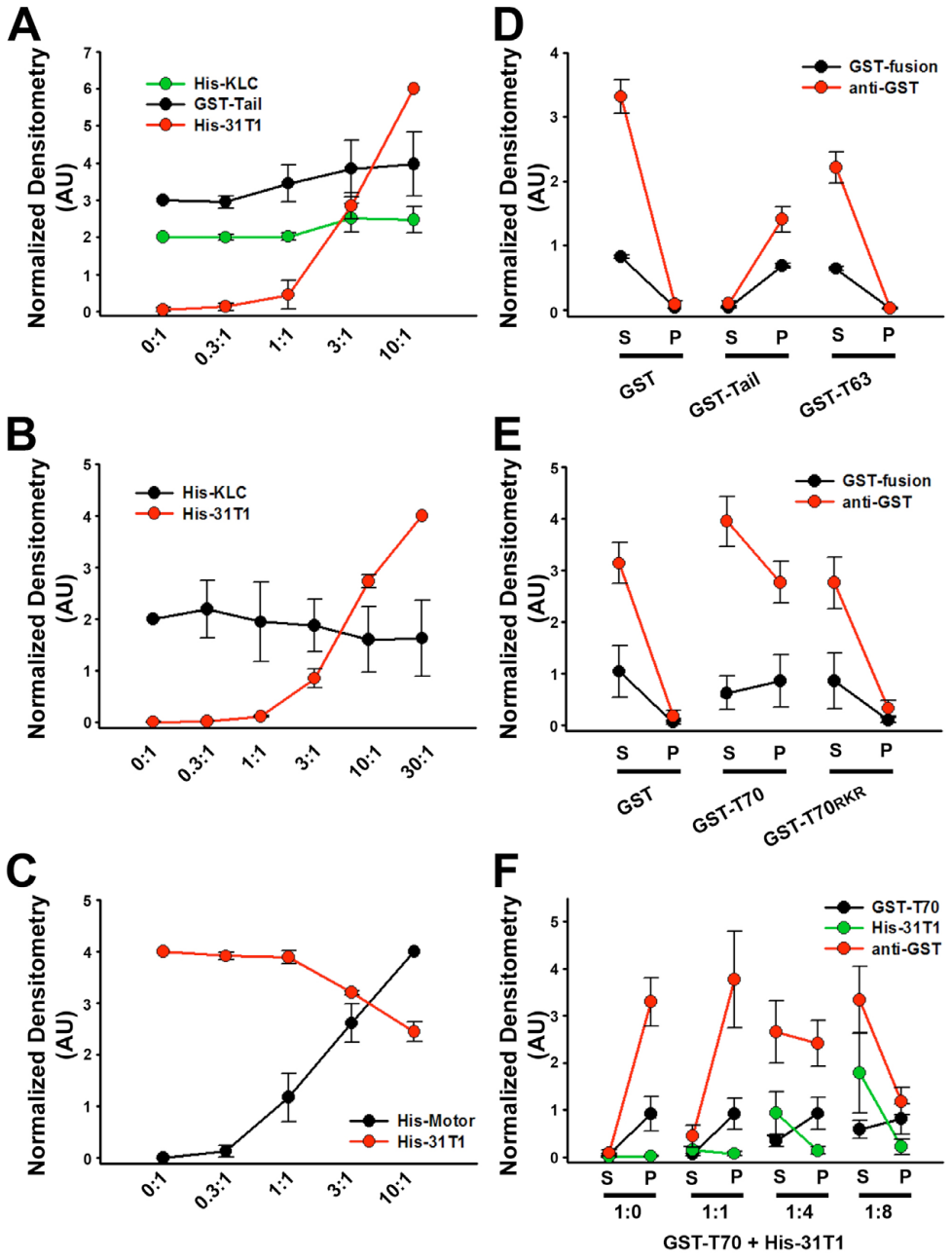

Fig. S1. Densitometry of the competition and microtubule pulldown assays. Three replicates for each western blot or protein gel were analyzed using NIH ImageJ. The intensity of each band was subtracted with the background and presented as mean±s.e.m. (**A**,**B**) His-31T1 did not compete with His-KLC1 for binding to GST-Tail. (A) This is the densitometry result for Fig. 4B. Bands of His-31T1 are normalized with the strongest band in the last lane (10:1) that is set to a value of 6 AUs (arbitrary units). His-KLC1 bands are normalized using the first lane set to a value of 2 AUs. GST-Tail bands are normalized using the first lane set to a value of 3 AUs. (B) This is the densitometry result for Fig. 4C. His-KLC1 bands are normalized using the first lane set to a value of 2 AUs, whereas His-31T1 bands are normalized using the last lane (30:1) set to a value of 4 AUs. (**C**) Increasing amounts of His-Motor competed away His-31T1 from GST-Tail. This is the densiometry result for Fig. 4D. His-Motor bands are normalized using the last lane set to a value of 4 AUs, whereas His-31T1 bands are normalized using the first lane set to a value of 4 AUs. (**D**,**E**) GST-Tail and GST-T70, but not GST, GST-T63 or GST-T70RKR bound to microtubules in the pellet. This is the densiometry results for Fig. 4E,F. (**F**) Increasing amounts of His-31T1 competed GST-T70 away from microtubules. This is the densitometry result for Fig. 4G. All the raw values in D–F are divided by 100 before presented in the chart. S = supernatant; P = pellet.

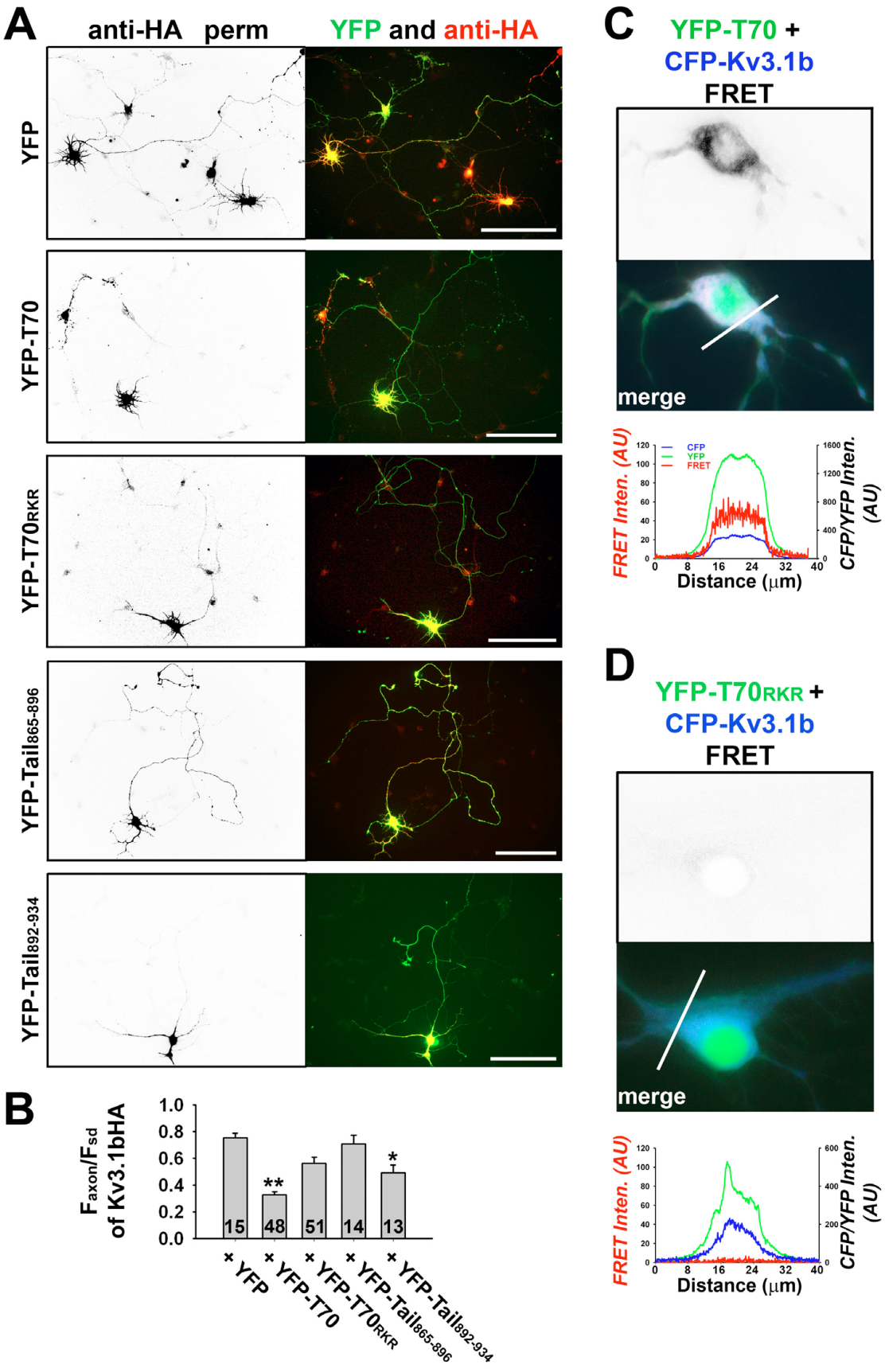

Fig. S2. KLC1 interacts with T63 but not T70 of KIF5B tail in live neurons. Hippocampal neurons were co-transfected with CFP- and YFP-tagged constructs and imaged two days later. Strong FRET signals were also detected in both axons (**A**) and soma (**B**) between expressed CFP-KLC1 (blue) and YFP-T63 (green) (35 out of 37 neurons had strong FRET signals). No FRET signal was detected in axons (**C**) and soma (**D**) between expressed CFP-KLC1 (blue) and YFP-T70 (green) (only 1 out of 19 cells has weak FRET signal). The FRET experiments were carried out under similar conditions with those of Fig. 5D,E.

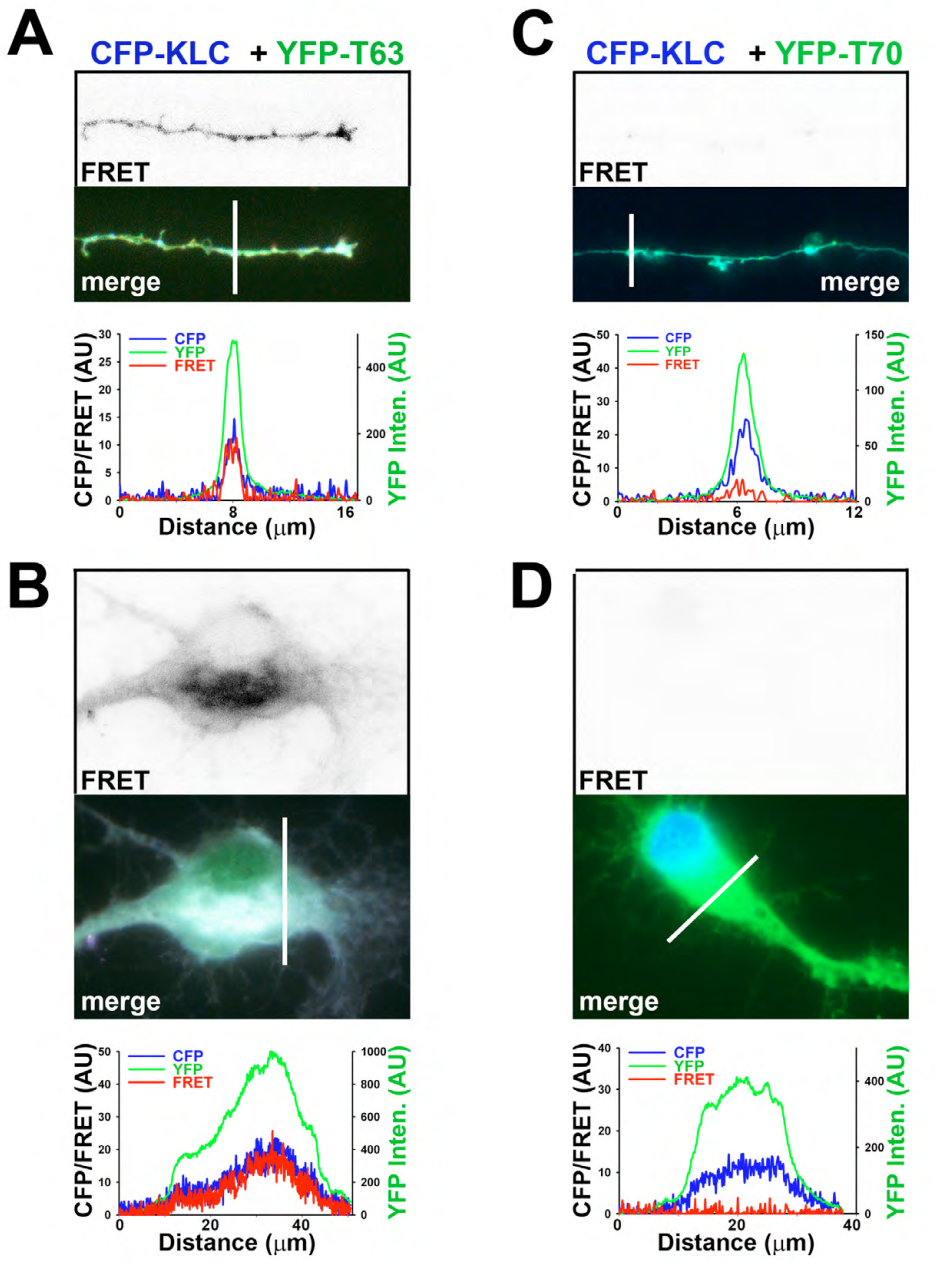

Fig. S3. Differential effects of KIF5B tail fragments on the Kv3.1bHA axonal level. (**A**) Effects of YFP, YFP-T70, YFP-T70$_{RKR}$, YFP-Tail$_{865\text{-}896}$, and YFP-Tail$_{892\text{-}934}$ on the axonal level of Kv3.1bHA. The YFP fusion proteins (green) were co-transfected with Kv3.1bHA (red) into cultured hippocampal neurons at 5 DIV. The neurons were fixed at 7 DIV and stained with an anti-HA antibody under permeabilized condition. (**B**) Summary of the effects of YFP fusion proteins on the axonal level (F_{axon}/F_{sd}) of Kv3.1bHA. Kv3.1bHA F_{axon}/F_{sd} ratios in the presence of YFP, YFP-T70, YFP-T70$_{RKR}$, YFP-Tail$_{865\text{-}896}$, or YFP-Tail$_{892\text{-}934}$, are provided as mean±s.e.m. "n" numbers are shown in the bars. Statistics were carried out with One-way ANOVA followed by Dunn's test. *P<0.05; **P<0.01. (**C**) FRET signals were detected in neuronal soma expressing YFP-T70 (green) and CFP-Kv3.1b (blue). The corrected FRET image is shown in inverted signals on the top and in red in merged image (middle). The fluorescence intensity profiles along the white line in the merged image are shown at the bottom. 14 out of 24 cells had strong FRET signals. (**D**) No significant FRET signals were detected between YFP-T70$_{RKR}$ and CFP-Kv3.1b. Only 2 out of 17 cells had weak FRET signals. Scale bars: 100 μm.

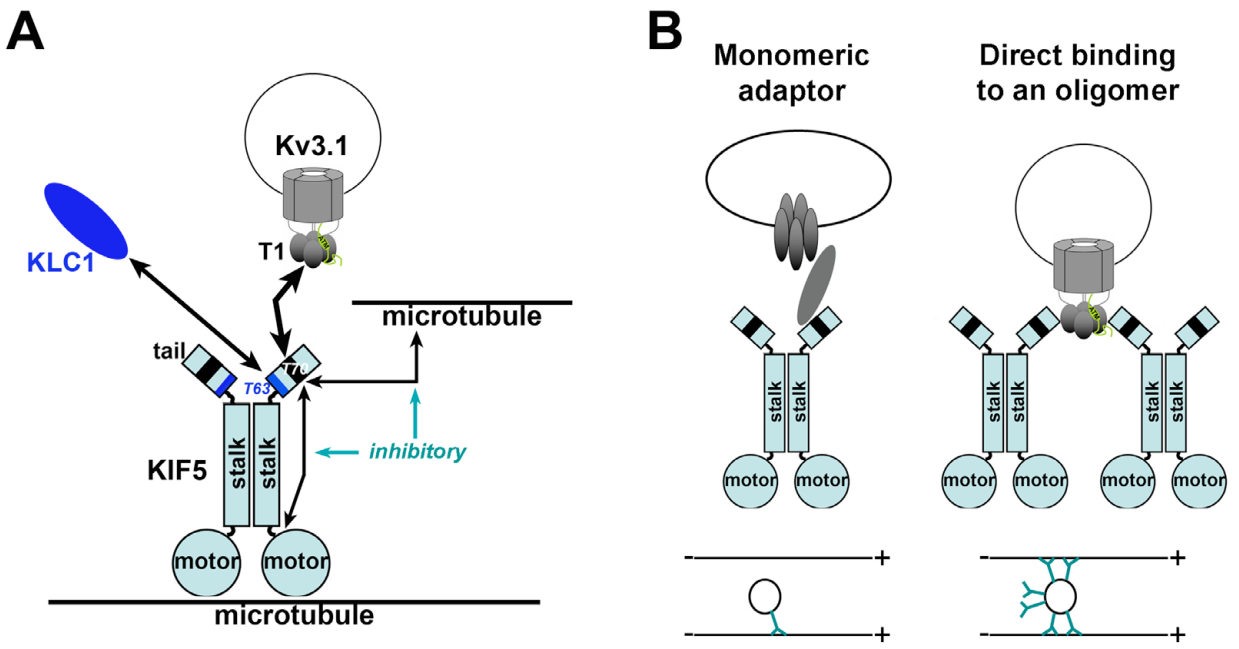

Fig. S4. Summary of the mechanism and consequence of the Kv3-KIF5 binding. (**A**) Interaction diagram of KIF5B, KLC1, Kv3.1 and microtubules. (**B**) Hypothetical diagrams. Direct binding to an oligomer, but not a monomeric adaptor, clusters and activates KIF5 motors, and regulates the motor number on a vesicle carrier.

Movie 1. A KIF5B-YFP-containing punctum traveling anterogradely along an axonal segment, as shown in Fig. 7A. The left side is towards soma and the right side is towards axonal growth cone. The total time is 198 sec and the total length is 40 μm. Signals are inverted.

Movie 2. Many KIF5B-YFP-containing puncta in different sizes traveling anterogradely along the axon of a neuron co-transfected with KIF5B-YFP and CFP-Kv3.1b. The presence of CFP-Kv3.1b was confirmed with imaging of the CFP fluorescence (not shown here). A segment of this axon (43 μm) is shown in Fig. 7B. The left side is towards soma and the right side is towards axonal growth cone. The total time is 198 sec. Signals are inverted.

Movie 3. A KIF5B-YFP-containing punctum traveling anterogradely along the axon of a neuron co-transfected with KIF5B-YFP and CFP-KLC1, as shown in Fig. 7C. The presence of CFP-KLC1 was confirmed with imaging of the CFP fluorescence (not shown here). The left side is towards soma and the right side is towards axonal growth cone. The total time is 198 sec and the total length is 40 μm. Signals are inverted.

Movie 4. KIF5B$_{RKR}$-YFP-containing puncta traveling along an axon. A segment of this axon (40 μm) is shown in Fig. 7D. The left side is towards soma and the right side is towards axonal growth cone. The total time is 198 sec. Signals are inverted.

Movie 5. KIF5B$_{RKR}$-YFP-containing puncta traveling anterogradely along the axon of a neuron co-transfected with KIF5B$_{RKR}$-YFP and CFP-Kv3.1b. A segment of this axon (40 μm) is shown in Fig. 7E. The presence of CFP-Kv3.1b was confirmed with imaging of the CFP fluorescence (not shown here). The left side is towards soma and the right side is towards axonal growth cone. The total time is 198 sec. Signals are inverted.

Movie 6. KIF5B$_{RKR}$-YFP-containing puncta traveling along the axon of a neuron co-transfected with KIF5B$_{RKR}$-YFP and CFP-KLC1. A segment of this axon (40 μm) is shown in Fig. 7F. The presence of CFP-KLC1 was confirmed with imaging of the CFP fluorescence (not shown here). The left side is towards soma and the right side is towards axonal growth cone. The total time is 198 sec. Signals are inverted.