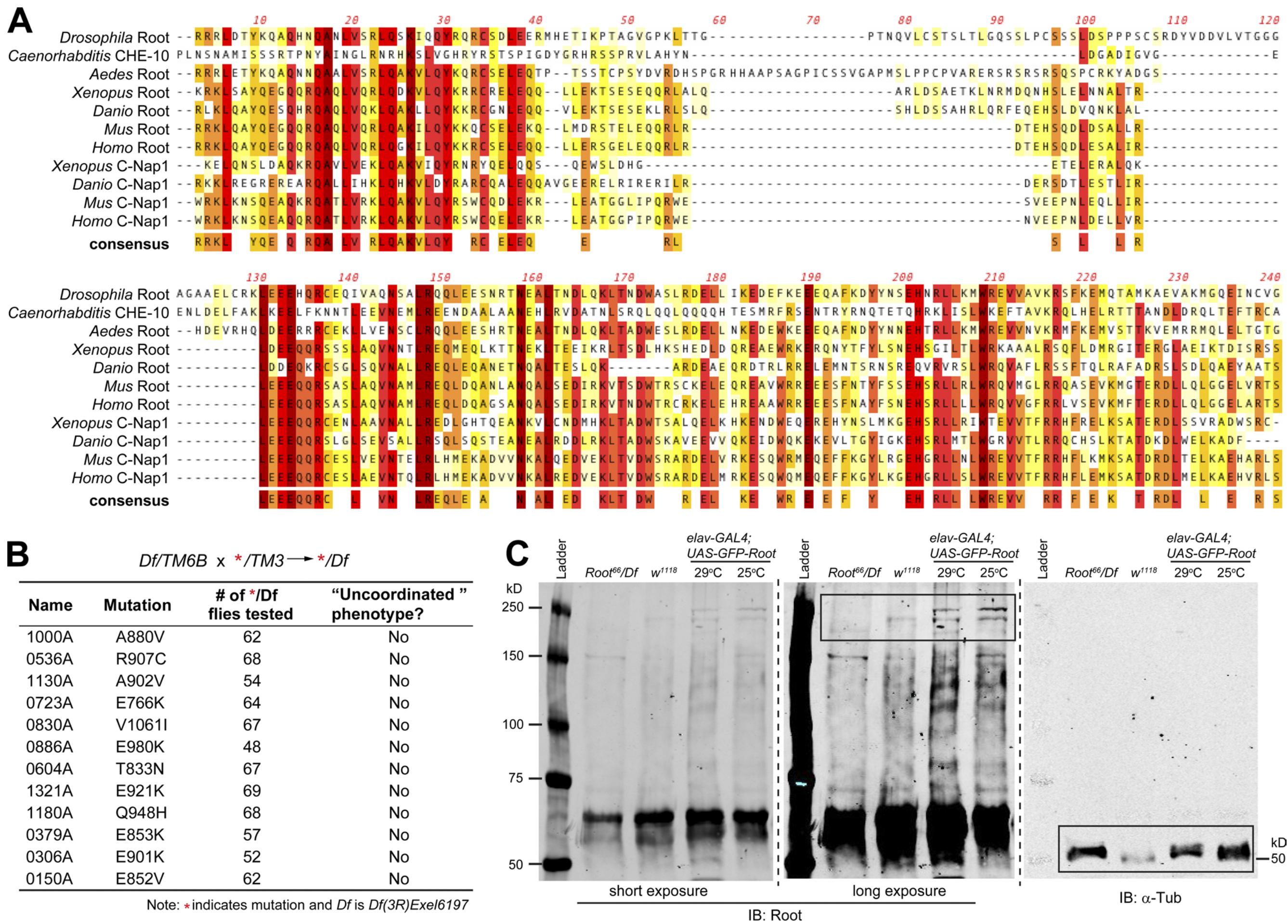

Name	Mutation	# of */Df flies tested	"Uncoordinated" phenotype?
1000A	A880V	62	No
0536A	R907C	68	No
1130A	A902V	54	No
0723A	E766K	64	No
0830A	V1061I	67	No
0886A	E980K	48	No
0604A	T833N	67	No
1321A	E921K	69	No
1180A	Q948H	68	No
0379A	E853K	57	No
0306A	E901K	52	No
0150A	E852V	62	No

Figure S1. **Homology of the Rootletin conserved domain in Root and C-Nap1 across species, and molecular genetic analysis of mutants and transgenes (related to Fig. 1).** (A) ClustalW alignment of the conserved Rootletin domain in Root and C-Nap1 across species. Accession: *Drosophila* Root, NP_651216.2; *Caenorhabditis* CHE-10, NP_494819.3; *Aedes* Root, XP_001654101.1; *Xenopus* Root, XP_012826292.1; *Danio* Root, XP_009300702.1; *Mus* Root, NP_742120.2; *Homo* Root, NP_055490.4; *Xenopus* C-Nap1, XP_004915842.1; *Danio* C-Nap1, XP_692550.3; *Mus* C-Nap1, NP_001123472.1; and *Homo* C-Nap1, NP_009117.2. (B) Coordination assessment of the 12 missense *Root* alleles recovered from TILLING. Mutation column describes the amino acid change at the corresponding position of the full-length protein. (C) Whole blots from the Western blot presented in Fig. 1. There is no apparent detection of the truncated protein (~77 kD) from *Root*^{66}. Boxes indicate cropped areas shown in Fig.1 E. Lysates from 40–50 antenna pairs were loaded in each lane.

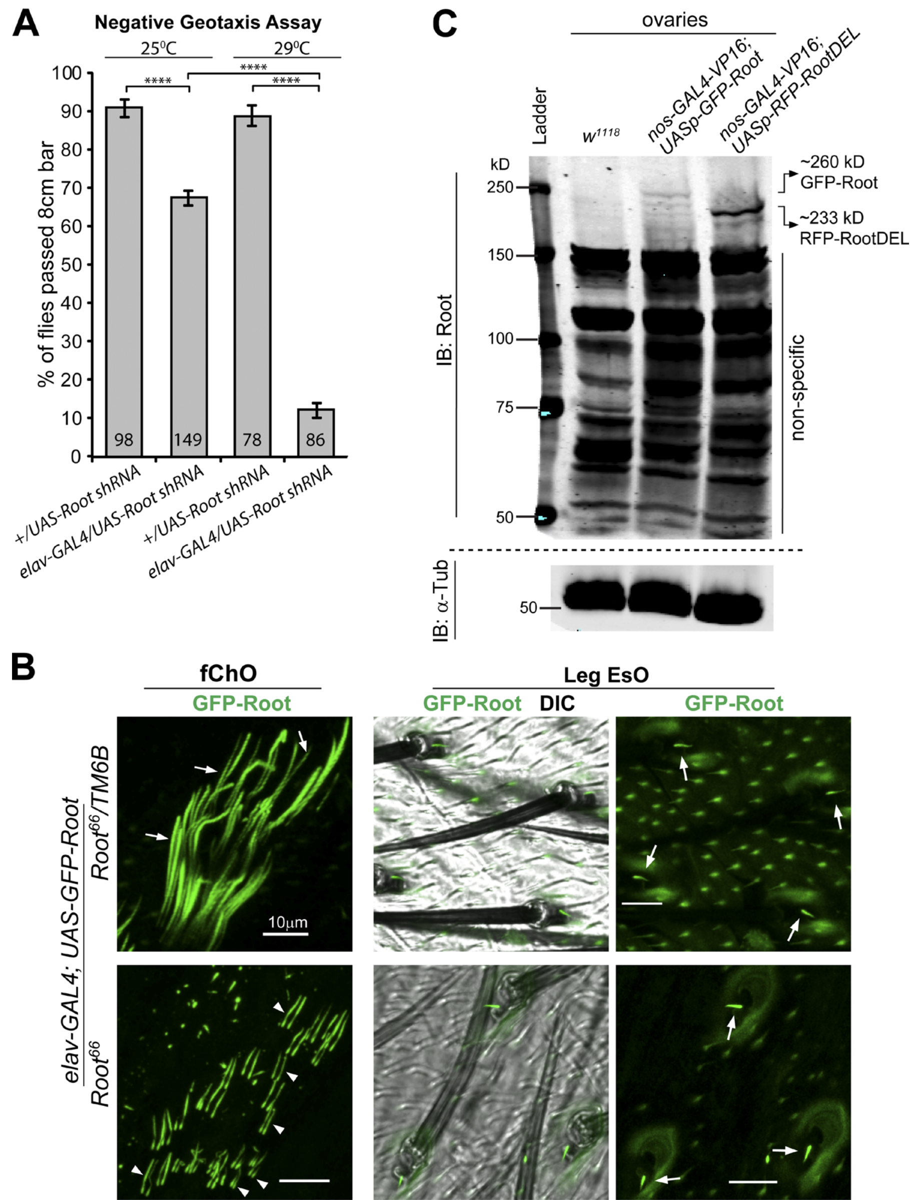

Figure S2. **In *Root*^{66} mutant, GFP-Root expression forms rootlets with normal length in Es organs, but it organizes shorter ones in ChOs (related to Fig. 4 and Fig. 5).** (A) Flies with Root knockdown by RNAi in the nervous system show severe defects in negative geotaxis behavior. Because GAL4-driven expression increases with temperature, producing higher RNAi knockdown at 29°C than 25°C, knockdown flies show a significantly greater impairment of negative geotaxis at 29°C compared with 25°C. Numbers inside the bars indicate the total number of males assayed; ****, $P \leq 0.0001$. (B) In Ch neurons of the fChO, ectopically expressed GFP-Root localizes to the normal-sized (~20–25 µm long) rootlets (arrows) in control *Root*^{66}/TM6B; in contrast, GFP-Root expression in the *Root*^{66} mutant, which produces significant rescue, organizes shorter rootlets (arrowheads) ~2–8 µm long. GFP-Root expression in Es organs in the *Root*^{66} mutant organizes rootlets (arrows) that are ~5 µm long and are similar to the *Root*^{66}/TM6B control. Bars, 10 µm. (C) Western blot detection of GFP-Root and RFP-RootDEL expressed from transgenes. The transgenes were expressed in ovaries and detected with anti–Root antibodies. Bands of the expected size were detected in transgene-expressing ovaries as indicated. Note that there is no endogenous Root (~233 kD, almost same size as RFP-RootDEL) in the ovaries. Lysates from three ovary pairs were loaded in each lane.

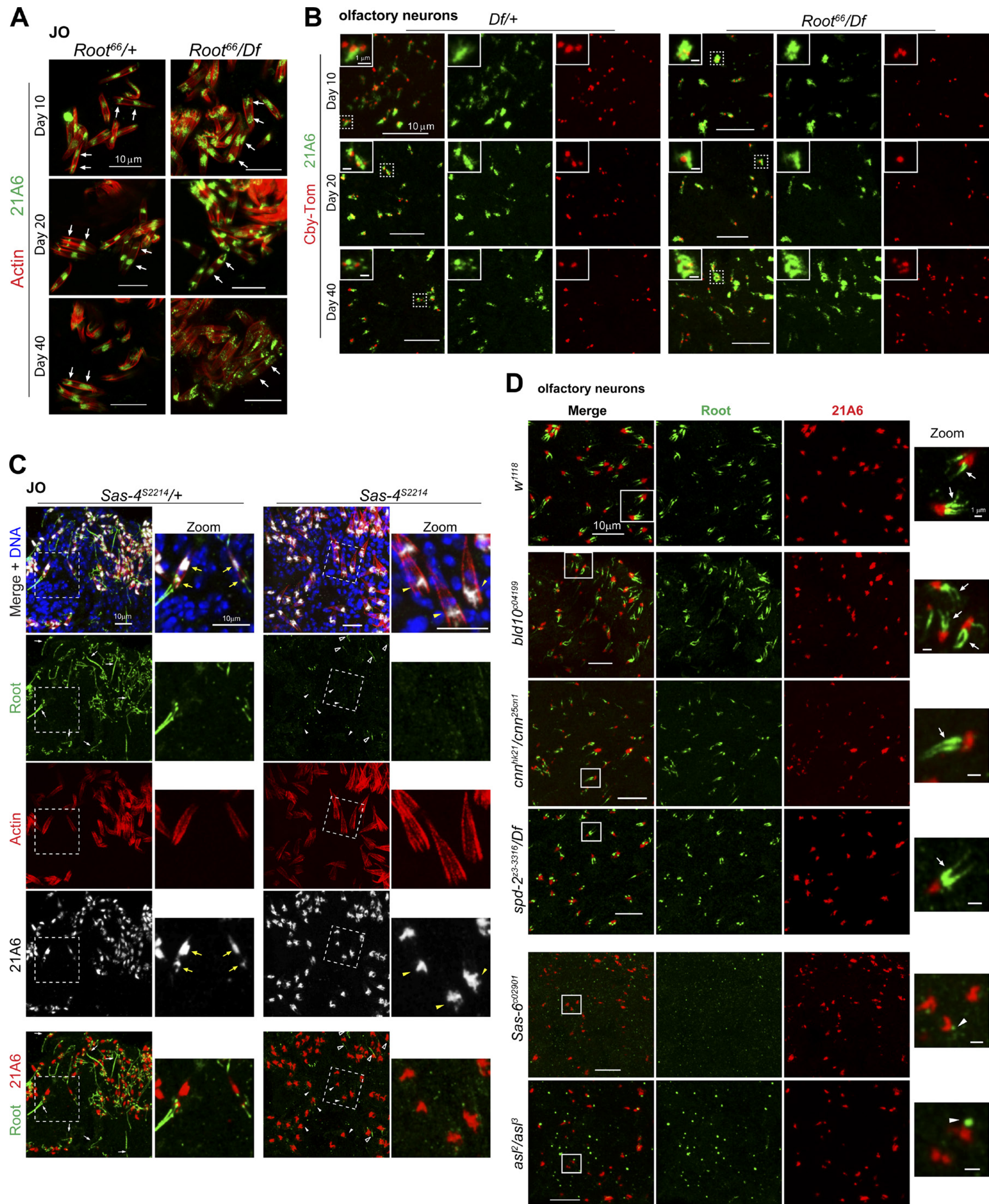

Figure S3. **The centriole is required for normal rootlet assembly in Ch neurons in the JO (related to Fig. 7).** (A and B) Assessment of cilia structure with age in *Root*^{66} neurons by 21A6 staining in JO neurons together with actin staining of the scolopale rods (A) and olfactory neurons together with Cby-Tomato (B). Arrows point to the pair of 21A6 signals typically found in each JO scolopidium. (C) The centriole is required for normal rootlet assembly in Ch neurons in the JO. Images show the staining of squashed antenna. In control *Sas-4/+ (TM6B)*, almost all the cells associated with Actin and 21A6 staining have rootlets that project from the base of the cilia marked by 21A6 (white arrows). However, rootlet structures are absent in most *Sas-4* mutant Ch neurons (white solid arrowheads). A few normal-looking rootlet-like filaments are observed in *Sas-4* mutant neurons (white open arrowheads). Note that 21A6 staining in the control localizes both at the base of the cilium and at the area below the ciliary dilation (yellow arrows in zoom view); whereas in *Sas-4* mutant neurons, it does not localize to the region below the ciliary dilation (yellow arrowheads in zoom view). Actin marks the scolopale rod. (D) Assessments of rootlet formation in mutants of centrosome proteins required for centriole/basal body assembly (*Sas-6* and *asl*), and those required for centrosome function but not basal body assembly (*bld10*, *cnn*, and *spd-2*). Apparently normal rootlets form in *bld10*, *cnn* and *spd-2* mutants in olfactory neurons (arrows). Rootlets fail to form in *Sas-6* and *asl* mutants, similar to the *sas-4* mutant shown in Fig. 7 E, and in these mutants, Root can sometimes be found localized to foci in neurons (arrowheads). A–D show images of the antenna squash. Boxed areas indicate the zoom-in regions. Bars: (A and C) 10 µm; (B) 10 µm; (B, insets) 1 µm; (D) 10 µm; (D, zoom) 1 µm.

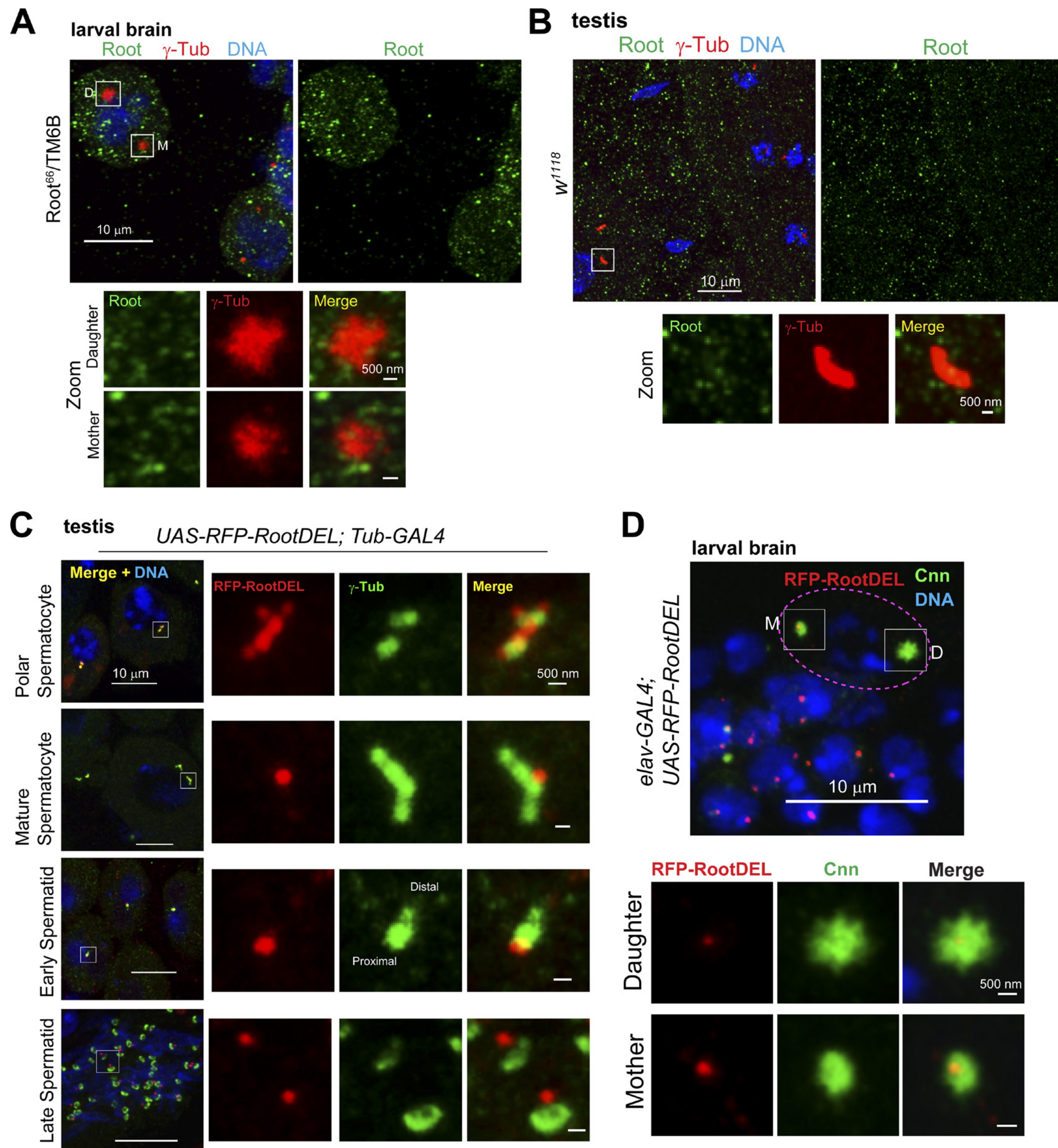

Figure S4. **Endogenous Root is undetectable in testes or larval brains, and ectopic RFP-RootDEL localizes to centrioles or centrosomes in testes and larval brains (related to Fig. 8).** (A and B) In larval brains (A) and testes (B), no endogenous Root protein is detected at centrioles or centrosomes with the affinity-purified Root antibody. γ-Tub marks the centrosomes in larval brains or centrioles in testes. Note: the background is high in these images because the gain on the green fluorescence signal was increased to detect any weak Root signal at centrioles. (C) In testis, RFP-RootDEL localizes to the proximal end of centrioles, similar to the localization of GFP-Root (see Fig. 8 B). γ-Tub marks the centrioles. (D) In NBs, RFP-RootDEL localizes preferentially to the mother centrosome, similar to the localization of GFP-Root (see Fig. 8 C). Cnn localizes significantly more at the daughter centrosome and is used to distinguish the daughter and mother. Dotted line outlines a single NB. Boxed areas indicate the zoom-in regions. Bars: (main) 10 µm; (zoom) 500 nm.

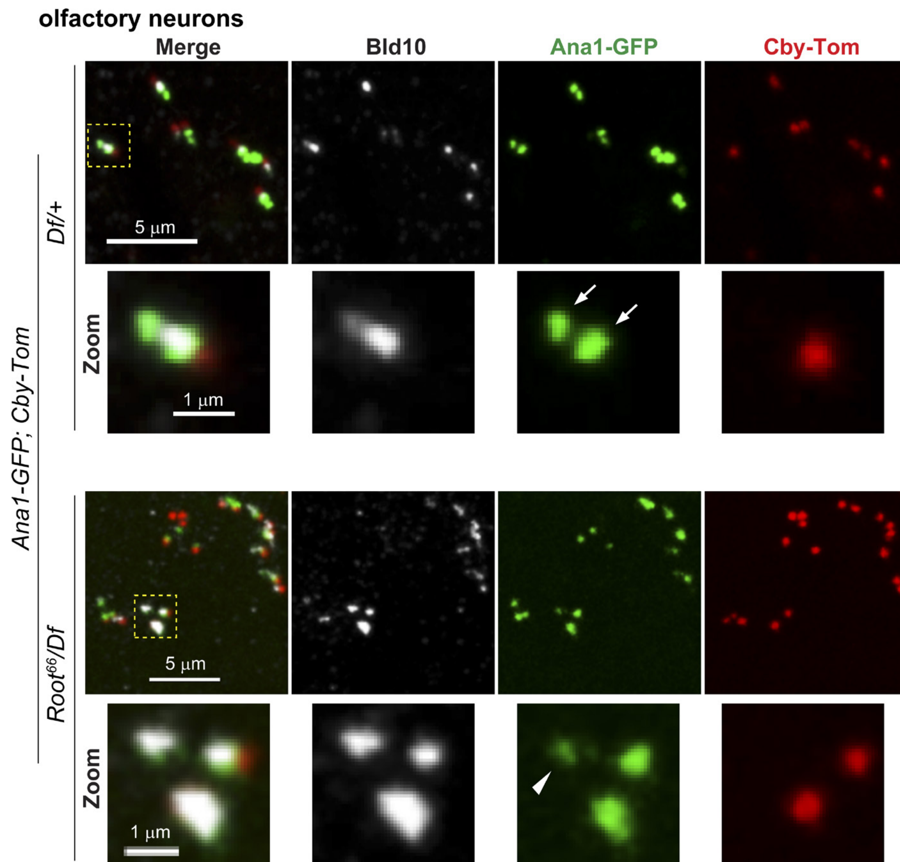

Figure S5. **Bld10 localization to centrioles is unaffected in *Root*^{66} olfactory neurons (related to Fig. 9).** Basal bodies are labeled with Ana1-GFP. Note that the basal bodies are less coherent in *Root*^{66} (arrowhead), compared with the control (arrows).

Video 1. ***Root*^{66} mutant flies show severe defects in startle response.** For each genotype, 10–12 (half males, half females) 4-d-old flies were placed on an apple juice agar plate the day before the experiment. The test was conducted at room temperature. Flies were rested for 3 min before being introduced with the mechanical disturbance generated by shaking the agar plates vigorously. Upon mechanical disturbance, control w^{1118} flies react, move, and/or jump around promptly, whereas most *Root*^{66} homozygous mutants do not respond. With GFP-Root expression in the nervous system driven by *elav*-GAL4 in *Root*^{66}, rescued flies have restored startle response comparable to the control. Genotypes are as follows: control is w^{1118}, mutant is *Root*^{66}, and GFP-Root rescue is *elav-GAL4;UAS-GFP-Root;Root*^{66}. Real-time movies were captured with a webcam video recorder (Logitech) at 15 frames/s.