Supplementary Information

Axin localizes to the centrosome and is involved in microtubule nucleation

Katsumi Fumoto, Moe Kadono, Nanae Izumi, and Akira Kikuchi

Supplementary Methods

Material and chemicals

MKN-1 (gastric cancer), HeLa S3 (uterine cancer), and U2OS (osteosarcoma) cells were kindly provided by Drs. W. Yasui (Hiroshima University , Hiroshima, Japan), K. Matsumoto (Nagoya University, Nagoya, Japan), and H. Saya (Keio University, Tokyo, Japan), respectively. Anti-GCP2 and anti-centrin 3 antibodies were kind gifts from Drs. Y. Ono (Kobe University, Kobe, Japan) and M. Bornens (Institut Curie, Paris, France), respectively. pCAG-SK/γ-tubulin-FLAG, pEGFP-N2/γ -tubulin, and rat monoclonal anti-Odf2 antibody were kind gifts from Dr. S. Tsukita (Osaka University, Osaka, Japan). The rabbit polyclonal anti-ninein antibody was generated as described previously (Fumoto *et al*, 2006). Mouse monoclonal anti-GSK-3β and anti-β-catenin antibodies were from Transduction Laboratories. Mouse monoclonal anti-β-tubulin, anti-γ-tubulin, and anti-FLAG antibodies and rabbit polyclonal anti-γ-tubulin antibody were from Sigma. Another mouse monoclonal anti-γ-tubulin (C-11) antibody was from SantaCruz. Other materials were from commercial sources.

Axin antibodies

Rabbit polyclonal anti-Axin antibody was purchased from Zymed (San Francisco, CA) and this antibody was used for immunocytochemical study and western blotting. In this study anti-Axin antibody from Zymed was mainly used. Mouse monoclonal anti-Axin antibody was from Chemicon (Billerica, MA) and this antibody was used for western blotting. Rabbit polyclonal anti-Axin body from Santacruz (Santa Cruz, CA) was tried for immunofluorescence analysis but it

was not suitable for immunocytochemical study. We generated two rabbit polyclonal antibodies by immunization with recombinant proteins of rAxin-(508-832) (Kishida *et al*, 2001) and rAxin-(1-229) (Yamamoto *et al*, 1999). The former was used for immunoprecipitation, and the latter was tried for immunofluorescence analysis but it was not suitable for immunocytochemical study.

Cell culture

MKN-1 cells were grown in RPMI1640 supplemented with 10% FBS (Yokozaki, 2000). HeLa S3, NIH3T3, and L cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS). U2OS and HEK293T cells were grown in DMEM/Ham's F12 supplemented with 10% FBS. Cells were transfected with plasmid DNA using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. The reason why MNK-1 cells were used in most of this study is that the centrosome is present between basal membranes (facing towards the dish) and the nucleus in the center of the cell, thereby the centrosome is observed clearly in contrast to the darkness of the nucleus.

siRNA sequence

The following siRNA duplexes were used: Axin, 5'-GGCAGCUACAGAUACUACUUCAAGA-3'; APC, 5'-GCGGCAGAAUGAAGGUCAATT-3' (Draviam *et al*, 2006); γ-tubulin, 5'-GGAGGACAUGUUCAAGGACTT-3' (Luders *et al*, 2006). The following double-strand RNA oligonucleotides were synthesized by using a CUGA *in vitro* small interference RNA synthesis kit (Nippon Gene) and the targeted sequences were as follows: β-catenin, 5'-GTCCTGTATGAGTGGGAAC-3' (Wong & Gumbiner, 2003).

Rescue constructs of Axin were generated by introducing silent substitutions in the target site of the siRNA. The primer used for this purpose was as follows: 5'-GGCTCTTATCGTTATTATTTCAAAA-3').

Immunoprecipitation

To show the complex formation of Axin with γ-tubulin at the endogenous level, HeLa S3 cells (in a 60-mm diameter dish) were lysed in 200 μl of Nonidet P-40 buffer (20 mM Tris/HCl, pH 7.5, 137 mM NaCl, 1% Nonidet P-40, 10% glycerol, 20 μg/ml leupeptin, 20 μg/ml aprotinin, and 5 mM phenylmethylsulfonyl fluoride). The lysates were immunoprecipitated with anti-γ-tubulin or anti-Axin antibody, and the immunoprecipitates were probed with anti-Axin and anti-γ-tubulin antibodies. To show the complex formation of Axin and ninein in centrosomes isolated by sucrose density gradient centrifugation, 200 μl of the centrosome fraction was suspended with 1 ml of Nonidet P-40 buffer and immunoprecipitated with anti-ninein antibody. The immunoprecipitates were probed with anti-ninein and anti-Axin antibodies.

To determine which region of Axin interacts with γ-tubulin, HEK293T cells (in a 60-mm diameter dish) were cotransfected with various deletion mutants of FLAG-tagged Axin and γ-tubulin-GFP and lysed in 200 μl of Nonidet P-40 buffer. The lysates were immunoprecipitated with anti-FLAG antibody, and the immunoprecipitates were probed with anti-FLAG and anti-GFP antibodies.

Quantitative RT-PCR

Total RNA was isolated from MKN-1 or HeLa S3 cells 72 hr after siRNA transfection. Each RNA sample (2 μg) was subjected to reverse transcription using murine leukemia virus reverse transcriptase (Applied Biosystems) in a total volume of 20 μl. Quantitative reverse transcription-PCR (RT-PCR) was performed by using a LightCycler instrument (Roche Molecular Biochemicals). Forward and reverse primers were as follows: Axin, 5'-AGCGAAGGCAGAGAGTT-3' and 5'- GTGCTGCTTACGGATCC-3'; Axin2, 5'-CTGGCTCCAGAAGATCACAAAG-3' and 5'-CATCCTCCCAGATCTCCTCAAA-3'; and glyceraldehyde-3-phosphate-dehydrogenase (GAPDH), 5'-CCTGTTCGACAGTCAGCCG-3' and 5'-CGACCAAATCCGTTGACTCC-3'.

Isolation of the centrosome fraction

The centrosome fraction was prepared from HeLa S3 cells basically according to the method described previously (Mitchison & Kirschner, 1986). Five confluent 100-mm dishes of HeLa S3 cells were treated with 10 μg/ml nocodazole (Sigma) and 5 μg/ml cytochalasin D (Sigma) for 90 min. After treatment, the cells were immediately washed with ice cold PBS, 0.1 x PBS containing 8% sucrose, 8% sucrose in distilled water, and LB (1 mM Tris/HCl (pH 7.5) and 0.1% β-mercaptoethanol). The cells were then lysed with 1 ml of LB containing 0.5% Nonidet P-40 per one 100-mm dish and agitated on ice for 10 min. The cells were collected and 1/50 volume of 50 x PE (500 mM PIPES/NaOH, pH 7.2, 50 mM EDTA, and 5% β-mercaptoethanol) was added. After centrifugation for 3 min at 1500 x *g* to remove debris, LB containing 0.5% Nonidet P-40 was added to the supernatants up to 8.4 ml. Then the sample was loaded onto a 12 ml discontinuous sucrose gradient consisting of 40% (1 ml), 50% (1 ml), and 70% (1.6 ml) (w/w) in gradient buffer (10 mM PIPES/NaOH at pH 7.2, 0.1% Triton X-100, and 0.1% β-mercaptoethanol). The gradient was then centrifuged at 112,000 x *g* for 2 h at 4°C in a CE60E ultracentrifuge (HITACHI, Tokyo, Japan) using a P40ST swinging rotor. The top supernatant was aspirated to nearly the 40% sucrose region and twenty fractions of 0.2 ml each were collected from the top of the gradient. The aliquots were subjected to SDS-PAGE.

Purification of γ-tubulin-FLAG /γ-TuRC

Lysates of U2OS cells stably expressing γ-tubulin-FLAG (three 10-cm diameter dishes) were prepared to purify γ-tubulin-FLAG/γTuRC as described previously (Izumi *et al*, 2008; Murphy *et al*, 2001). The lysates were immunoprecipitated with anti-FLAG antibody-conjugated Sepharose (300 μl of beads), and the beads were washed three times with HE (50 mM HEPES-NaOH, pH 7.4, 150 mM NaCl, 1 mM DTT, 1 mM EGTA, 1 mM $MgCl_2$, 0.25 mM GTP, and protease inhibitor mixture) containing 0.5% Triton X-100, once with HE containing 250 mM NaCl instead of 150 mM NaCl, and once with HE. The complex was eluted by incubation with 300 μl of 1 mg/ml FLAG peptide in HE for 3 h at 4°C. The presence of γ-tubulin-FLAG and GCP complex in the eluates was confirmed by immunoblotting with anti-γ-tubulin and anti-GCP2 antibodies.

Statistical analyses

The experiments were performed at least three times and the results were expressed as means ± S.E. Differences between the data were tested for statistical significance by *t*-test. *P* values less than 0.05 were considered statistically significant. Data of western blotting and cell staining were representative of at least three independent experiments. Relative proteins levels indicated in Figs. 2C, 3C, and 4F were means of three to five independent experiments.

Supplementary References

Draviam VM, Shapiro I, Aldridge B, Sorger PK (2006) Misorientation and reduced stretching of aligned sister kinetochores promote chromosome missegregation in EB1- or APC-depleted cells. *Embo J* **25:** 2814-2827

Fumoto K, Hoogenraad CC, Kikuchi A (2006) GSK-3β-regulated interaction of BICD with dynein is involved in microtubule anchorage at centrosome. *EMBO J* **25:** 5670-5682

Izumi N, Fumoto K, Izumi S, Kikuchi A (2008) GSK-3β regulates proper mitotic spindle formation in cooperation with a component of the gamma-tubulin ring complex, GCP5. *J Biol Chem* **283:** 12981-12991

Kishida M, Hino S-I, Michiue T, Yamamoto H, Kishida S, Fukui A, Asashima M, Kikuchi A (2001) Synergistic activation of the Wnt signaling pathway by Dvl and casein kinase Iε. *J Biol Chem* **276:** 33147-33155

Luders J, Patel UK, Stearns T (2006) GCP-WD is a gamma-tubulin targeting factor required for centrosomal and chromatin-mediated microtubule nucleation. *Nat Cell Biol* **8:** 137-147

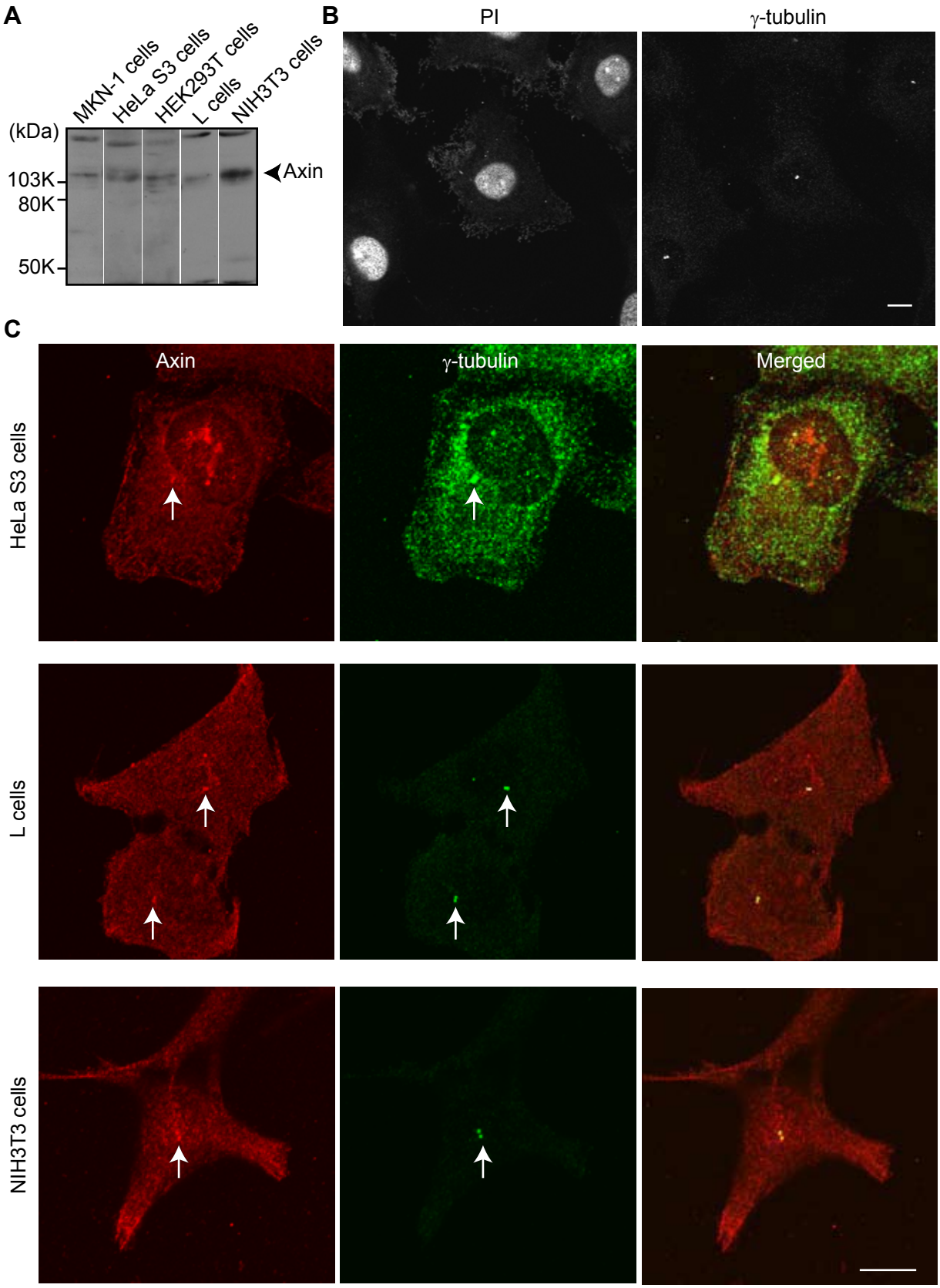
Mitchison TJ, Kirschner MW (1986) Isolation of mammalian centrosomes. *Methods Enzymol* **134:** 261-268

Murphy SM, Preble AM, Patel UK, O'Connell KL, Dias DP, Moritz M, Agard D, Stults JT, Stearns T (2001) GCP5 and GCP6: two new members of the human γ-tubulin complex. *Mol Biol Cell* **12:** 3340-3352

Wong AS, Gumbiner BM (2003) Adhesion-independent mechanism for suppression of tumor cell invasion by E-cadherin. *J Cell Biol* **161:** 1191-1203

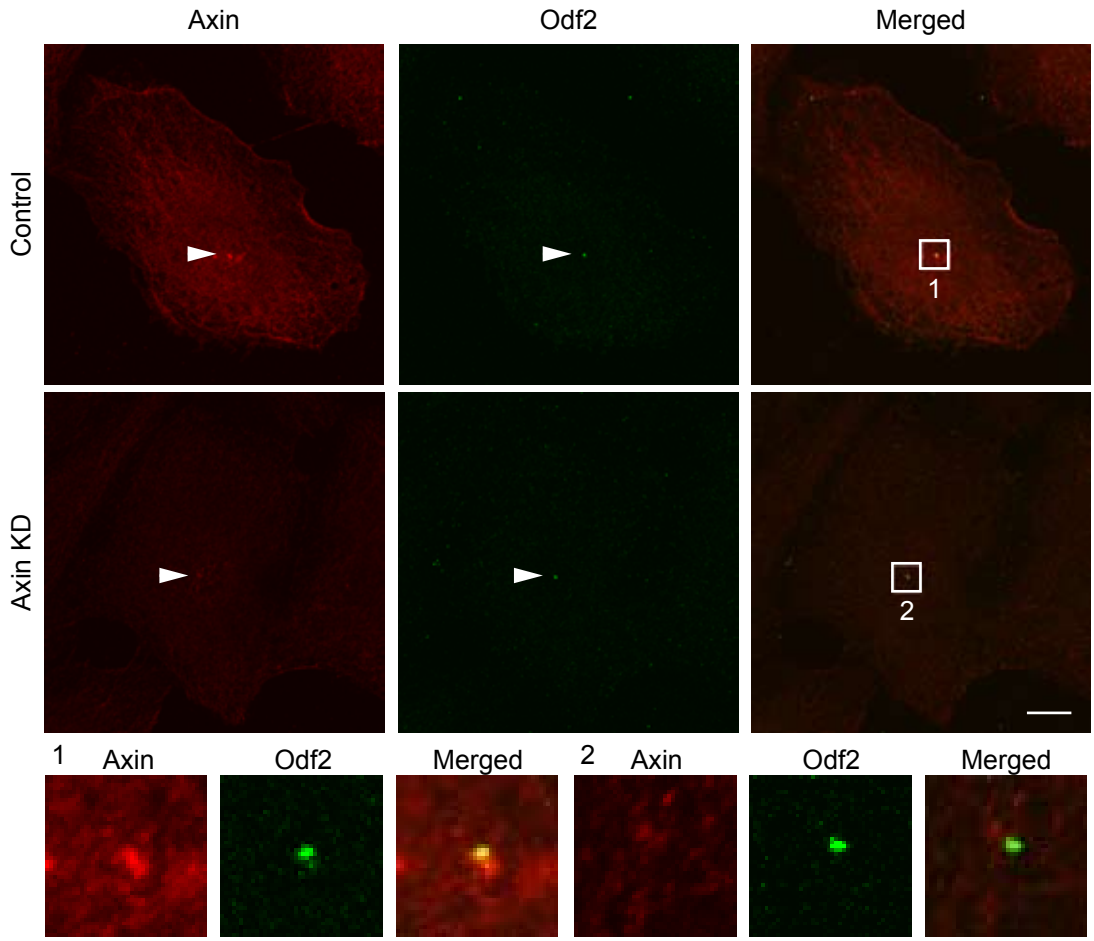
Yamamoto H, Kishida S, Kishida M, Ikeda S, Takada S, Kikuchi A (1999) Phosphorylation of Axin, a Wnt signal negative regulator, by glycogen synthase kinase-3β regulates its stability. *J Biol Chem* **274:** 10681-10684

Yokozaki H (2000) Molecular characteristics of eight gastric cancer cell lines established in Japan. *Pathol Int* **50:** 767-777



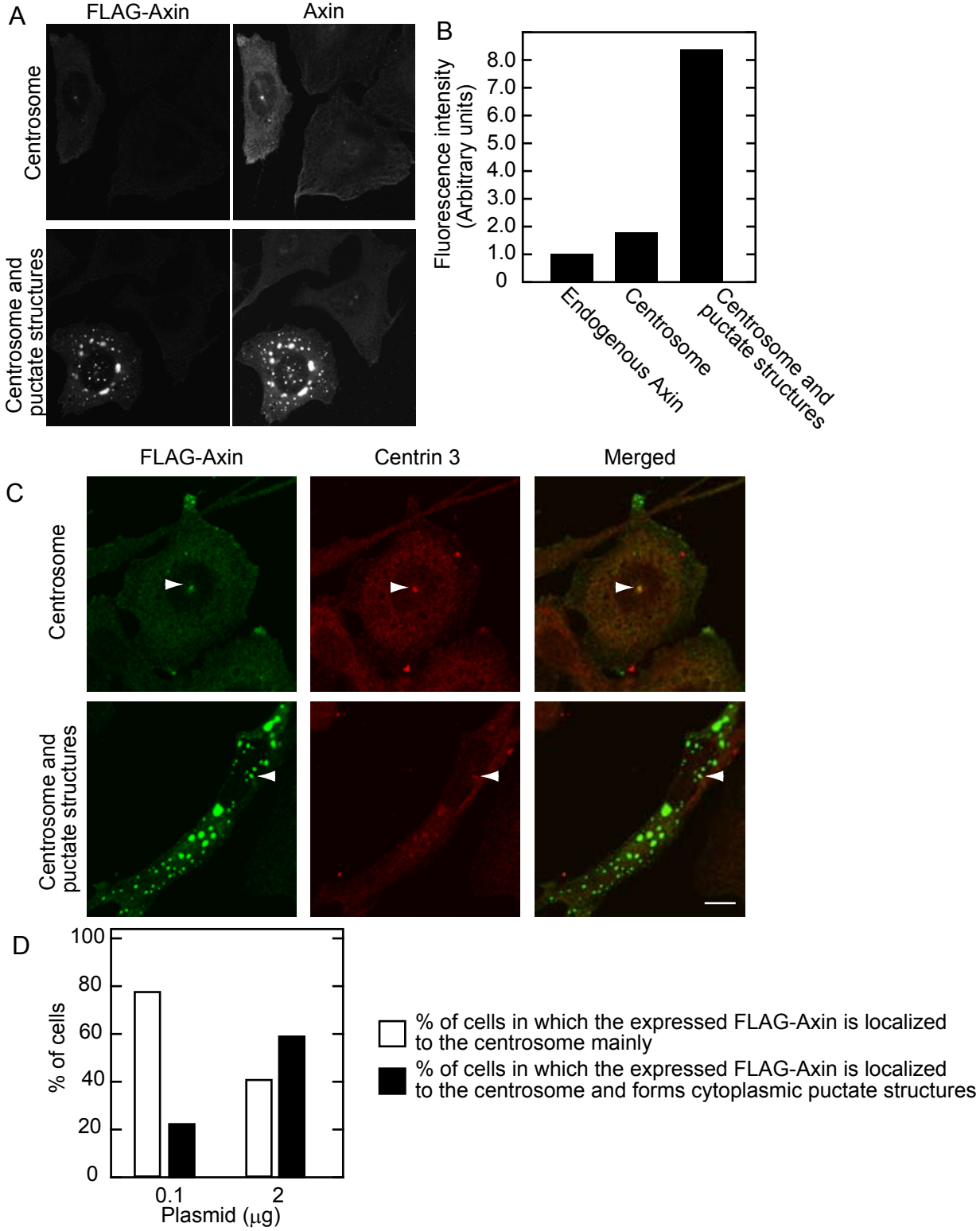
Supplementary Figure 1

(A) The lysates of MKN-1, HeLa S3, HEK293T, L, or NIH3T3 cells were probed with anti-Axin antibody. (B) MKN-1 were stained with γ-tubulin antibody and propidium iodine (PI). This indicates that cells in interphase were used in this study. Scale bar, 10 μm. (C) HeLa S3, L, or NIH3T3 cells were stained with anti-Axin (red) and anti-γ-tubulin (green) antibodies. Arrowheads indicate the centrosome. Scale bar, 10 μm.



Supplementary Figure 2

Control or Axin knockdown MKN-1 cells were stained with anti-Axin (red) and anti-Odf2 (green) antibodies. Centrosomal regions (1 and 2) were enlarged. Arrowheads indicate the centrosome. Scale bar, 10 μm.

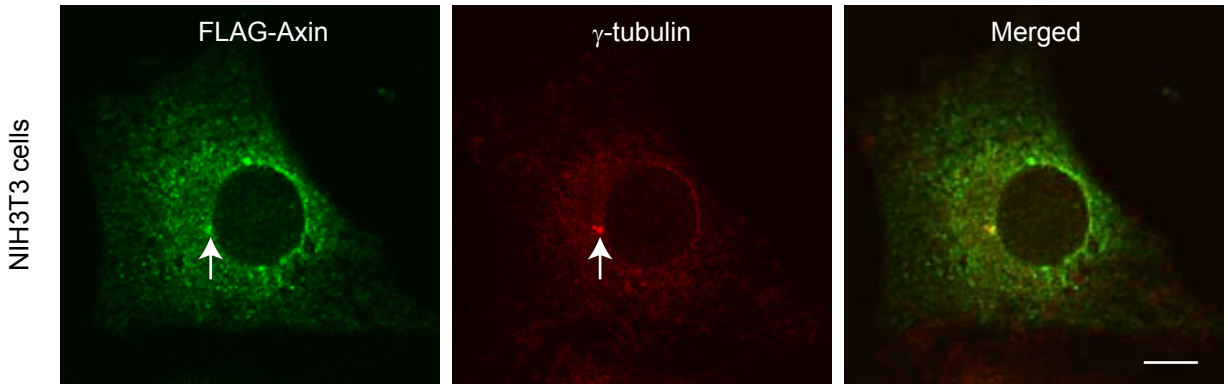


Supplementary Figure 3

(A) MKN-1 cells were transfected with FLAG-Axin cDNA and then the cells were stained with anti-FLAG and anti-Axin antibodies. “Centrosome” type indicates that the expressed FLAG-Axin is localized at centrosome maily and “Centrosome and punctate structures” type indicates that it is not only located at centrosome but also distributed as punctate structures in the cytoplasm. Scale bar, 10 μm. (B) FLAG-Axin expressing MKN-1 cells were stained with anti-FLAG and anti-Axin antibodies and classified as shown in (A). Fluorescence intensities of Axin from FLAG-Axin expressing cells were quantified and compared with that of endogenous Axin from the cells without Axin expression. (C) MKN-1 cells were transfected with different amounts (0.1 or 2 μg) of FLAG-Axin cDNA and then the cells were stained with anti-FLAG and anti-centrin 3 antibodies and classified as shown in (A). Scale bar, 10 μm.

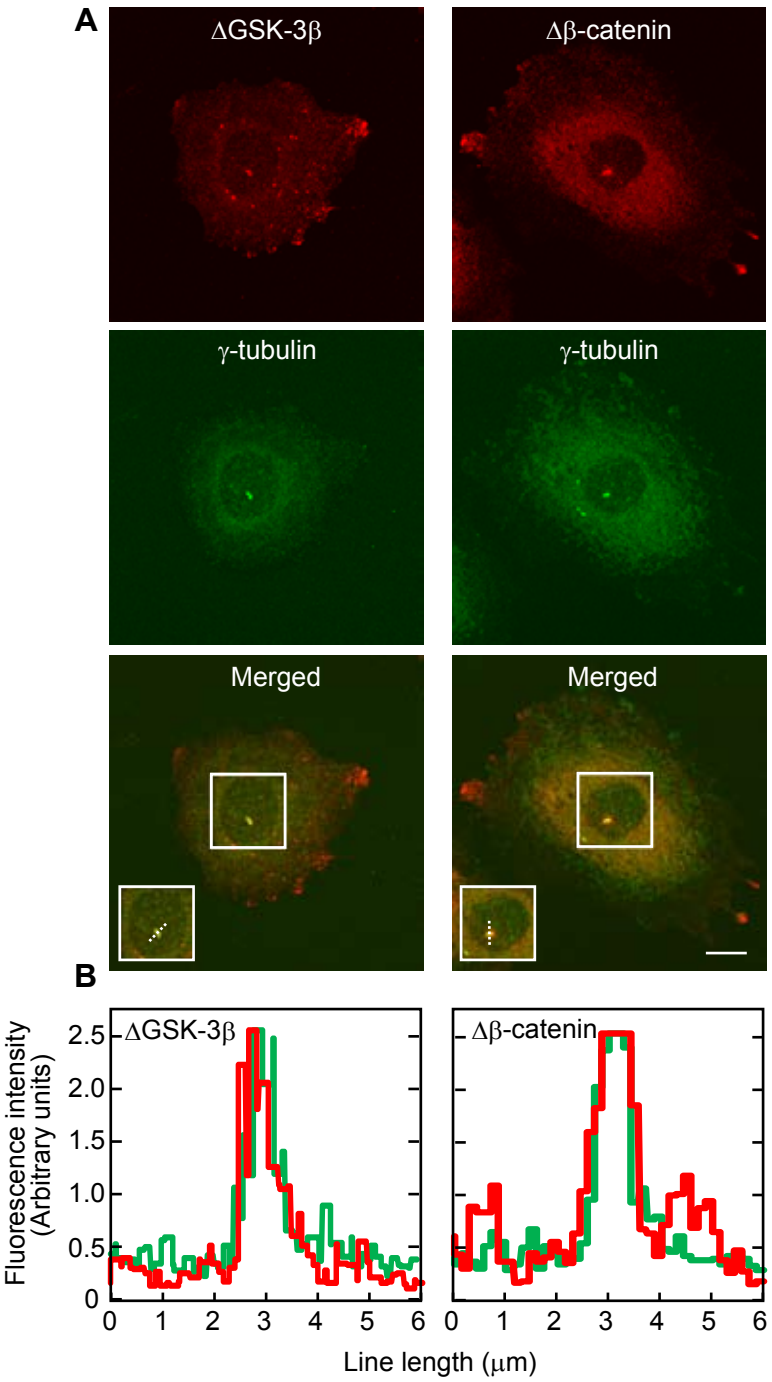
(D) MKN-1 cells were transfected with different amounts (0.1 or 2 μg) of FLAG-Axin cDNA and then the cells were stained and counted. White and black bars indicate the ratios (percentages) of the cells that shows the “Centrosome” or “Centrosome and punctate structures” types.

Fumoto et al. Supplementary Figure 4



Supplementary Figure 4

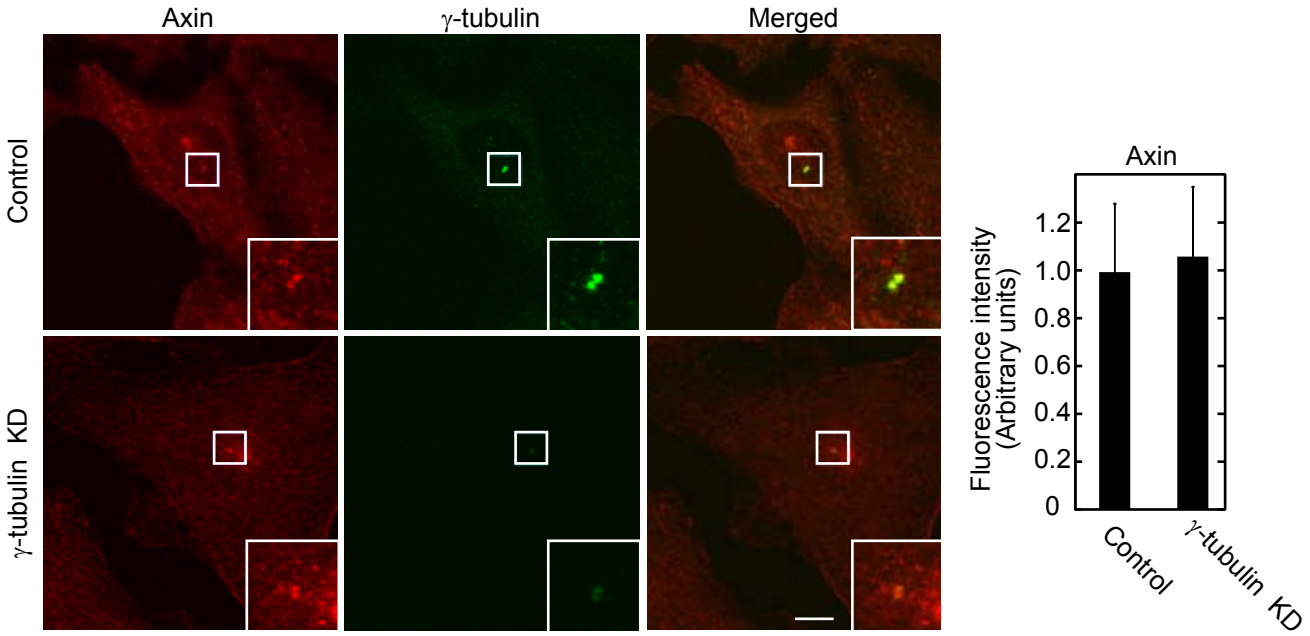
NIH3T3 cells expressing FLAG-Axin were stained with anti-FLAG (green) and anti-γ-tubulin (red) antibodies. Arrowheads indicate the centrosomes. Scale bar, 10 μm.



Supplementary Figure 5

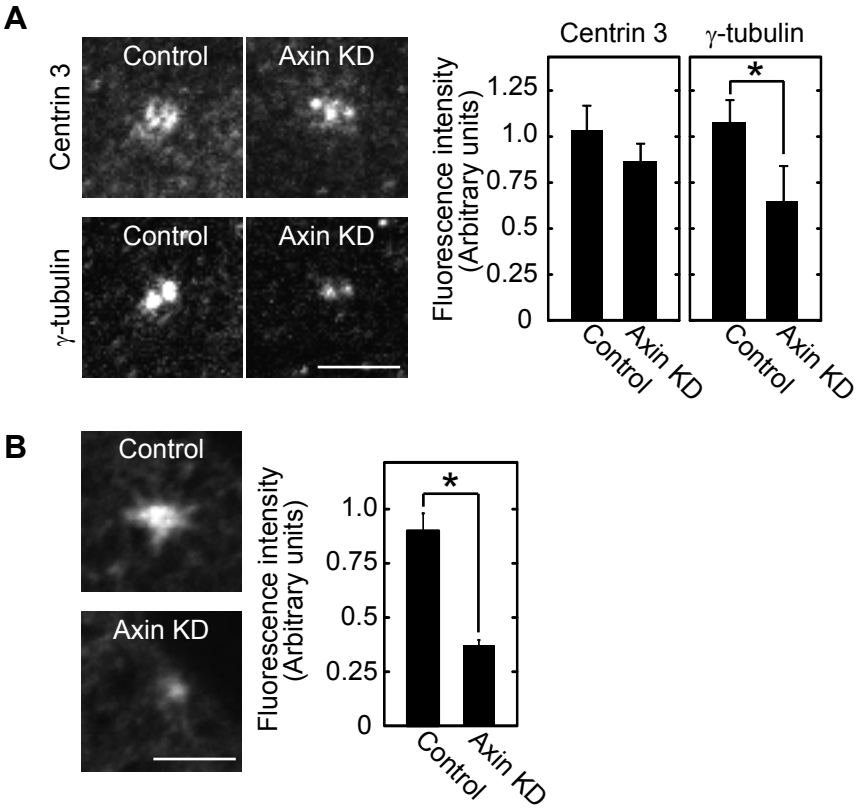
(A) MKN-1 cells expressing FLAG-Axin deletion mutants were stained with anti-FLAG (red) and anti-γ-tubulin (green) antibodies. Scale bar, 10 μm. (B) The distribution of fluorescence intensity was measured along the dashed line across the centrosome shown in the boxed area of (A). The fluorescence intensity was plotted as a function of the line length (μm).

Fumoto et al. Supplementary Figure 6



Supplementary Figure 6

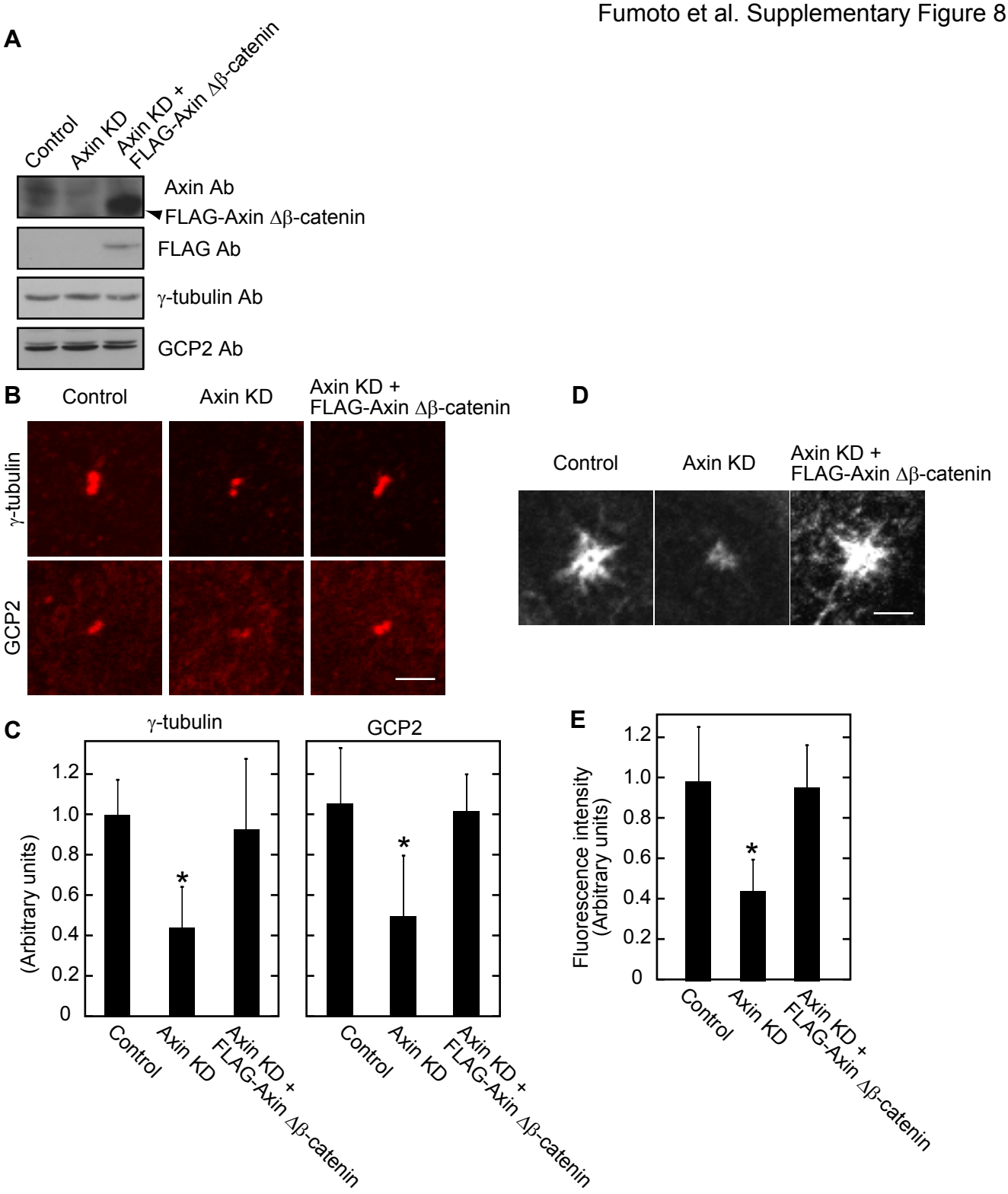
(A) MKN-1 cells were transfected with control or γ-tubulin siRNA and the cells were stained with anti-Axin and anti-γ-tubulin antibodies. The positions of γ-tubulin were boxed. Scale bar, 10 μm. (B) Fluorescence intensities from centrosomal Axin in control or γ-tubulin KD cells were quantified.



Supplementary Figure 7

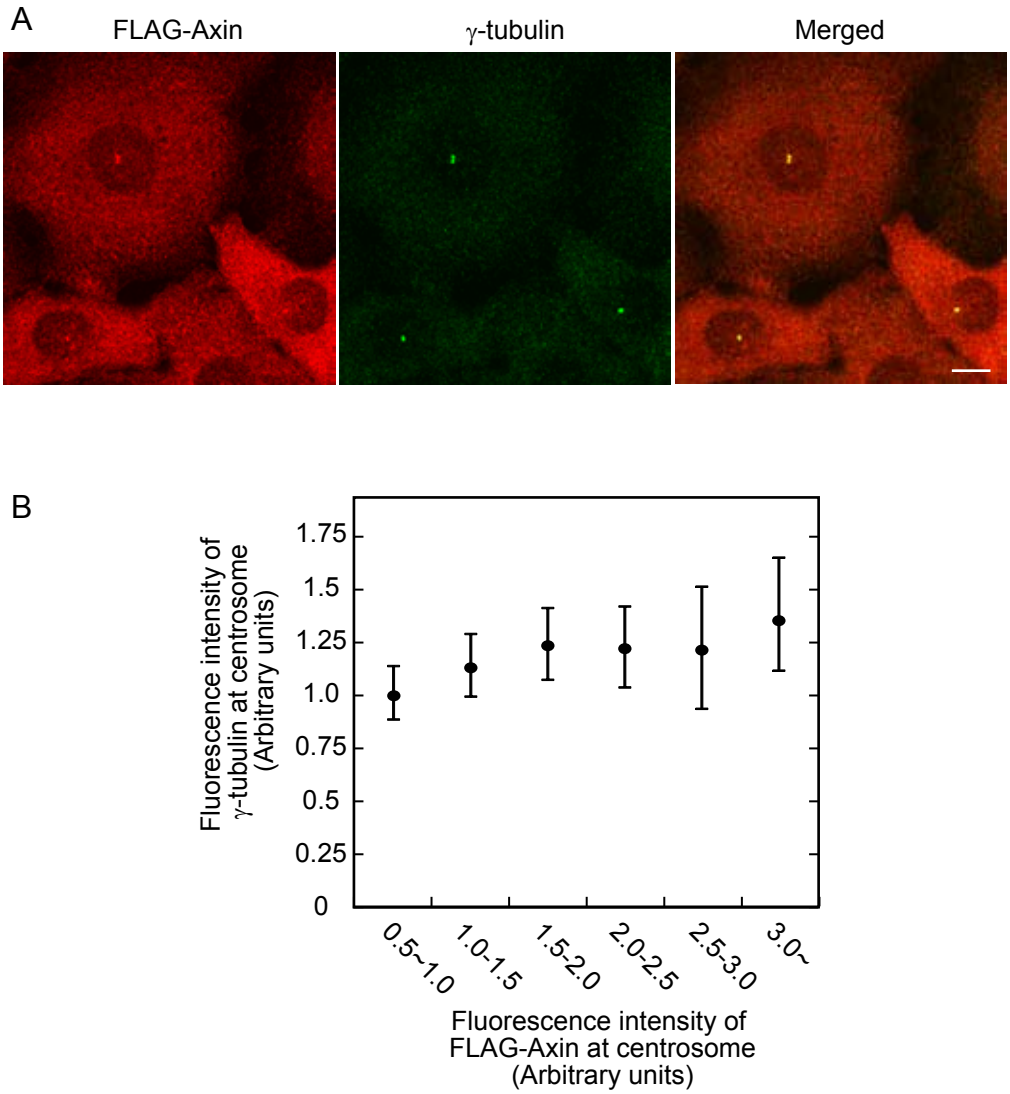
(A) Left panels, HeLa S3 cells were transfected with Axin siRNA and the cells were stained with the indicated antibodies. Right panels, fluorescence intensities from centrin 3 and γ-tubulin were quantified and the relative intensity in control and Axin KD cells was expressed as arbitrary units. KD, knockdown; *, P< 0.005. Scale bar, 5 μm.

(B) Left panels, HeLa S3 cells transfected with control or Axin siRNA were subjected to the microtubule regrowth assay and stained with anti-β-tubulin antibody at 5 min after regrowth. Right panel, fluorescence intensity of microtubule asters was quantified. KD, knockdown; *, P< 0.005. Scale bar, 5 μm.



Supplementary Figure 8

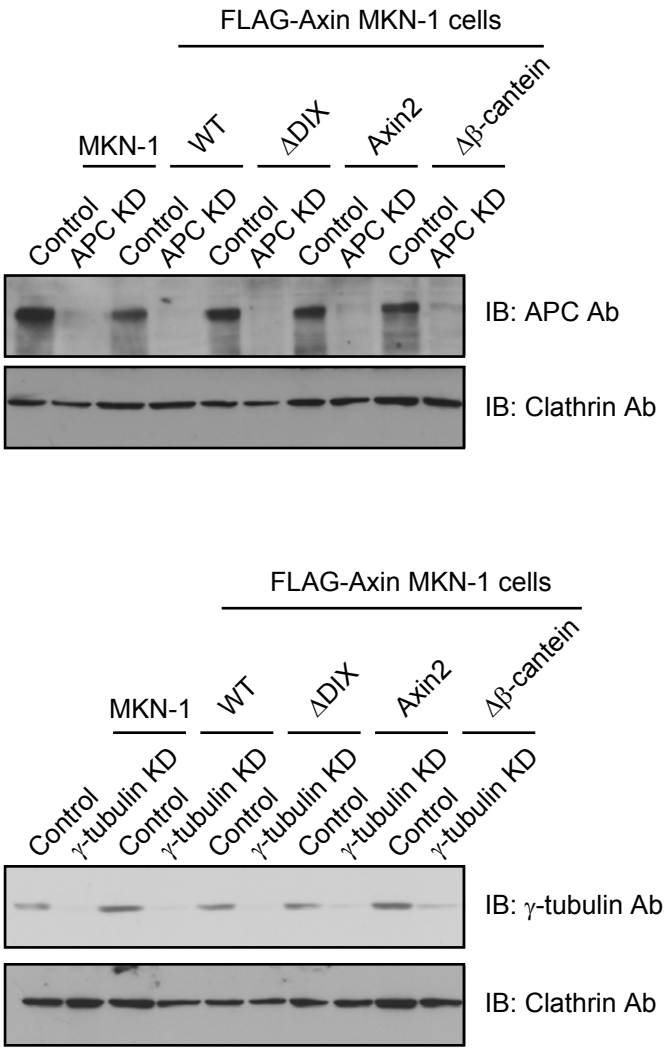
(A) Control MKN-1 or MKN-1 cells stably expressing FLAG-Axin-(Δβ-catenin) were transfected with control or Axin siRNA and the lysates were probed with the indicated antibodies. (B) The same cells prepared in (A) were stained with the indicated antibodies. Scale bar, 5 μm. (C) Fluorescence intensity from γ-tubulin and GCP-2 was quantified, and the relative intensity was expressed as arbitrary units. *, P< 0.01. (D) The same cells prepared in (A) were subjected to the microtubule regrowth assay and stained with anti-β-tubulin antibody at 5 min after regrowth. (E) Fluorescence intensity of microtubule asters in MKN-1 cells used in (D) was quantified. *, P< 0.01



Supplementary Figure 9

(A) MKN-1 cells stably expressing FLAG-Axin were transfected with Axin siRNA and stained with anti-FLAG (red) and γ-tubulin (green) antibodies. Scale bar, 10 μm. (B) To detemine the correlation between the expression level of FLAG-Axin and the degree of the γ-tubulin localization to the centrosome, fluorescence intensity from centrosomal γ-tubulin (y-axis) in cells showing different expression levels of centrosomal FLAG-Axin (x-axis) was measured, and the relative intensity was expressed as arbitrary units.

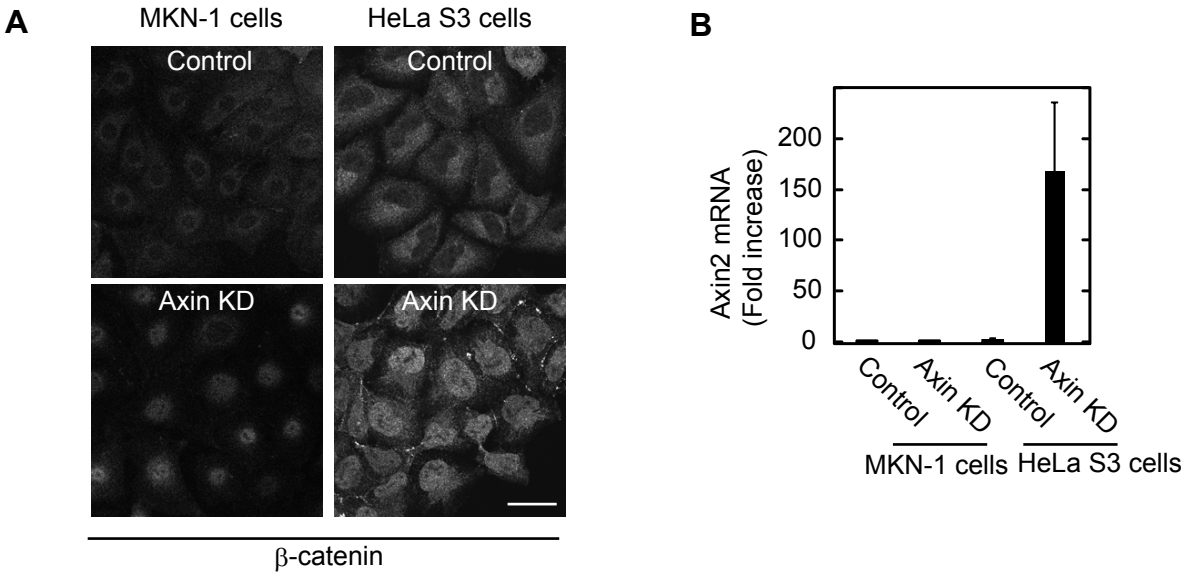
Fumoto et al. Supplementary Figure 10



Supplementary Figure 10

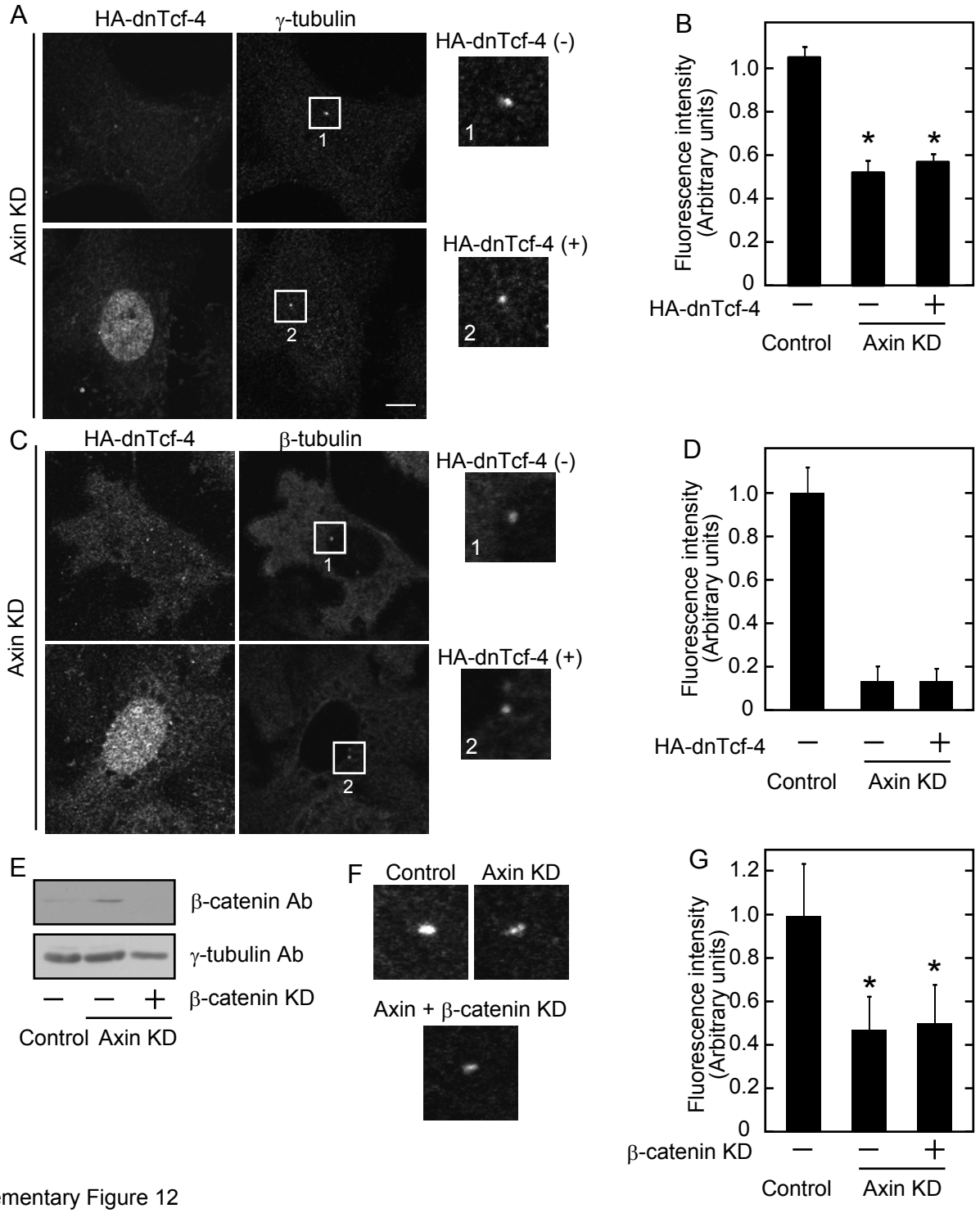
Wild type MKN-1 or MKN-1 cells stably expressing FLAG-Axin, FLAG-Axin-(ΔDIX), FLAG-Axin2, or FLAG-Axin-(Δβ-catenin) were transfected with APC (upper panels) or γ-tubulin (lower panels) siRNA and the lysates were probed with the indicated antibodies. Clathrin was used as a loading control.

Fumoto et al. Supplementary Figure 11



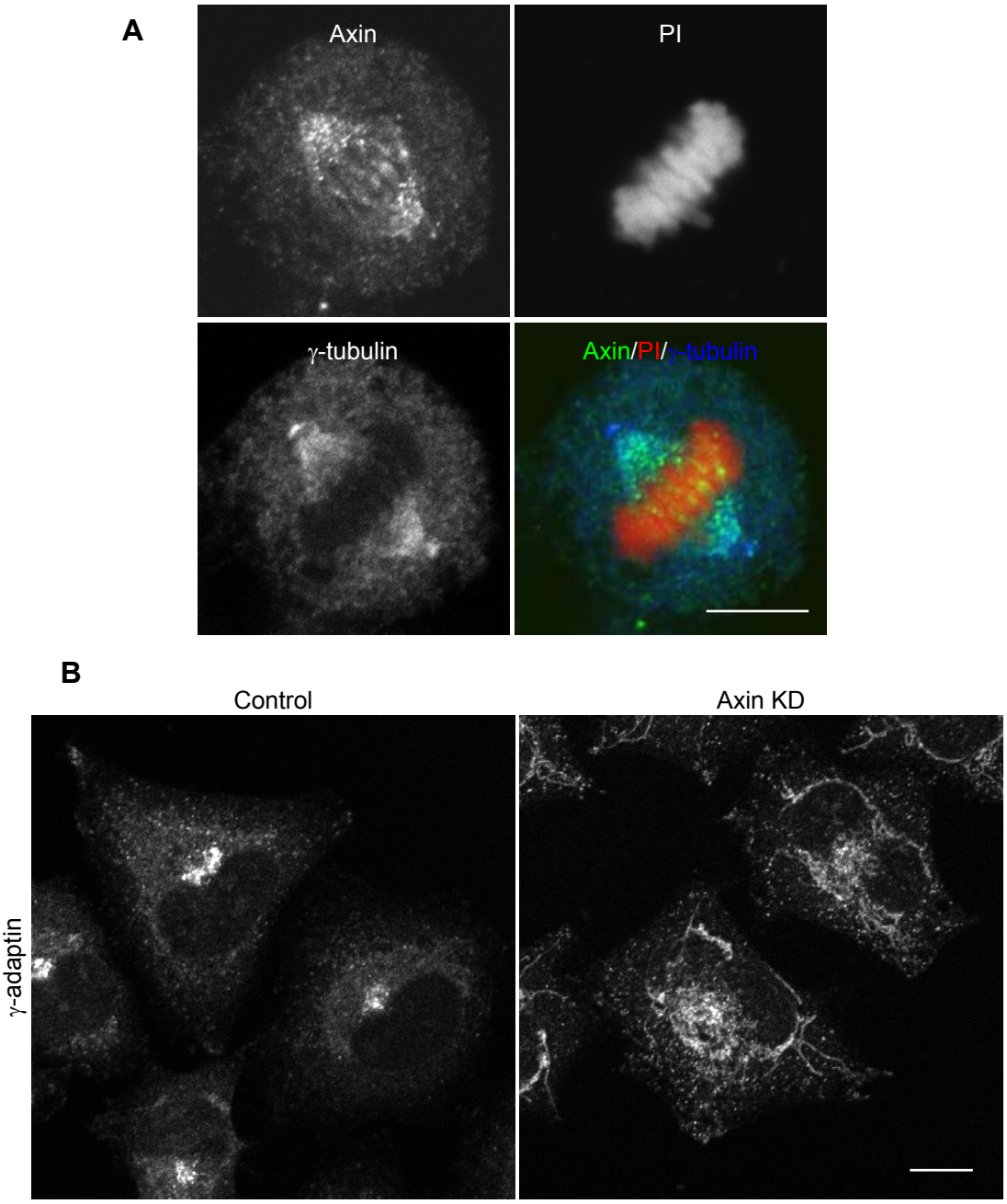
Supplementary Figure 11

(A) MKN-1 or HeLa S3 cells transfected with control or Axin siRNA were stained with anti-β-catenin antibody. KD, knockdown. Scale bar, 50 μm. (B) Total RNA from MKN-1 or HeLa S3 cells transfected with control or Axin siRNA was subjected to quantitative RT-PCR. The results shown were normalized by GAPDH mRNA levels, and the corresponding quantification results for the Axin2 mRNA levels were expressed as the fold increase compared with the control.



Supplementary Figure 12

(A) Axin knockdown (KD) MKN-1 expressing HA-tagged dominant negative Tcf-4 (HA-dnTcf-4) transiently were stained with anti-HA and anti-γ-tubulin antibodies. The γ-tubulin staining at the centrosomal regions was enlarged. 1, HA-dnTcf-4 unexpressing cells; 2, HA-dnTcf-4 expressing cells. Scale bar, 5 μM. (B) Fluorescence intensity from centrosomal γ-tubulin in MKN-1 cells used in (A) and control cells (data not shown) were quantified and the relative intensity was expressed as arbitrary units. *, P< 0.01. (C) Axin KD MKN-1 cells expressing HA-dnTcf-4 transiently were subjected to microtubule regrowth assay and stained with anti-HA and anti-β-tubulin antibodies at 5 min after regrowth. (D) Fluorescence intensity of microtubule asters in MKN-1 cells used in (C) and control cells (data not shown) was quantified and the relative intensity was expressed as arbitrary units. *, P< 0.01. (E) HeLa S3 cells were transfected with siRNA for Axin or Axin and β-catenin and the lysates were probed with the indicated antibodies. (F) The same cells prepared in (E) were stained with anti-γ-tubulin antibody. (G) Fluorescence intensity from γ-tubulin used in (F) was quantified. *, P< 0.01.



Supplementary Figure 13

(A) Mitotic HeLa S3 cells were stained with anti-Axin (green) and anti-γ-tubulin (blue) antibodies and propidium iodine (PI) (red). Scale bar, 10 μm. (B) Control or Axin KD MKN-1 cells were stained with anti-γ-adaptin antibody. γ-Adaptin is a marker for the Golgi apparatus. KD, knockdown. Scale bar, 10 μm.