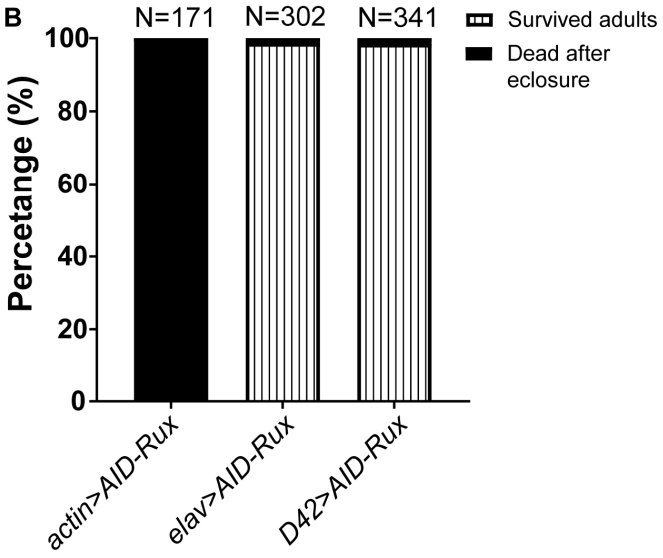
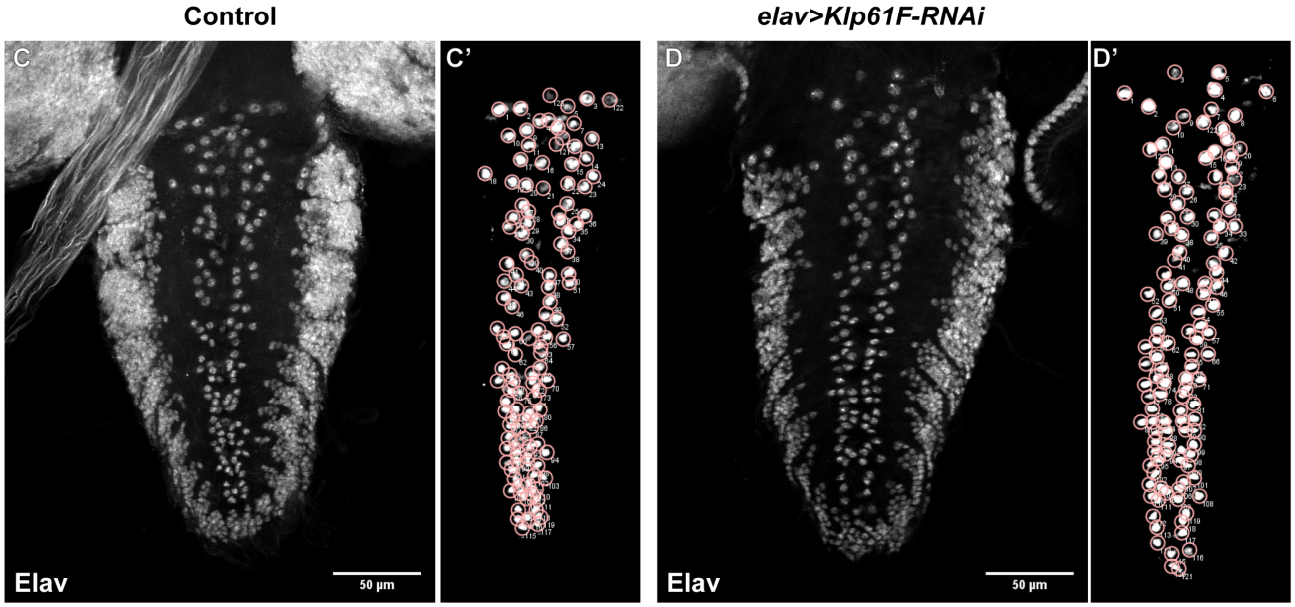
Supplemental Information

A

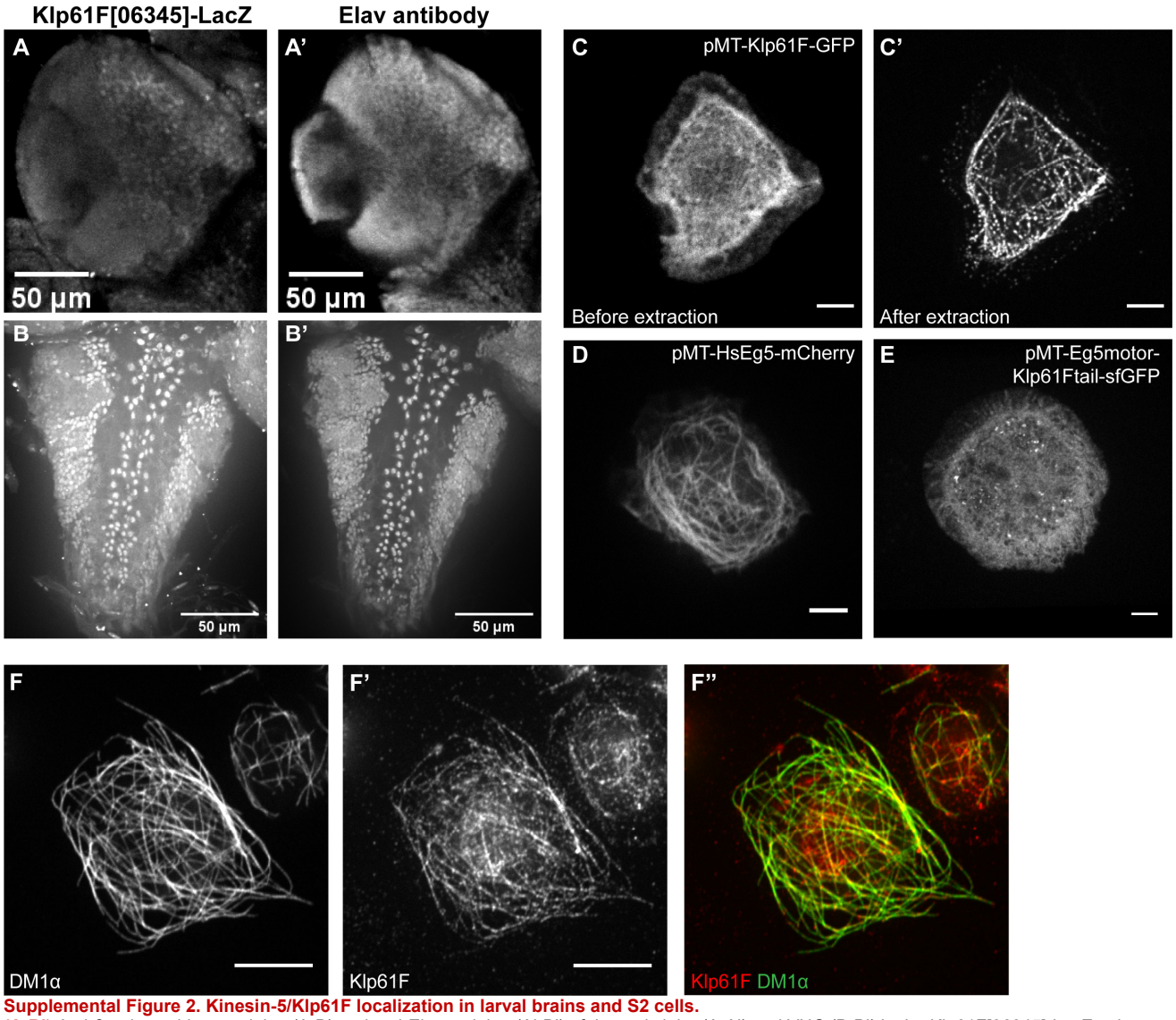
RNAi driven by *elav-Gal4* (early pan-neuronal)	Surviving from pupae to adult %
Klp61F-Valium22 (kinesin-5)	0% (N=514)
Klp67A-Valium22 (kinesin-8)	99.1% (N=101)
Klp10A-Valium20 (kinesin-13)	99.4% (N=632)
Ncd-Valium20 (kinesin-14)	100% (N=427)

RNAi driven by *actin5C-Gal4* (ubiquitous)	Surviving to adulthood %
Klp61F-Valium22 (kinesin-5)	0% (N=600)
Klp10A-Valium20 (kinesin-13)	0% (N=433)

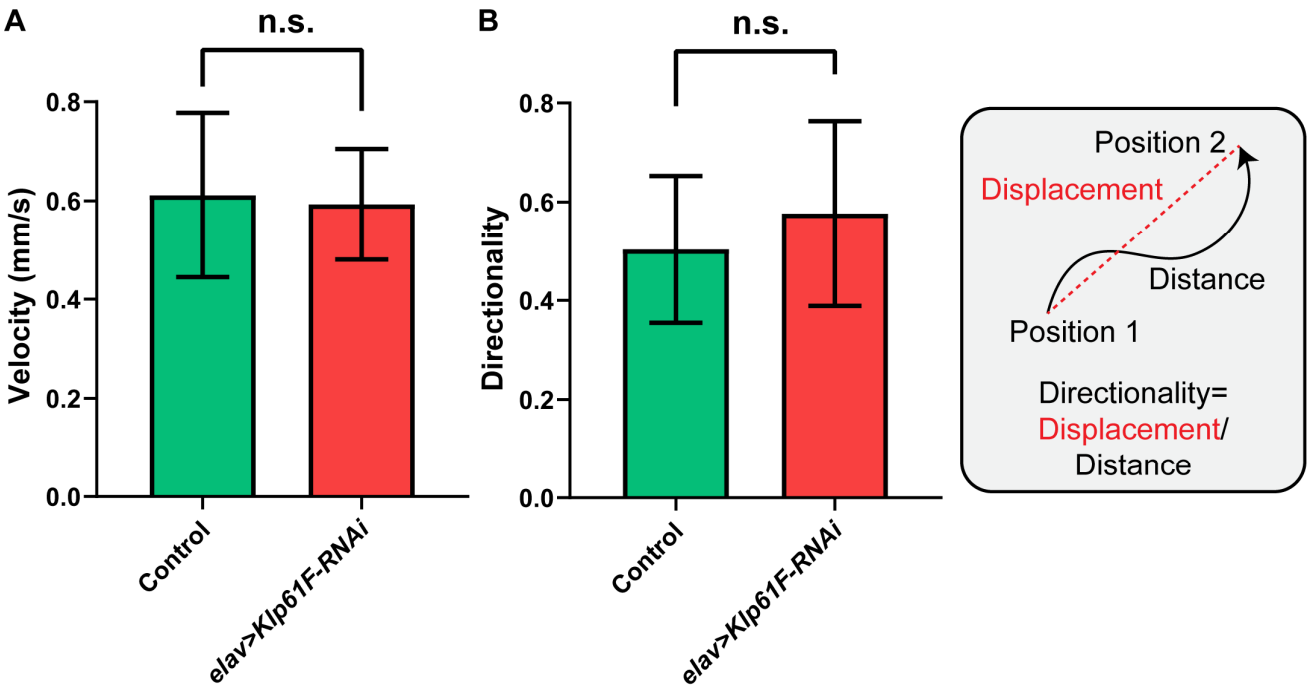




Supplemental Figure 1. Kinesin-5/Klp61F is required in neurons.
(A) Summary of adult survival rates following neuronal depletion of four mitotic motors, kinesin-5/Klp61F, kinesin-8/Klp67A, kinesin-13/Klp10A or kinesin-14/Ncd (top), and after ubiquitous depletion of kinesin-5/Klp61F or kinesin-13/Klp10A (bottom).
(B) Adult survival rates of flies expressing the CDK inhibitor, Roughex (Rux), driven by the ubiquitous *actin5C-Gal4*, pan-neuronal *elavP-Gal4* or pan-motor neuron *D42-Gal4* drivers.
(C-D') Neuronal nucleus labeled with anti-ELAV antibody in the 3rd instar larval VNC of control (C) and *elav>Klp61F-RNAi* (D). The manual tracking of the nuclei in the midline clusters of neurons is shown in (C'-D'). Scale bars, 50 µm.

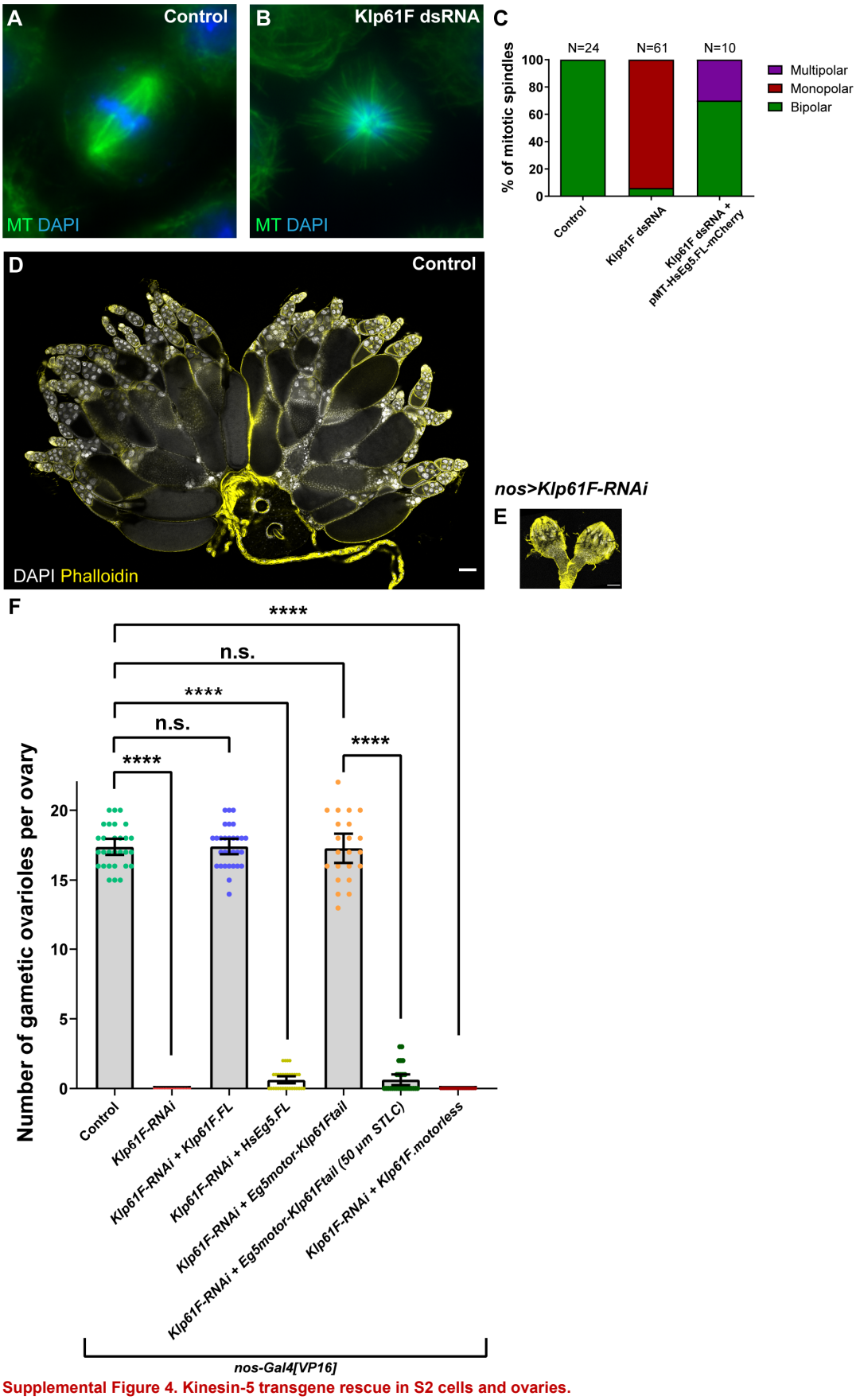


Supplemental Figure 2. Kinesin-5/Klp61F localization in larval brains and S2 cells.
(A-B') Anti-β-galactosidase staining (A-B) and anti-Elav staining (A'-B') of the optic lobe (A-A') and VNC (B-B') in the Klp61F[06345]-LacZ enhancer trap line. Scale bars, 50µm. See also Video 3.
(C-C') Representative images of an S2 cell transfected with pMT-Klp61F-GFP before (C) and after extraction (C'). Scale bars, 5µm.
(D-E) Representative images of S2 cells transfected with pMT-HsEg5-mCherry (D) and pMT-Eg5motor-Klp61Ftail-sfGFP (E). Scale bars, 5 µm.
(F-F'') Anti-α-tubulin (DM1α) staining (F), anti-Klp61F staining (F'), and the overlay of both staining channels (F'') in extracted S2 cells.



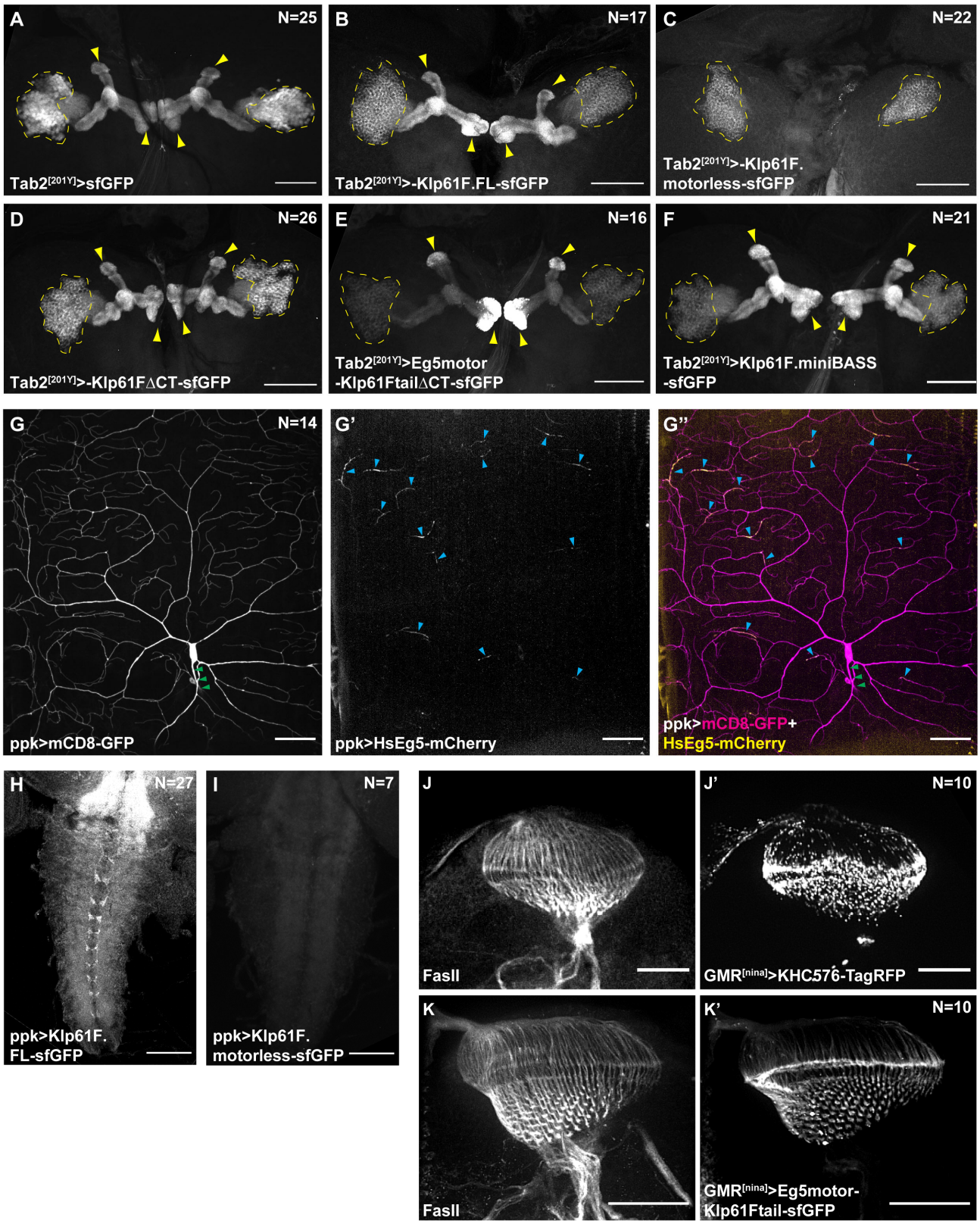
Supplemental Figure 3. Larval crawling is not affected by neuronal *Klp61F-RNAi*.

(A) Velocity and (B) directionality of crawling in 3rd instar larvae with control and neuronal *Klp61F* depletion. Directionality is calculated as the ratio of the displacement to the distance from the center (starting point) to the edge (ending point) of the petri dish. Data are presented as average ± 95% confidence intervals. Sample sizes for control and *Klp61F-RNAi* are N=18 and N=15, respectively. Unpaired t-tests with Welch's correction were performed between control and *Klp61F-RNAi* samples: Velocity, p= 0.8450 (not significant); Directionality, p=0.5226 (not significant). See “Larval crawling assay” in “Materials and Methods” for more details.



Supplemental Figure 4. Kinesin-5 transgene rescue in S2 cells and ovaries.

(A-B) Representative images of a bipolar mitotic spindle in a control S2 cell (A) and a monopolar mitotic spindle in an S2 cell treated with Klp61F dsRNA (B). (C) Percentage of mitotic spindle phenotypes in control, Klp61F dsRNA-treated, and Klp61F dsRNA-treated S2 cells transfected with pMT-HsEg5-mCherry.
(D-E) Representative images of a pair of control ovaries (D) and a pair of ovaries with *Klp61F* depletion driven by the early germline driver *nos-Gal4*$^{[VP16]}$ (E), shown at the same scale. Scale bars, 100 µm.
(F) Quantification of the number of gametic ovarioles per ovary in listed genotypes. Sample sizes: control, N=29; *Klp61F-RNAi*, N=24; *Klp61F-RNAi + Klp61F.FL* (Klp61F.FL rescue), N=30; *Klp61F-RNAi + HsEg5.FL* (HsEg5 rescue), N=32; *Klp61F-RNAi + Eg5motor-Klp61Ftail* (chimeric Eg5motor-Klp61Ftail rescue), N=22; *Klp61F-RNAi + Eg5motor-Klp61Ftail* raised with 50 µM STLC (chimeric Eg5motor-Klp61Ftail rescue with STLC), N=27; *Klp61F-RNAi + Klp61F.motorless* (Klp61F.motorless rescue), N=28. Unpaired t-tests with Welch's correction were performed for the following groups: control vs. *Klp61F-RNAi*, p<0.0001 (****); control vs. Klp61F.FL rescue, p=0.9576 (not significant); control vs. HsEg5 rescue, p <0.0001 (****); control vs. chimeric Eg5motor-Klp61Ftail rescue, p= 0.8538 (not significant); control vs. Klp61F.motorless rescue, p <0.0001 (****); chimeric Eg5motor-Klp61Ftail rescue with or without STLC, p <0.0001 (****). Notes: (1) 50 µM STLC was mixed into the cornmeal food where the flies were raised from embryos to adults (no obvious lethality was observed at this concentration of STLC); (2) In the HsEg5 rescue samples, only one or two egg chambers were observed per rescued gametic ovary, often with abnormal numbers of germline cells, indicating poor rescue of mitotic progression.

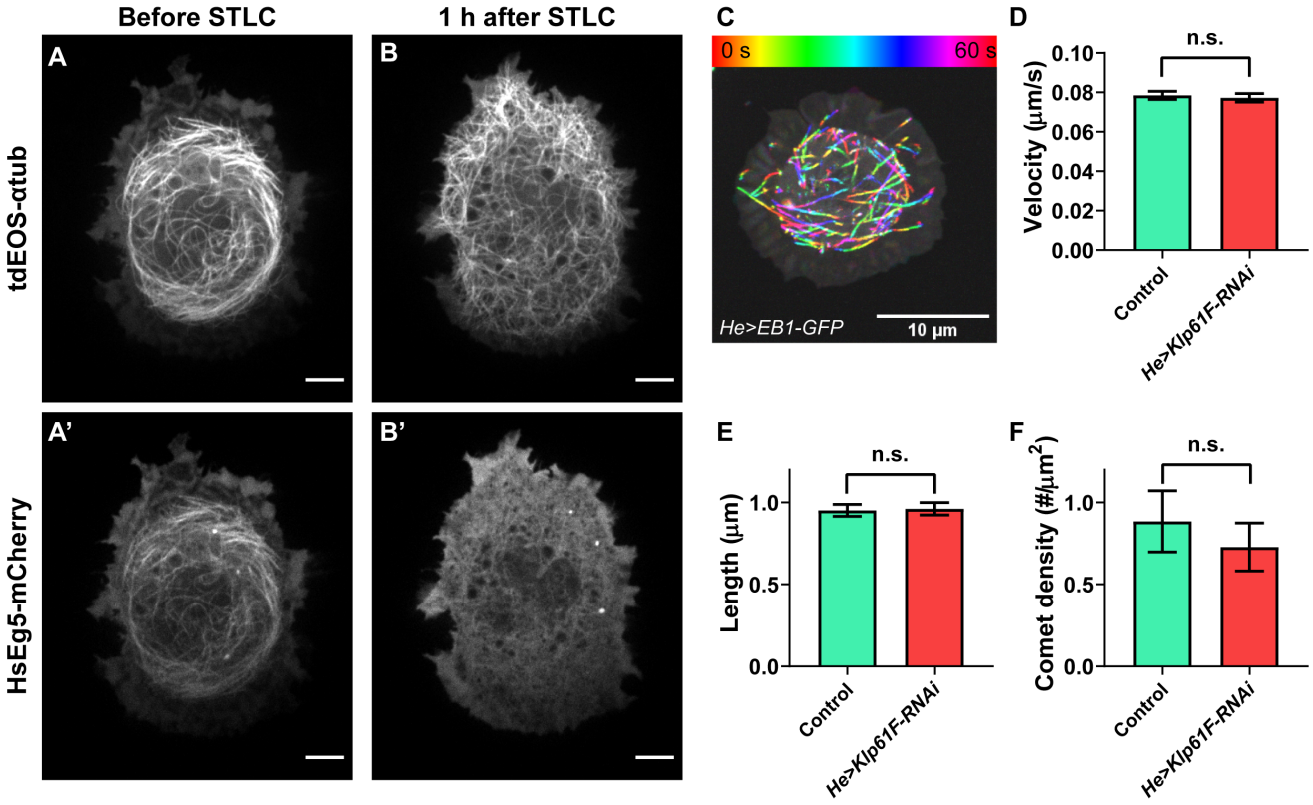


Supplemental Figure 5. Kinesin-5 transgene localization.

(A-F) Transgenic expression of soluble sfGFP (A), Klp61F.FL (B), Klp61F.motorless (C), Klp61FΔCT (D), Eg5motor-Klp61FtailΔCT (E), and Klp61F.miniBASS (F) in mushroom body neurons. Details of these transgene constructs are provided in Figure 4A and the "Molecular cloning" section of "Materials and Methods". All transgene expressions were driven by *Tab2[201Y]-Gal4*. The cell bodies of Kenyon cells are indicated by dashed lines, and their axon tips are indicated by yellow arrowheads. Scale bars, 50 μm.

(G-G'') Membrane labeling (mCD8-GFP) and localization of human Eg5 (HsEg5-mCherry) in a class IV da neuron. The proximal axon and the dendritic tips are marked by green and blue arrowheads, respectively. HsEg5 transgenic expression was driven by *ppk-Gal4*. Scale bars, 50 μm.

(H-I) Transgenic expression of Klp61F.FL (H) and Klp61F.motorless (I) in the axonal terminals of class IV da neurons in the VNC. Both transgene expressions were driven by *ppk-Gal4*. Scale bars, 50 μm.
(J-K') Anti-Fasciclin II (FasII) staining (J-K) and localization of either a constitutively active kinesin-1 truncation (KHC576) (J') or the chimeric Eg5motor-Klp61Ftail transgene (K'). Transgene expression was driven by *GMR$^{[nina]}$-Gal4*. Scale bars, 50 μm.



Supplemental Figure 6. Kinesin-5 prevents microtubule penetration independent of microtubule polymerization.
(A-B') Microtubule and human Eg5 localization before and after 2 µM STLC treatment in an S2 cell treated with Klp61F dsRNA. Scale bars, 5 µm.
(C) A temporal-color hyperstack of EB1-GFP movement in a *Drosophila* primary hemocyte. EB1-GFP expression was driven by *he-Gal4*. Scale bar, 10 µm.
(D-F) Tracking of EB1 comet velocity (D), length (E), and density (F) in control and *Klp61F-RNAi* hemocytes. Numbers of EB1 comet tracks: control, N=2101; *Klp61F-RNAi*, N=1836. Unpaired t-tests with Welch's correction were performed between control and *Klp61F-RNAi*: (D) velocity, p=0.4126 (not significant); (E) length, p=0.7163 (not significant); (F) comet density, p=0.1621 (not significant).

Supplementary Videos

Video 1. Adult fly climbing assay in control, *Klp61F-RNAi* and the chimeric motor rescue flies. From left to right: adult flies of control, *elav>Klp61F-RNAi* and *elav>Klp61F-RNAi+Eg5motor-Klp61Ftail-sfGFP*. The video is shown in real-time.

Video 2. Klp61F[07012]-LacZ and Elav staining in a larval VNC. Dual staining with anti-Elav (left) and anti-β-Gal (right) in the *Klp61F[07012]-LacZ* enhancer trap line. Scale bar, 50 μm.

Video 3. Klp61F[06345]-LacZ and Elav staining in a larval brain. Dual staining with anti-Elav (left) and anti-β-Gal (right) in the *Klp61F[06345]-LacZ* enhancer trap line. Scale bar, 50 μm.

Video 4. Microtubule movement and actin ruffling in control hemocyte. Microtubules (labeled with tdEOS-αtub84B, green, without photoconversion) and F-actin (labeled with LifeAct-Ruby, red) driven by *He-Gal4* in a control hemocyte. Scale bar, 5 μm.

Video 5. Microtubule penetration into the cell periphery in control. Microtubules (labeled with tdEOS-αtub84B, photoconverted to red) in a control hemocyte before and after 2.5 μM CytoD treatment. Scale bar, 5 μm.

Video 6. Microtubule penetration into the cell periphery in *Klp61F-RNAi*. Microtubules (labeled with tdEOS-αtub84B, photoconverted to red) in a *He>Klp61F-RNAi* hemocyte before and after 2.5 μM CytoD treatment. Scale bar, 5 μm.

Video 7. Microtubule penetration into the cell periphery in a *Drosophila* S2 cell upon STLC treatment. Microtubules (labeled with tdEOS-αtub84B, green channel, without photoconversion) in a *Drosophila* S2 cell treated with Klp61F dsRNA and ectopically expressing HsEg5-mCherry before and after 2 μM STLC treatment. Scale bar, 5 μm.