h at 4°C. After extensive washing with PBS containing 0.3% Triton X-100, samples were incubated in the dark with secondary antibodies labeled with Alexa 546 (1:400; Invitrogen) diluted in the blocking buffer for 3 h at 25°C. After washing with PBS containing 0.3% Triton X-100, the samples were stained with DAPI (0.5 μg/ml)/PBS/0.1% Triton X-100. After extensive washing with PBS containing 0.1% Triton X-100 and PBS, the samples were mounted in Vectashield (Vector Laboratories-Inc.) and observed under a confocal laser scanning microscope (OLYMPUS FLUOVIEW FV10i). Images were analyzed with the program MetaMorph Imaging System 7.7 (Molecular Devices Inc.). The use of this program made it possible to quantify the average and the standard error of fluorescence emission from nuclei of each fly strain.

For NMJ staining, third instar larvae were dissected in HL3 saline (49), and then fixed in 4% paraformaldehyde/PBS for 30 min. The blocking buffer contained 2% bovine serum albumin and 0.1% Triton X-100 in PBS. Fluorescein isothiocyanate-conjugated goat anti-horseradish peroxidase (HRP) (1:1000, MP Biochemicals) was used as the detection antibody. The samples were mounted and observed under a confocal laser scanning microscope (OLYMPUS FLUOVIEW FV10i). Images were analyzed with the program MetaMorph Imaging System 7.7 (Molecular Devices Inc.). The use of this program made it possible to quantify the average and the standard error of fluorescence emission from nuclei of each fly strain.

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Immunoblotting analysis

Protein extracts from the CNS of Drosophila carrying elav+/+, elav>UAS-Caz-IR, elav>UAS-Caz-IR/ter94 K15502, elav>UAS-Caz-IR/UAS-GFP and elav>UAS-Caz-IR/UAS-ter94 larvae were prepared as described previously (19). Briefly, the CNS was excised from third instar larvae and homogenized in a sample buffer containing 50 mM Tris–HCl (pH 6.8), 2% sodium dodecyl sulfate (SDS), 10% glycerol, 0.1% bromophenol blue and 1.2% β-mercaptoethanol. The homogenates were boiled at 100°C for 5 min and then centrifuged. The supernatants (extracts) were electrophoretically separated on SDS-polyacrylamide gels containing 12% acrylamide and then transferred to polyvinylidene difluoride membranes (Merck, Millipore, MA, USA). The blotted membranes were blocked with tris-buffered saline/0.05% Tween containing 5% skim milk for 1 h at 25°C, followed by incubation with rabbit polyclonal anti-Caz at a 1:5000 dilution for 16 h at 4°C. After washing, the membranes were incubated with HRP-conjugated anti-rabbit IgG (Thermo Scientific, IL, USA) at 1:10 000 dilution for 2 h at 25°C. Antibody binding was detected using ECL Western blotting detection reagents (Thermo Scientific) and images were analyzed using an ImageQuant™ LAS 4000 image analyzer (GE Healthcare Bioscience, Tokyo, Japan). To compare Caz protein levels in the CNS extracts of those larvae, densitometric quantification of the 45-kDa Caz protein bands was carried out. The relative band intensities were quantified and normalized to Coomassie Brilliant Blue staining, then expressed as the percentage of the band intensity derived from larvae carrying elav/+.

Scanning electron microscopy

Adult flies were anesthetized with 99% diethyl ether, mounted on stages and observed under an SEM V-7800 (Keyence Inc.) in the low vacuum mode (50). In every experiment, at least five adult flies were chosen from each line for scanning electron microscopy to assess the eye phenotype. For each experiment, there was no significant variation in eye phenotype among the five individuals from the same strain.

Longevity assay

Longevity assays were carried out in a humidified, temperature-controlled incubator set at 25°C and 60% humidity on a 12-h light and 12-h dark cycle; flies were maintained on standard fly food. Flies carrying elav/+ (n = 151), elav>UAS-Caz-IR (n = 123), elav>UAS-Caz-IR/ter94 K15502 (n = 120), elav>UAS-Caz-IR/UAS-GFP (n = 140) or elav>UAS-Caz-IR/UAS-ter94 (n = 140) were placed at 28°C, and newly eclosed adult male flies were separated and placed in vials at a low density (20 flies per vial). Every 3 days, they were transferred to new tubes containing fresh food and deaths were scored. The survival rate was determined by plotting a graph of the percentage of surviving flies among total flies at the starting point of each experiment versus days.

Climbing assay

Climbing assays were performed as described previously (29). Flies carrying elav+/+, UAS-Caz-IR/+; ter94 K15502/+, elav>UAS-Caz-IR, elav>UAS-Caz-IR/ter94 K15502, elav>UAS-Caz-IR/UAS-GFP and elav>UAS-Caz-IR/UAS-ter94 were placed at 28°C, and newly eclosed adult male flies were separated and placed in vials at a density of 20 flies per vial. Flies were transferred, without anesthesia, to a conical tube. The tubes were tapped to collect the flies to the bottom, and they were then given 30 s to climb the wall. After 30 s, the flies were collected at the bottom by tapping of the tube and were again allowed to climb for 30 s. Similar procedures, all of which were videotaped, were repeated five times in total. For each climbing experiment, the height to which each fly...
climbed was scored as score (height climbed); 0 (less than 2 cm), 1 (between 2 and 3.9 cm), 2 (between 4 and 5.9 cm), 3 (between 6 and 7.9 cm), 4 (between 8 and 9.9 cm) or 5 (greater than 10 cm). The climbing index for each fly strain was calculated as follows; each score was multiplied by the number of flies for which that score was recorded, and the products were summed up, then divided by five times the total number of flies examined. These climbing assays were carried out every 7 days until the 28th day after eclosion.

Data analysis
GraphPad Prism version 6.0 was used to perform each statistical analysis. The Mann–Whitney test was used for the assessment of the statistical significance of comparisons between two groups of data. For other assays, one-way analysis of variance (ANOVA) was used to determine the statistical significance of comparisons between groups of data. When the two-way ANOVA showed significant variation among groups, a subsequent Dunnett’s test was used for pairwise comparisons between groups. All data are shown as mean ± standard error (SE).

SUPPLEMENTARY MATERIAL
Supplementary Material is available at HMG online.

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Conflict of Interest statement. None declared.

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