

Figure S1. **Eg5 is not required for the organization of cytoplasmic microtubules and the Golgi complex in nonmitotic cells.** (A and B) HeLa cells were transfected with control (cont) siRNA or siRNA oligos specific for Eg5 (207 and 608). (A) 24 h after siRNA transfection, 2 mM thymidine was added to the medium and 24 h later the cells were fixed and visualized with anti- α -tubulin and anti-mannosidase II antibodies. Bar, 20 μ m. (B) 24 h after siRNA transfection, 2 mM thymidine was added to the medium and 24 h later the cells were treated with 15 μ M nocodazole (Noc) for 2 h. The cells were then washed and incubated in fresh medium. At the indicated time points, cells were fixed and visualized with anti- α -tubulin (green) and anti-TGN46 antibodies (red). Bar, 25 μ m. (C) HeLa cells were treated with 100 μ M monastrol (Mona) or DMSO for 15 min and then with 15 μ M nocodazole for 2 h. The cells were then washed and incubated in fresh medium containing 100 μ M monastrol. At the indicated time points, cells were fixed and visualized with anti- α -tubulin (green) and anti-TGN46 (red) antibodies. Bar, 25 μ m.

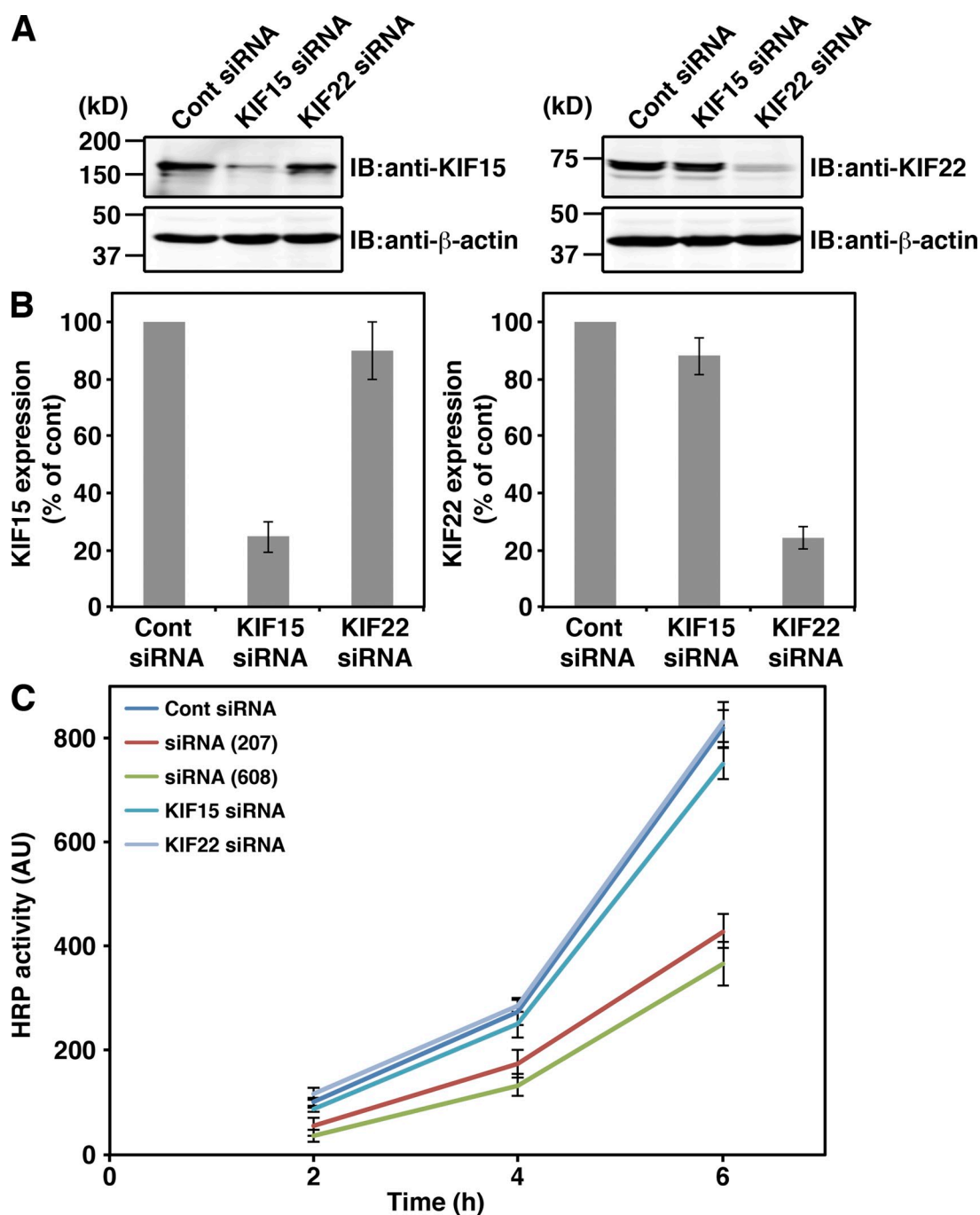


Figure S2. **Knockdown of Eg5 but not KIF15 or KIF22 inhibits HRP secretion in HeLa cells.** (A and B) HeLa-ssHRP cells were transfected with control (cont) siRNA or siRNA oligos specific for KIF15 or KIF22. 24 h later, 2 mM thymidine was added to the medium, and after 24 h of additional incubation the cell lysates were Western blotted with anti-KIF15, anti-KIF22, and anti-β-actin antibodies. (B) Quantification of the expression levels of KIF15 and KIF22 upon RNAi. The average values of three independent experiments are shown (mean ± SD). (C) HeLa-ssHRP cells were transfected with control (cont) siRNA or siRNA oligos specific for Eg5 (207 and 608), KIF15, or KIF22. HRP activity in the medium from control cells and Eg5, KIF15, and KIF22 knockdown cells was measured using ECL. HRP activity in the medium was normalized to the levels detected in the cell lysates. The average values of three independent experiments are shown (mean ± SD).

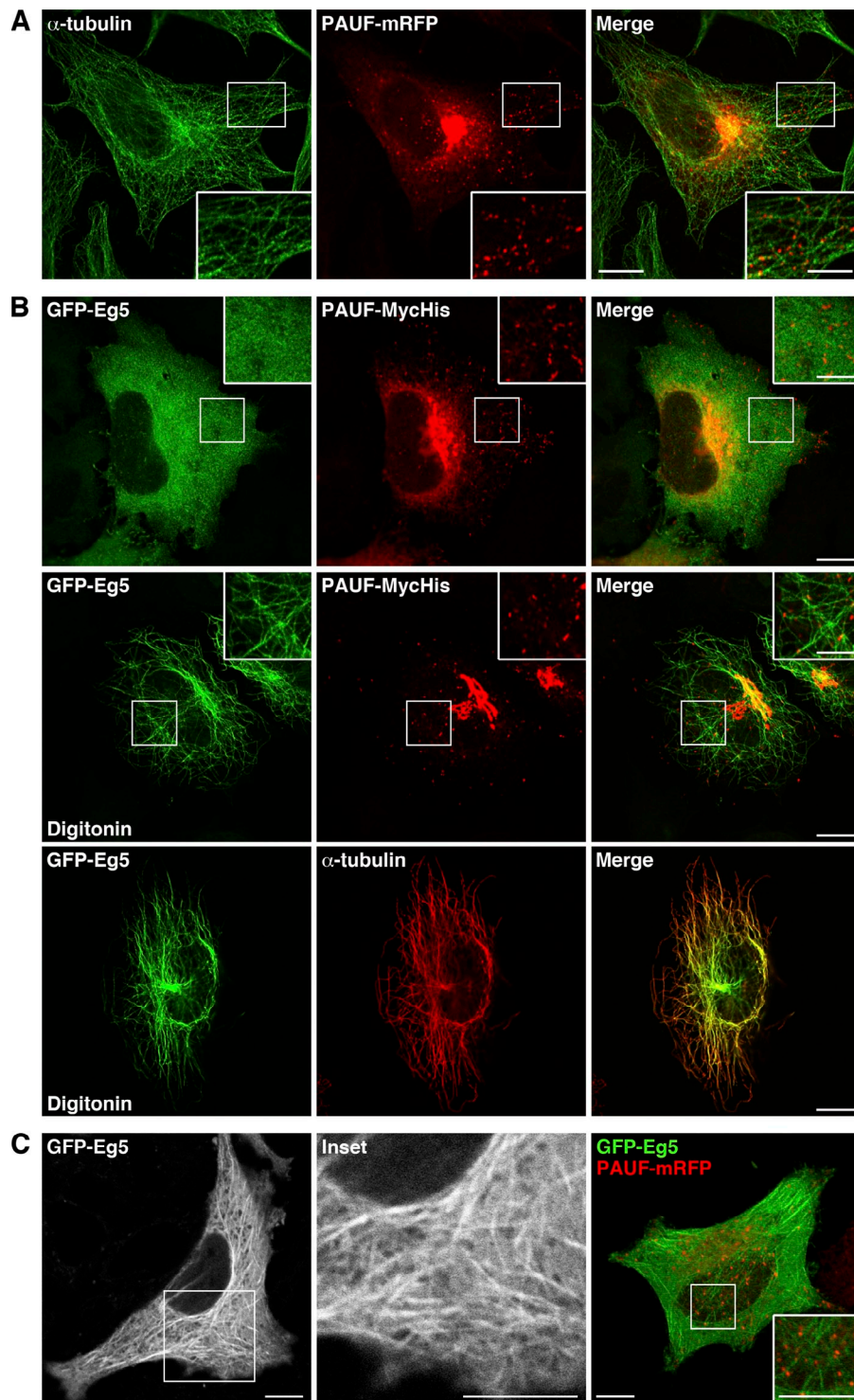
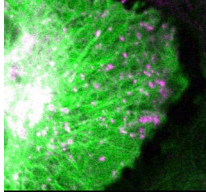
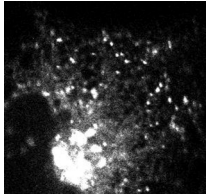


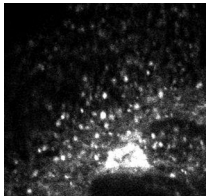
Figure S3. **Eg5 is located on microtubules during interphase.** (A) HeLa cells were transfected with a plasmid for PAUF-mRFP and visualized by fluorescent microscopy with anti- α -tubulin antibody. Bars, 10 μ m (inset, 5 μ m). (B) GFP-Eg5 was expressed alone (bottom row) or with PAUF-MycHis (top and middle rows) in HeLa cells. The cells were incubated with or without 30 μ g/ml digitonin, fixed, and visualized with anti-Myc or anti- α -tubulin antibody. The boxed areas are enlarged in the insets. Bars, 10 μ m (inset, 5 μ m). (C) HeLa cells expressing GFP-Eg5 alone or in combination with PAUF-mRFP were visualized by fluorescence microscopy without fixation. The boxed areas are enlarged in the insets. Bars, 10 μ m.



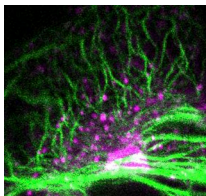
Video 1. **CARTS move on microtubules.** HeLa cells were cotransfected with plasmids for PAUF-mRFP (magenta) and GFP- α -tubulin (green). Images were acquired continuously with time intervals of 1 s between frames for ~ 3 min by a confocal microscope (Fluoview FV1000; Olympus) with a UPLSAPO 60 \times O NA 1.35 objective and FV10-ASW software.



Video 2. **Kinetics of PAUF-mRFP containing CARTS under normal conditions.** HeLa cells were cotransfected with plasmids for PAUF-mRFP and GFP-Eg5 T112N. The cells expressing PAUF-mRFP alone were imaged at 1-s interval for ~ 3 min by use of a confocal microscope (Fluoview FV1000; Olympus) with UPLSAPO 60 \times O NA 1.35 objective and FV10-ASW software. See also Fig. 5, A and B.



Video 3. **Kinetics of PAUF-mRFP containing CARTS in cells expressing GFP-Eg5 T112N.** HeLa cells coexpressing PAUF-mRFP and GFP-Eg5 T112N were imaged at 1-s interval for ~ 3 min by use of a confocal microscope (Fluoview FV1000; Olympus) with UPLSAPO 60 \times O NA 1.35 objective and FV10-ASW software. See also Fig. 5, A and B.



Video 4. **Dual-color live imaging of cells coexpressing PAUF-mRFP and GFP-Eg5 T112N.** The cell in Video 3 is shown in dual color: PAUF-mRFP (magenta) and GFP-Eg5 T112N (green).