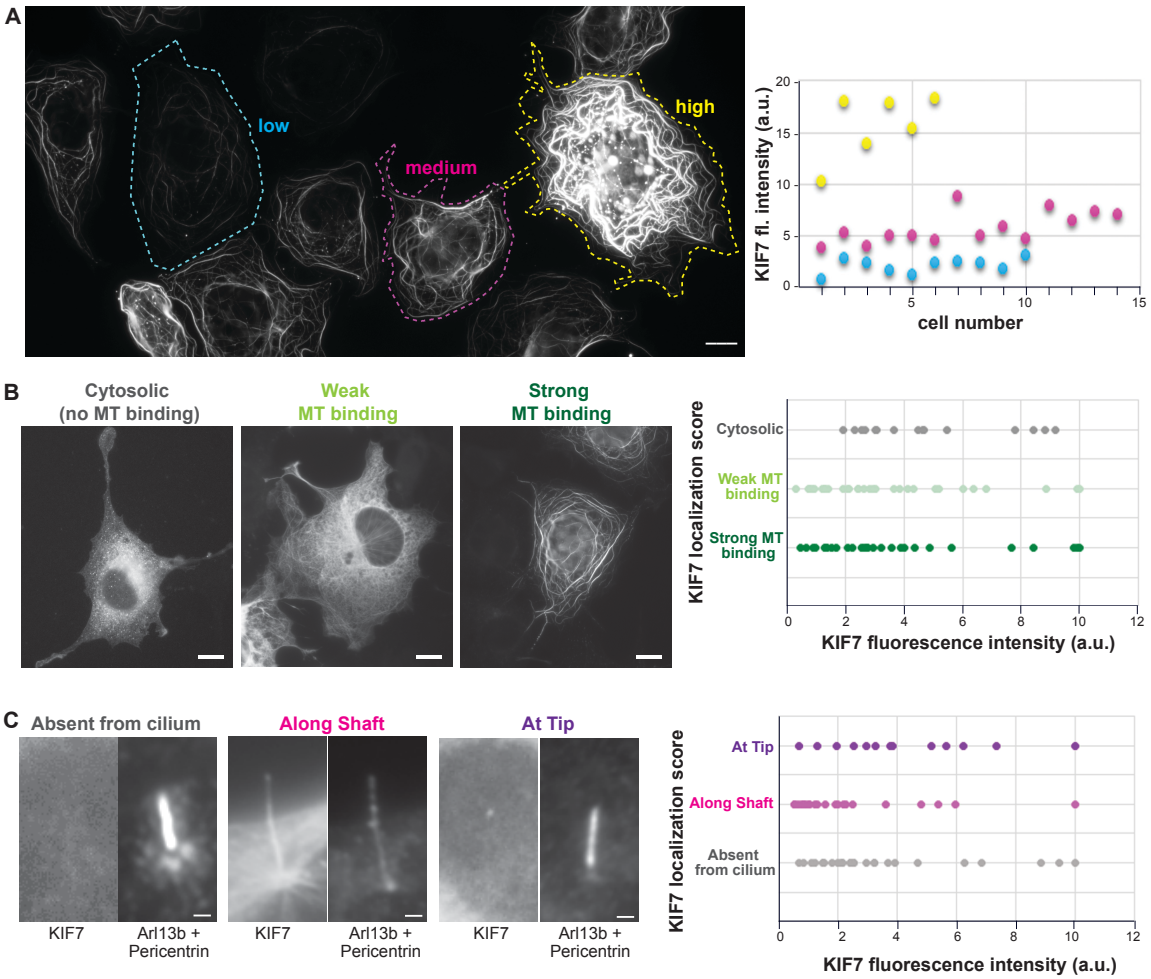

Figure S1. KIF7 localization does not correlate with level of expression. (A) Left, representative image of COS-7 cells expressing KIF7(1-1114). Scale bars, 10 μm. Right, plot of average KIF7(1-1114) fluorescence intensity per cell for 30 randomly chosen cells. Expression was defined as high, medium, or low expression and only cells with medium or low expression were included in the analysis. (B) Criteria for microtubule (MT) localization. Left, representative images of MT localization phenotypes in COS-7 cells. Scale bars, 10 μm. **I**f the expressed protein exhibited a cytoplasmic/diffuse localization, this was scored as 'no MT binding'. If the expressed protein could be seen both in the cytoplasm and on microtubules, this was scored as 'weak MT binding'. If the exogenous protein could only be seen on MTs, this was scored as 'strong MT binding'. Right, plot of average KIF7 fluorescence intensity across MT binding phenotypes. Each spot represents the average KIF7 fluorescence intensity of one cell across a total of 70 randomly selected cells. (C) Criteria for cilium localization. Left, representative images of cilium localization phenotypes in NIH-3T3 cells. Scale bars, 1 μm. If the expressed protein could not be detected in the cilium, this was scored as 'absent from cilium'. If the expressed protein was observed at the base of the cilium (defined by presence of pericentrin staining), this was scored as 'at base' (not shown). If the expressed protein was observed at the tip of the cilium (opposite end as pericentrin staining), the localization was scored as 'at tip'. If the expressed protein was distributed uniformly along the cilium, the localization was scored as 'along shaft'. Right, plot of average KIF7 fluorescence intensity across cilium localization phenotypes. Each spot represents the average KIF7 fluorescence intensity of one cell across a total of 60 randomly selected cells.

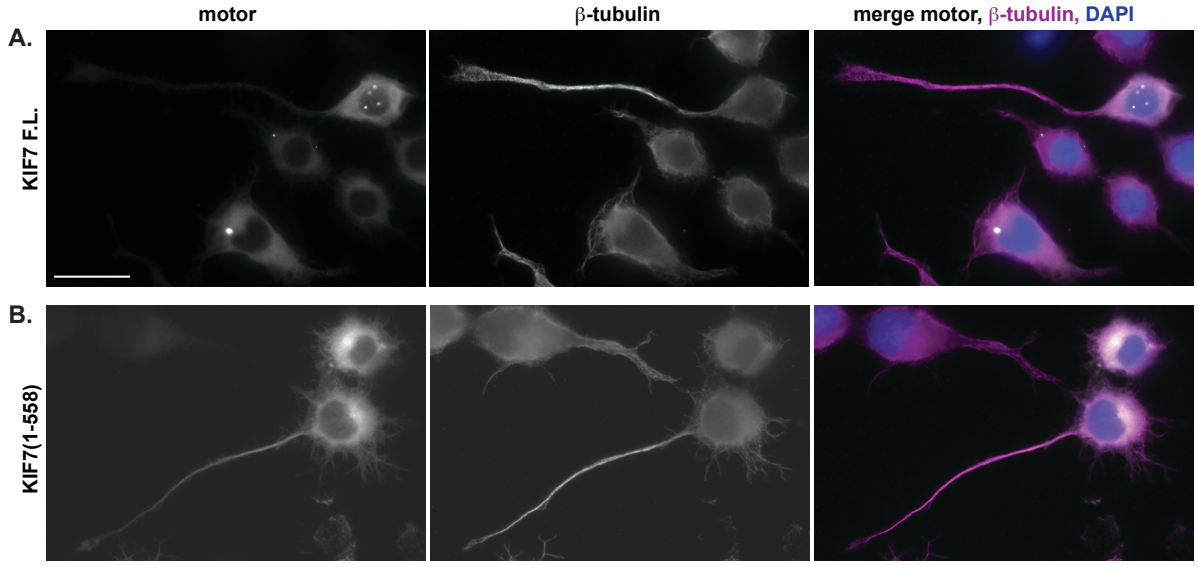

Figure S2. KIF7 is not a processive motor. Neuronal CAD cells expressing mCit-tagged versions of (A) full-length or (B) truncated (1-558) KIF7. The cells were fixed and stained with an antibody to β-tubulin to mark microtubules and with DAPI to mark nuclei. Scale bar, 20 µm.

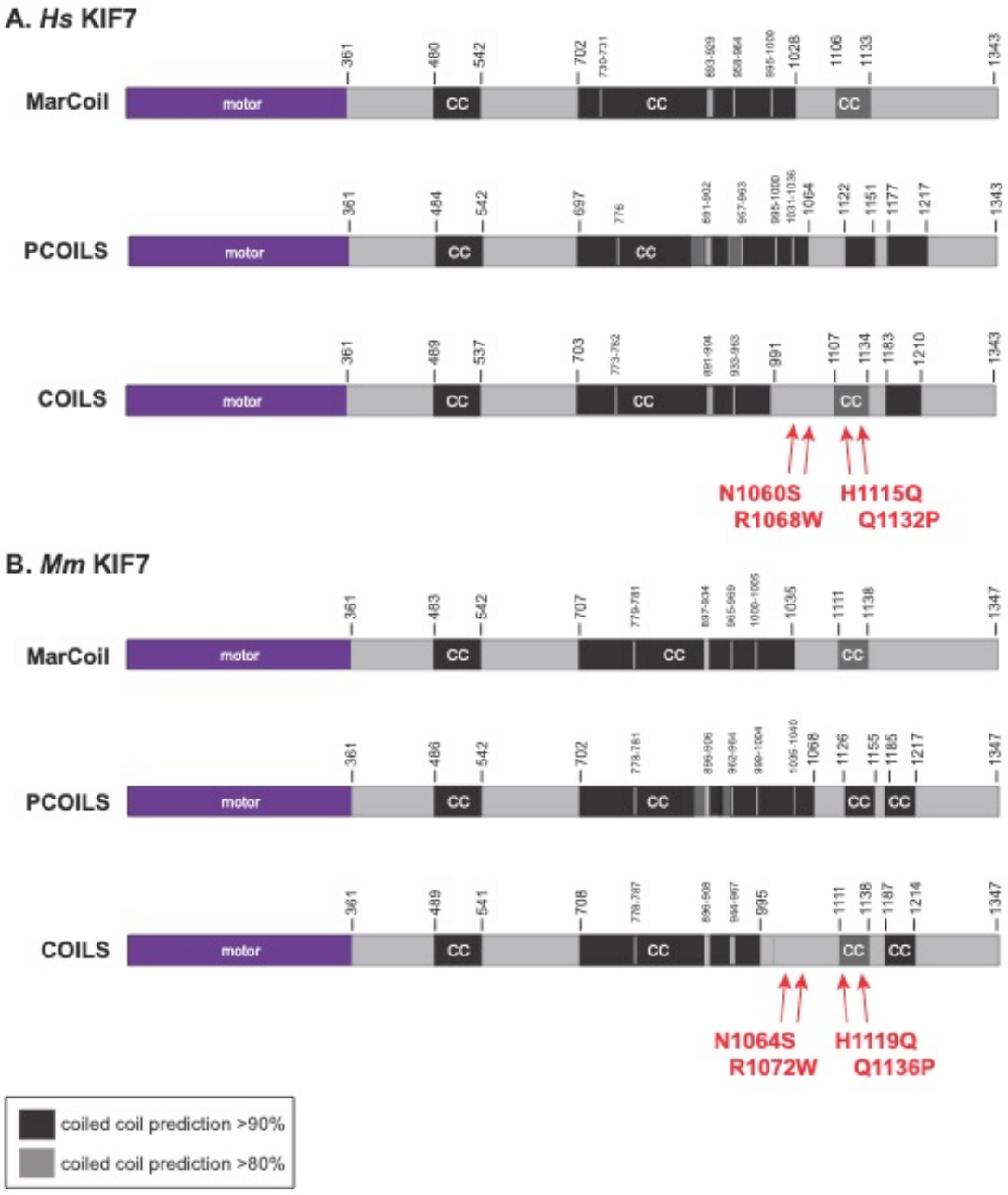

Figure S3. Coiled-coil predictions for human and mouse KIF7 using the indicated software

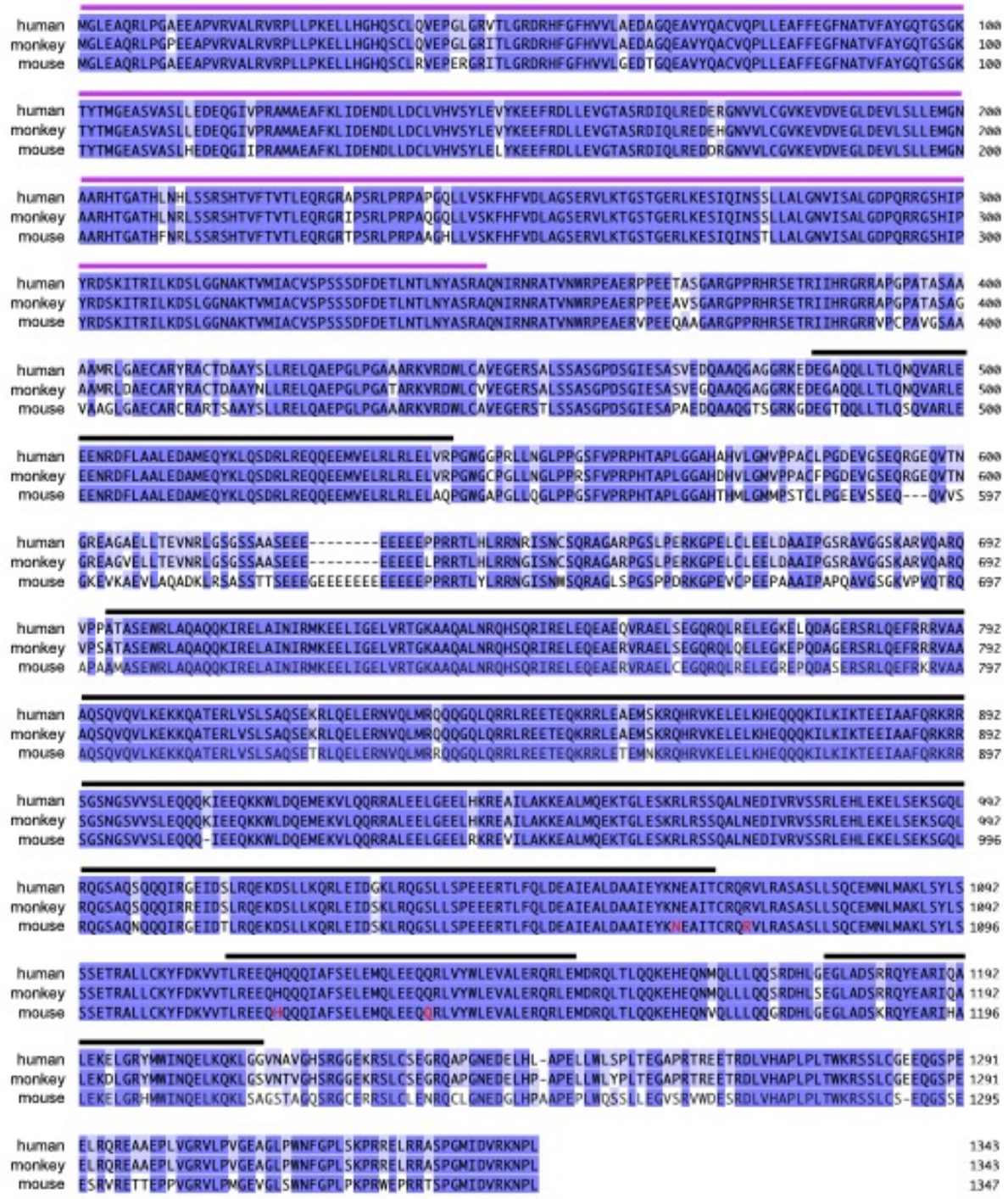

Figure S4. Alignment of the primary sequences of human (Homo sapiens NP_940927), green monkey (Chlorocebus sabaeus XP_007988548), and mouse (Mus musculus NP_034756) KIF7 proteins. Purple boxed resides indicate residues with high conservation across these mammalian species. Red text indicates disease-associated mutations introduced into mouse KIF7 in this manuscript. Pink overline indicates the kinesin motor domain, black overlines indicate predicted coiled-coil regions. Sequence alignments were carried out using T-COFFEE (version 11.00) and edited in JalView (2.11.1.4).

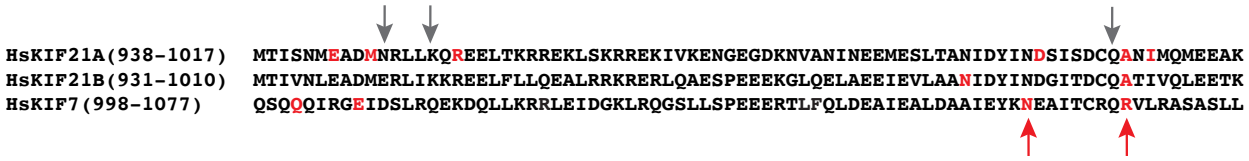

Figure S5. Sequence alignment of the rCC region across kinesin-4 family members KIF21A, KIF21B, and KIF7. Red text indicates mutations in *KIF21A* associated with CFEOM1 (Cheng et al., 2014; van der Vaart et al., 2013), in *KIF21B* associated with neurodevelopmental disorders associated with brain malformations including corpus callosum agenesis and microcephaly (Asselin et al., 2020), and in *KIF7* associated with Al-Gazali-Bakalinova and Bardet-Biedl syndromes (Ali et al., 2012; Putoux et al., 2012). Gray arrows indicate residues mutated in concert that relieve autoinhibition of full-length KIF21A (Bianchi et al., 2016). Red arrows indicate residues mutated individually in this study that do not relieve autoinhibition (Figure 5).

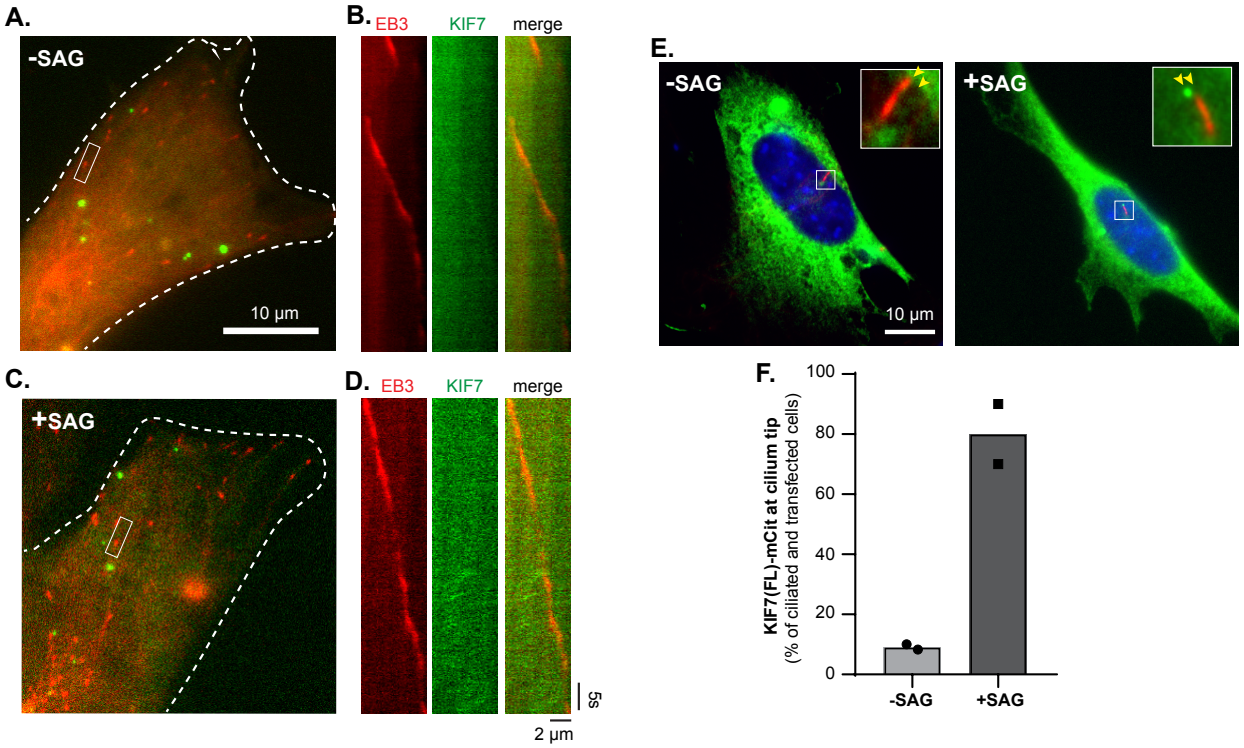

Figure S6. KIF7 does not track the plus ends of microtubules in cells. (A-D) NIH-3T3 cells were transfected with plasmids for co-expression of EB3-mCherry with KIF7-mCit. (A,C) Representative images in the (A) absence or (C) presence of 500 nM SAG treatment for 3-4 h. The periphery of the transfected cells is indicated by white dotted lines. Scale bar, 10 µm. (B,D) Representative kymographs of KIF7 and EB3-mCherry on the microtubule plus end indicated by the white boxed regions in (A) and (C). Time is on the y axis (scale bar, 5 sec); distance is on the x axis (scale bar, 2 µm). (E,F) Verification of Hedgehog pathway activation by SAG treatment. (E) Representative images of NIH-3T3 cells expressing KIF7-mCit in the absence (left) or presence of 500 nM SAG treatment for 3 h (right). The cells were fixed and stained with antibodies against acetylated-tubulin to mark the ciliary axoneme (red) and with DAPI to mark the nucleus (blue). Scale bar, 10 µm. Inserts show magnification of the area outlined with the white box with the fluorescence signals offset by 6 pixels for clarity. Yellow arrowheads indicate the tip of the cilium. (F) Quantification of the percent of cells exhibiting ciliary tip localization of KIF7 in the absence or presence of 500 nM SAG treatment for 3 h. Each spot indicates the result of one independent experiment and the bar indicates the average of the two experiments.

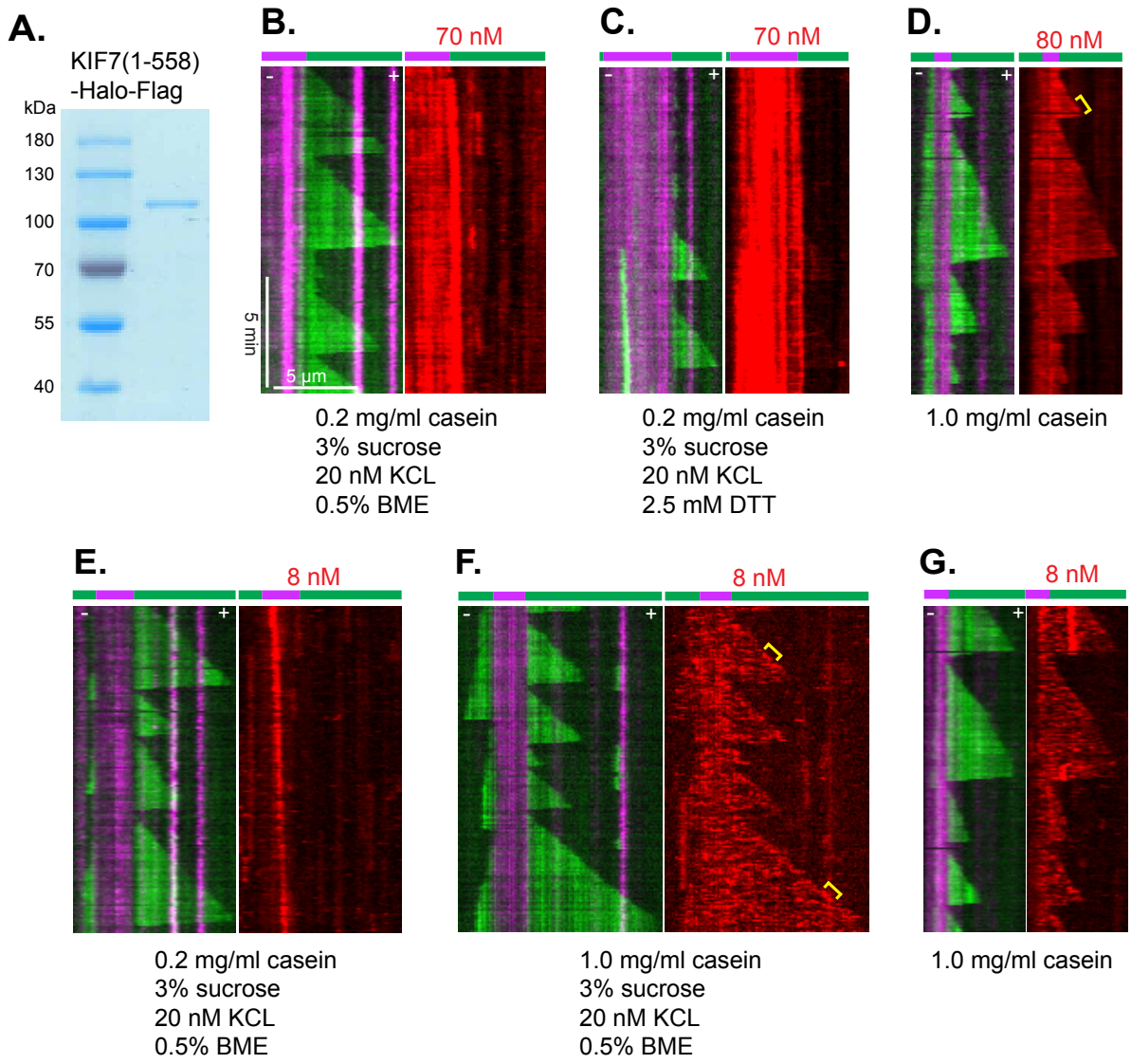

Figure S7. Comparison of KIF7(1-558)-Halo-Flag localization on growing microtubules *in vitro* under various buffer conditions. (A) Coomassie-stained gel of KIF7(1-558)-Halo-Flag protein purified from COS-7 cells. (B-G) Representative kymographs of microtubule dynamics in the presence of (B,C) 70 nM, (D) 80 nM or (E-G) 8 nM purified KIF7(1-558)-Halo-Flag protein. All assays were carried out in BRB80 buffer containing 18.8 µM tubulin (10% Hilyte488-labeled tubulin), 3 mM $MgCl_2$, 1 mM GTP, 1 mM ATP, 0.1 % methylcellulose, and 1 ul oxygen scavenger mix. Supplemental reagents that differ between the assays are indicated below each kymograph. Magenta= GMPCPP-containing microtubule seeds; green= growing microtubules; red= purified KIF7(1-558)-Halo-Flag protein (JF552 ligand). Time is on the y axis (scale bar, 5 min); distance is on the x axis (scale bar, 5 µm). Yellow brackets indicate events where KIF7(1-558)-Halo-Flag protein showed a slight enrichment at the plus end of a growing microtubule.