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The nuclear import of Frizzled2-C by Importins-β11 and α2 promotes postsynaptic development

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Abstract

Synapse-to-nucleus signaling is critical for synaptic development and plasticity. In *Drosophila*, the ligand Wingless causes the C-terminus of its Frizzled2 receptor (Fz2-C) to be cleaved and translocated from the postsynaptic density to nuclei. The mechanism of nuclear import is unknown and the developmental consequences of this translocation are uncertain. Here, we show that Fz2-C localization to muscle nuclei requires the nuclear import factors, Importin-β11 and Importin-α2 and that this pathway promotes the postsynaptic development of the subsynaptic reticulum (SSR), an elaboration of the postsynaptic plasma membrane. *importin-β11* and *dfz2* mutants have less SSR and some boutons lacking the postsynaptic marker Discs Large. These developmental defects in *importin-β11* can be overcome by expression of Fz2-C fused to a nuclear localization sequence that can bypass Importin-β11. Thus, Wnt-activated growth of the postsynaptic membrane is mediated by the synapse-to-nucleus translocation and active nuclear import of Fz2-C via a selective Importin-β11/α2 pathway.

Keywords

Synapse; Neuromuscular Junction; Wnt; *Drosophila*; Importin; Synapse-to-Nucleus Signalling; Frizzled2; Subsynaptic Reticulum; Postsynaptic Development

Introduction

Communication between the synapse and nucleus is essential for proper synaptic regulation and several pathways have been identified whereby synaptic signals are translated into nuclear instructions. Importins facilitate protein entry into the nucleus and can be critical in conveying these synapse-to-nucleus signals1, 2. In classical nuclear import, cargo binds an Importin-α and subsequent binding of an Importin-β allows the complex to pass through the nuclear pore3. In neurons, importins mediate signals from sites of axonal injury4, signals

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Author Contributions

TJM performed the experiments and analyzed the data. TJM and TLS designed the experiments and wrote the paper.

generated during LTP and other forms of synaptic plasticity, and signals required for photoreceptor axon targeting5, 6. Importins are emerging as a critical nexus of regulation between synaptic signals and the nucleus.

Wnt pathways are implicated in synaptic development and can include nuclear responses7. The best-studied nuclear response entails β-catenin, which does not require an importin for nuclear entry8. In other pathways, the *Drosophila* Wnt receptor Fz2 and the unrelated mammalian Wnt receptor, Ryk are cleaved and their liberated C-termini (Fz2-C or Ryk-C) translocate directly to the nucleus9, 10. The mechanism of nuclear import of these receptor-derived peptides is not known and yet Fz2-C is of particular interest as it potentially links activity-dependent release of Wingless (Wg, the *Drosophila* Wnt ligand) from the presynaptic motoneuron to synaptic plasticity10–12.

Deciphering the functional significance of nuclear Fz2-C translocation has been complicated by the myriad of other potential Wnt signaling pathways that may also be present. Although multiple phenotypes have been reported for *wg* and *dfz2* at the fly NMJ, including changes in synapse size and number, neuronal microtubule organization, and postsynaptic development11, 13, 14, it has not been possible to determine which, if any, can be attributed to nuclear translocation of Fz2-C. Recently, we showed that defects in bouton number, size and microtubules are accounted for by a local presynaptic pathway largely independent of Wg / Fz2 signalling in the muscle13. Wg signaling, however, is also implicated in some postsynaptic phenotypes that might be due to signaling in muscles. In addition to glutamate receptor localization, postsynaptic development includes formation of the subsynaptic reticulum (SSR), a complex array of folds in the plasma membrane that are potentially analogous to dendritic spines or the junctional folds of the vertebrate NMJ15. Mutations in *wg* reduce this SSR14. Therefore, understanding the precise contributions of pre- and postsynaptic Wnt signalling is critical for a thorough understanding of synapse development.

We now demonstrate that Importin-β11 and Importin-α2 can mediate synapse-to-nucleus signaling. Mutations in these importins selectively blocked the nuclear translocation of Fz2-C and revealed its requirement for Wg-stimulated growth of the SSR and postsynaptic specializations.

Results

Importin Expression in Drosophila Muscle

Nuclear import can proceed via an importin-α and -β acting jointly, or by an importin-β alone3. Our previous work determined that Importin-β11 was expressed in muscle nuclei (Fig. 1a,b)16, but importin-α expression in these muscles was not examined. Of the three *Drosophila* importin-α homologues17, neither Importin-α1 nor -α3 could be detected immunocytochemically in muscle nuclei (Fig. 1c,d and i,j). Importin-α2 immunoreactivity, however, was present in wild-type but not *importin-α2* mutant nuclei (Fig. 1e,f,g,h). Non-specific immunoreactivity was seen at the NMJ of both wild-type and *importin-α2* larvae (not shown), which prevented determining if Importin-α2 was also at the synapse.

Importins-β11 and α2 are Required for Fz2-C Nuclear Import

Muscle expression of Importins-α2 and -β11 led us to hypothesize that they may mediate the nuclear entry of Fz2-C. To begin this analysis, we first reexamined the question of Fz2 expression, cleavage, and translocation in the muscle. We used antibodies raised to the C-terminus of the receptor10 to examine the subcellular localization of Fz2 in larval muscle. As shown previously10, the antibody recognized neuromuscular junctions and puncta within muscle nuclei (Supplementary Fig. 1a,c). The specificity of the staining was confirmed by its absence in null *dfz2* mutants (Supplementary Fig. 1b)10, 18. To address the nuclear import of the C-terminus independently of the Fz2-C antibody, we overexpressed in muscle a transgenic Fz2 receptor with a C-terminal FLAG epitope. Anti-FLAG labeled synapses as well as puncta at the nuclear envelope and within the nucleus itself (Supplementary Fig. 1d,e,f). Further, when immunoblots of body-wall lysates from these larvae were probed with a FLAG antibody, two species were detected: a full-length, ~85 kDa band and a ~ 15 kDa band, likely corresponding to the cleaved C-terminal peptide. These findings confirmed previous observations that the C-terminus of the Fz2 receptor is cleaved and imported into the nucleus10.

To determine if Importins-β11 or -α2 were required for nuclear DFz2-C translocation, we examined mutants of each for defects in Fz2-C localization. Muscle nuclei of either *importin-β11 (imp-β11)* or *importin-α2* (*imp-α2*) larvae held 85% fewer puncta immunoreactive for Fz2-C than wild-type (Fig. 2a–h,k). In 40% of mutant nuclei, the absence of nuclear Fz2-C puncta was accompanied by the presence of puncta at or outside the nuclear envelope (Fig. 2f,h). This perinuclear accumulation, never seen in wild-type, is consistent with a deficiency in active nuclear import of Fz2-C. Moreover, in wild-type genetic backgrounds, the number of nuclear Fz2-C puncta could be nearly tripled by neuronal overexpression of the ligand Wg (Fig. 2k), consistent with previous observations10. In *imp-β11* larvae, however, neuronal Wingless overexpression could not restore Fz2-C to muscle nuclei (Fig. 2k, Supplementary Fig. 2a,b). In contrast to *imp-β11* or *imp-α2*, *imp-α1* mutants had normal levels of nuclear Fz2-C (WT = 0.93 ± 0.03 puncta; n = 3 animals, 143 nuclei, *Df(3L)α1S1* = 1.2 ± 0.10 puncta; n = 4 animals, 249 nuclei; $p > 0.3$).

Nuclear Fz2-C in *imp-β11* or *imp-α2* mutants could be restored to 71% and 85% respectively, of wild-type levels by expression of the corresponding importin in muscle (Fig. 2k, Supplementary Fig. 2c–f). Muscle overexpression of Ketel, the *Drosophila* homologue of Importin-β1, could not rescue the *imp-β11* phenotype, suggesting specificity for Importin-β11 (Fig. 2k). Similarly, expression of the importins in the nervous system did not restore Fz2-C to muscle nuclei (Fig. 2k). Thus, Fz2-C requires Importin-β11 and Importin-α2 in muscle for nuclear localization, as is consistent with their direct role in nuclear import of Fz2-C.

The *imp-β11* mutant could be selectively defective in Fz2-C import or generally impaired in nuclear import. We therefore examined three other proteins that translocate from the synapse to the nucleus. Nanos, an RNA binding protein19, was still present in *imp-β11* muscle nuclei (Fig. 2i,j). Similarly, pMad, the downstream effector of BMP signalling at the NMJ was properly imported into *imp-β11* nuclei16. In addition, the NF-κB transcription factor

Dorsal20 properly localized to the NMJ (not shown) and muscle nuclei (Supplementary Fig. 3a,b). This staining was specific for Dorsal, as it was undetectable in *dorsal* null mutants (Supplementary Fig. 3c). Other nuclear proteins were also imported in *imp-β11* mutants, including nonA, a pan-nuclear RNA-binding protein (Supplementary Fig. 3d,e), muscle-expressed GFP tagged with a nuclear localization sequence (Supplementary Fig. 3f,g), and Importins-α2 (Supplementary Fig. 3h,i) and -β1 (Supplementary Fig. 3j,k). Thus general nuclear import is not impaired in the *imp-β11* mutant and, although other unidentified proteins may require Importin-β11 for import, the absence of Fz2-C from muscle nuclei is a selective phenotype, even among synapse-to-nucleus signalling molecules.

Wnt Pathway Components Persist in Importin Mutants

The absence of nuclear Fz2-C localization need not be, *a priori*, a direct defect in nuclear import; mislocalization or loss of expression of Wnt signaling components in *imp-β11* or *imp-α2* could reduce nuclear Fz2-C as a secondary consequence. Therefore, we examined Wingless, Fz2 and dGRIP, a scaffold protein11, 14 at the fly NMJ and found no detectable change in their localization or levels of expression in either *imp-β11* or *imp-α2* (Fig. 3a–r). Therefore, the lack of nuclear Fz2-C in the *importin* mutants cannot be accounted for by the loss of upstream components of the pathway.

To determine whether receptor endocytosis was blocked, we examined localization of Fz2 with antibodies to its N-terminus (Fz2-N). Anti-Fz2-N labels both the NMJ and a ring that appears to be at or tightly associated with the nuclear envelope and is dependent upon endocytosis and receptor trafficking10. In all genotypes, this ring of Fz2-N staining outlined muscle nuclei (Fig. 3s–x); therefore endocytosis and relocation to the nuclear envelope of the Fz2-N portion occurs in the absence of Importin-β11 or Importin-α2. Moreover, cleavage of Fz2-C from Fz2-N also occurs in the mutants: the Fz2-N-associated nuclear envelope was not immunoreactive with anti-Fz2-C (compare Fig. 2 and Fig. 3). Fz2-C, though also transported to the vicinity of the nucleus in the absence of Importin-β11 or Importin-α2, is separately localized in puncta outside the nuclear envelope (Fig 2). As previously reported10, endogenous Fz2 levels were too low to be detected on immunoblots by available antibodies for further confirmation of receptor cleavage. Thus, importins appear specifically required for Fz2-C translocation across the nuclear envelope, but despite associating with receptors at the synapse, are not required for Fz2-C generation or movement through the cytoplasm.

An Importin / Fz2-C Complex at Synapses and in the Cytosol

The requirement for Importins-β11 and -α2 in Fz2-C nuclear import raised the possibility of their physical interaction at the synapse, in the cytosol, or at the nuclear pore. As the Importin-β11, -α2 and Fz2-C antisera were all from rabbits, immunocytochemical colocalization of endogenous proteins was not practical. We therefore co-expressed in larval muscles GFP-tagged Importin-β11 and Fz2 with a C-terminal FLAG-tag. Both tagged proteins retain their functions16, 21 and Fz2-FLAG distribution in muscle resembled that of endogenous Fz2 (Supplementary Fig. 1).

Expressed alone, Importin-β11-eGFP was diffusely cytoplasmic with occasional cytosolic puncta and prominent nuclear labeling (Fig. 4a–c). It was not detected at the NMJ (Fig. 4a–c, open arrowhead). In contrast, when coexpressed with Fz2-FLAG, the two extensively colocalized in the postsynaptic region (Fig. 4d–f, filled arrowheads); thus overexpression of Fz2-FLAG recruited Importin-β11 to the synapse.

Fz2-FLAG and Importin-β11-eGFP also colocalized in cytoplasmic puncta (Fig. 4d–f) that are likely to represent internalized C-terminal peptides en route to the nucleus10, 11. These FLAG / eGFP puncta were frequently near the nucleus (Fig. 4g–i, arrows). The colocalization of Importin-β11 and the FLAG tag at synapses, in the cytoplasm, and around the nucleus suggests that Fz2-C traffics in association with Importin-β11 to ensure nuclear entry.

We also examined whether the importins and receptor would co-immunoprecipitate. Anti-FLAG immunoprecipitated Importin-β11-GFP from third instar body-wall preparations that coexpressed Fz2-FLAG and Importin-β11-eGFP but not when Importin-β11-eGFP was expressed alone (Fig. 4j). Although co-immunoprecipitation of endogenous Importins-β11 and -α2 could not be detected from either S2 cells or wild-type body-walls (not shown), endogenous Importin-α2 did coprecipitate with Fz2-FLAG, but only when Importin-β11-eGFP was also expressed (Fig. 4j). Thus Fz2 can form a complex containing both importins and Importin-β11 may be necessary to stabilize the incorporation of Importin-α2 into the complex.

NLS-tagged Fz2-C is Imported Independently of Importin-β11

Although Importin-β11 was required for normal Fz2-C import, we attempted to bypass that requirement. We expressed in larval muscles the C-terminal 88 amino acids of Fz2-C with a nuclear localization signal (NLS) and a myc epitope (Fig. 5a) on its N-terminus, so as not to interfere with the PDZ-binding domain10. Attaching an NLS to cargoes can allow for import by alternative routes such as Importin-β122. In addition to diffuse cytoplasmic myc immunoreactivity, as seen previously10, we observed enrichment of myc-NLS-Fz2-C around synaptic boutons (Fig. 5b,c) likely due to binding via the PDZ-binding domain. We also observed nuclear myc-immunoreactivity in both wild-type and *imp-β11* mutants (Fig. 5d–f). The nuclear immunoreactivity was punctate but these puncta were smaller than endogenous Fz2-C puncta and most abundant near the nucleolus. Thus the NLS-fused Fz2-C (like NLS-GFP; Supplementary Fig. 3) enters the nucleus even in the absence of Importin-β11; this raised the possibility that myc-NLS-Fz2-C could functionally substitute for normal Fz2 signaling.

Nuclear Fz2-C does Not Regulate Presynaptic Structure

The ability to selectively prevent the nuclear entry of Fz2-C permitted the investigation of its developmental consequences, as distinguished from those of other Fz2-activated pathways. Previous work hypothesized that Fz2-C import controls presynaptic bouton size, number, and cytoskeletal organization through a retrograde, muscle-derived signal10. However, recent work revealed that Wg-related modulation of these parameters is predominantly explained by presynaptic Fz2 signaling13. We therefore examined *imp-β11*

and *imp-α2* mutants with regard to these phenotypes to ascertain if the nuclear Fz2-C pathway was involved.

Defective Wg signalling alters microtubules in synaptic boutons, as determined by immunostaining for Futsch, the *Drosophila* MAP1B homologue23, 24. Defects manifest as increased disorganized, unbundled Futsch and fewer Futsch-positive microtubule loops within boutons13, 14. To determine whether loss of nuclear Fz2-C causes these defects, we examined Futsch in *imp-β11* and *imp-α2* mutants and found that they did not recapitulate the *wg* phenotype. No significant differences were found in counts of boutons with unbundled Futsch or Futsch-immunoreactive loops (Supplementary Fig. 4). We conclude that impaired muscle Fz2-C import does not affect presynaptic microtubule organization.

Mutations in the Wnt pathway also decrease the number of synaptic boutons but increase the number of large (> 5 μm diameter) boutons10, 13, 14. While *imp-β11* mutants have 30% fewer boutons, this is due to a presynaptic function of Importin-β1116. Restoration of presynaptic Importin-β11 expression in *importin-β11* mutants restored bouton numbers despite the nearly complete absence of nuclear Fz2-C in the muscle. Furthermore, *imp-α2* mutants also lacked nuclear DFz2-C (Figure 2c,g,h,k), but displayed normal numbers of boutons (data not shown). Neither importin mutant had increased boutons of > 5 μm diameter (not shown). Thus, postsynaptic nuclear DFz2-C signalling appears not to govern the presynaptic phenomena of bouton number, bouton size, or microtubule organization.

wg and *dfz2* Mutants have Errors in Postsynaptic Specializations

Although no role for Fz2-C import in presynaptic development was detected, a postsynaptic role remained possible. In *Drosophila*, the postsynaptic NMJ is organized by Discs Large (DLG), the homologue of mammalian PSD-9525. Normally, postsynaptic DLG surrounds synaptic boutons and active zones align with glutamate receptor clusters26. Occasionally, however, so-called “ghost” boutons are seen where neither DLG nor glutamate receptors are concentrated opposite a bouton. These boutons contain synaptic vesicles, but largely lack active zones11. The *wg* pathway has been indirectly implicated in regulating ghost bouton frequency because muscle dGRIP RNAi increases their numbers and activity-dependent increases in ghost boutons are altered in *wg* mutants11, 12.

To test directly for an effect of the *wg* pathway and specifically Fz2-C nuclear import, we immunostained *wg* and *dfz2* larvae with anti-HRP and anti-DLG (Supplementary Fig. 5) and counted instances at muscles 6 and 7 where boutons lacked surrounding DLG. In wild-type, ghost boutons were occasionally seen (Figure 6j) and found to lack apposite glutamate receptors (by GluRIIC staining) and the active zone protein Bruchpilot, but contained Synaptotagmin I immunoreactivity. When Wg / Fz2 signalling was perturbed, ghost bouton frequency increased: in hypomorphic wg1 mutants, their frequency doubled while in the more severe wg^{TS} mutant, when shifted to a non-permissive temperature, their frequency was increased 4-fold (Supplementary Fig. 5). Similarly, in *dfz2* mutants, ghost boutons increased nearly 3-fold (Supplementary Fig. 5). Neuronal overexpression of Wingless (Fig. 2)10 and hyperactivity mutations like $eag^{1}Sh^{KS133}$ double mutants12 increase Wg signaling and nuclear Fz2-C. In these genotypes, ghost boutons were significantly fewer than in wild-type (Supplementary Fig. 5). Thus manipulations that decreased Wg / Fz2 signaling

increased the frequency of ghost boutons and those that enhanced signaling diminished their occurrence.

We also examined muscles 6 and 7 expressing myc-NLS-Fz2-C via the BG487 GAL4 driver in a *dfz2* mutant background. This expression completely rescued the increase in ghost boutons associated with the loss of Fz2 function (Supplementary Fig. 5g), indicating that the C-terminus of Fz2 was sufficient to restore proper postsynaptic development. Thus Wg, Fz2, and particularly Fz2-C are required for preventing errors in postsynaptic specializations.

Nuclear Fz2-C Promotes Correct Postsynaptic Specializations

We examined *imp-β11* and *imp-α2* mutants to determine whether ghost boutons arise from a failure of nuclear Fz2-C import (Fig. 6) and found their frequency increased more than threefold over controls (Fig. 6j). Though *imp-β11* mutants have fewer boutons16, an increase in ghost boutons is not a secondary consequence of fewer boutons: null mutants in *wishful thinking*, the type II BMP receptor had 50% fewer boutons27, 28 but no increase in ghost boutons (Fig. 6j).

To determine whether the lack of Importins-β11 and -α2 in the nerve or muscle gave rise to this phenotype, we restored their expression in a tissue-specific manner. Muscle expression, but not neuronal expression, corrected the ghost bouton phenotype (Fig. 6j). Ketel (Importin-β1) was unable to substitute for Importin-β11, as ghost boutons were increased over threefold in *imp-β11* mutants overexpressing Ketel in muscle (Fig. 6j). Therefore, Importin-β11 and Importin-α2 are required in the muscle to prevent ghost boutons.

If nuclear Fz2-C promotes proper postsynaptic development, restoring it to *imp-β11* mutant muscles should restore normal development. Whereas transgenic Importin-β11 expression likely restored import of all β11-dependent cargoes, expression of myc-NLS-Fz2-C should restore only Fz2-C nuclear import (Fig. 5). Consistent with this hypothesis, muscle expression of myc-NLS-Fz2-C completely prevented the increase in ghost boutons in *imp-β11* (Fig. 6j). This rescue was specific to Fz2-C; NLS-tagged GFP expression did not rescue the ghost bouton phenotype (Fig. 6j). These findings support a model wherein Fz2-C nuclear import by Importins-β11 and α2 promotes proper postsynaptic development.

importin-β11 and *dfz2* Mutants Have a Thinner SSR

While the significant increase in ghost boutons indicates a role for Fz2-C import in postsynaptic development, ghost boutons represent a small percentage of the boutons at the NMJ (approximately 1% in wild-type and 3% in the *importin* or *dfz2* mutants). Their occurrence, therefore, was unlikely, in and of itself, to have a major impact on synaptic function. We hypothesized instead that the complete lack of DLG immunoreactivity at the ghost boutons reflected only the most extreme example of a more widespread alteration. An indication of a more general consequence of Wg signaling has been reported for wg^{TS} mutants raised with a temperature shift during larval development: at third instar, a third of their synaptic boutons lacked a subsynaptic reticulum (SSR) as determined by electron microscopy (EM)14. We therefore examined synaptic boutons by EM for defects resulting from impaired nuclear import of Fz2-C.

Presynaptic boutons in *imp-β11* mutants were ultrastructurally normal with regard to many parameters (Table 1). However, the SSR surrounding *imp-β11* and *dfz2* boutons (Fig. 7a–c) was only 58% as thick as in controls (Fig. 7d). Similar reductions were seen in SSR area and the ratio of SSR area to bouton area, but not bouton area itself (Supplementary Fig. 6a–c). The density, though, of SSR folds was unchanged (Table 1). Moreover, the phenotypes of *imp-β11* and *dfz2* were statistically indistinguishable. Two of 26 *dfz2* boutons lacked any surrounding SSR (Supplementary Fig. 7b), potentially an ultrastructural correlate of ghost boutons and similar to those observed in wg^{TS} larvae14. Although no *imp-β11* boutons were seen that completely lacked SSR, many had very little and might have been scored as ghost boutons by immunocytochemistry (Supplementary Fig. 7a). This loss of SSR was not due to a general defect in muscle growth or membrane traffic. Muscle size, input resistance and miniature EPSP amplitude in *imp-β11* larvae were all unchanged16. Moreover, despite thinner SSR, glutamate receptor levels were normal (Supplementary Fig. S8) and receptors correctly apposed active zones16 everywhere except the ghost boutons. Thus, the SSR reduction is not an indication of broadly compromised muscles.

The reduced SSR in *imp-β11* and *dfz2* mutants suggested that import of Fz2-C promotes SSR growth. To address this hypothesis, we expressed myc-NLS-Fz2-C in *imp-β11* mutant muscles. As with the ghost bouton phenotype (Fig. 6d), this transgene nearly completely rescued the SSR defects (Fig. 7d, Supplementary Fig. 7c,d). The ability of myc-NLS-Fz2-C to permit nuclear localization of Fz2-C and thereby overcome the *imp-β11* phenotype demonstrates that import of Fz2-C, and not other possible Importin-β11 cargoes, is required for full SSR development.

The role of nuclear Fz2-C and the identity of potential genes it might regulate are unknown. To address possible downstream factors in SSR growth, we immunocytochemically assayed a number of NMJ components previously shown to affect SSR thickness through either loss-of-function mutations or overexpression, including Wsp. dPix, dPak, Syndapin, PAR-1 and $pDLG^{S797}$, the target of PAR-129–32. The fluorescence intensities of immunoreactivity for each of these were not detectably changed between wild-type and *imp-β11* mutant NMJs (Supplementary Fig. 9) nor were protein levels in body-wall homogenates (not shown). The SSR phenotype of *dfz2* and *imp-β11* mutants is likely to develop slowly during the third instar and may therefore arise from a relatively modest alteration in these or other factors governing SSR growth.

We also examined α-spectrin at the NMJ, as postsynaptic pools of α- and β-spectrin are required for SSR development and spectrin is enriched around each bouton32, 33. The location of this spectrin relative to the membranes of the SSR is unknown, but spectrin may be an organizing scaffold for the SSR cytoskeleton. We observed a 23% reduction in the intensity of α-spectrin immunoreactivity at the NMJ in *imp-β11* larvae, compared to controls (Supplementary Fig. 10a–d, i). This decrease was accompanied by a 42% reduction in the thickness of α-spectrin staining surrounding the type Ib boutons (Supplementary Fig. 10e–h, j–l). This thinning of α-spectrin is consistent with that of the SSR measured by EM and corroborates the finding of impaired postsynaptic development in the *imp-β11* mutant.

Discussion

Both pre- and postsynaptic events shape the properties of synapses during their development and subsequent plastic changes. In the present study, we examined a synapse-to-nucleus pathway that governs the anatomical maturation of the *Drosophila* neuromuscular junction, a model glutamatergic synapse. By analyzing the phenotypes of *importin-β11* and *importin-α2* mutants, we have elucidated the mechanism by which an activity-dependent synaptic signal, the C-terminus of the Wg receptor Fz2, enters postsynaptic nuclei. By selectively blocking this process, we determined that nuclear translocation causes growth of the SSR, a specialized post-synaptic membrane. By circumventing the importins with an NLS-tagged C-terminal peptide (NLS-Fz2-C) we demonstrated that this phenotype depends on this signal and not other unidentified nuclear cargoes that might use these importins.

Wg signaling at the Drosophila NMJ is an activity-dependent process that involves secretion of the Wg ligand from nerve terminals12, 14. Activation of Fz2 receptors on the postsynaptic membrane may give rise to multiple signals in the muscle, but the selective block of one such signal, the nuclear import of Fz2-C, gave rise to a thinner SSR surrounding the terminals and more boutons not surrounded by the scaffolding protein Discs Large. The SSR is an exceptional structure in which the sarcolemma is extensively invaginated15. The function of the SSR is unclear, but it may create biochemically isolated postsynaptic compartments, similar to dendritic spines34. By increasing the extracellular volume at the synapse, it may also provide a “sink” into which released glutamate can diffuse to terminate synaptic responses15. The SSR may filter the electrophysiological consequences of receptor activation35 or otherwise alter electrical signals36. The SSR is also closely associated with ribosomes and may be a site of local glutamate receptor translation37. The SSR is absent from newly formed embryonic synapses and grows during the larval stages38. The number of boutons also increases during larval life and increased neuronal activity causes further increases in bouton number39. Thus SSR growth entails the elaboration of postsynaptic membranes in an on-going process at both existing synapses and newly added boutons. The activity-dependent secretion of Wg and nuclear translocation of Fz2-C may therefore assist in matching SSR growth to synapse expansion. The SSR is reduced by only 42% in the absence of nuclear Fz2-C, however. Therefore, this signaling pathway serves to up-regulate a process likely initiated by other signals. At present, the nuclear targets of Fz2-C are unknown and, in addition to the potentially subtle modulation of SSR component expression, may include unrelated targets that require modulation by presynaptically released Wg.

Diminished SSR did not represent a broader impairment of muscle growth or membrane trafficking. Many muscle parameters, such as size, input resistance and quantal response16, as well as glutamate receptor clustering (Supplementary Fig. 8) were normal. This latter observation is consistent with previous studies showing that receptors cluster at embryonic synapses prior to formation of the SSR40 and independent of transmitter release41. Considerable receptor addition also occurs later as synapses are strengthened and new boutons form. This process was not blocked by *imp-β11* mutations or the loss of nuclear Fz2-C. Thus, the reduction in SSR is not likely due to impaired muscle health, but rather, to the specific impairment of one aspect of synaptic maturation.

Nuclear Import of Fz2-C Requires an Active Process

Both Importin-β11 and Importin-α2 are required in the muscle for Fz2-C import and are detected in a complex with Fz2, suggesting a direct role for both importins in nuclear Fz2-C import. Although Importin-α is chiefly found to interact with Importin-β1, in the present case Importin-α2 partnered with Importin-β11 and Importin-β1 could not substitute for it. Thus particular combinations of α and β importins may provide specificity to nuclear translocation of signals42. Interestingly, mutations in *importin-13* also have diminished SSR43. We observed increased ghost boutons and decreased nuclear Fz2-C in *importin-13* larvae (not shown), suggesting that the active nuclear import complex may also contain this importin. It has not yet been possible to demonstrate a biochemical association of Importin-13 with the Fz2 receptor, so its exact relationship remains uncertain.

Active nuclear import is not mandated by the size of Fz2-C (8 kDa), which is below the diffusionary limit for the nuclear pore3. Diffusion may account for the ~10% of normal levels of Fz2-C puncta that are observed in the absence of the importins but insufficient to preserve normal SSR growth. The inadequacy of free diffusion may indicate that cytosolic Fz2-C is restrained by a binding partner and an active import system may help regulate the pathway. Such a step is implied by the finding that Fz2 is constitutively cleaved, but thought to require an unidentified Wg-triggered activation step for nuclear entry 10. Recruitment of the importins in response to Wg could represent this activation step. Indeed, when expressed in S2 cells, Fz2-C is cleaved and can interact with dGRIP but, in the absence of Wg, neither enters the nucleus10 nor binds the importins (Supplementary Fig. 11)11.

Postsynaptic vs. Presynaptic Fz2-C Phenotypes

Mutations of the Wnt pathway cause several NMJ phenotypes, including changes in bouton number, shape, and presynaptic microtubule arrangements, in addition to the appearance of ghost boutons and the disruption of SSR formation reported here. Determining the mechanisms for each has therefore been complicated. Mutation of the Fz2 cleavage site10, for example, may disrupt other downstream pathways because the cleavage site is also a Disheveled (Dsh) binding site44. Mathew and colleagues10 suggested that a broad array of defects arose from the failure of DFz2-C nuclear import. However, more recent work13 showed that bouton number, size, and microtubule organization was predominantly, though not necessarily exclusively, controlled by a presynaptic and local Wnt signalling pathway similar to the mammalian cerebellum45. This left unresolved the significance of nuclear Fz2-C and other potential Fz2-derived muscle signals. Our identification of a nuclear import mechanism for Fz2-C allowed a separation of postsynaptic pathways and a directed assessment of nuclear Fz2-C in synaptic development (Supplementary Fig. 12).

Both *imp-β11* and *imp-α2* mutants caused errors in postsynaptic development that resembled defects associated in *wg* and *dfz2*: the increase in boutons lacking DLG (Fig. 6, Supplementary Fig. 5) and the under-development of the SSR (Fig. 7). In contrast, neither *imp-β11* nor *imp-α2* had the altered bouton size or cytoskeleton seen in *wg* and *dfz2* (Supplementary Fig. 4). Only development of the SSR was consistently linked to failures in Fz2-C nuclear import. The restoration of normal postsynaptic development by NLS-Fz2-C

in *dfz2* and *imp-β11* confirmed that nuclear DFz2-C is necessary and sufficient for normal postsynaptic development in these mutant backgrounds (Supplementary Fig. 12).

Fz2-C import may not fully account for Wg signaling in the muscle; nearly 1/3 of *wg* boutons completely lacked SSR14 while nearly all *dfz2* and *imp-β11* boutons retained some SSR. A likely explanation is the presence of a parallel pathway mediated by another Wg receptor, Fz, and Dsh. Dsh is present in muscle13 and we observed Fz immunoreactivity at the NMJ (not shown). Indeed, partial redundancy of Fz and Fz2 exists in embryonic patterning18. Because Armadillo/β-catenin, the nucleus-targeted element of the canonical Wnt pathway, is not detectable in the muscle13, the Fz/Dsh pathway may be local and cytoplasmic. It is tempting to speculate that the two pathways work in parallel, with the Fz/Dsh-dependent pathway driving SSR formation locally and Fz2-C in the nucleus supporting it by transcriptional changes.

The nuclear translocation of a synaptic Fz2-C signal illustrates the importance of synapse-to-nucleus signaling and active nuclear import for synaptic development. This mode of signalling also occurs for learning and memory models and axonal regeneration2, 4. Receptor cleavage and nuclear translocation are well-established for many developmental signals, but have chiefly been identified in single-pass transmembrane proteins9, 46. For the 7-transmembrane domain Fz receptors, however, the *Drosophila* NMJ is the only known case. Wnt signaling influences synapse formation in mammalian brain, and Fz5 and Fz8, the closest mammalian homologues of *Drosophila* Fz2, are strongly expressed in the hippocampus47. Both Fz5 and Fz8 are highly conserved with *Drosophila* Fz2 in the vicinity of the cleavage site and also have extended C-terminal sequences48. Potentially, the signaling pathway that promotes postsynaptic development in *Drosophila* via Wnt signaling, receptor cleavage, and active nuclear import of the C-terminus may also occur in mammals. As at the fly NMJ, mammalian nuclear import factors are likely regulators of synapse-to-nucleus signals rather than passive facilitators of transit through the nuclear pore. By associating with synaptic receptors in a manner likely to depend on receptor activation, importins offer a means to coordinate gene regulation with synaptic activity.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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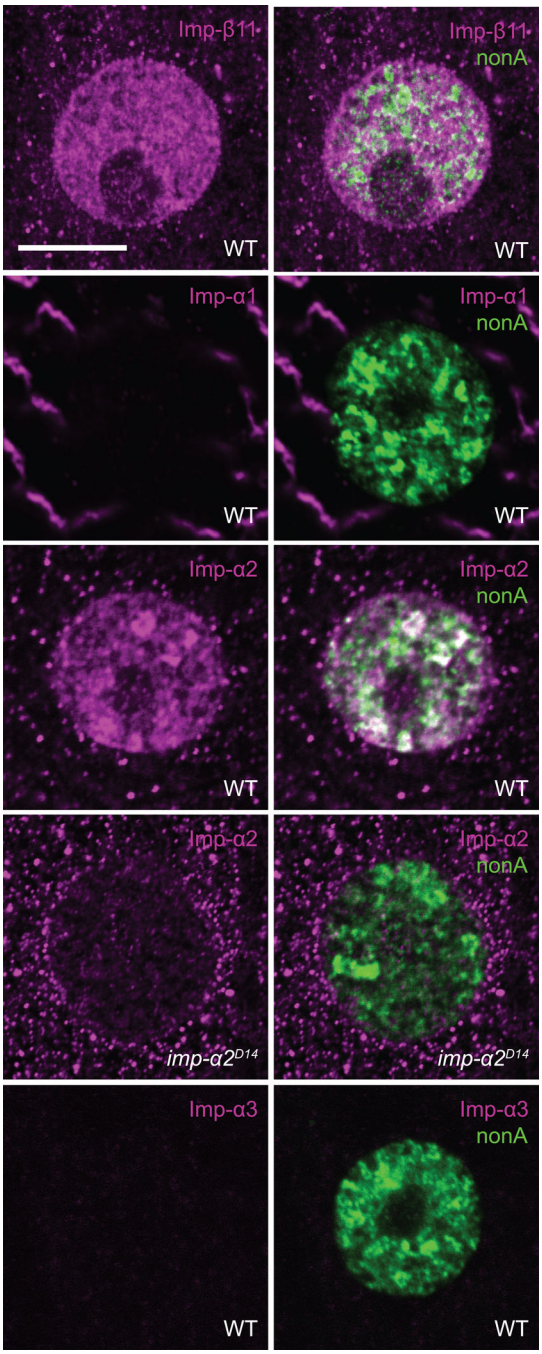

Fig. 1. Importins-β11 and -α2 are Expressed in Drosophila Muscle Nuclei

Representative confocal images of wild-type (*y,w; FRT42D; +; +*) muscles (a-f, I, j) or *importin-α2* null mutant (*y,w; imp-α2^{D14}; +; +*) nuclei (g,h) stained with antibodies to an importin (magenta) and to the nuclear marker nonA (green). (a,b) Anti-Importin-β11 staining is observed at the nuclear envelope and within the nucleus. (c,d) Anti-Importin-α1 nonspecifically labels Z-bands but is undetectable in the nucleus. (e,f) Anti-Importin-α2 staining is observed within the nucleus. (g,h) In *Importin-α2* null mutant nuclei anti-Importin-α2 does not label muscle nuclei, indicating that the nuclear staining in e and f is

specific for importin-α2. Nonspecific puncta in the cytoplasm persist, however in the mutant. (i,j) Wild-type muscle was not immunoreactive for anti-Importin-α3 although the antibody did recognize Importin-α3 in neuronal tissues known to express Importin-α3 (data not shown). Scale bar = 10 μm.

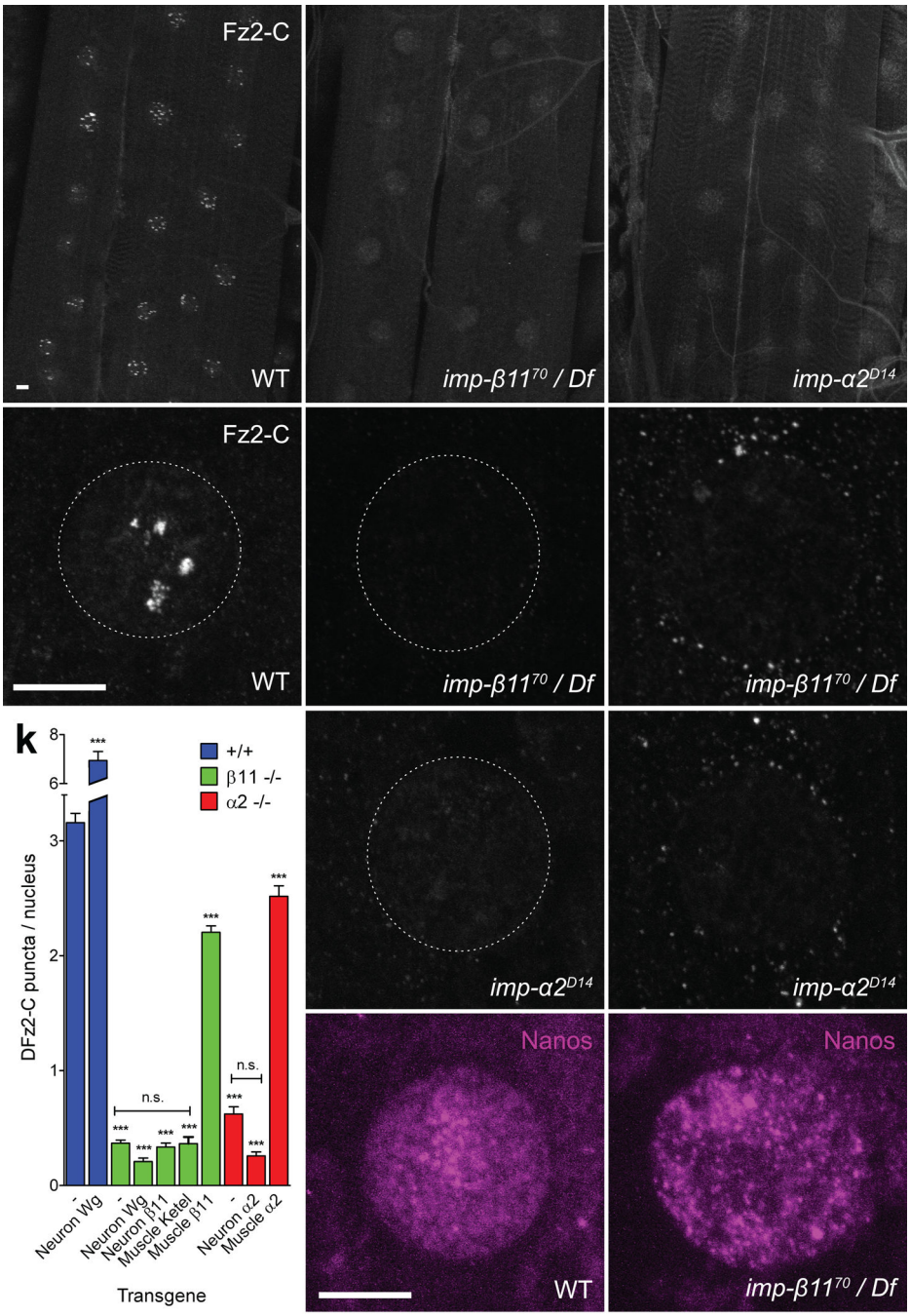

Fig. 2. Importins-β11 and -α2 are Required for Proper Nuclear Import of Fz2-C

(a–c) Representative wide-field image stacks of muscles 6 and 7 in (a) wild-type (*y,w; FRT42D; +; +*), (b) *importin-β11* mutant (*y,w; imp-β11^{70} / Df; +; +*), and (c) *importin-α2* mutant (*y,w; imp-α2^{D14}; +; +*) third instar larvae stained with antibodies to the C-terminus of Fz2. Immunoreactive puncta are evident in nuclei (dashed circle) in wild-type but not *imp-β11* or *imp-α2* mutants. (d–h) Representative individual nuclei stained with an antibody to Fz2-C. The nuclear puncta of Fz2-C present in wild-type (d) were absent from *imp-β11* (e,f) or *imp-α2* (g,h) mutant nuclei. Though internal DFz2-C puncta, some mutant nuclei had

punctate immunoreactivity at the nuclear envelope, like those shown in (f) and (h). (i,j) Nuclei immunostained for Nanos (magenta), an RNA binding protein present at the NMJ and in nuclei of both wild-type (i) and *imp-β11* mutants (j) illustrating the specificity of the Fz2-C phenotype. Scale bars = 10 μm. (k) Quantification of Fz2-C nuclear puncta in third instar larval muscles demonstrates the mutant phenotypes as well as their rescue by the transgenic expression of the specific importin in the muscle. Colors indicate the genetic background in which the marked transgenes are expressed. Genotypes and specific values are as follows: +/+ (*y,w; FRT42D; +; +*) = 3.2 ± 0.1 puncta, *n* = 12 animals, 1101 nuclei; Neuron Wg (*y,w; +; elav-GAL4 / UAS-Wingless*; +) = 6.9 ± 0.4 puncta; *n* = 4 animals, 139 nuclei; β11 −/− (*y,w; imp-β11^{70} / Df; +; +*) = 0.37 ± 0.03 puncta, n = 15 animals, 1159 nuclei; β11 −/− Neuron Wg (*y,w; imp-β11^{70} / Df; elav-GAL4 / UAS-Wingless*; +) = 0.21 ± 0.03 puncta, *n* = 4 animals, 374 nuclei, *p* vs. β11 −/− > 0.2; β11 −/− Neuron β11 (*y,w; imp-β11^{70} / Df; elav-GAL4 / UAS-importin-β11-eGFP;* +) = 0.34 ± 0.04 puncta, *n* = 6 animals, 567 nuclei *p* vs. β11 −/− > 0.2; β11 −/− Muscle β11 (*y,w; imp-β11^{70} / Df; 24B–GAL4 / UAS-importin-β11-eGFP*; +) = 2.2 ± 0.06 puncta, *n* = 10 animals, 907 nuclei, *p* vs. +/+ or β11 −/− > 0.0001; β11 −/− Muscle Ketel (*y,w; imp-β11^{70} / Df; 24B–GAL4 / UAS-Ketel*; +) = 0.37 ± 0.06 puncta; n = 3 animals, 244 nuclei, *p* vs. β11 −/− > 0.2; α2 −/− (*y,w; imp-α2^{D14}; +; +*) = 0.62 ± 0.06 puncta, *n* = 6 animals, 377 nuclei α2 −/− Neuron α2 (*y,w; imp-α2^{D14}; elav-GAL4 / UAS-importin-α2*; +) = 0.58 ± 0.06 puncta, n = 5 animals, 377 nuclei, *p* vs. α2 −/−>0.2; α2 −/− Muscle α2 (*y,w; imp-α2^{D14}; 24B–GAL4 / UAS-importin-α2*; +) = 2.5 ± 0.09 puncta; n = 7 animals, 497 nuclei, *p* vs. +/+ or α2 −/− > 0.001. Substantial rescue is observed by restored muscle, but not neuronal, expression of either importin in the respective mutant background. *** $p < 0.0001$ compared to +/+.

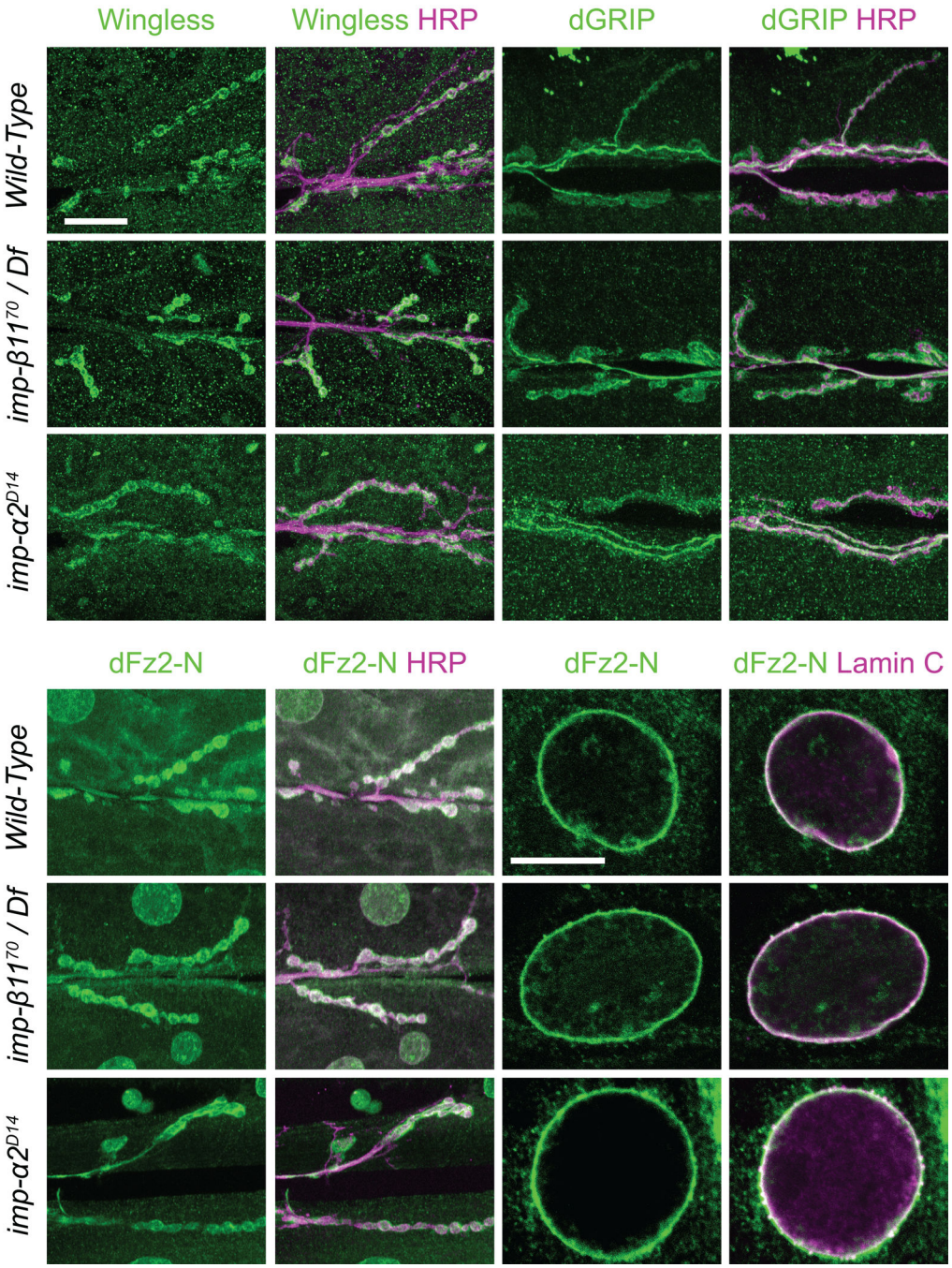

Fig. 3. Wnt Pathway Components are Properly Localized in Importin Mutants

(a–r) Representative confocal images of the NMJ at muscles 6 and 7 in the indicated genotypes, and immunostained as indicated, with anti-HRP (magenta) to mark the neuronal endings. Neither importin mutant prevented Wingless ligand localization to synaptic boutons, dGRIP localization to synapses, or Frizzled2 receptor localization to the synapse. Antibodies to the N-terminal (Fz2-N) and C terminus (Fz2-C; data not shown) gave equivalent results. (s–x) Single confocal slices through the equator of individual third-instar muscle nuclei in the indicated genotypes and stained with the DFz2-N antibody (green) and

an antibody to Lamin C (magenta) to mark the nuclear envelope. Perinuclear staining in each genotype indicates endocytosis and trafficking of the DFz2 receptor to the nuclear envelope. In all images, scale bar = 10 μm. Genotypes: Wild-type, (*y,w; FRT42D; +; +*), *imp-β11^{70} / Df* (*y,w; imp-β11^{70} / Df; +; +*), *imp-α2^{D14}* (*y,w; imp-α2^{D14}; +; +*).

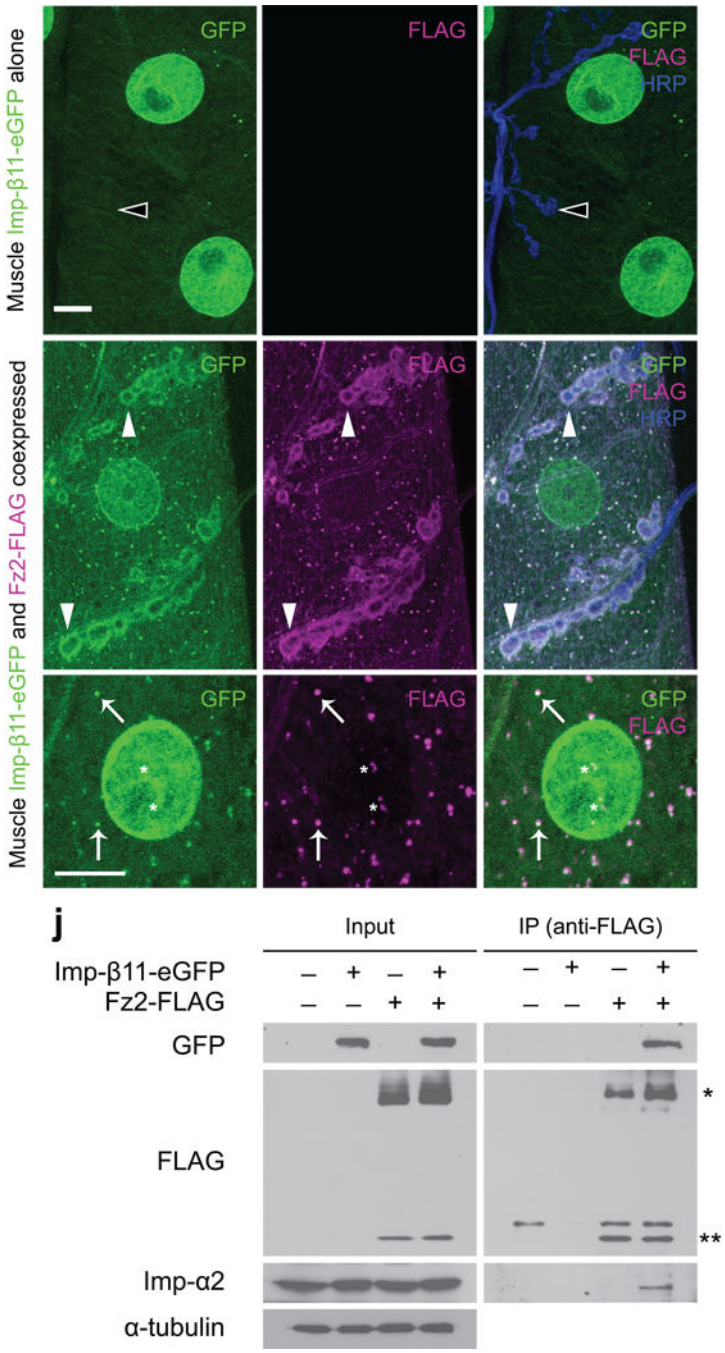

Fig. 4. Fz2 Colocalizes with and Binds Importin-β11 and Importin-α2

(a–c) Muscles expressing Importin-β11-eGFP (green) alone in larvae of the genotype *y,w; +; 24B–GAL4 / UAS-Importin-β11-eGFP*; +. Importin-β11-eGFP localizes to muscle nuclei and diffusely in the cytoplasm but is not concentrated at the NMJ (stained with anti-HRP; blue, open arrowhead). (d–f) In muscles expressing both Importin-β11-eGFP (green) and Fz2-FLAG (magenta) of the genotype *y,w; UAS-Fz2-FLAG / +; 24B–GAL4 / UAS-importin-β11-eGFP*; +, Importin-β11 and Fz2 colocalize at the NMJ (filled arrowheads). (g–h) A single muscle nucleus from a larva co-expressing Importin-β11-eGFP and Fz2-FLAG as in

(d–f). Each FLAG-positive Fz2 punctum colocalizes with a GFP-positive Importin-β11 punctum (arrows), including puncta in the cytoplasm, at the nuclear envelope and within the nucleus (asterisk). For all images, scale bar = 10 μm. (j) Immunoblots of larvae coexpressing Importin-β11-eGFP and DFz2-FLAG. The proteins were immunoprecipitated with anti-FLAG and detected with anti-GFP, FLAG or anti-Importin-α2. Here, expressed Importin-β11-eGFP and endogenous Importin-α2 proteins were coimmunoprecipitated with the receptor. Both the full-length Fz2 (marked by *) and the C-terminus (marked by **) are detected by anti-FLAG. Anti-FLAG also detected a non-specific background species (middle band). Alpha-tubulin was used as a loading control. All blots save Importin-α2 were cropped; the full-length blots are presented in Supplementary Figure 13.

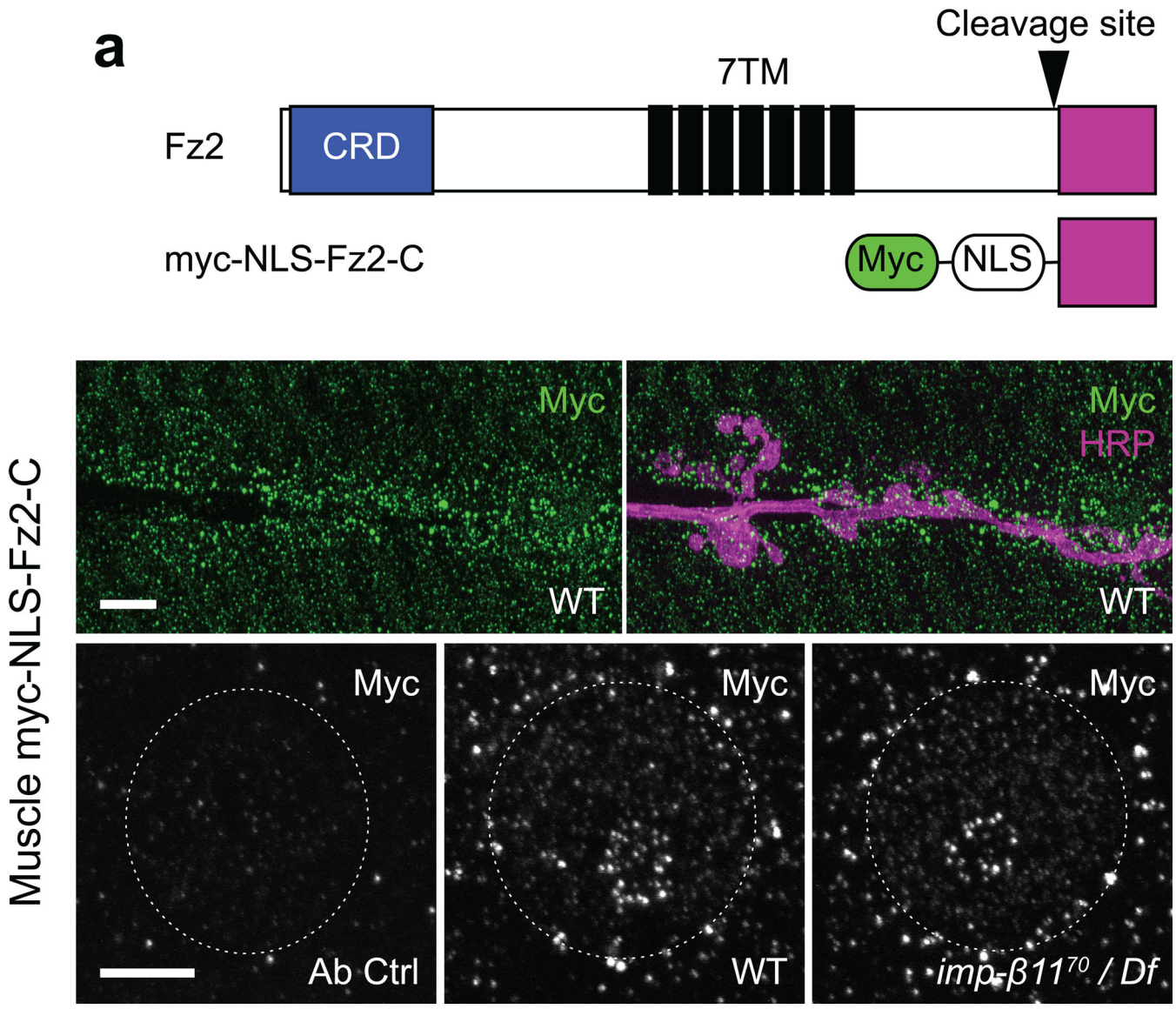

Fig. 5. An NLS-tagged, truncated DFz2-C Construct Localizes to Synapses and is Imported Independently of Importin-β11

(a) Organization of the full-length Frizzled2 protein and the truncated C-terminal construct. For this construct, the sequence after the cleavage site (arrowhead) was cloned behind a classical, monopartite nuclear localization signal and a myc epitope10. CRD = cysteine rich domain and TM = transmembrane domain. (b,c) Representative confocal images of third-instar larval NMJs of the genotype *w, UAS-myc-NLS-DFz2-C; +; 24B–GAL4* / +; + expressing myc-NLS-DFz2-C in the muscle and stained with an anti-myc (green) and anti-HRP (magenta). Myc immunoreactivity appears concentrated near boutons. Scale bar = 5 μm. (d–f) Representative single confocal slices through the equator of muscle nuclei (dashed circles) stained with anti-myc. (d) In the absence of the transgene (*y,w; FRT42D; +; +*), immunoreactivity is absent. (e,f) When myc-NLS-DFz2-C is expressed in wild-type (*w, UAS-myc-NLS-DFz2-C; +; 24B–GAL4* / +; +) or *importin-β11* mutant larvae (*w, UAS-myc-*

NLS-DFz2-C; imp-β11^{70} */ Df; 24B–GAL4* / +;+) nuclear staining is observed. Scale bars = 10 μm.

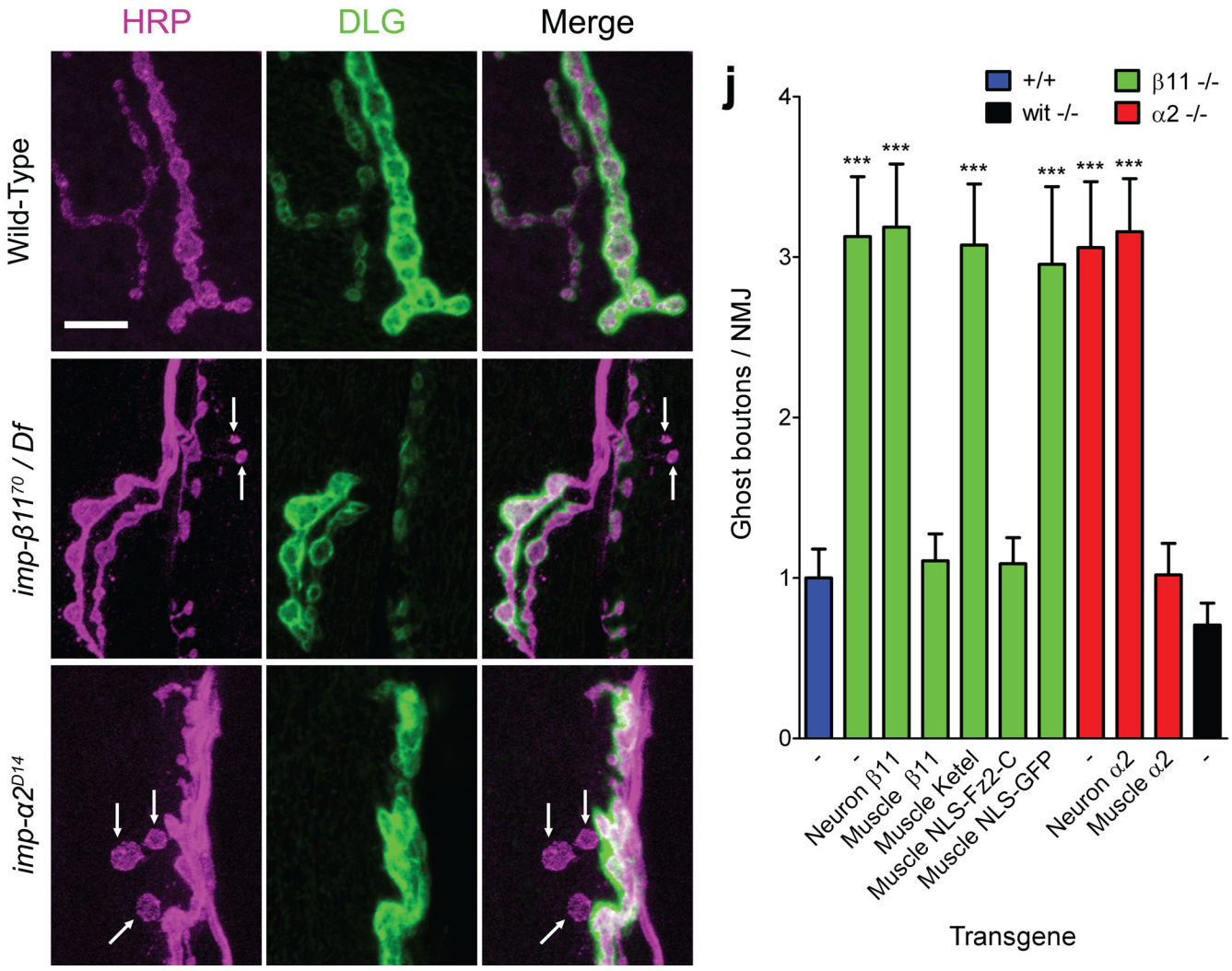

Fig. 6. Ghost Boutons are More Frequent at importin-β11 and -α2 Mutant NMJs

(a–i) Antibodies to HRP (magenta) and Discs Large (DLG, green) marked pre- and postsynaptic compartments of NMJs at muscles 6 and 7 of the indicated genotypes. Typically, each wild-type presynaptic bouton correctly faces a postsynaptic concentration of DLG but, particularly in the mutant genotypes, there is an increased frequency of presynaptic boutons that have no opposite postsynaptic DLG (arrows). At other mutant boutons, DLG staining is weaker but still present. Wild-type = (*y,w; FRT42D; +; +*), *importin-β11* = (*y,w; imp-β11^70 / Df; +; +*), *importin-α2* mutant = (*y,w; imp-α2^D14; +; +*) Scale bar = 5 μm. (j) Loss of either Importin-β11 or Importin-α2 caused a threefold increase in ghost boutons. The frequency of ghost boutons could be returned to control levels by expression of the particular importin in the muscle, but not neurons. Also, muscle expression of NLS-Fz2-C rescued the *importin-β11* phenotype but a control NLS-tagged GFP transgene did not. Ghost boutons were quantified; genotypes (as indicated by colors) and specific values are as follows: +/+ (*y,w; FRT42D; +; +*) = 1.0 ± 0.18 ghost boutons / NMJ, *n* = 10 animals, 59 NMJs; β11 −/− (*y,w; imp-β11^70 / Df; +; +*) = 3.1 ± 0.4 *n* = 13 animals, 78 NMJs; α2 −/− (*y,w; imp-α2^D14; +; +*) = 3.1 ± 0.4, *n* = 11 animals, 65 NMJs, *p* vs. *imp-*

β11^{70} */ Df* >0.8; wit −/− (*y,w; +; wit*$^{A12/B11}$; +) = 0.71 ± 0.14, *n* = 11 animals, 65 NMJs, *p* vs. +/+ > 0.2; β11 −/− Neuron β11 (*y,w; imp-β11*^{70} */ Df; elav-GAL4 / UAS-importin-β11-eGFP*; +) = 3.2 ± 0.39 ghost boutons per NMJ, *n* = 8 animals, 48 NMJs, *p* vs. β*11* −/− > 0.8; β11 −/− Muscle β11 (*y,w; imp-β11*^{70} */ Df; 24B–GAL4 / UAS- importin-β11-eGFP*; +) = 1.1 ± 0.17, *n* = 11 animals, 65 NMJs, *p* vs. +/+ > 0.5, vs. *imp-β11*^{70} */ Df* < 0.0001; α2 −/− Neuron α2 (*y,w; imp-α2*^{D14}*; elav-GAL4 / UAS-importin-α2*; +) = 3.2 ± 0.33, *n* = 10 animals, 57 NMJs, *p* vs. α*2*−/− > 0.4; α2 −/− Muscle α2 (*y,w; imp-α2*^{D14}*; 24B–GAL4 / UAS-importin-α2*; +) = 1.0 ± 0.19, *n* = 9 animals, 52 NMJs, *p* vs. +/+ > 0.8, vs. *imp-α2*^{D14} < 0.0001; β11 −/− Muscle Ketel = *y,w; imp-β11*^{70} */ Df; 24B–GAL4 / UAS-Ketel*; + (3.1 ± 0.38, *n* = 7 animals, 40 NMJs, *p* vs. β*11* −/− >0.7; β11 −/− Muscle NLS-Fz2-C (*w, UAS-myc-NLS-DFz2-C / + or Y; imp-β11*^{70} */ Df; 24B–GAL4* / +; +) = 1.1 ± 0.16 ghost boutons per NMJ, *n* = 14 animals, 78 NMJs, *p* vs. +/+ > 0.7, vs. *imp-β11*^{70} */ Df* < 0.0001; β11 −/− Muscle NLS-GFP (y,w; imp-β11^{70} / Df; UAS-NLS-GFP / 24B–GAL4; +) = 3.0 ± 0.48, *n* = 8 animals, 45 NMJs, *p* vs. *imp-β11*^{70} */ Df* > 0.4. *** *p* < 0.0001 vs. + / +.

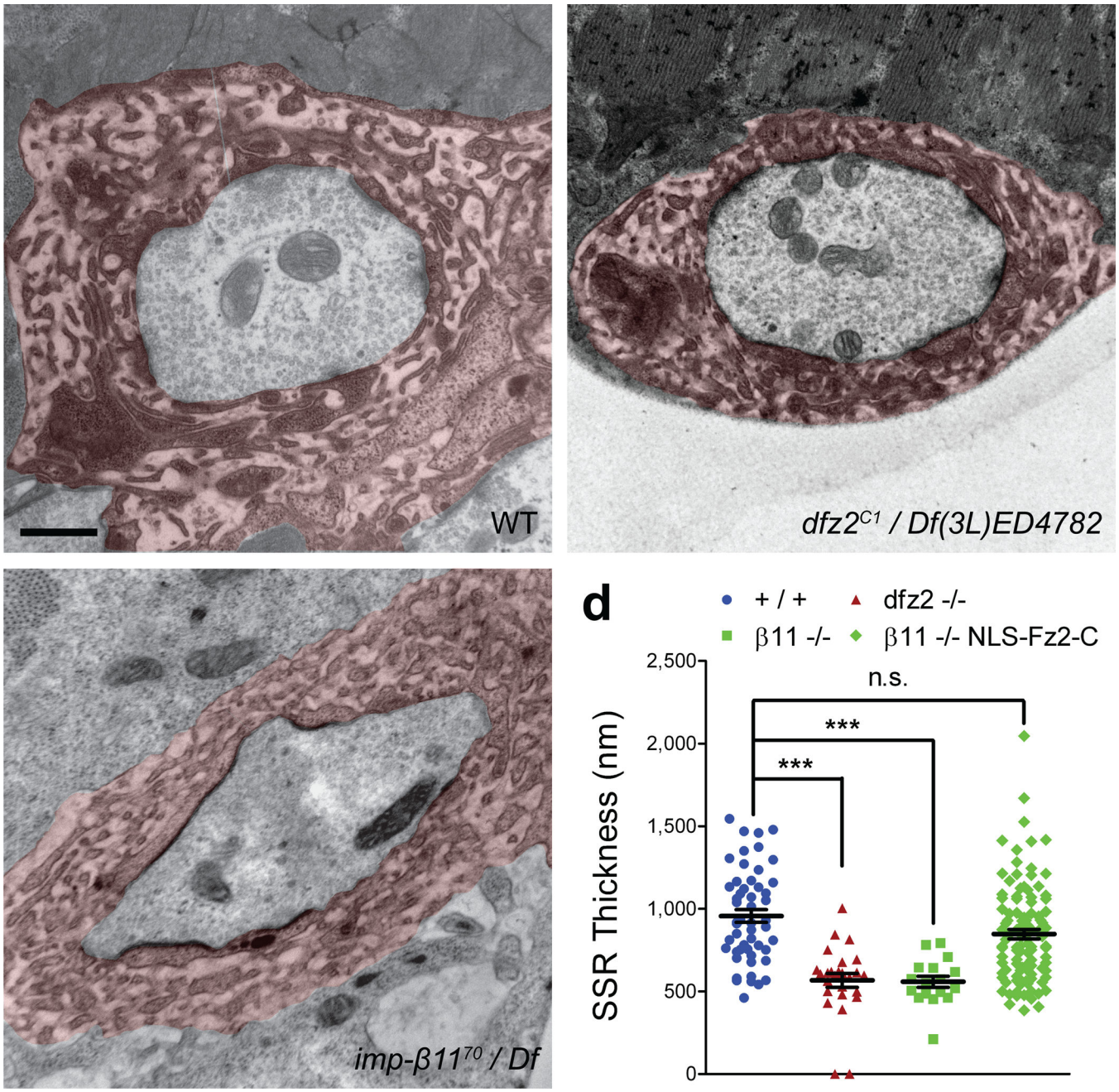

Fig. 7. Loss of Nuclear DFz2-C Leads to Reduced SSR Thickness at the EM Level

(a–c) Representative electron micrographs of type Ib boutons on muscles 6 and 7 in segment A2 of wild-type (*y,w; FRT42D; +; +*), *importin-β11* mutants (*y,w; imp-β11*^{70} */ Df; +; +*) and *dfz2* mutants (*y,w; +; dfz2*^{C1} */ Df(3L)ED4782*; +). Both *importin-β11* and *dfz2* mutants display regions of subsynaptic reticulum (SSR, false colored in red) that are thinner than wild-type controls. In all images, scale bar = 500 nm. (d) Scatterplot quantification of the thickness of the SSR in wild-type (blue circles, *y,w; FRT42D; +; +*)*dfz2* mutants (crimson triangles, *y,w; +; dfz2*^{C1} */ Df(3L)ED4782*; *+*), *importin-β11* mutants (green squares, *y,w; imp-β11*^{70} */ Df; +; +*), and *importin-β11* mutants expressing NLS-Fz2-C in the muscle

(green diamonds, *w, UAS-myc-NLS-Fz2-C; imp-β11^{70} / Df; 24B–GAL4* / +). Each symbol represents the average thickness around a single bouton. Restoration of nuclear Fz2-C in the *importin-β11* mutant rescues SSR thickness to nearly wild-type levels. Specific values are as follows: +/+ = 956.4 ± 38.66 nm, *n* = 5 animals, 52 boutons; β11 −/− (y,w; *imp-β11^{70} / Df;* *+; +*) = 558.5 ± 33.74 nm, *n* = 5 animals, 17 boutons; dfz2 −/− (y,w; +; *dfz2^{C1} / Df(3L)ED4782; +)* = 567.1 ± 41.71 nm, *n* = 3 animals, 26 boutons, *p* vs. *imp-β11^{70} / Df* > 0.8; β11 −/− Muscle NLS-Fz2-C = 841.3 ± 28.72 nm, *n* = 5 animals, 103 boutons, *p* vs. +/+ > 0.2, vs. *imp-β11^{70} / Df* < 0.0001. *** *p* < 0.0001.

Table 1

Quantification of EM Bouton Parameters in Third Instar Control and *imp-ß11* Larvae

Genotype	Vesicle Density	PSD Length	%Mito. Area	Vesicle Diameter	SSR Crossings	%Active ZoneLength	T-Bars / ActiveZone
Wild-Type	184.4 ± 22.55	610.0 ± 38.79	7.1 ± 0.80	33.17 ± 0.8109	0.013 ± 0.0013	31 ± 2.3	0.33 ± 0.094
imp-ß11^{70}/Df	185.0 ± 23.99	577.8 ± 54.28	8.3 ± 2.0	34.52 ± 0.6966	0.015 ± 0.00079	35 ± 2.3	0.26 ± 0.067
	$p > 0.9$	$p > 0.6$	$p > 0.7$	$p > 0.2$	$p > 0.2$	$p > 0.2$	$p > 0.5$

Quantification of these parameters from type Ib boutons imaged by TEM. n > 10 boutons for both genotypes.