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Age-dependent deterioration of locomotion in *Drosophila melanogaster* deficient in the homolog of *Amyotrophic Lateral Sclerosis 2*

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Short title: *Drosophila* ALS2 mutant

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Abstract

Recessive mutations in the *amyotrophic lateral sclerosis 2 (ALS2)* gene have been linked to juvenile-onset ALS2. Although one of the molecular functions of the ALS2 protein is clearly the activation of Rab5, the mechanisms underlying the selective dysfunction and degeneration of motor neurons in vivo remain to be fully understood. Here we focused on the *ALS2* homolog of *Drosophila melanogaster*, isolated two independent deletions, and systematically compared phenotypes of the mutants with those of animals in which Rab5 function in identified neurons was abrogated. In the *dALS2* mutant flies, we found that the stereotypic axonal and dendritic morphologies of neurons shared some features with those in Rab5-deficient flies, but the *dALS2* mutant phenotypes were much milder. We also found that the abrogation of Rab5 function in motor neurons strongly depressed the locomotion activity of adults, resembling the behavior of aged *dALS2* mutants. Importantly, this age-dependent locomotion deficit of *dALS2* mutants was restored to normal by expressing the *dALS2* transgene in a wide range of tissues. This finding provided a platform where we could potentially identify particular cell types responsible for the phenotype by tissue-specific rescue experiments. We discuss our results and the future utility of the *dALS2* mutant as a new ALS model.

Introduction

Motor neuron diseases (MNDs) are a group of progressive neurological disorders. They are caused by degeneration of upper motor neurons (UMNs) of the motor cortex in brain and lower motor neurons (LMNs) in brainstem and spinal cord. One of the best-characterized forms of MND is amyotrophic lateral sclerosis (ALS), in which loss of UMNs with or without LMN involvement leads to muscle atrophy, fasciculations, and spasticity (Robberecht & Philips. 2013; Boillee *et al.* 2006; Pasinelli & Brown 2006). To elucidate the molecular pathogenesis for these diseases, the functions of the causative genes have been investigated. One of these is *ALS2*, which was originally discovered through genetic mapping and the identification of recessive mutations in juvenile-onset ALS families, and its clinical features include spasticity of limb and facial muscles accompanying distal amyotrophy of hands and feet (Ben Hamida *et al.* 1990; Hadano *et al.* 2007; Cai *et al.* 2008; Kato 2008).

Human *ALS2* protein (often designated as Alsln) comprises several domains that are characteristic of guanine nucleotide exchange factors (GEFs): an RCC1-like domain (RLD), the Dbl homology and pleckstrin homology (DH/PH), and the vacuolar protein sorting 9 (VPS9) domain. The VPS9 domain of *ALS2* specifically binds to Rab5 and functions as its GEF (Otomo *et al.* 2003; Kunita *et al.* 2004). The majority of *ALS2* mutations generate premature termination codons, resulting in protein instability and loss of function of *ALS2* (Yamanaka *et al.* 2003). Cell biological assays

suggest that *ALS2* dysfunction affects multiple contexts of endosomal trafficking through a Rab5-mediated mechanism (Devon *et al.* 2006; Hadano *et al.* 2006; Otomo *et al.* 2008; Lai *et al.* 2009), including formation of amphisomes, the hybrid-organelle formed upon fusion between autophagosomes and endosomes (Otomo *et al.* 2011). Although it has been hypothesized that this loss of GEF function could result in neuronal dysfunction and degeneration, mechanisms that lead to a selective dysfunction and degeneration of motor neurons *in vivo* remain to be fully understood.

To study the underlying pathologic mechanisms of *ALS2* deficiency *in vivo*, *ALS2* knockout mice have been generated and characterized (Cai *et al.* 2005; Devon *et al.* 2006; Hadano *et al.* 2006; Yamanaka *et al.* 2006). Unexpectedly, the KO mice do not necessarily recapitulate clinical or neuropathological phenotypes consistent with the motor neuron disease. The KO mice do not show apparent overall locomotion deficits in their lifetime, except for a mild motor deficit in the Rotarod test (Deng *et al.* 2007) and slowed movement (Yamanaka *et al.* 2006); and no significant difference has been observed in the numbers of cortical or spinal motor neurons when compared to wild-type control mice (Cai *et al.* 2008). On the other hand, the KO mice are more vulnerable to oxidative stress than the wild-type controls (Cai *et al.* 2005), and expression of a pathogenic form of SOD1 (superoxide dismutase 1) in the *ALS2* KO background aggravates the *SOD1*-associated disease symptoms such as body weight loss, motor dysfunction, and the accumulation of insoluble ubiquitinated proteins in the nervous system,

leading to earlier death (Hadano *et al.* 2010).

Here we attempted to dissect the *in vivo* roles of ALS2 in the disease by taking advantage of another model organism, *Drosophila melanogaster*, for two main reasons. First, many of the human genes responsible for neurodegenerative diseases are conserved in the *Drosophila* genome, including *ALS2* (see below). Second, a number of versatile *Drosophila* genetic tools and knowledge of well-characterized stereotypic morphologies of neuronal subtypes can be leveraged to provide mechanistic insights on MND (Lessing & Bonini 2009; Jaiswal *et al.* 2012). For example, the *Drosophila* *TBPH* encodes a human homolog of TDP-43 that is found in cytoplasmic inclusions in ALS (Feiguin *et al.* 2009), and studies of its tissue-specific knockdown suggest that that impaired pre-synaptic transmission is one of the earliest events in TDP-43-related pathogenesis (Diaper *et al.* 2013). We focused on the *Drosophila* homolog of *ALS2*, isolated its mutants, characterized both anatomical and behavioral phenotypes, and compared these to what has been shown in human patients and KO mice.

Results & Discussion

CG7158 encodes a homolog of ALS2

Annotated genes in the genome of *Drosophila melanogaster* include homologs of four human GEFs and two GTPase activating proteins (GAPs) (Figure 1A and S1). Among them, the predicted protein product of a single gene *CG7158* shows similarities to ALS2/Alsin. Hereafter, *CG7158* and its protein product are designated as *dALS2* and dALS2 for simplicity, respectively. Both ALS2/Alsin and dALS2 contain four domains in the same order from their amino-terminals (Figure 1A), among which the “membrane occupation and recognition nexus (MORN)” motifs and the carboxyl-terminal “vacuolar protein sorting 9 (VPS9)” domain in human ALS2 are necessary for its selective GEF activity for Rab5 in vitro (Otomo *et al.* 2003). One distinctive difference between dALS2 and ALS2/Alsin is that dALS2 lacks a DH domain, which is adjacent to the PH domain in human ALS2 and provides a basis of its GEF activity for Rac1 (Kunita *et al.* 2007). Thus dALS2 could be a more Rab5-specific GEF compared to ALS2.

CG7158/dALS2 has a GEF activity for Rab5

To examine whether dALS2 does possess GEF activity or not, we co-expressed dALS2 and a fluorescence resonance energy transfer (FRET) probe, Raichu-Rab5 in *Drosophila* S2 cells (Kitano *et al.* 2008). The probe comprised Venus (a modified YFP), the amino-terminal Rab5-binding domain of EEA1, SECFP (a modified CFP), and Rab5. In this probe design, the increase

in the emission ratio reflects an increase in the active GTP-bound form of Rab5 relative to the inactive GDP-bound form in living cells (Kitano *et al.* 2008). The emission ratio was increased significantly when dALS2 was co-expressed (the blue bar of “dALS2[WT]” in Figure 1B) compared to the transfection of the vector (the blue bar labeled “Control” in Figure 1B). This increase in the ratio was indeed dependent on the conversion from the GDP-bound form to the GTP-bound one, as shown by the fact that the emission ratio of Raichu-Rab5[S34N], a constitutive GDP-bound form (Entchev *et al.* 2000), was unchanged even in the presence of dALS2 (the red bar labeled “dALS2[WT]” in Figure 1B). We also studied the effects of substitutions of two conserved amino acid residues in the VPS9 domain (Figure 1A and S2), which are necessary for full GEF activity of ALS2 *in vitro* (Otomo *et al.* 2003). The two mutant forms of dALS2 (dALS2[P1425A] and dALS2[L1439A]) increased the FRET efficiency of Raichu-Rab5, but not to the same extent as the wild-type form (Figure 1B). Collectively, these results showed that the wild-type form of dALS2 has GEF activity for Rab5.

***dALS2* mutants are viable and fertile**

To study the *in vivo* consequences of *dALS2* dysfunction, we mobilized an existing transposable element that was inserted 120 bp upstream of the 1st ATG of the *dALS2* coding sequence and isolated two independent alleles (*Ex44* and *Ex54*) that delete about 30% of the coding sequence, including the start codon and the entire RLD domain (Figure 2A). We also obtained precise jumpers, where the transposon excision restored the exact contiguous WT

sequence, and used homozygotes of two of these (*Ex101/Ex101* and *Ex95/Ex95*) for subsequent analysis as controls or the wild-type animals. Homozygotes of either *Ex44* or *Ex54* were viable and fertile; and the adults looked morphologically normal. Thus *dALS2* may be dispensable for viability in flies, as is the case in mice (Cai *et al.* 2005; Devon *et al.* 2006; Hadano *et al.* 2006; Yamanaka *et al.* 2006). For further phenotypic analysis, we mostly employed *Ex44* with a larger deletion than *Ex54*, unless described otherwise, and designated *Ex44* homozygotes as *dALS2*^{-/-}.

RT-PCR analysis confirmed the deletion of the amino-terminal coding sequence of *dALS2* in adult *dALS2*^{-/-} flies (Figure 2B). To address whether truncated polypeptides might be made by translation initiation from internal ATG codons downstream of the deletion in *dALS2*^{-/-}, we generated antibodies to the carboxyl-terminal VPS9 domain. Unfortunately however, our antibodies failed to detect endogenous *dALS2* with high sensitivity (data not shown), thus we could not exclude the possibility of the generation of the truncated polypeptides. Nonetheless, it is known that *ALS2* without the RLD domain no longer associates with endosomes (Yamanaka *et al.* 2003), so the truncated *dALS2* polypeptides, if synthesized from *Ex44* and *Ex54* alleles, would most likely not be functional.

It is shown that *Rab5* is widely expressed, albeit with different expression levels in different tissues including larval and adult nervous systems and imaginal discs (Chan *et al.* 2011). *dALS2* is also expressed in a broad range of tissues including the nervous system and the epidermis in the embryo (Figure 2C), larval central nervous system, adult brains, and larval

and adult guts (St Pierre *et al.*).

Morphological analysis of axon terminals and dendritic arborization of identified neurons in the *dALS2* mutant and in wild-type flies expressing a dominant-negative form of Rab5

We searched for neurological disorders in the *dALS2*^{-/-} mutant. In parallel, we addressed how far we could explain the detected phenotypes of the mutants as the dysregulation of Rab5 activity. To establish a basis for comparison, we expressed a dominant negative form of Rab5[S34N] (Entchev *et al.* 2000) in wild-type neurons and addressed whether this caused effects reminiscent of the mutant phenotypes (Figure 3).

We initially focused on presynaptic terminals of motor axons that innervate abdominal body wall muscles and form neuromuscular junctions (NMJs) with stereotyped connectivity (Kohsaka *et al.* 2012; Menon *et al.* 2013). To visualize presynaptic terminals in larvae, we double-labeled presynaptic membranes and active zones (anti-HRP and anti- Bruchpilot (Brp), respectively; Coyle *et al.* 2004) and quantified the number of Brp-positive boutons (Figure 3A). The number of the boutons that was normalized by the area of the innervated muscle was not significantly different between the wild type and the mutant (Figure 3B). We did not find a noticeable difference in the number of postsynaptic marker (Mhc-CD8:YFP:Sh; Zito *et al.* 1999) -positive boutons; neither did we find a significant difference in the size of the muscles imaged in Figure 3A and 3C (data not shown).

We also imaged motor axons in whole-mount adults using a transgenic

marker (Figure 3E; Hebbar *et al.* 2006) and found that there was no noticeable defect in axonal growth in mutants at the ages of 3-4 days and 3 weeks. We further measured the area of each presynaptic arbor and counted the number of boutons at the ventral NMJ in 2 week-old adults (the magenta-line polygons of Figure 3F, and Figure 3G-3I). Although the normalized bouton number was not significantly different between the two genotypes (Figure 3I), the absolute value of the bouton number per terminal was increased in the mutant (Figure 3H).

To express Rab5[S34N] in the motor neuron, we used *OK371-Gal4*, an enhancer trap line where the *Gal4* gene is inserted in the gene encoding a vesicular glutamate transporter promoter (Mahr & Aberle 2006). Rab5[S34N] expression resulted in an increase in the bouton number of presynaptic terminals of motor axons in larvae and adults (Figure 3C, 3D, 3J, and 3K), and the Rab5[S34N] effect was more dramatic than the phenotype in the *dALS2* mutant. In addition to the increase in the number of boutons, each bouton became smaller than that of the control axon terminals at larval NMJs (Figure 3C). From earlier experiments with Rab5[S34N], it is known that Rab5 controls synaptic transmission at larval NMJs (Wucherpfennig *et al.* 2003). In that study, Rab5[S34N] expression was kept low during embryonic and early larval stages so that it did not affect morphological development of NMJs.

We employed dendritic arborization (da) neurons as another assay system (Figure S3A; Jan & Jan 2010; Shimono *et al.* 2009). Previously, we showed that Rab5[S34N] expression or a *rab5* null mutation strongly downsizes and simplifies dendritic arbors of class IV da neurons (Sato *et al.* 2008). Our

quantifications of the da neurons in the *dALS2* mutant showed mild defects in dendrite morphogenesis (Figure S3B and S3C): the total dendritic length and the number of branch endings per cell decreased by 11 % and 19%, respectively, relative to wild type, while the coverage index was not altered (Figure S3C). The overexpression of *dALS2*[WT] did not cause a significant effect on morphological development of the NMJs or the dendritic arbors (Figure 3D and data not shown).

Collectively, the *dALS2* mutant exhibited much milder phenotypes compared to the severe effects of the abrogation of Rab5 function. This might be explained by a contribution of at least one of the other Rab5 GEFs, Rabex5, (Figure S1) to regulation of synaptic vesicles and dendrite morphogenesis (Mori *et al.* 2013; Sann *et al.* 2012).

Mutant adults showed a lowered locomotion activity

In addition to the cell morphological phenotypes, we found that Rab5[S34N] expression in the motor neurons strongly depressed the climbing ability of adults (Figure S4A and supplemental movies 1 and 2). This prompted us to perform the climbing assay with the *dALS2*^{-/-} mutant adults, which revealed the most prominent phenotype, and it was an age-dependent locomotion deficit that was causally related to loss of *dALS2* function (Figure 4 and 5).

The locomotion assay employed was the negative geotaxis assay, which is an established method to test the climbing ability of *Drosophila* (Figure S4B-S4D; Ali *et al.* 2011; see details in Experimental procedures). We reared the *dALS2*^{-/-} mutants with either of the controls (*Ex101/Ex101* or

Ex95/Ex95) at the same time, and examined the climbing ability of the mutant flies (Figure 4). We found that the adult mutants showed a locomotion defect at 2 weeks after eclosion, but not at 1 week or younger ages. At 4 weeks after eclosion, most of the mutant females failed to reach a fixed height within the allowed time (Figure S4C and S4D).

The locomotion deficit of the mutant was rescued by *dALS2* transgene expression

We addressed whether or not this adult-onset climbing disability could be restored by tissue-specific expression of the wild-type *dALS2* cDNA. We started with broad expression, because *dALS2* is also expressed outside the nervous system (St Pierre *et al.*), and because it has been suggested, but not rigorously demonstrated, that the motor neuron is the primary locus of ALS2 action in mice.

To realize broad expression, we used *Ubiquitin (Ubi)-Gal4* to drive expression of the wild-type *dALS2* transgene in a wide range of tissues (Figure 5). As a negative control for the transgene expression, we tested the effect of *mmRFP* expression on the climbing ability. 2 week-old *dALS2*^{-/-} adults showed lowered climbing ability, compared to wild-type, and this phenotype was restored to normal by *dALS2* transgene expression in both females and males (compare blue and green data points in Figure 5B). These results showed that the age-dependent locomotion deficit is indeed a loss-of-function phenotype of *dALS2*. This phenotype is reminiscent of the moderate, age-dependent deficit in motor coordination in *ALS2*-null mice

(Cai *et al.* 2005).

We next attempted to address whether the cell-type-specific *dALS2* transgene expression in motor neurons could rescue the mutant phenotype (Figure 6). In this experiment, we employed *OK371-Gal4*. This rescue strategy, however, made the assessment of the recovery difficult. This is primarily because the *dALS2* expression in the wild-type motor neurons caused a deleterious effect on the climbing activity (compare red data points with orange ones in Figure 6). The *dALS2*-expressing aged mutants of both females and males tended to improve the climbing ability when compared to *mRFP*-expressing aged-matched adults (compare blue points with green ones in Figure 6B), although our results did not reach statistical significance.

The above results can be interpreted in several ways. First, *dALS2* is indeed required in the motor neuron; however, the motor neuron *GAL4* driver (*OK371-Gal4*) failed to correct the phenotype significantly because this *Gal4*-driven expression of *dALS2* far exceeds the physiological range and disturbs precise spatial-temporal regulation of Rab5 activity. Second, *dALS2* is supplied in the motor neuron at larval stages by *Ubi-GAL4* and a portion of the proteins persist and function at the adult stage (we found that *Ubi-GAL4* is expressed in the motor neuron in larvae, but not in adults). Third, *dALS2* is critically required in cell types other than the motor neuron to prevent the deterioration of locomotion during aging (e.g., the presumptive “upper” motor neuron in flies); and *Ubi-GAL4*, not *OK371-Gal4*, is expressed in that cell type. Use of the rich resource of *GAL4* stocks (Jenett *et al.* 2012) and searches for the stocks that realize appropriate expression levels of

dALS2 would allow us to distinguish these possibilities.

Aging, longevity, and sensitivity to oxidative stress of the *dALS2* mutant

It is known that even wild-type flies decline in climbing performance over time (Ganetzky & Flanagan 1978; Le Bourg & Lints 1992). Therefore, we suspected that aging might be accelerated in the *dALS2* mutant and/or that the mutant might be short-lived. As a part of normal aging, the expression of several essential autophagy genes decline in fly neural tissues, which is causative of accumulations of ubiquitinated proteins and p62/Ref(2)P (Bartlett *et al.* 2011; Simonsen *et al.* 2008; Tonoki *et al.* 2009). This has been shown by the facts that ubiquitin-positive puncta precociously appear in *atg8* mutants, which are defective in autophagy, and that enhanced neuronal *Atg8a* expression suppresses the accumulation of insoluble ubiquitinated proteins (Simonsen *et al.* 2008). Therefore we examined levels of ubiquitinated proteins and p62 in the wild-type and the *dALS2* mutant adults at distinct ages (Figure S5).

At the age of 3-4 days after eclosion, ubiquitin-positive puncta were barely detected immunohistochemically in any regions of the brains of the wild type or the *dALS2* mutant, whereas they were found in 2 week-old adults irrespective of the genotypes (Figure S5A). Via western blot analysis of whole lysates, we did not find overt differences in the levels of ubiquitinated proteins and p62, between the genotypes, at either of the two ages investigated (Figure S5B); and we did not find a noticeable difference in the amount of insoluble ubiquitinated proteins (IUP), either (data not shown). In contrast to the *dALS2* mutant, ubiquitin-positive puncta precociously appeared in *atg8* mutants as

previously reported (data not shown). Thus we did not find evidence for accelerated aging in the *dALS2* mutant and our results suggest that *dALS2* plays a relatively minor role in protein homeostasis, compared to the core machinery of autophagy.

Unexpectedly, the mutant (*Ex44/Ex44*) exhibited a significantly longer life span than either of the two wild type stocks (*Ex101/Ex101* or *Ex95/Ex95*) under our experimental protocol (Figure S6A-S6D). However, the *Ubi-Gal4*-dependent *dALS2* transgene expression failed to normalize the life span of *Ex44/Ex44* (data not shown); and homozygous females of another deletion allele (*Ex54/Ex54*) did not exhibit a similarly significant longevity phenotype (Figure S6E-S6F). Thus we could not conclude that the longevity phenotype of *Ex44/Ex44* was due to the loss of *dALS2* function. Nonetheless, it is fairly safe to conclude that the loss of *dALS2* function does not reduce the life span.

Intrigued by the fact that the *ALS2* KO mice are more sensitive to oxidative stress than the wild-type controls (Cai *et al.* 2005), we recorded survival of the wild type and *dALS2* mutant adult flies (*Ex44/Ex44* or *Ex54/Ex54*) when they were exposed to hydrogen peroxide-containing food (Vrailas-Mortimer *et al.* 2011). Under this condition, *Ex44/Ex44* females, but not *Ex44/Ex44* males, were more sensitive to the stress than the wild-type females, whereas we did not obtain reproducible results when *Ex54/Ex54* adults were tested. Again, we were unable to restore the supersensitive phenotype of *Ex44/Ex44* females by the *Ubi-Gal4*-dependent *dALS2* rescue experiment (data not shown).

Future utility of the *Drosophila* ALS models

In this study, we pursued the possibility that the *Drosophila dALS2* mutant could become a new animal model for investigating mechanistic insights of the disease. Our discovery of the age-dependent locomotion deficit has provided a platform, where we could potentially specify the responsible cell types, verify the common belief that the primary focus of *dALS2* action is the UMN, and screen genetic backgrounds or drugs that could alleviate or possibly prevent the onset of the symptoms.

In addition to animal behaviors and neuronal cell morphologies, the absence of *dALS2* function could impact synaptic transmission. Control of Rab5 activity is required for normal development of NMJ as described above; in addition, Rab5 regulates the efficacy of the evoked neurotransmitter release once the NMJ is formed (Wucherpfennig *et al.* 2003). So NMJs in the *dALS2* mutant could be a target of physiological and ultrastructural investigations. Other future targets are pre-motor interneurons that control the neurotransmitter release at NMJ and further upstream neural circuits, which are functional counterparts of UMNs in mammals. Identification of such neurons and technical accessibility to those would allow us to readdress whether the markers of neuronal aging and/or the *Drosophila* homolog of TDP-43 are accumulated in those particular neuronal classes, and this approach may validate *Drosophila* as a tractable model of not only *ALS2* but also other genetic causes of ALS.

Experimental procedures

Fly stocks

We used the Gal4-UAS system (Brand & Perrimon 1993) to express transgenes and to visualize motor neurons and da neurons. Gal4 driver strains employed were *Gr-Gal4* lines (Weiss *et al.* 2011), *OK371-Gal4* (Mahr & Aberle 2006), and *Ubi-Gal4* (the Bloomington Stock Center). UAS stocks and others were *UAS-mCD8:GFP* and *UAS-myr-mRFP* (*mmRFP*; the Bloomington Stock Center), *UAS-mCD8:3xEGFP* (Kakihara *et al.* 2008), *UAS-Rab5[S43N]* (Entchev *et al.* 2000), *Mhc-CD8:YFP:Sh* (Zito *et al.* 1999), and *atg8²* (the Bloomington Stock Center). The P element of *CG7158^{G4607}* (the Bloomington Stock Center) was mobilized, and a total of one hundred and fifty excision lines were established. Genomic deletions within the *CG7158* locus were identified by genomic PCR using primers (sequences of the two primers in Figure 2A are 5'-GCGTATGCAAACCAGCAGTA-3' and 5'-CCCGAAATCTTTGAGCTGAG-3') and subsequent direct sequencing. Transgenic flies were generated by a standard method.

Molecular cloning

The FRET probes, Raichu-Rab5[WT] and Raichu-Rab5[S34N], are described (Kitano *et al.* 2008) and the DNA fragment encoding *Raichu-Rab5* was inserted into a pUAST-based vector. The full-length coding sequence (CDS) of *dALS2/CG7158* was amplified using pOT2-LD33266 (Berkeley Drosophila Genome Project) as a template and inserted into pUAST-3xHA to generate

pUAST-3xHA:dALS2[WT]. CDSs of *dALS2[P1425A]* and *dALS2[L1439A]* were generated by site-directed mutagenesis using two single-primer reactions. The primers were 5'-GGTGAGCGCTATCATGTTGT-3' and 5'-ACAACATGATAGCGCTCACC-3' for *dALS2[P1425A]*, and 5'-ACCAGCGTGGGGAACAGCA-3' and 5'-TGCTGTTCCCCACGCTGGT-3' for *dALS2 [L1439A]*. The CDSs of *dALS2[P1425A]* and *dALS2[L1439A]* were also inserted into pUAST-3xHA.

Drosophila S2 cells and FRET imaging

S2 cells were cultured at 25°C in Schneider's Drosophila medium (Gibco) containing 10% fetal bovine serum (Gibco) and penicillin/streptomycin, and passaged every 3 to 4 days. S2 cells were cotransfected with pDA-Gal4 (*actin*-promoter Gal4; a gift from Fumio Matsuzaki's lab), pUAST-Raichu-Rab5, and either of pUAST-3xHA:dALS2[WT], pUAST-3xHA:dALS2[P1425A], or pUAST-3xHA:dALS2[L1439A] by using HilyMax (Dojindo), and used for microscopic analysis 2 days after transfection. FRET imaging was performed as described (Tsuyama *et al.* 2013).

RT-PCR and in situ hybridization

We isolated total RNA from wild type and *dALS2^{-/-}* adults with an RNeasy kit (QIAGEN). After cDNA was synthesized by using Ready-To-GO RT-PCR Beads (GE Healthcare UK), expression of *dALS2* and *Actin5c* was detected by using the following primers: 5'-TCTGCAGCAGTCCAGCACATAC-3' and 5'-CATTTTGCCGAAACCTTCAATG-3' for *dALS2*, and 5'-

GTGCTGTGTGGATACTCC-3' and 5'-TGTGGTGCCAGATCTTCTCC-3' for *Actin5c*. RNA probes for in situ hybridization were made by using a cDNA clone LD33266 (Berkeley Drosophila Genome Project) as a template and hybridized with fixed embryos essentially as described (Matsubara *et al.* ; Yamamoto *et al.* 2006; Hattori *et al.* 2007).

Production of antibodies and western blotting

Guinea pigs were immunized with a GST protein that had been fused to the N-terminal 410 amino acids of dALS2 to generate anti-N1 antibody, and the C-terminal 196 amino acids to generate anti-C1 antibody. We solubilized dALS2-expressing S2 cells by sonication in the sample buffer for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The antibodies recognized dALS2 that was endogenously expressed in S2 cells and the one overexpressed by *Ubi-Gal4* in transgenic flies (data not shown). Adult heads were homogenized in the sample solution and lysates were boiled immediately. The lysates were separated on 8% polyacrylamide gels in WIDE RANGE Gel Preparation Buffer for SDS-PAGE (Nacalai) and transferred to PVDF membranes (Millipore). To detect ubiquitinated proteins and p62 proteins, lysates equivalent to 7.5 fly heads were run per lane (Figure S5B). The primary antibodies were used at the following concentrations: guinea pig anti-dALS2 (1:1000), rabbit anti-p62 (1:4000; Pircs *et al.* 2012), mouse anti-polyubiquitin FK2 (1:1000; COSMO BIO), and mouse anti-actin MAB1501 (1:1000; Millipore). Signals were detected with Chemi-Lumi One Super (Nacalai).

Immunohistochemistry

Wandering third instar larvae were dissected in PBS, fixed in PBS containing 3.7% formaldehyde for 10 min, incubated in primary antibodies overnight at 4°C, and in secondary antibodies for 1-2 days at 4°C. Adult brains were fixed in PBS containing 3.7% formaldehyde for 1h. The primary antibodies were used at the following concentrations: rabbit anti-HRP (1:300; Jackson ImmunoResearch), mouse NC82 (1: 100; Developmental Studies Hybridoma Bank [DSHB] at the University of Iowa), mouse anti-polyubiquitin FK2 (1:500; COSMO BIO), and rabbit polyclonal antibody against *Drosophila* p62 protein (1:2000; Piracs *et al.* 2012).

Imaging and quantification

In fillet preparations of wandering 3rd instar larvae, we counted the numbers of boutons of motor nerve endings at muscles 6/7 in segment A2 as previously described (Colyle *et al.* 2004). To acquire images of adult abdominal NMJs, we mounted abdomens as described (Shimono *et al.* 2009) and imaged motor nerve endings in the ventral plate. We quantified the area size of the nerve endings and the total number of boutons in segments A4 and A5 as described by Hebbar *et al.* (2006). Images of larval da neurons were acquired and quantitatively analyzed essentially as described (Matsubara *et al.* 2011; Hattori *et al.* 2007; Parrish *et al.* 2009).

Negative geotaxis assay, longevity, and sensitivity to oxidant exposure

Flies of each genotype were collected 1 day after eclosion, and mated for 1 day.

After this mating, males and females were separated, and sorted into ten groups of ten individuals of each gender. These groups of individual genotypes were reared at the same time in the same incubator and used for the assays. In the negative geotaxis assay, we transferred each group of ten flies into an empty test vial, gently tapped the flies to the bottom of the vial, and scored the number of flies that could climb above the 8-cm mark by 10 seconds after the tap (Figure S4B). This test was repeated ten times for each group and the mean (average climbing pass rate) was plotted as a single data point (Figures 4-6). We basically followed published protocols of assays for negative geotaxis (Ali *et al.* 2011), longevity (Linford *et al.* 2013), and sensitivity to oxidant exposure, (Vrailas-Mortimer *et al.* 2011).

Databases

For homology and domain searches, we used Homologene in the NCBI BioSystems Database (Geer *et al.* 2010) and Pfam27.0 (Punta *et al.* 2012), respectively. To search for genomic information of *Drosophila melanogaster*, we used FlyBase (St Pierre *et al.*).

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Figure legends

Figure 1 Domains of human ALS2 and its Drosophila homolog (dALS2) and an analysis of dALS2 GEF activity.

(A) hALS2 has an RCC1-like domain (RLD domain) that is composed of five RCC1-like repeats (amino acids 93-122, 109-165, 170-216, 527-574, and 579-625), a Dbl homology domain (DH domain; amino acids 695-882), a pleckstrin homology domain (PH domain; amino acids 935-1003), seven MORN repeats (amino acids 1049-1071, 1072-1094, 1100-1122, 1123-1143, 1151-1166, 1198-1220, and 1221-1244), and a VPS9 domain (amino acids 1552-1654). dALS2 has an RLD domain that is composed of two RCC1-like repeats (amino acids 258-361 and 308-361), a PH domain (amino acids 617-719), six MORN repeats (amino acids 744-765, 766-786, 789-805, 817-835, 839-855, and 863-885), and a VPS9 domain (amino acids 1373-1481). The Pfam program does not predict a DH domain in dALS2. P1425A and L1439A indicate substitutions of amino acids in the VPS9 domain of dALS2. (B) Analysis of GEF activity of dALS2 in S2 cells (n > 70 cells for each transfection). Each of the dALS2 forms indicated was co-expressed with a FRET probe (Raichu-Rab5[WT]) or its negative control probe (Raichu-Rab5[S34N]) in S2 cells, and the FRET/CFP ratio was determined. See details in the text. Error bars indicate standard deviations. ***: P<0.001 (ANOVA with Bonferroni) and P=0.852 (student's t-test). Two other experiments gave results similar to this dataset; in every experiment, the ratio was increased by 40 % or more when Raichu-Rab5[WT] was co-

expressed with *dALS2*[WT].

Figure 2 The *dALS2* locus and its protein.

(A) Exon/intron organization of the *dALS2* locus and genomic structures of mutant alleles. Gray and orange boxes are UTRs and CDSs, respectively. A blue arrow on the wild-type genome indicates the position of the transposon insertion, and black arrows indicate primers that were used for genomic PCR. Deleted regions in individual alleles are shown with allele numbers. Green lines indicate primers that were used in RT-PCR. (B) RT-PCR detection of *dALS2* mRNA from adults of one of the precise jumpers (*Ex101/Ex101*) and from those of *dALS2*^{-/-} (*Ex44/Ex44*). (C) In situ hybridization analysis of *dALS2* expression in embryos at stage 13 (top) or stage 16 (bottom). Ventral views. (Left) Embryo hybridized with a sense probe. (Middle and right) Embryo hybridized with an anti-sense probe. The boxed regions in the middle panels are magnified in the right. *dALS2* is expressed in a wide-range of tissues including the epidermis (arrowheads) and the ventral nerve cord (boxed regions). Arrows mark unidentified cell types in the ventral nerve cord that express *dALS2* at a high level.

Figure 3 Phenotypes of NMJs in the *dALS2* mutant and those elicited by Rab5[S34N] expression in motor neurons.

(A-D) Phenotypes in wandering third instar larvae. (A and C) NMJs at muscles 6/7 (A) and muscle 4 (C) in segment A2 that were stained for HRP (green) and Brp (magenta merged with green look white). (B and D)

Quantitative analysis. The numbers of synaptic boutons were normalized by the surface areas of individual muscles. Bars indicate means. **: $P < 0.01$ ***: $P < 0.001$ (ANOVA with Tukey's HSD posthoc test). Relevant genotypes in A and B are *Ex101/Ex101* (wild type) and *Ex44/Ex44* (*dALS2^{-/-}*); and those in C and D are [*OK371-Gal4/UAS-mmRFP*] (Control), [*OK371-Gal4/+; UAS-Rab5[S34N]/+*] (*Rab5^{S34N}*), [*OK371-Gal4/+; UAS-GFP:Rab5[WT]/+*] (*Rab5^{WT}*), [*OK371-Gal4/UAS-3HA:dALS2[WT]*] (*dALS2^{WT}*). Scale bars: 20 μm .

(E-K) Phenotypes of motor axons in abdomens of 3-4 day-old adult females (E, J, K) and 2 w-old adult females (F-I). (E-I) A low-power view of the lateral and ventral plates of the wild type (E). The boxed region in E indicates an example of motor axon terminals, and blue arrows mark spiracles. (F) High-power images of axon terminals that innervate ventral abdominal muscles of segment A5 of the wild type and the mutant at 2 week-old. Note that neither of the images in F is a magnification of the boxed region in E. The area of each presynaptic arbor was defined as a polygon outlined with a magenta broken line, which was quantitatively analyzed in G-I. Quantifications of the area size (G), the number of boutons in each area (H), and the number of normalized boutons (I). **: $P < 0.01$ (student's t-test). (J and K) Low-power ventral views along the midlines (magenta broken lines). The boxed regions highlight distinct growth of axon terminals between the two genotypes. Scale bars: 50 μm (E, J, K) and 20 μm (F). Relevant genotypes in E-I are [*OK371-Gal4 UAS-mCD8:3xEGFP/OK371-Gal4 UAS-mCD8:3xEGFP*] (E), [*OK371-Gal4 UAS-mCD8:3xEGFP/OK371-Gal4 UAS-mCD8:3xEGFP; Ex101/Ex101*]

(wild type in F-I), [OK371-Gal4 UAS-mCD8:3xEGFP/OK371-Gal4 UAS-mCD8:3xEGFP; Ex44/Ex44] (*dALS2*^{-/-} in F-I); and those in J and K are [OK371-Gal4 UAS-mCD8:3xEGFP/UAS-mmRFP] (J), and [OK371-Gal4 UAS-mCD8:3xEGFP/+; UAS-Rab5[S34N]/+] (K).

Figure 4 The *dALS2* mutant exhibited age-dependent locomotor dysfunction. Wild type and *dALS2*^{-/-} were assayed for negative geotaxis at the indicated ages, scoring each gender separately. The genotypes of the wild type in A and B are *Ex101/Ex101* and *Ex95/Ex95*, respectively, while the genotype of the mutants is *Ex44/Ex44*. *** $P < 0.001$ (student's t-test).

Figure 5 The locomotion deficit of the mutant was rescued by widely expressing the *dALS2* transgene

Rescue experiment of the locomotion deficit of the *dALS2*^{-/-} mutant by using *Ubi-Gal4*. Females and males of the four genotypes were assayed for negative geotaxis at 1 week (A) or 2 weeks (B) after eclosion. Relevant detailed genotypes (from left to right in each graph) were [*Ubi-Gal4/UAS-mmRFP; Ex101/Ex101*] (*Ubi*>*RFP*; WT), [*Ubi-Gal4/UAS-dALS2; Ex101/Ex101*] (*Ubi*>*dALS2*; WT), [*Ubi-Gal4/UAS-mmRFP; Ex44/Ex44*] (*Ubi*>*RFP*; *dALS2*^{-/-}), and [*Ubi-Gal4/UAS-dALS2; Ex44/Ex44*] (*Ubi*>*dALS2*; *dALS2*^{-/-}). Magenta asterisks indicate statistically significant differences of the cohort from the wild type (*Ubi*>*RFP*; WT); and blue asterisks from the mutant (*Ubi*>*RFP*; *dALS2*^{-/-}). *: $P < 0.05$; **: $P < 0.01$; NS: Statistically not significant ($P > 0.05$). ANOVA with Tukey's HSD posthoc test. In the top right graph, the

significance of the difference between *Ubi>RFP; dALS2^{-/-}* and *Ubi>dALS2; dALS2^{-/-}* is marginal, so the exact P value is provided.

Figure 6 Locomotion deficit rescue experiment using a motor neuron-specific *Gal4* driver.

Females and males of the four genotypes were assayed for negative geotaxis at 1 week (A) or 2 weeks (B) after eclosion. Relevant detailed genotypes (from left to right in each graph) were [*OK371-Gal4/UAS-mmRFP; Ex101/Ex101*] (*OK371>RFP; WT*), [*OK371-Gal4/UAS-dALS2; Ex101/Ex101*] (*OK371>dALS2; WT*), [*OK371-Gal4/UAS-mmRFP; Ex44/Ex44*] (*OK371>RFP; dALS2^{-/-}*), and [*OK371-Gal4/UAS-dALS2; Ex44/Ex44*] (*OK371>dALS2; dALS2^{-/-}*). Magenta asterisks indicate statistically significant differences of the cohort from the wild type (*OK371>RFP; WT*); and blue asterisks from the mutant (*OK371>RFP; dALS2^{-/-}*). *: $P < 0.05$; **: $P < 0.01$; NS: Statistically not significant ($P > 0.05$). ANOVA with Tukey's HSD posthoc test.

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Supplementary information

Figure S1 Human and Drosophila Rab5-GEFs and GAPs.

Human Gapex-5 and its Drosophila homolog, CG1657, have RasGAP (blue boxes) and VPS9 (red boxes) domains. Rabex-5 and dRabex-5 have A20-like zinc finger (pink boxes) and VPS9 domains. Rin1 and its Drosophila homolog Sprint have Src homology 2 (SH2; aqua blue boxes), VPS9, and RA (brown boxes) domains. RN-tre and RabGap-5 have Tre-2, Bub2, and Cdc16 (TBC; purple boxes) domains. RabGap-5 and its Drosophila homolog, CG11241, have Src homology 3 (SH3; green boxes) and RUN domains.

Figure S2 Multiple sequence alignment of VPS9 domains of Rab5 GEFs.

Amino-acid sequences of Human ALS2 and Drosophila CG7158 are marked with asterisks. Residues that are conserved among all of the eight GEFs are highlighted by white letters in black boxes, while residues conserved among five to seven GEFs are by white letters in gray boxes. Those conserved among only three or four GEFs are shown by black letters in gray boxes. The red boxes represent the residues that are mutated in this study (Figure 1).

Figure S3 Phenotypes of class IV da neurons in the *dALS2* mutant.

(A and B) Class IV da neurons (ddaC) in wandering 3rd instar larvae of the wild type (A) or the *dALS2* mutant (B). The area of the dendritic arbor (light

blue-colored region) and that of the dorsal body surface (box outlined in red) are superimposed on original images. The total length (TL) and coverage index (CI) are documented. Genotypes are [*Gr28b.c-Gal4 UAS-mCD8:GFP/+; Ex101/Ex101*] (A) and [*Gr28b.c-Gal4 UAS-mCD8:GFP/+; Ex44/Ex44*] (B). (C) Quantifications of total branch lengths of the neurons (left), the numbers of branch endings (middle), and the coverage index (right). *: $P < 0.05$; **: $P < 0.01$ (student's t-test). Scale bars: 100 μm .

Figure S4 Negative geotaxis assay.

(A) Snap shots that were taken 5 seconds after the tap. (Left) Climbing control flies (*OK371-Gal4 UAS-mCD8:3xEGFP/UAS-mmRFP*). (Right) *Rab5[S34N]*-expressing flies (*OK371-Gal4 UAS-mCD8:3xEGFP/+; UAS-Rab5[S34N]/+*) hardly climbed. (B) A schematic representation of the negative geotaxis assay. The score of the climbing pass rate is 70% in this example. (C and D) 4 week-old wild-type (*Ex101/Ex101*) and *dALS2^{-/-}* (*Ex44/Ex44*) flies, respectively. The pictures were taken at 0, 5, and 8 seconds after the tap. Individual tracks of the flies, traced from the video movie, are shown by different colors.

Figure S5 Aging was not accelerated in the *dALS2* mutant.

(A) Confocal images of whole-mount brains of 3-4 day old or 2-week-old flies, which were stained for polyubiquitin. (B) Total lysates of 1-week or 3-week old adult heads were probed with anti-polyubiquitin, anti-actin, or anti-p62 antibodies. An arrow identifies p62, which co-migrated with the 100

kDa marker protein. Genotypes are *Ex101/Ex101* (wild type) and *Ex44/Ex44* (*dALS2^{-/-}*).

Figure S6 Life span assay

Adult survivorship of the wild type (magenta) and *dALS2^{-/-}* (blue). Males and females were reared separately after one-day mating. (A, C, and E) males and (B, D, and F) females. The genotypes of the wild type are *Ex101/Ex101* (A and B) and *Ex95/Ex95* (C-F). The genotypes of *dALS2^{-/-}* mutants are *Ex44/Ex44* (A-D) and *Ex54/Ex54* (E and F). Survivorship data were analyzed using the log-rank test and p values are indicated in individual panels.

Supplemental movie 1

Climbing ability of the control adults (0-3 days old). The genotype is *OK371-Gal4/UAS-mmRFP*.

Supplemental movie 2

Climbing ability of *Rab5[S34N]-expressing* adults (0-3 days old). After the tap, the flies were recorded longer than 10 seconds. The genotype is *OK371-Gal4/+; UAS-Rab5[S34N]/+*.