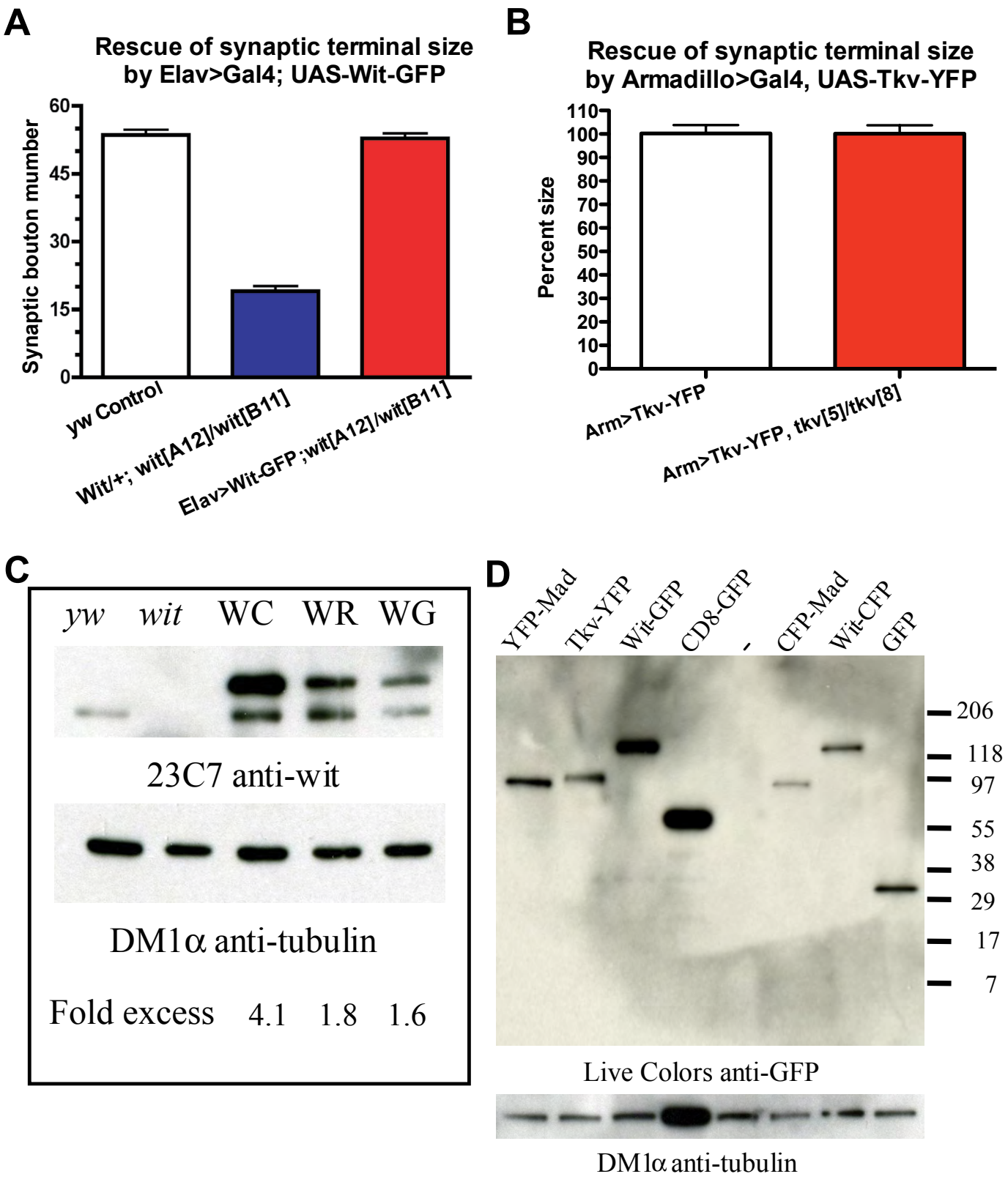

Fig. S1. Fluorescent fusion proteins are functional and show normal levels and molecular weight. (A) Elav>Gal4 expressing Wit-GFP rescued bouton number in *wit* mutants (*UAS>wit*/+; wit^{A12}/wit^{B11}, the UAS transgene is silent in this genotype due to the absence of Gal4 driver) to levels not significantly different from control ($P<0.0001$). (B) Arm>Gal4 expressing Tkv-YFP rescued embryonic lethality to pupal stages and fully rescued the synaptic terminal size in muscle 4 in *tkv* mutants (tkv^5/tkv^8). (C) Western blot analysis of larval CNS shows low to moderate levels of OK6 overexpression of Wit fluorescent fusion proteins (top band in upper panel, absent in non transgenic controls *yw*) when compared to endogenous Wit (bottom band in upper panel, absent in *wit* mutants). Fold excess expression of the transgenes to the endogenous gene was as follows: Wit-CFP (WC) 4.1; Wit-mCherry (WR) 1.8; Wit-GFP (WG) 1.6. (D) Larval CNS were analyzed to verify that no unconjugated XFP was present in animals expressing the fluorescent fusion proteins. Note free GFP band in lane 8 and absence of GFP-containing cleavage products.

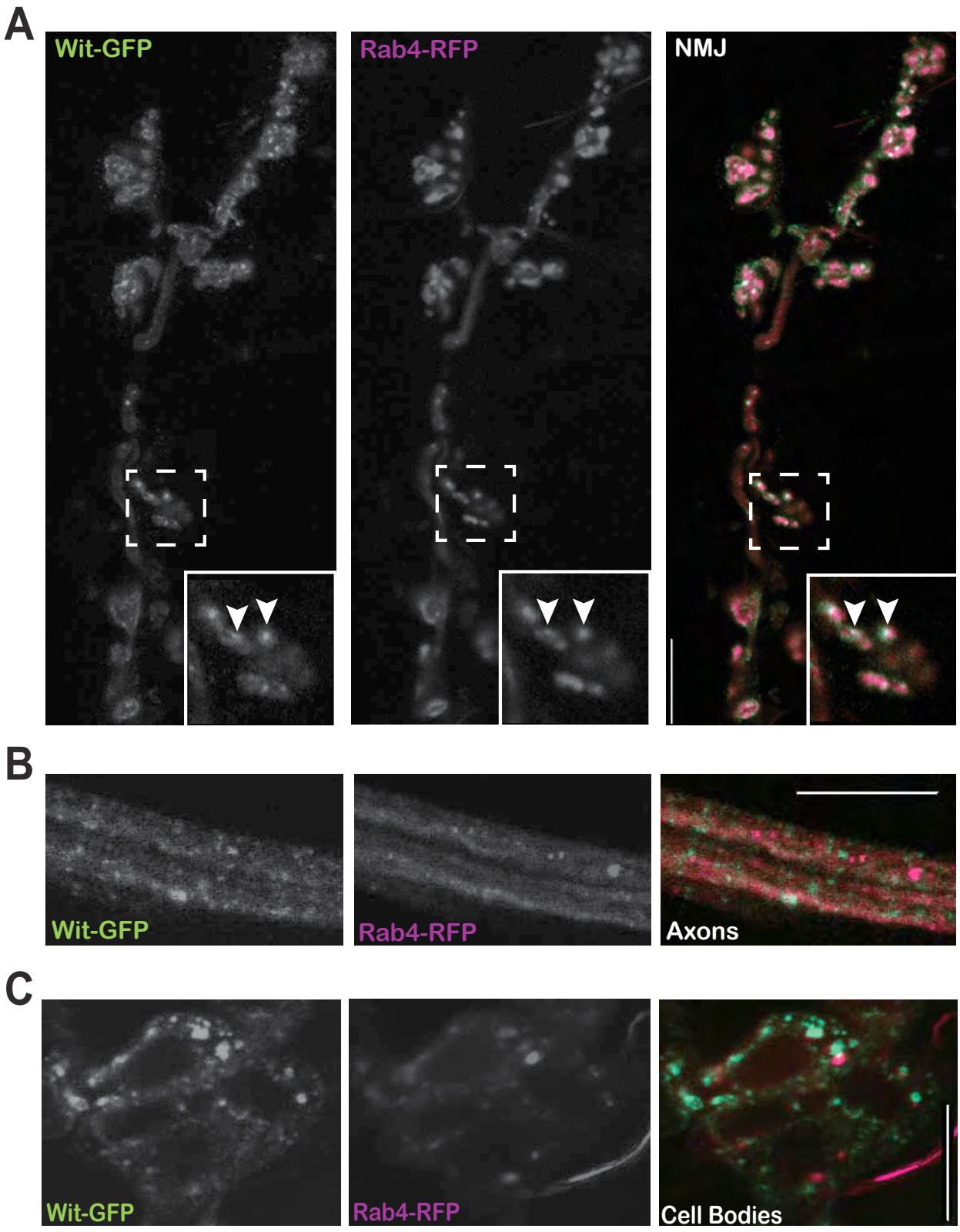

Fig. S2. Wit-GFP and Rab4-RFP colocalize at the NMJ but not in cell bodies or axons. (A) Wit-GFP (green) and Rab4-RFP (magenta) partially colocalize at the NMJ. Wit-GFP and Rab4-RFP do not colocalize along the axon (B) or in motoneuron cell bodies (C). Note Wit-GFP plasma membrane localization in A. Scale bars: 10 μm.

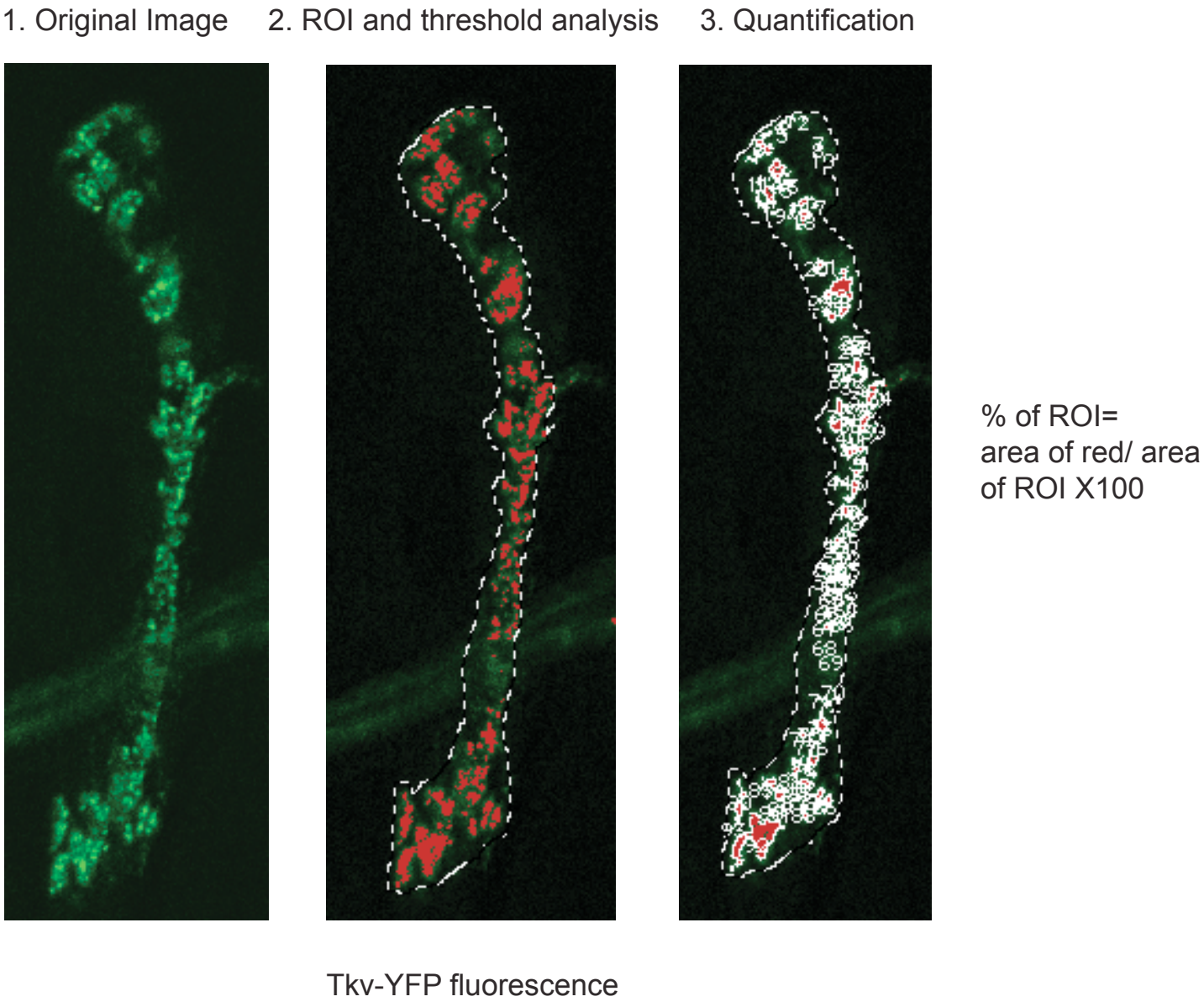

Fig. S3. Quantifying synaptic fluorescence. To quantify Tkv-YFP fluorescence at the synaptic terminal (1) the following steps were taken. An ROI was drawn around the synaptic terminal in the HRP channel (2) and then transferred to theTkv-YFP channel. (3) Threshold analysis was conducted to identify the Tkv-YFP punctae, and the area of regions within the ROI that had signal intensity above a given threshold was added. The combined area of the regions divided by the ROI total area is the normalized amount (in arbitrary units) of Tkv-YFP in the synaptic terminal. pMad fluorescence was quantified in a similar manner.

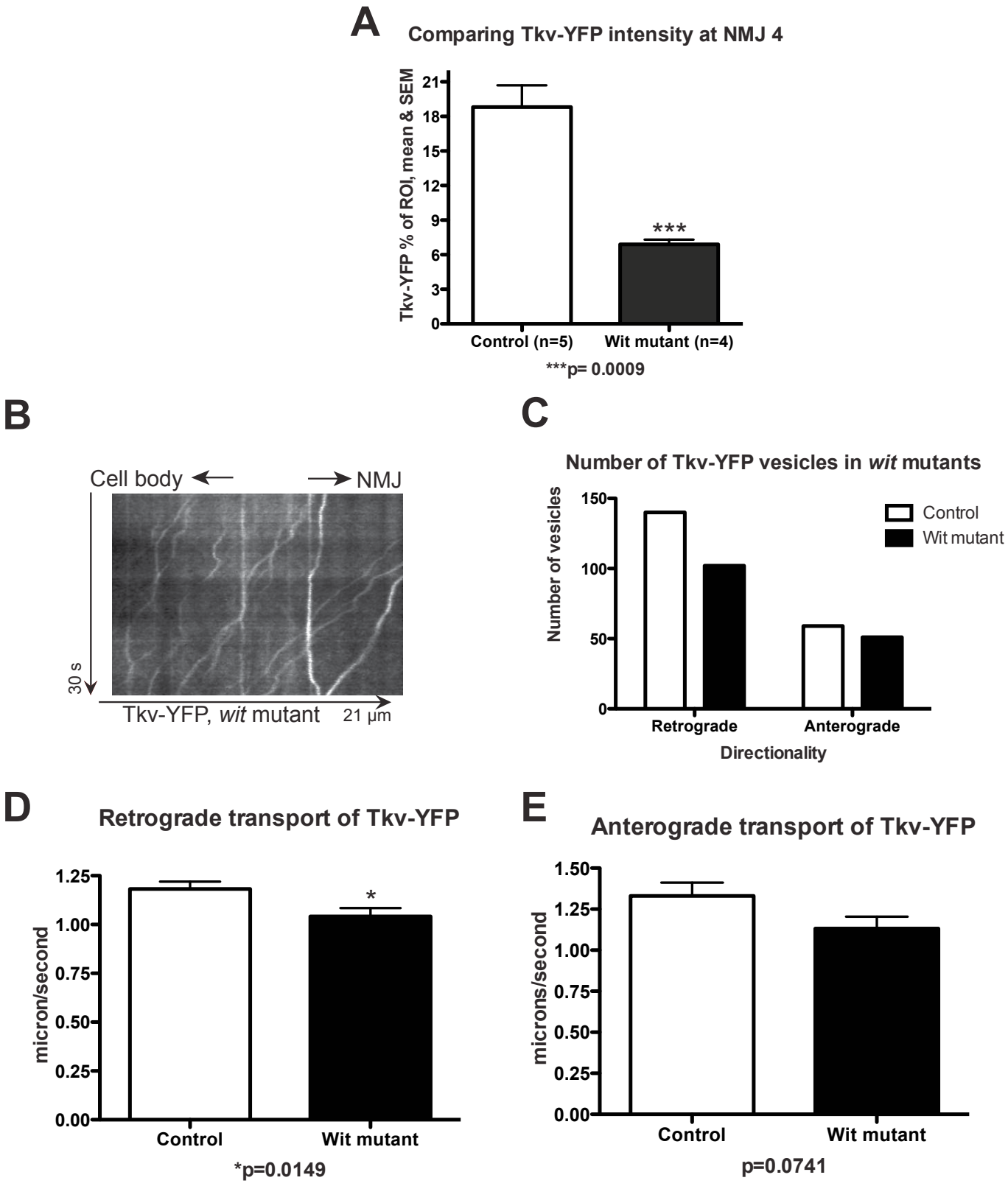

Fig. S4. Distribution and movement of Tkv-YFP in *wit* mutants. (A) Synaptic terminal muscle four images were quantified for fluorescence and measured for comparisons between control and *wit* mutants. Compared to control (n=5), *wit* mutants (n=4) had a 63% decrease in Tkv-YFP intensity at the synaptic terminal. Compared to control (*n*=5), *wit* mutants (*n*=4) had a 63% decrease in Tkv-YFP intensity at the synaptic terminal. (B) Kymograph analysis of Tkv-YFP retrograde vesicular traffic (ascending slope projections) in a *wit* mutant. (C) Analysis of movement directionality of Tkv-YFP shows a decrease (although not reaching significance) in the number of retrograde vesicles in the *wit* mutant compared to Tkv-YFP vesicles in control. Anterograde traffic is barely affected by loss of *wit.* The velocity of Tkv-YFP vesicles along the axon was measured in control and *wit* mutants. (D) The retrograde speed of Tkv vesicles was slightly reduced in *wit* mutants (*P*=0.0149). (E) The anterograde speed was also slightly reduced in the mutant, but it was not significantly different from control (*P*=0.0741).

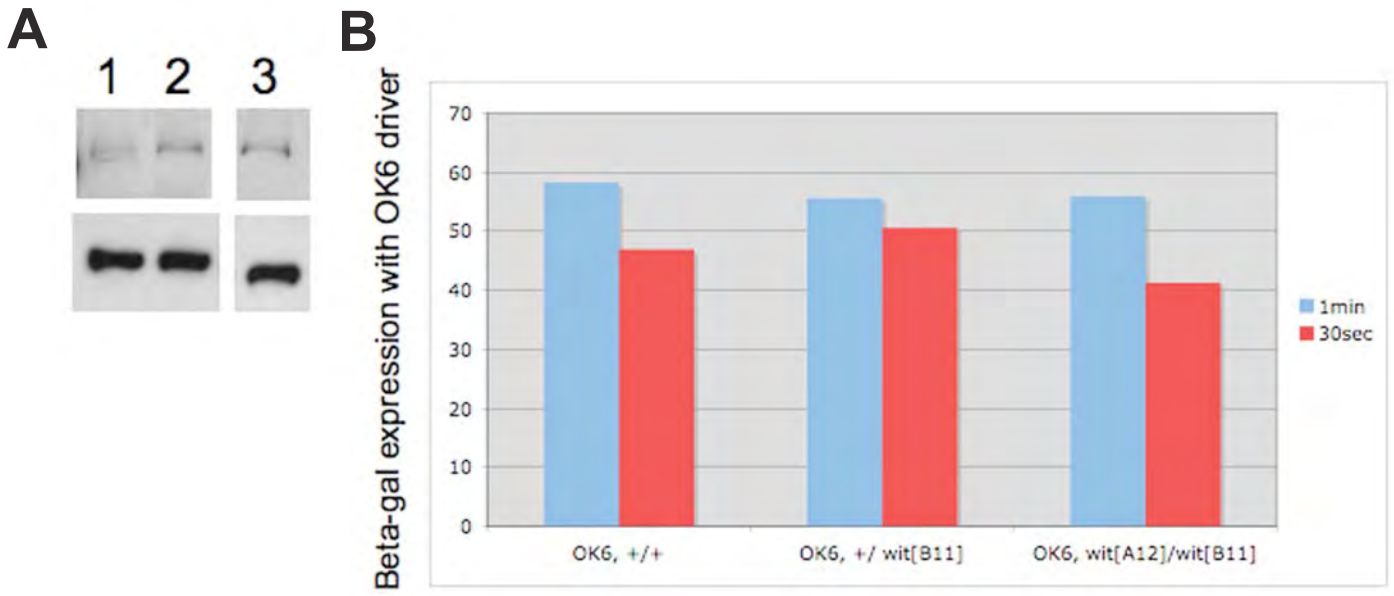

Fig. S5. OK6 driver expression is not affected by BMP signaling. (A) Western blot of β-Gal expressed by driver OK6. Third-instar larval brains with the following genotypes were analyzed by western blot: 1. Control: OK6>Gal4/UAS>β-Gal; +/+. 2. *wit* heterozygote: OK6>Gal4/UAS>β-Gal; +/ wit^{B11}. 3. *wit* mutant: OK6>Gal4/UAS>β-Gal; wit^{A12}/ wit^{B11}. Each lane contains the equivalent of 2 larval brains and Tubulin (lower panel) was used as loading control. (B) Quantification of the western β-Gal bands normalized to tubulin signal with exposure times of 1 min (blue) or 30 sec (red).

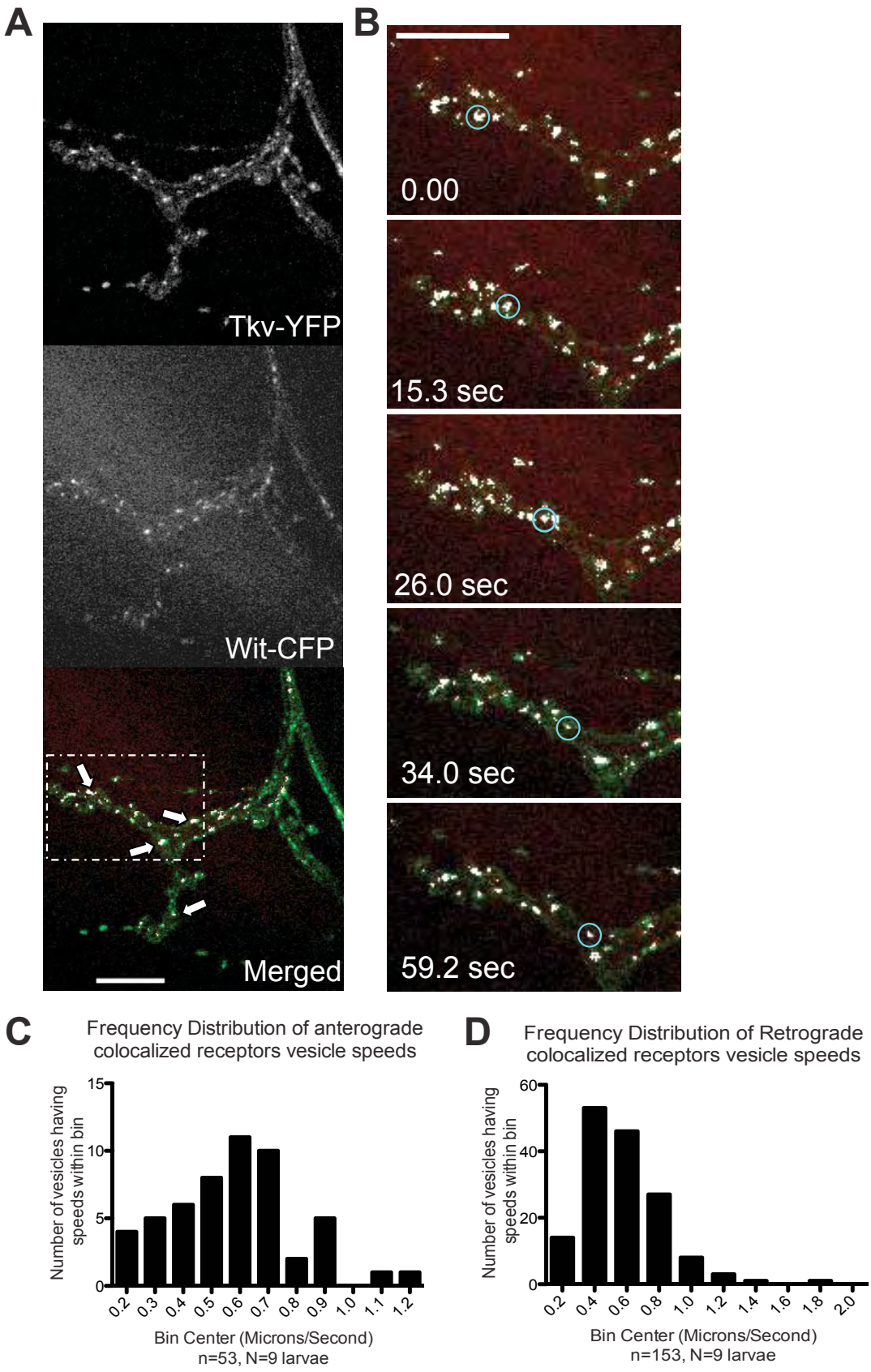

Fig. S6. Wit and Tkv colocalize at the synaptic terminal in dynamic vesicles that move bidirectionally along the axon. (A) Expression of Tkv-YFP and Wit-CFP in motoneurons shows receptor colocalization (white in bottom panel, arrows) at the synaptic terminal. (B) Enlarged frames of a time-lapse movie (box in merged panel in A) show that vesicles move at the synaptic terminal. The highlighted vesicle (blue circle) is moving in a retrograde direction, away from the distal end of the synaptic terminal. Tkv-YFP and Wit-CFP were expressed with motoneuron drivers BG380 and OK6. Scale bar: 10 μm. (C) Speed distribution of colocalized Wit-CFP/Tkv-YFP axonal anterograde traffic, range 0.20–1.18 μm/s. (D) Speed distribution of colocalized Wit-CFP/Tkv-YFP axonal retrograde traffic, range 0.23–1.82 μm/s.