Mutations in the human ALS2 gene cause recessive juvenile-onset amyotrophic lateral sclerosis and related motor neuron diseases. Although the ALS2 protein has been identified as a guanine-nucleotide exchange factor for the small GTPase Rab5, its physiological roles remain largely unknown. Here, we demonstrate that the Drosophila homologue of ALS2 (dALS2) promotes postsynaptic development by activating the Frizzled nuclear import (FNI) pathway. dALS2 loss causes structural defects in the postsynaptic subsynaptic reticulum (SSR), recapitulating the phenotypes observed in FNI pathway mutants. Consistently, these developmental phenotypes are rescued by postsynaptic expression of the signaling-competent C-terminal fragment of Drosophila Frizzled-2 (dFz2). We further demonstrate that dALS2 directs early to late endosome trafficking and that the dFz2 C terminus is cleaved in late endosomes. Finally, dALS2 loss causes age-dependent progressive defects resembling ALS, including locomotor impairment and brain neurodegeneration, independently of the FNI pathway. These findings establish novel regulatory roles for dALS2 in endosomal trafficking, synaptic development, and neuronal survival.

**Introduction**

Mutations in the human ALS2 gene are associated with multiple early-onset motor neuron diseases (MNDs), including juvenile amyotrophic lateral sclerosis 2 (ALS2), juvenile primary lateral sclerosis, and infantile-onset ascending hereditary spastic paraplegia (Chen et al., 2013; Eymard-Pierre et al., 2002; Hadano et al., 2001; Yang et al., 2001). These ALS2-deficient MNDs are commonly characterized by progressive degeneration of motor neurons in the central nervous system, indicating an important role for the ALS2 gene product (ALS2/alsin) in motor neuron survival and maintenance. Consistently, the ALS2 protein is expressed primarily in central nervous system neurons, including motor neurons of the cortex and spinal cord (Devon et al., 2005; Otomo et al., 2003).

A large proportion of ALS2 mutations in MND patients leads to premature termination of protein translation or decreased protein stability (Sato et al., 2018; Yamanaka et al., 2003), implying a loss-of-function disease mechanism. ALS2<sup>−/−</sup> mice develop mild motor dysfunction and distal axonopathy in the corticospinal tract (Deng et al., 2007; Julien and Kriz, 2006; Yamanaka et al., 2006). Although biochemical and cell biological studies suggest that ALS2 regulates endosomal fusion and trafficking by activating the small GTPase Rab5 (Otomo et al., 2003; Topp et al., 2004), the roles of ALS2 in axon/synaptic maintenance and their potential link to endosomal trafficking are unknown.

Wnt signaling plays key roles in synaptic development, and its dysregulation is increasingly recognized as an important mechanism of synaptic loss in neurodegenerative diseases (Dickins and Salinas, 2013). At the Drosophila glutamatergic neuromuscular junction (NMJ), the Wnt homologue Wingless (Wg) is secreted from presynaptic terminals and binds the Drosophila Frizzled-2 (dFz2) receptor, which is expressed on pre- and postsynaptic membranes (Packard et al., 2002). In postsynaptic muscles, Wg activates the noncanonical Frizzled nuclear import (FNI) pathway by binding to dFz2 and inducing its endocytosis and cleavage (Mathew et al., 2005). The cleaved C-terminal fragment (dFz2-C) is imported into muscle nuclei in an Importin-β1-dependent manner to promote postsynaptic differentiation (Mathew et al., 2005; Mosca and Schwarz, 2010); however, the intracellular compartment in which dFz2 is cleaved to produce dFz2-C remains unknown.

In the present study, we investigate the physiological roles of a Drosophila homologue of human ALS2 (dALS2) in the larval development, and neuronal survival.
NMJ and adult brain. We demonstrate that loss of dALS2 impairs normal development of the subsynaptic reticulum (SSR), a network of postsynaptic membrane invaginations at the NMJ. Genetic interaction data suggest that dALS2 promotes postsynaptic development by regulating the FNI pathway. We also demonstrate that dALS2 ablation causes a general defect in receptor trafficking from early to late endosomes. Our results also indicate that dFz2-C cleavage occurs in the late endosome/lysosome compartment. Finally, we show that loss of dALS2 causes adult-onset, progressive neurodegeneration resembling ALS in an FNI pathway–independent manner. Together, these findings demonstrate an unexpected role for dALS2-mediated endosomal trafficking in postsynaptic dFz2 signaling and highlight the potential contribution of impaired receptor and endosomal trafficking to the pathogenesis of ALS2-associated MNDs.

**Results**

**dALS2 is required for normal postsynaptic development**

To explore the in vivo function of dALS2 protein at synapses, we disrupted the dALS2 gene using two different approaches. First, we imprecisely excised a P-element insertion (G4607) in the first exon of dALS2 and isolated the deletion dALS2Δ73, which deletes a portion of the first exon, including the translation start site (Fig. S1 A). Second, we performed CRISPR/Cas9 mutagenesis to isolate a larger deletion, dALS2Δ, which removed most of the protein-coding region, including the translation start site (Fig. S1 A). Transheterozygotes of dALS2Δ and dALS2Δ73 were null for dALS2 expression, but normally expressed the adjacent gene CG11248 (Fig. S1 B). Homozygotes and transheterozygotes of dALS2Δ and dALS2Δ73 were viable and fertile.

We next examined NMJs 6/7 of third instar larvae of WT (wt192) and dALS2Δ/dALS2Δ73 animals using synaptic markers. Bouton number, bouton size, and muscle size did not differ between WT and dALS2 mutant larvae (Fig. S1, C–F). Furthermore, Futsch-labeled synaptic microtubules were normal in dALS2 mutants (Fig. S1, G and H); however, we observed a 4.2-fold increase of synaptic boutons lacking the SSR marker Discs-alds (ΔdALS2) compared with WT controls, membrane layers of ghost boutons were less complex, with lower SSR membrane thickness (WT: 13.69 ± 1.08; dALS2Δ/dALS2Δ73: 8.35 ± 0.28; P < 0.001; Fig. 2, A and B). In addition, we observed striking expansion of the postsynaptic pocket (pocket depth; WT: 0.14 ± 0.001 µm; dALS2Δ/dALS2Δ73: 0.24 ± 0.02 µm; P < 0.001; Fig. 2, A and C), a postsynaptic area immediately apposed to an active zone containing amorphous material (Packard et al., 2002); however, SSR thickness was unchanged (Fig. 2 D). These data suggest that dALS2 is required for normal postsynaptic differentiation at the NMJ.

**dALS2 regulates postsynaptic ultrastructure**

We next examined the ultrastructure of NMJ synapses in dALS2 mutants using transmission EM. Presynaptic terminals, including synaptic vesicle density and size, number of active zones per bouton area, number of T-bars per active zone, and number of mitochondria per bouton, were normal in dALS2Δ/dALS2Δ73 mutants (data not shown); however, the SSR, the network of infolded postsynaptic membrane at the NMJ, was structurally defective. Because ghost boutons are completely devoid of postsynaptic structure (Ataman et al., 2006; Packard et al., 2002), our analysis was focused on mature boutons surrounded by SSR. Compared with WT controls, membrane layers in dALS2 mutants were less complex, with lower SSR membrane density (WT: 13.69 ± 1.08; dALS2Δ/dALS2Δ73: 8.35 ± 0.28; P < 0.001; Fig. 2, A and B). In addition, we observed striking expansion of the postsynaptic pocket (pocket depth; WT: 0.14 ± 0.001 µm; dALS2Δ/dALS2Δ73: 0.24 ± 0.02 µm; P < 0.001; Fig. 2, A and C), a postsynaptic area immediately apposed to an active zone containing amorphous material (Packard et al., 2002); however, SSR thickness was unchanged (Fig. 2 D). These data suggest that dALS2 is required for normal postsynaptic differentiation at the NMJ.

**dALS2 mutations alter synaptic function**

To assess the functional consequence of dALS2 mutations at the NMJ, we performed electrophysiological measurements of excitatory junctional potentials (EJPs). The amplitude of spontaneously occurring miniature EJPs (mEJPs) was significantly reduced in dALS2Δ/dALS2Δ73 mutants relative to WT (WT: 1.54 ± 0.06 mV; dALS2Δ/dALS2Δ73: 0.86 ± 0.05 mV; P < 0.001; Fig. 3, A and B), while mEJP frequency was normal (Fig. 3 C). This was

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Figure 1. **dALS2 regulation of postsynaptic development.** (A) Confocal images of NMJ 6/7 stained with anti-HRP and anti-Dlg antibodies are shown for WT (w1118), dALS2Δ1/dALS2Δ73, UAS-HA-dALS2, dALS2Δ1/D42-GAL4, dALS2Δ73 (dALS2 rescue-pre), UAS-HA-dALS2, dALS2Δ1/24B-GAL4, dALS2Δ73 (dALS2 rescue-post), UAS-HA-hALS2/+; dALS2Δ1/24B-GAL4, hALS2Δ73 (hALS2 rescue-post); and UAS-Myc-NLS-dFz2-C/+; dALS2Δ1/24B-GAL4, dALS2Δ73 (NLS-dFz2-C rescue-post) third instar larvae. Arrowheads and arrows mark ghost boutons formed at type Ib and Is terminals, respectively. (B) Quantification of ghost bouton number per NMJ 6/7 in indicated genotypes. dALS2 OE (overexpression)-post indicates UAS-HA-dALS2/24B-GAL4, and NLS-dFz2-C OE-post indicates UAS-Myc-NLS-dFz2-C/+; 24B-GAL4/+; n = 30 NMJs. (C) Confocal images of third instar NMJ 6/7 of the indicated genotypes stained with anti-HRP and anti-GluRIIB antibodies. (D) Quantification of GluRIIA, and GluRIIB immunoreactivities normalized to HRP. n = 18–46 NMJ branches. (E) Quantification of the ratio of mean GluRIIB to GluRIIA fluorescence intensities. n = 42 NMJ branches. Data are presented as mean ± SEM. Comparisons are with WT or 24B-GAL4/+ control unless otherwise indicated. Scale bars: 20 µm (A); 5 µm (C). ***, P < 0.001; *, P < 0.05.
consistent with the finding that synaptic levels of GluRIIB relative to GluRIIA are increased in dALS2 mutants, as ion conductivity is decreased in GluRIIB-expressing receptor complexes relative to GluRIIA-expressing receptor complexes (DiAntonio et al., 1999). Decreased mEJP amplitude phenotype was significantly rescued by postsynaptic, but not presynaptic, expression of HA-dALS2 in dALS2 mutants (Fig. 3, A and B). Finally, despite decreased mEJP amplitude, the amplitude of evoked EJPs remained normal in dALS2 mutants due to a compensatory increase in presynaptic neurotransmitter release (quantal content; WT: 11.16 ± 0.84; dALS2Δ/Δ dALS2Δ/Δ rescue-pre: 19.37 ± 1.05; P < 0.001; Fig. 3 F). The increased quantal content phenotype was also rescued by muscular expression of HA-dALS2, suggesting that a retrograde (post to presynaptic) homeostatic mechanism is involved in this compensatory process.

**dALS2 regulates the FNI Wg signaling pathway in the muscle**

The synaptic defects of dALS2 mutants, including excessive ghost bouton formation and abnormal SSR development, are strikingly similar to those of animals deficient in the postsynaptic FNI Wg pathway or in dGRIP, a PDZ protein required for dFz2 intracellular trafficking (Ataman et al., 2006; Harris et al., 2016; Kamimura et al., 2013; Mosca and Schwarz, 2010; Packard et al., 2002). We therefore hypothesized that the synaptic defects in dALS2-null mutants could be due to dysregulation of the postsynaptic FNI pathway. To test this possibility, we first examined genetic interactions between dALS2 and wg or dGRIP. Heterozygosity for dALS2, wg, or dGRIP did not affect postsynaptic development (Fig. 4, A and B); however, the number of ghost boutons was significantly increased in larvae transheterozygous for dALS2 and wg or dGRIP relative to WT controls (Fig. 4, A and B), suggesting functional linkage between dALS2 and the postsynaptic FNI pathway during synaptic development.

Next, we determined if loss of dALS2 affects nuclear accumulation of the cleaved dFz2-C. In WT larvae, dFz2-C immunoreactivity was detected in a punctate pattern in muscle nuclei (Fig. 4 C), as previously reported (Mathew et al., 2005). Importantly, the number of nuclear dFz2-C puncta was
significantly decreased in dALS2Δ1/dALS2Δ73 mutants (WT: 4.28 ± 0.42; dALS2Δ1/dALS2Δ73: 1.36 ± 0.33; P < 0.001; Fig. 4, C and D).

Muscular expression of HA-dALS2 or HA-hALS2 in dALS2 mutants rescued decreased nuclear accumulation of dFz2-C puncta (Fig. 4, C and D), supporting a role for dALS2 in the postsynaptic FNI pathway.

Previous studies have shown that expression of Myc-NLS-dFz2-C, a Myc epitope fused with a nuclear localization signal (NLS) and the C-terminal 88 aa of dFz2, can bypass the requirement of the FNI pathway for postsynaptic development (Mosca and Schwarz, 2010). We therefore tested if Myc-NLS-dFz2-C is able to rescue the synaptic defects of dALS2 mutants. Muscle-specific expression of Myc-NLS-dFz2-C in dALS2Δ1/dALS2Δ73 mutants decreased the number of ghost boutons to that of WT larvae and, furthermore, rescued other key dALS2 phenotypes, including synaptic elevation of GluRIIB expression, decreased SSR complexity, expansion of postsynaptic pockets, and decreased mEJP amplitude (Figs. 1, 2, and 3). These findings are consistent with the model that dALS2 regulates postsynaptic development by modulating the FNI pathway.

dALS2 regulates dFz2 transport from early to late endosomes

The mammalian homologue of dALS2 is localized to Rab5+ early endosomes (Otomo et al., 2003; Topp et al., 2004). To investigate the mechanism by which dALS2 regulates dFz2 signaling, we examined subcellular localization of dALS2 in postsynaptic muscles. Because our polyclonal anti-dALS2 antibody could not detect endogenous dALS2 in immunomicroscopy, we expressed low levels of UAS-HA-dALS2 in muscles by leaky expression from Mhc-GS-GAL4 in the absence of the steroid RU486 (Osterwalder et al., 2001). In muscles, Rab5+ early endosomes preferentially distributed around synaptic boutons, while Rab5+/Rab7+ intermediate endosomes and Rab7+ late endosomes were more common in the perinuclear area (Fig. S2 A). The HA-dALS2 fusion protein primarily appeared in reticular and punctate patterns. HA-dALS2 puncta overlapped with a subset of Rab5+...
early endosomes and Rab5+/Rab7+ intermediate endosomes, but rarely colocalized with Rab7+ late endosomes (Fig. S2, A and B).

The endosomal localization of dALS2 led us to hypothesize that dALS2 regulates the FNI pathway by facilitating dFz2 endocytic trafficking. To test this possibility, we first generated a UAS transgene of a Flag-dFz2-Myc construct containing a Flag epitope directly following the signal peptide and a Myc epitope fused to the C terminus of full-length dFz2. When this transgene was expressed in postsynaptic muscles, anti-Myc signals were detected in the postsynaptic region of larval NMJs as well as punctate structures in the cytoplasm and nuclei (Fig. S2, C and D). Furthermore, muscular expression of Flag-dFz2-Myc completely suppressed increased ghost bouton numbers in dfz2 mutants (Fig. S2, E and F), suggesting that Flag-dFz2-Myc behaves similarly to endogenous dfz2 protein in vivo.

Figure 4. **dALS2 regulation of Wg/dFz2-mediated postsynaptic development.** (A) Confocal images of NMJ 6/7 labeled with anti-HRP and anti-Dlg are shown for WT, dALS2Δ1/+;, wg17/+;+; dGRIP+36/+; dALS2Δ1/+; and dGRIP+36/+; dALS2Δ1/+; third instar larvae. Arrowheads indicate ghost boutons. (B) Quantification of ghost bouton number (n = 30 NMJs). Note transheterozygous interactions between dALS2 and wg or dGRIP during synaptic development. (C) Confocal images of muscle nuclei stained with anti-dFz2-C and DAPI are shown for WT, dALS2Δ1/dALS2Δ73, UAS-HA-dALS2, dALS2Δ1/24B-GAL4, dALS2Δ73 (dALS2 rescue-pre), and UAS-HA-dALS2, dALS2Δ1/24B-GAL4, dALS2Δ73 (dALS2 rescue-post) third instar larvae. Arrowheads indicate dFz2-C nuclear puncta. (D) Quantification of dFz2-C puncta per nucleus (n = 36 nuclei). Data are presented as mean ± SEM. All comparisons are with WT unless otherwise indicated. Scale bars: 20 µm (A); 5 µm (C). *** P < 0.001; *, P < 0.05.
Subsequently, we used the UAS-Flag-dfz2-Myc transgene to assess the role of dALS2 in dfz2 internalization and endosomal trafficking. In this assay, flies of WT and dALS2Δ1/dALS2Δ273 larvae expressing UAS-Flag-dfz2-Myc in postsynaptic muscles were prelabeled with an anti-Flag antibody at 4°C, a temperature that blocks endocytosis. Trafficking was then initiated by incubating fillets at RT. After permeabilization, three-color immunofluorescence was performed to monitor colocalization of internalized Flag-dFz2-Myc receptors with Rab5 and Rab7. In WT muscles, 10 min after prelabeling, most internalized Flag-dFz2-Myc receptors localized to Rab5+ puncta closely associated with synaptic boutons (Fig. 5, A and C). At this time point, only a few receptors were associated with perinuclear Rab5+/Rab7+ and Rab7+ endosomes (Fig. 5, B and C). Thirty minutes after prelabeling WT muscles, internalized Flag-dFz2-Myc was slightly increased in periboutonal Rab5+ puncta, but substantially increased in perinuclear Rab7+ puncta (Fig. 5, A–C), demonstrating successful transport of Flag-dFz2-Myc from periboutonal early endosomes to perinuclear late endosomes. In dALS2 mutant muscles imaged 10 min after prelabeling, the amount and distribution of internalized Flag-dFz2-Myc were similar to those of WT muscles (Fig. 5, A–C). By sharp contrast, 30 min after prelabeling, the level of Flag-dFz2-Myc in periboutonal Rab5+ puncta was significantly higher, and the level of Flag-dFz2-Myc in perinuclear Rab7+ puncta was significantly lower, in dALS2 mutant larvae relative to WT (Fig. 5, A–C). These results indicate that dALS2 loss selectively impairs dfz2 trafficking to late endosomes, which is associated with transport from NMJ synapses to the perinuclear area.

To further solidify these findings, we tested the impact of dALS2 knockdown on endosomal trafficking of Wg in BG2-c2 Drosophila neuronal cells. We found that transient expression of Flag-dFz2-Myc in BG2-c2 cells potently induced Wg uptake and that depletion of dALS2 did not affect dfz2-mediated Wg internalization (Fig. S3 A–C). We then assessed trafficking of internalized Wg in control and dALS2 knockdown cells coexpressing Flag-dFz2-Myc and GFP-Rab5 or GFP-Rab7. In control cells, 10 min after Wg treatment, a majority (~66%) of intracellular Wg puncta were labeled by GFP-Rab5, while only ~34% of puncta overlapped with GFP-Rab7 (Fig. S3, D, E, and H). In control cells, after 30 min of Wg treatment, Wg puncta preferentially colocalized with GFP-Rab7 rather than GFP-Rab5 (Fig. S3, F–H), suggesting the progression of Wg from early to late endosomes. However, in dALS2 knockdown cells at the same time point, Wg puncta remained highly associated with GFP-Rab5, and Wg colocalization with GFP-Rab7 was significantly decreased relative to control cells (Fig. S3, F–H). The sizes of GFP-Rab5+ and GFP-Rab7+ endosomes remained unchanged by dALS2 knockdown (Fig. S3 I). Collectively, these results confirm that dALS2 loss impairs dfz2 trafficking from early to late endosomes without grossly affecting endosomal dynamics.

Internalization of maleylated BSA (mBSA) mediated by the class C scavenger receptor SR-CI occurs via clathrin-mediated endocytosis (Gupta et al., 2009), while the BMP receptor Thickveins (Tkv) is internalized via macropinocytosis upon stimulation by the BMP ligand Glass bottom boat (Gbb; Kim et al., 2019). To examine the general role of dALS2 in intracellular receptor trafficking, we addressed the effect of dALS2 knockdown on endosomal trafficking of mBSA and Tkv. First, we pulsed SR-CI-expressing BG2-c2 cells for 1 min with fluorescently labeled mBSA and assessed mBSA trafficking at different chase time points. As assessed at the 4-min chase time point, dALS2 knockdown did not impair internalization of mBSA into Rab5+ early endosomes (Fig. S4, A and D); however, mBSA did not efficiently progress to Rab7+ late endosomes in dALS2 knockdown cells during a longer chase time (19 min; Fig. S4, B–D). Next, we performed an Myc-Tkv trafficking assay in BG2-c2 cells. dALS2 knockdown did not affect Gbb-induced Myc-Tkv enrichment on Rab5+ early endosomes (Fig. S4, E and H), but strongly impaired its progression to Rab7+ late endosomes (Fig. S4, F–H). Thus, dALS2 is required for early to late endosome trafficking of multiple endocytosed receptors, regardless of their internalization routes.

dALS2-mediated transport of dfz2 to late endosomes is required for cleavage of its C terminus

To determine the significance of dALS2-mediated endosomal transport in the FNI pathway, we examined the effect of dALS2 deficiency on cleavage of the dfz2-C using the Flag-dfz2-Myc transgene. Western blots of body wall lysates from WT and dALS2Δ1/dALS2Δ273 larvae overexpressing Flag-dFz2-Myc specifically in muscles were probed with anti-Myc antibody, and two protein bands were detected: a ~75-kD band corresponding to full-length Flag-dFz2-Myc and a ~9.9-kD band corresponding to the cleaved C-terminal fragment (Fig. 6 A). The ratio of cleaved dfz2-C-Myc to full-length Flag-dFz2-Myc was significantly reduced in dALS2 mutant larvae relative to WT controls (Fig. 6 B). Thus, defective transport of dfz2 from Rab5+ early endosomes to Rab7+ late endosomes in dALS2-null larvae is accompanied by decreased cleavage of the dfz2 receptor.

To directly test whether dfz2-C cleavage requires progression of dfz2 to late endosomal compartments, we knocked down Rabβ or Mon1, a critical component of guanine-nucleotide exchange factor for late endosomal Rabβ (Nordmann et al., 2010), in Flag-dFz2-Myc-overexpressing postsynaptic muscles and measured the effect on steady-state levels of dfz2-C-Myc by Western blot. Muscle-specific expression of a UAS-RNAi construct targeting Rabβ or Mon1 using BG57-GAL4 significantly reduced the level of dfz2-C in postsynaptic muscles (Fig. 6, C and D), indicating that C-terminal cleavage of dfz2 requires its trafficking to Rab7+ late endosomes. In addition to the Rab5–to–Rabβ switch, endosomal maturation involves the formation of intraluminal vesicles, which is initiated by the endosomal sorting complex required for transport-0 complex, and endosomal acidification by vacuolar H+-ATPase (V-ATPase; Huotari and Helenius, 2011). Expression of a UAS-RNAi construct construct targeting the endosomal sorting complex required for transport-0 complex Hrs or the V-ATPase subunit VhaAC9-J in Flag-dFz2-Myc-overexpressing postsynaptic muscles decreased dfz2-C-Myc levels (Fig. 6, C and D), supporting that dfz2-C cleavage requires progression of dfz2 to late endosomal compartments. Consistently, presynaptic but not postsynaptic knockdown of Rabβ, Mon1, Hrs, and VhaAC9-J aberrantly increased the formation of ghost boutons (Fig. 6, E and F), as in dALS2-null mutants.
Figure 5. dALS2 loss impairs dFz2 transport from early to late endosomes in postsynaptic muscles. Filets of UAS-Flag-dFz2-Myc/+; 24B-GAL4/+ (WT) and UAS-Flag-dFz2-Myc/+; dALS2Δ1/dALS2Δ73, 24B-GAL4 (dALS2Δ1/dALS2Δ73) larvae expressing Flag-dFz2-Myc in muscles were prelabeled with anti-Flag antibody at 4°C and subjected to a Flag-dFz2-Myc trafficking assay. (A) Single confocal sections of NMJs 6/7 in WT and dALS2Δ1/dALS2Δ73 larvae at 10 or 30 min after anti-
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To further confirm dFz2-C cleavage in late endosomal compartments, Flag-dFz2-Myc-expressing BG2-c2 cells were subjected to a receptor trafficking assay in which the two epitope tags of internalized Flag-dFz2-Myc receptors in comparison with endosomal markers were monitored by immunofluorescence. Ten minutes after Wg treatment, anti-Flag and anti-Myc signals were fully overlapped on Rab5+ early endosomes, but to a much lesser extent on Rab7+ late endosomes (Fig. 7, A and D). At later times (30 and 90 min), overlapping anti-Myc and anti-Flag signals dissociated from Rab5+ early endosomes, colocalizing instead with Rab7+ late endosomes (Fig. 7, B and D), indicating progression of Flag-dFz2-Myc to the late endosomal compartment. Even 90 min after Wg treatment, anti-Flag and anti-Myc signals remained associated on Rab5+ early endosomes. By sharp contrast, Rab7+ late endosomes also contained anti-Myc signal, but progressively lost anti-Flag signal, generating Flag−Myc+ puncta (Fig. 7, B and D). Importantly, a Flag-dFz2-Myc mutant—Flag-dFz2ΔSKTLESW-Myc—with deletion at the site of predicted dFz2-C cleavage by glutamyl-endopeptidase (Mathew et al., 2005) generated Flag−Myc−Rab7− endosomes but failed to form Flag−Myc+Rab7− endosomes (Fig. 7, C and D), suggesting progressive formation of Flag−Myc+Rab7+ late endosomes results from physiologically relevant dFz2-C cleavage. Flag−Myc+ puncta also colocalized with the lysosomal marker Lysotracker (Fig. 7 E). Finally, Flag−Myc+ puncta, but not Flag+Myc+ puncta, colocalized with Importin-β1 (Fig. 7 F), which mediates nuclear import of dFz2-C (Mosca and Schwarz, 2010). Collectively, these data strongly suggest that the C terminus of dFz2 is cleaved in late endosome/lysosome compartments.

Subsequently, we tested if mammalian ALS2 has a conserved role in promoting the FNI pathway. We focused on Frizzled-5 (Fz5), the closest mammalian homologue of dFz2 containing a conserved sequence (KTLES) for glutamyl-endopeptidase cleavage in the intracellular domain (Mathew et al., 2005). We produced a human Fz5 construct with a GFP tag fused to the Fz5 C terminus (Fz5-GFP) and transfected mouse motor neuron lines with a construct in the presence or absence of ALS2 siRNA (Fig. S5 A). In unstimulated control cells, Fz5-GFP was detected predominantly in the cytoplasm as diffuse and punctate patterns (Fig. S5 B). The Fz5 ligand, Wnt5a, stimulated accumulation of Fz5-GFP in nuclear puncta (Fig. S5 B). The number of cells with nuclear Fz5-GFP puncta was significantly increased by 5.9-fold upon Wnt5a stimulation relative to control (Fig. S5 C). Importantly, ALS2 knockdown decreased the number of nuclear Fz5-GFP+ cells by 47% in the presence of Wnt5a stimulation (Fig. S5 C). Western blot analysis of whole-cell lysates probed with anti-GFP antibody confirmed that, upon Wnt5a treatment, Fz5-GFP was cleaved to produce a band of ∼32 kD (Fig. S5 D), corresponding to the cleaved dFz5 C terminus fused to GFP. This band was also detected in the nuclear fraction of cells stimulated with Wnt5a, but not in that of unstimulated control cells (Fig. S5 E). Finally, ALS2 knockdown impaired Wnt5a-induced nuclear accumulation of the 32-kD band (Fig. S5, E and F). Together, these results imply that mammalian ALS2 plays a critical role in promoting the FNI pathway.

dALS2 regulates locomotor activity and neuronal survival

The hallmark of ALS is progressive degeneration of cortical and spinal motor neurons, leading to locomotor defects (Taylor et al., 2016). To ascertain the role of dALS2 in locomotor performance, we performed a negative-geotaxis climbing assay in adult flies (20 d).

dALS2Δ1/dALS2Δ73 mutant flies exhibited significantly decreased climbing ability relative to WT and revertant controls (P < 0.001; Fig. 8, A and B). This locomotion deficit in mutant animals was rescued by neuronal expression of dALS2 or hALS2, but not NLS-dFz2-C. To determine if the locomotion deficit of dALS2 mutants was associated with neurodegeneration, we analyzed fly brain morphology by performing hematoxylin and eosin (H&E) staining. Brains of newly eclosed dALS2Δ1/dALS2Δ73 mutants were anatomically and histologically normal; however, aged dALS2 mutants exhibited numerous vacuoles in the cortex and neuropils (Fig. 8 C), a hallmark of degeneration in the Drosophila brain (Muqit and Feany, 2002). At 10 and 20 d of age, the average number of brain vacuoles in dALS2 mutant flies was significantly increased by 2.4-fold and 2.9-fold, respectively, relative to WT controls (P < 0.001; Fig. 8 D). The brains of 30- and 40-d-old dALS2 flies exhibited further increased vacuolization, while age-matched controls were well preserved (Fig. 8 D). To characterize age-related degeneration in dALS2 mutants, we assessed apoptotic cell death in 20-d-old brains. The number of TUNEL+ cells per brain was significantly increased in dALS2 mutant flies relative to WT (WT: 12.92 ± 1.44; dALS2Δ1/dALS2Δ73: 25.50 ± 1.64; P < 0.001; Fig. 8, F and G). To determine the nature of apoptotic cells, dALS2 mutant brains were stained with TUNEL or anti-cleaved death caspase-1 (Dcp-1) in combination with antibodies against the neuronal marker anti-Elav and the glial marker Repo. TUNEL+ and Dcp-1+ cells expressed Elav, but not Repo (Fig. 8 H), suggesting that apoptotic cells are predominantly neurons. Notably, dALS2 deficiency also induced apoptotic cell death in OK6-GAL4/UAS-NLS-mCherry–labeled motor neurons residing in the ventral nerve cord (VNC; Fig. 8, I and J). Finally, neuronal expression of dALS2 or hALS2, but not NLS-dFz2-C, in dALS2 mutants rescued the phenotypes of brain vacuolization and neuronal cell death (Fig. 8, E and G). This finding suggests that dALS2 regulates neuronal cell survival in a dFz2-independent manner. Together, these results demonstrate a causal relationship between locomotor deficits and neurodegeneration in aged dALS2 mutants.
Discussion

In the present study, we establish a role for dALS2 in synaptic maturation at the NMJ, a model of glutamatergic synapses. We demonstrate that dALS2 regulates postsynaptic development by activating the FNI pathway of noncanonical Wg signaling. First, we showed that loss of dALS2 led to postsynaptic phenotypes, including excess ghost boutons and a structurally abnormal SSR, which have previously been associated with disruption of the postsynaptic FNI pathway (Ataman et al., 2006; Harris et al., 2016; Mathew et al., 2005; Mosca and Schwarz, 2010). Second,
Figure 7. The dfz2 C-terminal fragment is cleaved in late endocytic compartments. 

(A–C) Single confocal slices through the middle of BG2-c2 cells. Surface Flag-dFz2-Myc (A and B) or Flag-dFz2ΔSGKTLESW-Myc (C) receptors in live BG2-c2 cells were prelabeled with anti-Flag antibody in Wg-conditioned medium (100 ng/ml Wg) at 4°C for 30 min, returned to RT to allow for receptor internalization, and fixed after 10 min (A) or 90 min (B and C). After permeabilization, cells were stained with anti-Myc and anti-Rab5 or anti-Rab7 and subsequently with fluorescent secondary antibodies. Arrowheads indicate Flag⁺Myc⁺Rab⁺ endosomal structures, and arrows indicate Flag⁺Myc⁺Rab⁺ endosomal structures. (D) Quantification of the number of Flag⁺Myc⁺ and Flag⁺Myc⁺ structures associated with Rab5 or Rab7 for cells shown in A–C. n = 12 cells. Notably, internalized Flag-dFz2-Myc receptors lost anti-Flag signals on Rab7⁺ late endosomes in a manner dependent on the sequence SGKTLESW. (E) Single confocal slice of Flag-dFz2-Myc-transfected BG2-c2 cells from experiments performed as in B. Cells were labeled with 1 µM Lysotracker for 5 min before cell fixation and subsequently immunostained for Flag and Myc. Flag⁺Myc⁺ structures also colocalize with the lysosomal marker Lysotracker (arrow). (F) Single confocal slice of Flag-dFz2-Myc/Importin-β11-HA–transfected BG2-c2 cells from experiments performed as in B. Flag-Myc⁺ puncta colocalize with Importin-β11-HA (arrow). Scale bar: 5 µm.
Figure 8. Loss of \(dALS2\) function in neurons causes locomotor dysfunction and progressive brain neurodegeneration. (A) Distance climbed by 20-d-old WT, \(dALS2^{Δ1}/dALS2^{Δ73}\), and C155-GAL4/; UAS-HA-\(dALS2/dALS2^{Δ1}/dALS2^{Δ73}\) (\(dALS2\) rescue) flies over a 30-s period (\(n = 9\) trials, 100 flies/trial). (B) Quantification of average distance climbed by WT, revertant, \(dALS2^{Δ1}/dALS2^{Δ73}\), C155-GAL4/; UAS-HA-\(dALS2/dALS2^{Δ1}/dALS2^{Δ73}\) (\(dALS2\) rescue), C155-GAL4/; UAS-HA-\(hALS2/\); \(dALS2^{Δ1}/dALS2^{Δ73}\) (\(hALS2\) rescue), and C155-GAL4/UAS-Mycl-NLS-dfz2-C; \(dALS2^{Δ1}/dALS2^{Δ73}\) (NLS-dFz2-C rescue) flies (\(n = 9\) trials). (C) Frontal sections (5-µm thickness) of brains from 20-d-old WT and \(dALS2^{Δ1}/dALS2^{Δ73}\) flies were H&E stained. Vacuoles are marked by arrowheads. (D) Quantification of vacuoles with diameter greater than 5 µm in WT and \(dALS2^{Δ1}/dALS2^{Δ73}\) brains at indicated ages (\(n = 10\) brains). (E) Quantification of brain vacuolization in 20-d-old flies of indicated genotypes (\(n = 10\) brains). (F) Confocal images of TUNEL-stained 20-d-old WT and \(dALS2^{Δ1}/dALS2^{Δ73}\) brains. Arrowheads indicate TUNEL+ cells. (G) Quantification of TUNEL+ cells in brains of 20-d-old flies of the indicated genotypes (\(n = 12\) brains). (H) Confocal single slices of 20-d-old \(dALS2^{Δ1}/dALS2^{Δ73}\) brains costained with TUNEL or anti-cleaved Dcp-1, anti-Elav, and anti-Repo. TUNEL+ or cleaved Dcp-1+ cells coexpress the neuronal cell marker Elav (arrowheads) but not the glial cell marker Repo. (I) Single confocal slice of TUNEL-stained 20-d-old OK6-GAL4/UAS-NLS-mCherry; \(dALS2^{Δ1}/dALS2^{Δ73}\) VNCs. Arrowheads indicate a TUNEL+mCherry+ motor neuron. (J) Quantification of TUNEL+mCherry+ cell number per VNC (\(n = 9\) VNCs). Data are presented as mean ± SEM. Comparisons are with WT unless otherwise indicated. Scale bars: 50 µm (C and F); 10 µm (H and I). ***, \(P < 0.001\); **, \(P < 0.01\); *, \(P < 0.05\).
**dALS2** exhibited transheterozygous interactions with wg and **dGRIP**, resulting in excess ghost bouton formation, and decreased nuclear levels of dFz2-C. Third, an NLS-tagged dFz2-C completely rescued the postsynaptic phenotypes of **dALS2** mutants. Together, these findings suggest that impairment of the FNI pathway is responsible for postsynaptic defects observed in **dALS2** mutants.

**dALS2** mutants exhibit normal evoked EJP amplitude, despite decreased mEJP amplitude, indicating a homeostatic increase in presynaptic neurotransmitter release (quantal content). The balance between GluRIIA and GluRIIB and the association of GluR with synaptic homeostasis at NMJ terminals have recently been further elucidated (Hong et al., 2020; Zhao et al., 2020). GluRIIB overexpression reduces the level of GluRIIA (Zhao et al., 2020), while **dALS2** loss selectively increases GluRIIB levels without affecting GluRIIA. Moreover, T-bar clustering elicited by reducing the GluRIIC level (Hong et al., 2020) is not present in **dALS2** mutants, suggesting that canonical homeostatic control may be also perturbed by **dALS2** loss.

How might **dALS2** regulate the FNI pathway? Studies have shown that mammalian **ALS2** regulates early endosomal fusion and trafficking by activating Rab5 (Devon et al., 2006; Otomo et al., 2003; Topp et al., 2004). We thus assessed the potential role of **dALS2** in endocytic dFz2 trafficking. Although dFz2 transport from synapses to the nucleus is essential for activation of the postsynaptic FNI pathway (Ataman et al., 2006), the exact nature of this retrograde transport remains unclear. In the present study, we demonstrate that retrograde dFz2 transport occurs via trafficking from Rab5+ early endosomes to Rab7+ late endosomes. We further demonstrate that **dALS2** is required for Rab5-to-Rab7 conversion of dFz2 endosomes.

The above findings raise a key question as to how the Rab5-to-Rab7 conversion of dFz2 endosomes influences the FNI pathway. Our data suggest that cleavage of the signaling-competent C-terminal fragment of dFz2 depends on its trafficking to Rab7+ late endosomes. First, muscle-specific depletion of central regulators of late endosome formation and maturation (Hrs, Rab7, Mon1, and VhaAC39-1) inhibited dFz2-C cleavage and increased ghost bouton formation at the NMJ. Second, in a receptor trafficking assay, Flag-dFz2-Myc receptors internalized in response to Wg stimulation, progressively inducing formation of Flag Myc+ dFz2-C puncta colocalizing with Rab7 or Lyso-tracker, but not Rab5, implying physical separation of the N and C termini of dFz2 in the late endosome/lysosome compartment. However, a Flag-dFz2-Myc mutant with deletion at the site of dFz2-C cleavage formed Flag Myc+Rab7+ endosomes but did not form Flag Myc+Rab7– endosomes, indicating that Flag Myc+ dFz2-C puncta arise from physiologically relevant cleavage of Flag-dFz2-Myc. Finally, Flag Myc+ dFz2-C puncta overlapped with Importin-β11, which mediates nuclear import of dFz2-C (Mosca and Schwarz, 2010). We thus propose that C-terminal cleavage of dFz2 occurs in late endocytic compartments, such as late endosomes and lysosomes.

It is presently unclear why C-terminal cleavage of dFz2 requires progression of dFz2 to late endocytic compartments; however, the necessity of V-ATPase implies that acidic luminal pH in late endosomes and lysosomes creates an environment that is favorable for proteolytic dFz2 cleavage. V-ATPase-mediated acidification is thought to promote γ-secretase proteolysis (S3 cleavage) of Notch by facilitating ectodomain shedding, which can be accomplished by inducing denaturation or acid hydrolase–dependent degradation of the Notch extracellular domain (Schnute et al., 2018). Future investigations will determine whether ectodomain shedding is required for C-terminal cleavage of dFz2.

We found that **dALS2** mutant flies recapitulated some key phenotypes of **ALS**, including adult-onset motor impairment and brain neurodegeneration. Intriguingly, these defects were suppressed by neuronal expression of **dALS2**, but not NLS-dFz2-C, indicating that **dALS2** regulates neuronal cell survival in a dFz2-independent manner. These findings raise an important question as to the cellular mechanisms of neuronal cell death in **dALS2** mutant flies and **ALS** patients. Increasing evidence suggests a central role for impaired receptor and endosomal trafficking in the pathogenesis of **ALS** (Burk and Pasterkamp, 2019). For example, modulation of endosomal trafficking by constitutively active Rab5 or chemokine modulators of Rab5 effectors ameliorates neurodegeneration caused by an intronic GGGGCC hexanucleotide repeat expansion in the C9ORF72 gene (Shi et al., 2018), which is the most common cause of **ALS** and frontotemporal dementia (Renton et al., 2014). Moreover, loss-of-function mutations in charged multivesicular body protein 2B and the valosin-containing protein (VCP or ALS14) gene, both of which are involved in endosome maturation, have also been linked to **ALS** (Cox et al., 2010; Johnson et al., 2010), further supporting the potential connection between endosomal trafficking defects and neurodegeneration in **ALS**. Given the generalized role of **dALS2** in controlling endosomal trafficking, defining the detailed mechanism by which endosomal trafficking defects trigger or contribute to neuronal cell death in **ALS** is of paramount importance.

**Materials and methods**

**Drosophila stocks**

All *Drosophila* stocks were maintained at 25°C on normal food. The WT flies were w1118. The wg17, Df(3L)ED4782 (a deficiency uncovering the dfz2 locus), UAS-NLS-mCherry, UAS-Rab7-RNAi, UAS-Hrs-RNAi, and UAS-VhaAC39-1-RNAi flies were obtained from the Bloomington *Drosophila* Stock Center. UAS-*dALS2-*RNAi and UAS-**Mon1**-RNAi flies were obtained from the Vienna *Drosophila* Resources Center. The UAS-myc-NLS-dFz2-C flies were a kind gift from Thomas Schwarz (Boston Children’s Hospital, Boston, MA). The dfz2c1 flies were obtained from Makoto Sato (Kanazawa University, Kanazawa, Japan). Stephan Sigrist (Freie Universität Berlin, Berlin, Germany) provided the dGRIP106 flies. Transgenic expression was driven by the following GAL4 drivers: D42-GAL4, OK6-GAL4, 24B-GAL4, B57-GAL4, and Mhc-GS-GAL4 (Brand and Perrimon, 1993; Budnik et al., 1996; Osterwalder et al., 2001; Sanyal, 2009; Yeh et al., 1995).

A **dALS2**-null allele, dfz2Δ1, was generated as described previously via a CRISPR/Cas9-assisted nonhomologous end-joining strategy (Gratz et al., 2013). Briefly, two pU6-Bbsl-

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subcloned into the pcDNA3.1 (Invitrogen) and pEGFP-N2 (Clontech) full-length cDNAs for human For transient expression in mouse motor neuron (Kim et al., 2019). To generate a DNA template for in vitro (dsRNA) for transcription, primers containing the T7 promoter sequence  ATCCATTGA-3 were quantified by PCR using the following primers: dALS2, S’-CGCATC AACTCATTGGCGCC-3’ and 5’-GATGCGGAAGATGCTGCT-3’; CG1248: S’-CAAAGGCTCAAGGGCAG-3’ and 5’-GGCGAAATCCGCCTGGC-3’; and rp49: S’-CACCAGTCGATGATGTCGAT-3’ and 5’-CAGTTTGTGCACGAAGACT-3’.

Cell transfection and production of Wg-conditioned medium

For molecular characterization of dALS2 deletions, the following primers were used for genomic PCR: 5’-CAGTTGCTC ATATGCGCCCTCAGC-3’ and 5’-GTCACCTTTTACCCGAAACCC CTTCAC-3’. To assess the effect of dALS2 deletions on the expression of dALS2, CG1248, and rp49, total RNA isolated from larvae was reverse transcribed into cDNA. cDNAs were quantified by PCR using the following primers: dALS2, S’-CGCATC AACTCATTGGCGCC-3’ and 5’-GATGCGGAAGATGCTGCT-3’; CG1248: S’-CAAAGGCTCAAGGGCAG-3’ and 5’-GGCGAAATCCGCCTGGC-3’; and rp49: S’-CACCAGTCGATGATGTCGAT-3’ and 5’-CAGTTTGTGCACGAAGACT-3’.

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control of BG57-GAL4. Samples were homogenized in SDS sample buffer (62.5 mM Tris–HCl, pH 6.8, 10% glycerol, 2% SDS, 2.88 mM β-mercaptoethanol, and 0.02% bromophenol blue), boiled for 5 min, and centrifuged at 13,000 g for 5 min. Supernatant samples were subjected to 12% SDS-PAGE and transferred to polyvinylidene fluoride membrane (Merck Millipore). Blots were incubated overnight at 4°C with mouse anti-Flag (1:2,000; Cat# F1804; Sigma-Aldrich), rabbit anti-Myc (1:1,000; Cat#: 2278; Cell Signaling Technology), or rabbit anti-β-actin (1:1,000; Cat#: A2066; Sigma-Aldrich) antibodies, and then for 1 h at RT with HRP-conjugated anti-rabbit or anti-mouse secondary antibody (1:4,000; Cat#: 111–035–144 or 715–035–150; Jackson ImmunoResearch Laboratories) in blocking solution (5% skim milk/0.1% Tween-20/TBS). Protein bands were visualized by ECL reagents (iNtRON Biotechnology).

NSC-34 cells were transfected with pEGFP-Fz5 and either pcDNA-Wnt5a or empty vector in the presence of control or ALS2 siRNA. For whole-cell lysates, cells were lysed in lysis buffer (50 mM Tris–HCl, pH 7.4, 0.5% Triton X-100, 150 mM NaCl, and protease inhibitor mixture). Nuclear fractions were isolated using a NE-PER nuclear and cytoplasmic extraction reagent kit (Thermo Scientific Scientific). Whole-cell lysates and nuclear fractions were analyzed by Western blotting using rabbit anti-GFP (1:1,000; Cat#: Al1122; Invitrogen), rabbit anti-Lamin B1 (1:1,000; Cat#: ab16048; Abcam), and rabbit anti-GAPDH (1:1,000; Cat#: sc-25778; Santa Cruz Biotechnology).

**Larval NMJ immunohistochemistry and quantification**

Wandering third instar larvae were dissected in Ca2+-free HL3 saline (70 mM NaCl, 5 mM KCl, 20 mM MgCl2, 10 mM NaHCO3, 5 mM trehalose, 5 mM Hepes, pH 7.2) and fixed for 30 min in 4% formaldehyde/PBS or for 7 min in Bouin’s fixative (Sigma-Aldrich). Fixed larvae were washed in 0.1% Triton X-100/PBS and then sequentially incubated with primary and secondary antibodies in 0.1% Triton X-100/PBS containing 0.2% BSA as described previously (Kim et al., 2019). The following primary antibodies were used: mouse anti-Dlg (1:500; Cat#: 4F3; DSHB), mouse anti-Brp (1:10; Cat#: nc82; DSHB), mouse anti-GluRIIA (1:50; Cat#: 8B4D2; DSHB), mouse anti-Futsch (1:5; Cat#: 22C10; DSHB), mouse anti-Synapsin (1:25; Cat#: 3C11; DSHB), mouse anti-Csp (1:50; Cat#: 1G12; DSHB), rabbit anti-Rab5 (1:200; Cat#: ab31261; Abcam), mouse anti-Rab7 (1:50; Cat#: Rab7; DSHB), rat anti-HA (1:200; Cat#: 11867423001; Roche), mouse anti-Myc (1:200; Cat#: 5S1101; BD Biosciences), rabbit anti-GluRIIA (1:2,000; custom-produced by Afromier), rabbit anti-GluRIIIC (1:2,000; custom-produced by Afromier), and rabbit anti-dFz2-C (1:200; a kind gift from Vivian Budnik, University of Massachusetts, Worcester, MA). The following secondary antibodies from Jackson ImmunoResearch Laboratories were used at 1:200: FITC-, Cy3-, and Cy5-conjugated donkey anti-mouse (Cat#: 715–095–151, 715–165–151, and 715–175–151, respectively); FITC-, Cy3-, and Cy5-conjugated donkey anti-rabbit (Cat#: 711–095–152, 711–165–152, and 711–175–152, respectively); FITC- and Cy3-conjugated donkey anti-rat (Cat#: 712–095–153 and 712–165–153, respectively); and FITC-, Cy5-, and DyLight405-conjugated goat anti-HRP (Cat#: 123–195–021, 123–175–021, and 123–475–021, respectively). Labeled larvae were mounted in SlowFade mounting medium (Molecular Probes), and images of NMJs and muscle nuclei were acquired as confocal z stacks with an LSM 800 confocal microscope (Carl Zeiss) with a C-Apo 40× 1.2 NA W, Plan-Apo 63× 1.4 NA Oil, or Plan-Apo 100× 1.4 NA Oil objective at RT. Images were processed with the Zen microscopy software and Adobe Photoshop. For comparison between genotypes, samples were processed simultaneously and imaged using identical acquisition settings.

For quantification of NMJ morphology, NMJs on muscles 6/7 from segment A3 were selected for analysis. To quantify bouton numbers, ghost bouton numbers, and bouton size, maximum intensity projection images of NMJs 6/7 contained with anti-Dlg or anti-Csp and anti-HRP were produced from confocal z stacks collected at 1-μm intervals. Larval muscle size was not significantly different among the genotypes evaluated in this study. Ghost boutons were identified by the presence of anti-HRP-stained boutons lacking postsynaptic Dlg staining. Bouton size was quantified by measuring the bouton diameter for one terminal and four adjacent nonterminal Ib boutons on two different NMJ 6/7 branches per animal using ImageJ. The proportion of Ib boutons with Futsch-immunoreactive loops was quantified using anti-HRP and anti-Futsch containing. Synaptic levels of GluR receptors were quantified using anti-HRP and anti-GluRIIA or anti-GluRIIB containing. The synaptic area was delimited by HRP immunoreactivity, and the average GluRIIA or GluRIIB signal intensity within the synaptic area was normalized to the average HRP signal intensity. Nuclear dFz2-C was quantified in muscle 6 by counting distinct dFz2-C immunoreactive spots in the three nuclei closest to the NMJ, as described previously (Mathew et al., 2005). The nuclear boundary was defined by DAPI staining.

**Fluorescence-based internalization and trafficking studies**

The Flag-dFz2-Myc trafficking assay in dissected third instar larvae was performed as described previously (Ataman et al., 2006; Mathew et al., 2005). Briefly, WT and dAls2ΔdAls2Δ larvae expressing Flag-dFz2-Myc specifically in muscles were dissected in HL3 solution containing 0.1 mM Ca2+ and incubated at 4°C in 0.1 mM Ca2+/HL3 containing 5 μg/ml rat anti-Flag (Cat#: 637302; BioLegend) for 1 h to label surface Flag-dFz2-Myc receptors. Samples were then washed with 0.1 mM Ca2+/HL3 to remove unbound antibody and incubated at RT for 10 or 30 min to allow internalization of the labeled receptors. Samples were fixed in 4% formaldehyde/PBS for 30 min and incubated with 20 μg/ml anti-rat IgG secondary antibody (Cat#: 712–005–150; Jackson ImmunoResearch Laboratories) under non-permeant conditions to mask external Flag-dFz2-Myc receptors. Samples were permeabilized in 0.1% Triton X-100/PBS and incubated with rabbit anti-Rab5 (1:200; Cat#: ab31261; Abcam) and mouse anti-Rab7 (1:50; Cat#: Rab7; DSHB), followed by incubation with FITC-conjugated donkey anti-rat, Cy3-conjugated donkey anti-rabbit, and Cy5-conjugated donkey anti-mouse secondary antibodies, together with DyLight405-conjugated goat anti-HRP antibody (1:200; Cat#: 712–095–153, 713–165–152, 715–175–151, and 123–475–021, respectively; Jackson ImmunoResearch Laboratories). After 10 and 30 min of internalization, the majority of internalized Flag-dFz2-Myc was detected in discrete punctate

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structures. For each sample, images were captured with an LSM 800 confocal microscope using a Plan-Apo 63× 1.4 NA Oil objective at RT. To quantitatively monitor endosomal trafficking of internalized Flag-dFz2-Myc, the number of Flag-immunoreactive puncta (green) containing Rab5 (red) and/or Rab7 (blue) was manually counted in a 1-µm area surrounding anti-HRP-stained synaptic branches (peribouton area) and a 5-µm area surrounding the nucleus (perinuclear area) using ImageJ.

The Flag-dFz2-Myc trafficking assay in BG2-c2 cells was performed to determine the intracellular location of dFz2-C cleavage. BG2-c2 cells were transiently transfected with pAc-Flag-dFz2-Myc or pAc-Flag-dFz2ΔSCKTLESW-Myc—a dFz2 mutant deleting the coding sequences for the glutamyl-endopeptidase cleavage site—in the absence or presence of pAc-Importin-β-Myc. At 48 h post-transfection, cells were incubated for 6 h in serum-free M3 medium containing 100 µg/ml cycloheximide (Sigma-Aldrich), a protein synthesis inhibitor. Cells were incubated in Wg-conditioned medium (100 ng/ml Wg) containing 5 µg/ml rat anti-Flag (Cat# 637302; BioLegend) at 4°C for 30 min to label surface Flag-dFz2-Myc receptors. Cells were then rinsed in M3 medium and incubated at RT for 10, 30, or 90 min to allow for internalization of labeled receptors. In some experiments, LysoTracker (Molecular probe) was added to a final concentration of 1 µM, 85 min after Wg stimulation. Cells were fixed in 4% formaldehyde/PBS for 10 min and subsequently permeabilized with 0.2% Triton X-100/PBS for 10 min. Cells were incubated overnight with the following combinations of primary antibodies in 0.2% BSA/PBS at 4°C: mouse anti-Myc (1:100; Cat# 551101; BD Biosciences); mouse anti-Myc (1:100; Cat# 551101; BD Biosciences) and rabbit anti-Rab5 (1:200; Cat# ab31261; Abcam); rabbit anti-Myc (1:1,000; Cat# 2278; Cell Signaling Technology) and mouse anti-Rab7 (1:50; Cat# Rab7; DSHB); and mouse anti-Myc (1:100; Cat# 551101; BD Biosciences) and rabbit anti-HA (1:200; Cat# 3724; Cell Signaling Technology). After washing with PBS, cells were incubated with FITC-conjugated donkey anti-rat, Cy3- or Cy5-conjugated donkey anti-rabbit, and Cy3- or Cy5-conjugated donkey anti-mouse secondary antibodies (1:200; Cat# 702–095-153, 711–165–152, 711–175–152, 715–165–151, and 715–175–151, respectively; Jackson ImmunoResearch Laboratories) in 0.2% BSA/PBS for 1 h. For each cell, a z stack of an optical section (0.23-µm thickness) was captured with an LSM 800 confocal microscope using a Plan-Apo 63× 1.4 NA Oil objective at RT. The numbers of intracellular Flag-Myc‘Rab5’, Flag-Myc‘Rab7’, or Flag-Myc‘Rab7’ puncta per cell were manually counted using ImageJ.

For the Wg trafficking assay, BG2-c2 cells were transfected with pAc-Flag-dFz2-Myc in the presence or absence of dALS2 dsRNA. Cells at 48 h post-transfection were serum starved in M3 medium for 6 h and subsequently incubated at 4°C with 2.5 µg/ml mouse anti-Myc antibody or rabbit anti-Myc antibody for 30 min to label surface Myc-Tkv. Cells were treated at RT for 1 min with Gbb-conditioned medium (50 ng/ml Gbb) and further incubated for 4 or 19 min in serum-free M3 medium. Cells were stained for Rab5 and Rab7 and imaged for quantification of colocalization between mBSA and Rab5 or Rab7, as described for the Wg trafficking assay.

For the Myc-Tkv receptor trafficking assay, BG2-c2 cells were transfected with pAc-Myc-Tkv in the presence or absence of dALS2 dsRNA. Cells at 48 h post-transfection were serum starved in M3 medium for 6 h and subsequently incubated at 4°C with 2.5 µg/ml mouse anti-Myc antibody or rabbit anti-Myc antibody for 30 min to label surface Myc-Tkv. Cells were treated at RT for 1 min with Gbb-conditioned medium (50 ng/ml Gbb) and further incubated for 4 or 19 min in serum-free M3 medium. Cells were stained for Myc and Rab5 or Rab7 and imaged for quantification of colocalization between Myc-Tkv and Rab5 or Rab7, as described for the Wg trafficking assay.

For quantitative analysis of nuclear Fz5-GFP, NSC-34 cells were transfected with pEGFP-Fz5 and empty vector or pcDNA-Wnt5a in the presence of control or ALS2 siRNA. Cells at 72 h post-transfection were fixed, stained for nuclei (1:10,000; Hoechst; Thermo Fisher Scientific), and imaged for quantification of cells with nuclear GFP signal among all GFP+ cells. Images of NSC-34 cells were acquired as confocal z stacks with a Leica TCS SP8 confocal microscope (Leica) with an HCX Apo 63× 0.9 NA W objective at RT. Images were processed with the Leica LASX software and Adobe Photoshop.

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Wandering third instar larvae were dissected in Ca²⁺-free HL3 saline at RT. Larval fillets were fixed in 1% glutaraldehyde/4% paraformaldehyde/0.1 M cacodylic acid (pH 7.2) solution at 4°C for 12 h. The samples were then washed with 0.1 M cacodylic acid (pH 7.2) solution three times, post-fixed in 1% OsO4/0.1 M cacodylic acid (pH 7.2) solution at RT for 3 h and subjected to
Wandering third instar larvae were dissected in ice-cold Ca2+-free HL3 saline and subsequently incubated with 0.5 mM Ca2+-HL3 for 5–10 min. Whole-muscle recording was performed on muscle 6 in segment A3 as described previously (Yao et al., 2017). Intracellular electrodes with a resistance of ∼40 MΩ filled with 3 M KCl solution were used for recording. We analyzed recordings from muscles with resting membrane potential less than −60 mV and input resistance more than 5 MΩ. mEPJs were elicited by 0.2-Hz stimulation. Stimulus pulses were fixed at 0.5-ms duration using pClamp 10.6 software (Axon Instruments). Spontaneous mEPJs were recorded for 3 min. Both mEPJs and mEPs were amplified with an Axoclamp 900A amplifier (Axon Instruments) under bridge mode and filtered at 10 kHz. The mean amplitude of mEPJs and the mean amplitude and frequency of mEPs were analyzed using Clampfit software (Molecular Devices).

Histology, TUNEL staining, and immunostaining of adult brains
Paraffin sectioning and H&E staining of adult brains were performed as previously described (Heo et al., 2017). Heads from adult flies at 2, 10, 20, 30, and 40 d posteclosion were fixed with 3 M KCl solution were used for recording. We analyzed recordings from muscles with resting membrane potential less than −60 mV and input resistance more than 5 MΩ. mEPJs were elicited by 0.2-Hz stimulation. Stimulus pulses were fixed at 0.5-ms duration using pClamp 10.6 software (Axon Instruments). Spontaneous mEPJs were recorded for 3 min. Both mEPJs and mEPs were amplified with an Axoclamp 900A amplifier (Axon Instruments) under bridge mode and filtered at 10 kHz. The mean amplitude of mEPJs and the mean amplitude and frequency of mEPs were analyzed using Clampfit software (Molecular Devices).

For immunostaining of adult brains (Fig. 8 H), 20-d-old adult fly brains were stained as previously described (Nahm et al., 2013). Briefly, dissected brains were fixed in 4% formaldehyde/PBS for 20 min at RT. Brains were then permeabilized in 0.3% Triton X-100/PBS, blocked with 5% BSA in 0.3% Triton X-100/PBS at RT for 1 h, and incubated with primary antibodies in blocking buffer for 2 d at 4°C. Samples were then incubated in 0.3% Triton X-100/PBS containing 5% BSA with secondary antibodies overnight at 4°C. To stain brains with TUNEL and antibodies, brains were incubated in 0.1% Triton X-100/PBS containing 100 mM sodium citrate for 30 min at RT and incubated with TUNEL reaction mixture in the dark for 1 h at 37°C before incubation with primary antibodies. The following antibodies were used in this study: rat anti-Elav (1:10; Cat# 7E8A10; DSHB), mouse anti-Repo (1:10; Cat# 8D12; DSHB), rabbit anti–cleaved Dcp-1 (1:100; Cat# 9578; Cell Signaling Technology), and donkey secondary antibodies (1:200) from Jackson ImmunoResearch Laboratories, including FITC-conjugated anti-rabbit (Cat# 711–095–152), Cy3-conjugated anti-rat (Cat# 712–095–153), and Cy5-conjugated anti-mouse (Cat# 715–175–151).

Adul behavioral analysis
Adult locomotor function was assessed using a climbing ability test (Nahm et al., 2013). For each genotype, ~100 flies were collected within 1 d after eclosion and aged for 20 d. Aged flies were placed into an empty glass cylinder, allowed to acclimate for 5 min, and gently tapped to the bottom. The distance climbed by individual flies after 30 s was measured.

Statistical analysis
Data are presented as mean ± SEM. Statistical significance was calculated using a one-way ANOVA followed by pairwise Tukey’s tests (for multiple group comparisons) or using a two-tailed Student’s t test (for two-group comparisons).

Online supplemental material
Fig. S1 shows characterization of dALS2 mutants and extends Fig. 1. Fig. S2 shows the subcellular localization of HA-dALS2 in larval muscles and demonstrates that Flag-dFz2-Myc behaves similarly to endogenous dFz2 protein in vivo. Fig. S3 shows the impact of dALS2 depletion on intracellular trafficking of Wg in BG2-c2 cells. Fig. S4 shows the impact of dALS2 depletion on intracellular trafficking of mBSA and Tkv in BG2-c2 cells. Fig. S5 shows that depletion of mammalian ALS2 impairs Wnt5a-induced nuclear translocation of the Fz5 receptor in mouse motor neuron–like NSC-34 cells.

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Figure S1. Characterization of dALS2 gene and mutants. (A) Schematic of the genomic dALS2 (CG7158) locus. The position of the transposon G4607 used to generate the dALS2 allele dALS2Δ73 via transposase-mediated excision is indicated by the inverted triangle. The gRNA target sites used to generate the dALS2Δ1 allele via the CRISPR/Cas9 genome editing system are marked by arrows. Exon-intron organization of dALS2 and the neighboring CG11248 gene is shown in the middle. Introns are indicated by horizontal lines, untranslated regions by white boxes, translated regions by black boxes, and translation initiation sites by arrows. Shown below are deletion breakpoints for dALS2Δ1 and dALS2Δ73. (B) RT-PCR analysis of dALS2, CG11248, and rp49 transcripts in WT and dALS2Δ1/dALS2Δ73 third instar larvae. rp49 is used as a loading control. (C–H) Multiple aspects of presynaptic development remain unchanged in dALS2 mutants. (C) Confocal images of NMJ 6/7 stained with antibodies against HRP (green) and cysteine string protein (Csp; red) are shown for WT and dALS2Δ1/dALS2Δ73 third instar larvae. (D–F) Quantification of total bouton number (D), bouton size (E), and muscle 6/7 area (F). n = 16 NMJs. (G) Confocal images of anti-Futsch–stained NMJ 6/7 in WT and dALS2Δ1/dALS2Δ73 third instar larvae. Arrowheads indicate Futsch-immunoreactive loops. (H) Quantification of boutons with Futsch loops (n = 18 NMJs). (I and J) Characterization of ghost boutons in dALS2-null mutants. (I) Confocal images of NMJ 6/7 stained with anti-HRP, anti-Brp, and anti-GluRIIC antibodies for WT and dALS2Δ1/dALS2Δ73 third instar larvae. Ghost boutons (arrowheads) are identified as HRP-labeled varicosities lacking Brp and GluRIIC immunoreactivities. (J) Confocal images of NMJ 6/7 labeled with anti-HRP, anti-Synapsin (Syn), and anti-GluRIIC antibodies are shown for WT and dALS2Δ1/dALS2Δ73 third instar larvae. Synapsin is still present in dALS2-induced ghost boutons (arrowheads). (K and L) Postsynaptic, but not presynaptic, dALS2 knockdown results in excessive ghost bouton formation. (K) Confocal images of NMJ 6/7 labeled with anti-HRP and anti-Dlg antibodies are shown for D42-GAL4/+; UAS-dALS2-RNAi-pre, D42-GAL4/+; UAS-dALS2-RNAi-post, 24B-GAL4/+, and UAS-dALS2-RNAi/+; 24B-GAL4/+ (dALS2-RNAi-post) third instar larvae. Right panels show higher magnification views of areas marked by white boxes. Arrowheads indicate ghost boutons. (L) Quantification of ghost bouton number (n = 30 NMJs). Data are presented as mean ± SEM. Comparisons are with WT (D–F and H) or D42-GAL4 unless otherwise indicated. Scale bars: 20 µm (C, G, and K); 5 µm (I and J). ***, P < 0.001.
Figure S2. Characterization of transgenic HA-dALS2 and Flag-dFz2-Myc expression in larval muscles. (A) Single confocal sections of the peribouton area (upper panels) at NMJ 6/7 and the perinuclear area (lower panels) in muscle 6 are shown for Mhc-GS-GAL4/UAS-HA-dALS2 third instar larvae stained with anti-HA, anti-Rab5, and anti-Rab7. Arrowheads indicate endosomal compartments double positive for HA-dALS2 and Rab5, while arrows indicate endosomal compartments triple positive for HA-dALS2, Rab5, and Rab7. Note that HA-dALS2 rarely colocalizes with Rab7. (B) Quantification of the number of HA-dALS2+ puncta associated with Rab5 (red), Rab7 (blue), or Rab5/Rab7 (magenta) in the peribouton or perinuclear area (n = 7 muscles). (C and D) Flag-dFz2-Myc localizes similarly to endogenous dFz2 in the body wall muscles. (C) Confocal image of UAS-Flag-dfz2-Myc/+; 24B-GAL4/+ third instar larval NMJs stained with anti-Myc. (D) Single confocal sections through the equators of muscle nuclei (dashed circles) stained with anti-Myc. Upper panel: Immunoreactivity is not detected in the absence of the UAS-Flag-dfz2-Myc transgene (24B-GAL4/+). Lower panel: Nuclear staining is observed when the UAS-Flag-dfz2-Myc transgene is expressed in body wall muscles (UAS-Flag-dfz2-Myc/+; 24B-GAL4/+). (E and F) Muscle expression of Flag-dfz2-Myc rescues the dfz2 NMJ phenotype. (E) Confocal images of third instar NMJ 6/7 stained with antibodies against HRP (green) and Dlg (red) are shown for WT, dfz2^{C1}/Df(3L)ED4782, and UAS-Flag-dfz2-Myc/+; dfz2^{C1}/Df(3L)ED4782,24B-GAL4 (Flag-dFz2-Myc rescue-post) larvae. Right panels show higher magnification views of areas marked by white boxes. Arrowheads indicate ghost boutons. (F) Quantification of ghost bouton number per NMJ 6/7 (n = 15–39 NMJs). Data are presented as mean ± SEM. Comparisons are relative to WT unless otherwise indicated. Scale bars: 5 µm (A and D), 20 µm (C and E). **, P < 0.01. n, muscle nucleus.

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Figure S3. dALS2 is required for Wg trafficking to Rab7+ late endosomes in BG2-c2 cells. (A) RT-PCR analysis of dALS2 expression in control and dALS2 dsRNA BG2-c2 cells using rp49 as a loading control. (B and C) Depletion of dALS2 in BG2-c2 cells does not impair DFz2-mediated Wg internalization. (B) Representative confocal images for control and dALS2 knockdown cells allowed to uptake Wg for 10 min. Cells were transfected with pAc-Flag-dFz2-Myc in the presence or absence of dALS2 dsRNA, serum starved for 6 h, and pulsed with Wg-conditioned medium (100 µg/ml Wg) at 4°C for 30 min. Cells were further incubated in serum-free M3 medium at RT for 10 min and fixed for immunostaining with anti-Wg antibody. Notably, Wg internalization into BG2-c2 cells occurs in a receptor-mediated manner. (C) Quantification of the amount of internalized Wg per cell (n = 15 cells). (D–H) Depletion of dALS2 impairs Wg trafficking from Rab5+ early endosomes to Rab7+ late endosomes. (D–G) Representative confocal images for control and dALS2 knockdown cells allowed to uptake Wg for 10 (D and E) or 30 min (F and G). Cells were cotransfected with pAc-Flag-dFz2-Myc together with either pAc-GFP-Rab5 (D and F) or pAc-GFP-Rab7 (E and G) in the presence or absence of dALS2 dsRNA were treated with Wg-conditioned medium as in B. Cells were further incubated in serum-free medium for 10 or 30 min at RT before immunostaining with anti-Wg and anti-GFP antibodies. Arrowheads indicate Wg puncta overlapping with GFP-Rab5 (D and F) or GFP-Rab7 (E and G). (H) Quantification of Wg-GFP-Rab5 and Wg-GFP-Rab7 colocalization control and dALS2 knockdown cells (n = 15 cells). (I) Quantification of the average areas of Rab5-GFP and Rab7-GFP puncta per cell. Data are presented as mean ± SEM. Comparisons are with the mock-treated control. Scale bars: 5 µm. ***, P < 0.001.
Figure S4. Depletion of dALS2 disrupts the transport of mBSA and the BMP receptor Tkv from early to late endosomes. (A–D) Knockdown of dALS2 impairs progression of mBSA from early to late endosomes. (A–C) BG2-c2 cells were transfected with a Sr-CI construct in the absence (control) or presence of dALS2 dsRNA and pulsed with Cy3-mBSA for 1 min in serum-free M3 medium containing 1.5 mg/ml BSA. The pulse was chased for 4 min (A) and 19 min (B and C) in serum-free medium. After fixation, cells were immunostained for endogenous Rab5 (A and B) or Rab7 (C). Single confocal sections through the middles of cells are shown. Arrowheads indicate mBSA puncta colocalizing with Rab5 (A and B) or Rab7 (C). (D) Quantification of mBSA-Rab5 or mBSA-Rab7 colocalization in control and dALS2 knockdown cells (n = 15 cells). (E–H) dALS2 knockdown impairs progression of Myc-Tkv from early to late endosomes. (E–G) BG2-c2 cells were transfected with a Myc-Tkv construct alone (control) or together with dALS2 dsRNA. Cells were serum starved for 6 h, prelabeled with anti-Myc antibody at 4°C for 30 min, and subsequently stimulated with Gbb-conditioned medium (50 ng/ml Gbb) at RT for 1 min. After further incubation in serum-free M3 medium for 4 min (E) or 19 min (F and G), cells were immunostained for endogenous Rab5 (E and F) or Rab7 (G). Single confocal sections through the middles of cells are shown. Arrowheads indicate Myc-Tkv puncta colocalizing with Rab5 (E and F) or Rab7 (G). (H) Quantification of Myc-Tkv-Rab5 and Myc-Tkv-Rab7 colocalization (n = 10 cells). Data are presented as mean ± SEM. Comparisons are with mock-treated control. Scale bars: 5 µm. ***, P < 0.001.
Figure S5. Depletion of mammalian ALS2 impairs Wnt5a-induced nuclear translocation of the Fz5 receptor in motor neuron–like NSC-34 cells. (A) RT-PCR analysis of ALS2 expression in NSC-34 cells treated with control or ALS2 siRNA using GAPDH as a loading control. (B–F) NSC-34 cells were transfected with pEGFP-Fz5 and empty vector (−Wnt5a) or pcDNA-Wnt5a (+Wnt5a) in the presence of control or ALS2 siRNA. (B) Representative confocal images for Fz5-GFP–expressing cells under indicated conditions. Notably, Wnt5a-induced nuclear localization of Fz5-GFP is abrogated by ALS2 knockdown. (C) Quantification of the percentage of cells with nuclear GFP signal among all GFP+ cells. Approximately 50 cells were counted per genotype in each of three replicate experiments. (D) Western blot analysis of whole-cell lysates using anti-GFP and anti-GAPDH (loading control). Markers are shown in kilodaltons. Notably, a ∼34-kD band corresponding to the cleaved C-terminal peptide of Fz5-GFP is decreased in ALS2 knockdown cells relative to controls. (E) Western blot analysis of nuclear extracts using anti-GFP, anti–Lamin B1, and anti-GAPDH. The purity of nuclear fractions was confirmed by detection of Lamin B1 (a nuclear fraction marker), but not GAPDH (a cytoplasmic fraction marker). (F) Quantification of cleaved Fz5-GFP levels by densitometric measurements from three independent experiments. Notably, Wnt5a–induced nuclear accumulation of cleaved Fz5-GFP is abrogated by ALS2 knockdown. Data are presented as mean ± SEM. Scale bar: 10 µm. ***, P < 0.001; **, P < 0.01; *, < 0.05.