Supplementary Information

Dishevelled, a Wnt signaling component, is involved in mitotic progression in cooperation with Plk1

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Supplementary Methods

cDNA constructs and cloning procedures

Deletion mutants or point mutants of Dvl2 were generated using PCR amplification of fragments of interest and subcloning into pEGFP-C1, pEGFP-N3, or pMALC2. All PCR products were confirmed by sequencing.

In vitro kinase assay

All *in vitro* kinase assays were performed with recombinant proteins purified from bacteria. Bacteria harboring each MBP-Dvl2 construct were collected by centrifugation and resuspended in lysis buffer (50 mM Tris–HCl at pH 8.0, 150 mM NaCl, 1 mM DTT, 200 μg/ml lysozyme, and 1 mM phenylmethylsulfonylfluoride, 1 μg/ml leupeptin, and 1 μg/ml aprotinin). After incubation for 30 min on ice, TritonX-100 was added up to 1%, and lysates were placed on a rotary mixcer at 4°C for 20 min. After centrifugation, the supernatant was collected, and a 50% slurry of amylose resin (New England Biolabs, USA) was added. After 1 h mixing at 4°C, the beads were washed extensively with wash buffer (20 mM Tris-HCl, pH 7.5 and 1 mM DTT). Wild-type or mutant forms of MBP-Dvl2 were incubated with GST–Plk1 in kinase reaction buffer (10 mM Tris-HCl at pH 7.5, 10 mM $MgCl_2$, 1 mM DTT, 10 μM [γ-^{32}P]ATP (specific activity, 1000-1500 cpm/pmole) for 30 min at 30°C, followed by SDS–PAGE and autoradiography.

Immunoblotting and immunoprecipitation

Methods for immunoblotting and immunoprecipitation were described previously (Elowe *et al*, 2007; Hino *et al*, 2005). To immunoprecipitate endogenous proteins, cells were washed once with PBS and lyzed in lysis buffer (20 mM Tris-HCl at pH 7.5, 150 mM NaCl, 2 mM EGTA, 0.5% NP40, protease inhibitors (1 mM phenylmethylsulfonylfluoride, 1 μg/ml leupeptin, and 1 μg/ml aprotinin), and phosphatase inhibitors (5 mM sodium fluoride, 1 mM sodium orthovanadate, and 10 mM β-glycerophosphate) for 30 min on ice. After

centrifugation, the supernatant was collected, and incubated with appropriate antibodies. After incubation, 30 μl of protein A or G Sepharose (50% slurry) was added, and the mixtures were placed on a rotary mixer for 1 h at 4°C. The beads were then washed five times with lysis buffer and finally resuspended in Laemmli's sample buffer.

Supplementary Figure Legends

Supplementary Figure S1 Subcellular localization of Dvl2 in mitosis. (A) Lysates from HeLaS3 expressing GFP-Dvl2 and U2OS cells expressing GFP-Dvl2 were probed with an anti-Dvl2 antibody. (B) A single still image of GFP-Dvl2 in living mitotic U2OS cells. Scale bar, 10 μm.

Supplementary Figure S2 Mitotic phosphorylation of Dvl2. (A) HeLaS3 cells were arrested at prometaphase by thymidine-nocodazole block and then released into normal medium. Lysates were probed with anti-Dvl2, anti-Cyclin B1, and anti-clathrin heavy chain (CHC) antibodies. CHC was used as a loading control. (B) Procedure for synchronization by the double thymidine-MG132 block. Cells were synchronized by double thymidine block and released from G_1/S block (G1/S). At 7.5 h after release, cells were arrested at metaphase (M) by treatment with 10 μM MG132 for 2 h. CHC was used as a loading control. (C) Cells were synchronized by double thymidine block and released from G_1/S block (G1/S). At 7.5 h after release, cells were treated with buffer or recombinant hDkk1 (500 ng/ml) in the presence of 10 μM MG132 for 2 h. (C) Lysates from HEK293 cells co-expressing HA-Dvl2 with Myc-Aurora A, GFP-Cdk1, or GFP-Cdk1 constitutive active form (CA) were immunoprecipitated with anti-HA, anti-Myc, or anti-GFP antibody, and the immunoprecipitates were probed with anti-HA, anti-Myc, and anti-GFP antibodies.

Supplementary Figure S3 Complex formation of Dvl2 and Plk1. (A) Lysates from HEK293 cells co-expressing HA-Dvl2 with GFP-Plk1 full length (Full), GFP-Plk1 N-terminal region (N-ter), or GFP-Plk1 C-terminal region (C-ter) were immunoprecipitated with anti-GFP antibody, and the immunoprecipitates were probed with anti-HA and anti-GFP antibodies. (B) Lysates from HEK293 cells co-expressing various deletion mutants of GFP-Dvl2 with HA-Plk1 were immunoprecipitated with an anti-HA antibody, and the immunoprecipitates were probed with anti-GFP and anti-HA antibodies.

Supplementary Figure S4 Phosphorylation of Dvl2 by Plk1. (A) Possible phosphorylation sites of Dvl2 by Plk1 are shown. Sequences around T206 (bold) were highly conserved from Drosophila to human. Hs; human, Pt; chimpanzee, Cf; dog, Mm; mouse, Gd; chicken, Dr; zebrafish, X; Xenopus, Dro; Drosophila. (B) MBP-Dvl2(1-433) (1.5 μg of protein) was incubated with the indicated concentrations of GST–Plk1^{WT} or GST-Plk1^{K82R} in kinase reaction buffer. (C) MBP-Dvl(140-358), MBP-Dvl2(140-358)^{T216A}, MBP-Dvl2(140-358)^{T260A}, or MBP-Dvl2(140-358)$^{S227/228A}$ (1.5 μg of each protein) was incubated with GST–Plk1^{WT} in kinase reaction buffer. [^{32}P], Autoradiography. CBB, Coomassie brilliant blue staining.

Supplementary Figure S5 Involvement of Dvl2 in spindle orientation. (A) Plk1 was stained together with ACA and PI in control or Dvl2-depleted HeLaS3 cells. (B) When no other cell was adjacent to the dividing cell, images of the mitotic Dvl2-depleted HeLaS3 cells were acquired every 3 min. (C) Control or Dvl2-depleted HeLaS3 cells at metaphase were classified in terms of the spindle angle, and the percentages in each category are shown in a histogram (n=50).

Supplementary Figure S6 Localization of DVl2^{WT} and Dvl2^{T206A} in Dvl2-depleted cell. (A) Mouse Dvl2 (mDvl2) cDNA or mDvl2^{T206A}, which is resistant to siRNA against human Dvl2, was expressed in Dvl2-depleted HeLaS3 cells, and lysates were probed with the indicated antibodies. (B) GFP-mDvl2^{WT} was expressed in Dvl2-depleted mitotic HeLaS3 cells, and cells were stained for GFP-mDvl2 (red), β-tubulin (blue), or ACA (a KT maker, green). (C) GFP-mDvl2^{T206A} was expressed in Dvl2-depleted mitotic HeLaS3 cells, and cells were stained for GFP-mDvl2 (red), β-tubulin (blue), or ACA (green). Top and middle panels, pictures of sections with each spindle pole. Bottom panels, pictures from the middle section of the cell.

Supplementary Figure S7 Dvl2 was not involved in the amount of spindles, the spindle pole distances, or MT nucleation. (A) Sum of spindle intensity was calculated by measuring the intensity of the spindle MT area. (B) Spindle pole distance between the two poles was calculated by measuring the linear distance (X μm) and the vertical distance (Z μm) in control or Dvl2-depleted HeLaS3 cells at metaphase (n=50). (C) EB1 intensity at centrosomes was quantified based on Centrin3 staining in control or Dvl2-depleted HeLaS3 cells at metaphase (n=50).

Supplementary Figure S8 Depletion of APC in HeLaS3 cells. HeLaS3 cells were transfected with siRNAs for Dvl2 and APC, and then the lysates were probed with the indicated antibodies. CHC was used as a loading control.

Supplementary Figure S9 Increase in the prometaphase cell populations in Dvl2-depleted cells. More than 1,000 cells in asynchronous culture for mitotic control or Dvl2-depleted HeLaS3 cells were counted.

Supplementary Figure S10 Involvement of Dvl2 in SAC activation. (A) After GFP-mDvl2^{WT} or GFP-mDvl2^{T206A} was expressed in Dvl2-depleted HeLaS3 cells, the cells were treated with 200 ng/ml nocodazole for 1 h. Then cells were stained for GFP and centromere (ACA). (B) GFP-mDvl2^{WT} or GFP-mDvl2^{T206A} was expressed in Dvl2-depeleted U2OS cells treated with 500 ng/ml nocodazole for 24 h, and mitotic index was measured.

Supplementary Figure S11 Dvl2 did not form a complex with BubR1 or APC. (A) Control or BubR1-depleted HeLaS3 cells were synchronized as in Supplementary Figure S2B. Lysates were probed with the indicated antibodies. β-Actin was used as a loading control. (B) Control or Dvl2-depleted HeLaS3 cells were synchronized as in Supplementary Figure S2B. Lysates were probed with the indicated antibodies. β-Actin was used as a loading control. (C) Dvl2-depleted HeLaS3 cells expressing GFP-mDvl2 were treated with 200

ng/ml nocodazole for 1 h to activate SAC. For Bub1 and BubR1 staining, the cells were fixed with 2% paraformaldehyde for 10 min, permeabilized with 0.2 % Triton X-100 for 15 min, blocked with 1% BSA in PBST for 20 min, and then stained with indicated antibodies. (D) The outer KT structure was stained with anti-Hec1 antibody together with ACA in control or Dvl2-depleted HeLaS3 cells. (E) Lysates from asynchronous (A) and thymidine-nocodazole blocked (M) HeLaS3 cells were immunoprecipitated with the indicated antibodies, and the immunoprecipitates were probed with anti-Dvl2 and anti-BubR1 antibodies. (F) Lysates from asynchronous (A) and thymidine-nocodazole blocked (M) HeLaS3 cells were immunoprecipitated with control IgG or anti-APC antibody, and the immunoprecipitates were probed with anti-APC and anti-Mps1 antibodies.

Supplementary Figure S12 Phosphorylation of LRP6 in mitosis. (A) Interphase or mitotic HeLaS3 cells transiently expressing GFP-LRP6 were stained with anti-GFP (green) and anti-pS1490 (red) antibodies. Chromosomes were stained with PI (blue). (B) Cells were synchronized by double thymidine block and released from G_1/S block (G1/S). At 7.5 h after release, cells were treated with buffer or recombinant hDkk1 (500 ng/ml) in the presence of 10 μM MG132 for 2 h**.** The lysates were probed with the indicated antibodies. (C) LRP6 or Fz2 was depleted in HeLaS3 cells. After the cells were arrested at mitosis by thymidine-nocodazole block, the lysates were probed with an anti-Dvl2 antibody. CHC was used as a loading control.

Supplementary Figure S13 Fz2 or Lrp6 was not required for MT-KT attachment. (A) LRP6 or Fz2 was depleted in HeLaS3 cells, and the cells were treated with 12 ng/ml nocodazole for 2 h in the presence of 10 μM MG132. The numbers of cells in which chromosomes were aligned or misaligned on the metaphase plate were counted. The results were expressed as a percentage of chromosome aligned cells in metaphase cells. (B) LRP6 or Fz2 was depleted in HeLaS3 cells. After the cells were treated with 12 ng/ml nocodazole

for 2 h in the presence of 10 μM MG132, they were stained for β-tubulin (green) and ACA (red).

Supplementary Figure S14 Effect of depletion of Dvl1 or Dvl3 on spindle axis and mitotic index. (A) Dvl1, Dvl2, or Dvl3 was depleted in HeLaS3c ells, and the lysates were probed with anti- Dvl1, anti-Dvl2, anti-Dvl3, anti-Mps1, anti-Bub1, anti-BubR1, and anti-β-actin antibodies. (B) HeLaS3 cells were treated with siRNA for Dvls as indicated, and the spindle angle was measured. (C) Control or Dvl-depleted HeLaS3 cells were treated with 100 ng/ml nocodazole or 10 nM taxol for 24 h and then stained with an anti-Mpm2 antibody and PI. The ratios of mitotic cells (mitotic index) among more than 100 cells were calculated.

Supplementary Movies

Supplementary movie 1 Time-lapse imaging in control HeLaS3 cells.

Supplementary movie 2 Time-lapse imaging in Dvl2-depleted HeLaS3 cells.

Supplementary movie 3 Time-lapse imaging of GFP-EB3 in mitotic control HeLa cells.

Supplementary movie 4 Time-lapse imaging of GFP-EB3 in mitotic Dvl2-depleted HeLa cells.

Supplementary movie 5 Time-lapse imaging of GFP-Cenp-A in mitotic control HeLa cells.

Supplementary movie 6 Time-lapse imaging of GFP-Cenp-A in mitotic Dvl2-depleted HeLa cells.

Supplementary References

Elowe S, Hummer S, Uldschmid A, Li X, Nigg EA (2007) Tension-sensitive Plk1 phosphorylation on BubR1 regulates the stability of kinetochore microtubule interactions. *Genes Dev* **21:** 2205-2219

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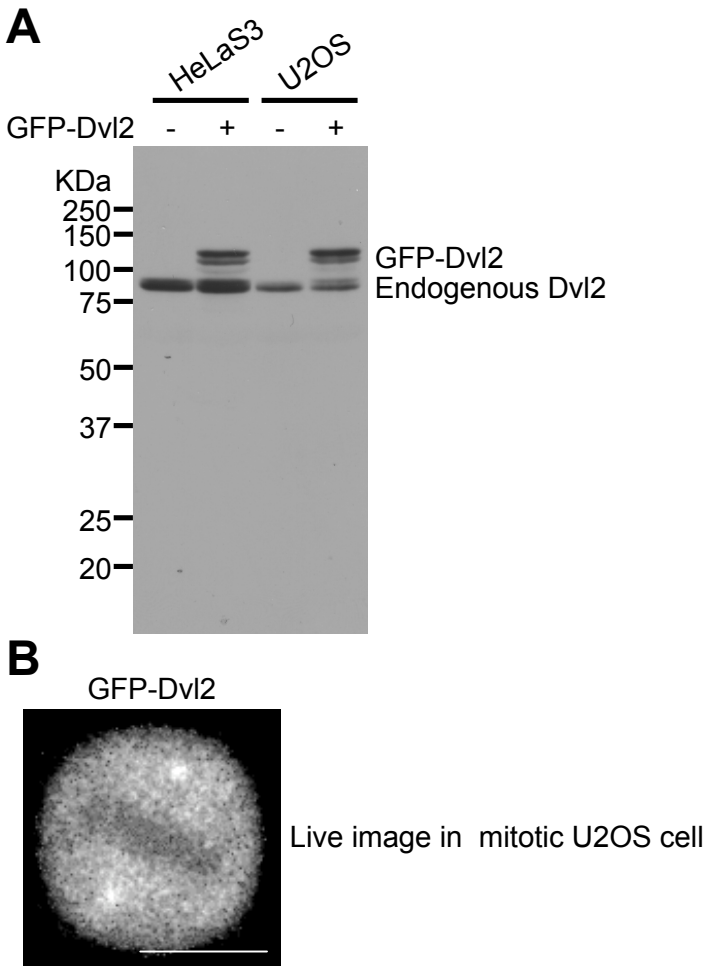
Kikuchi_Fig. S1



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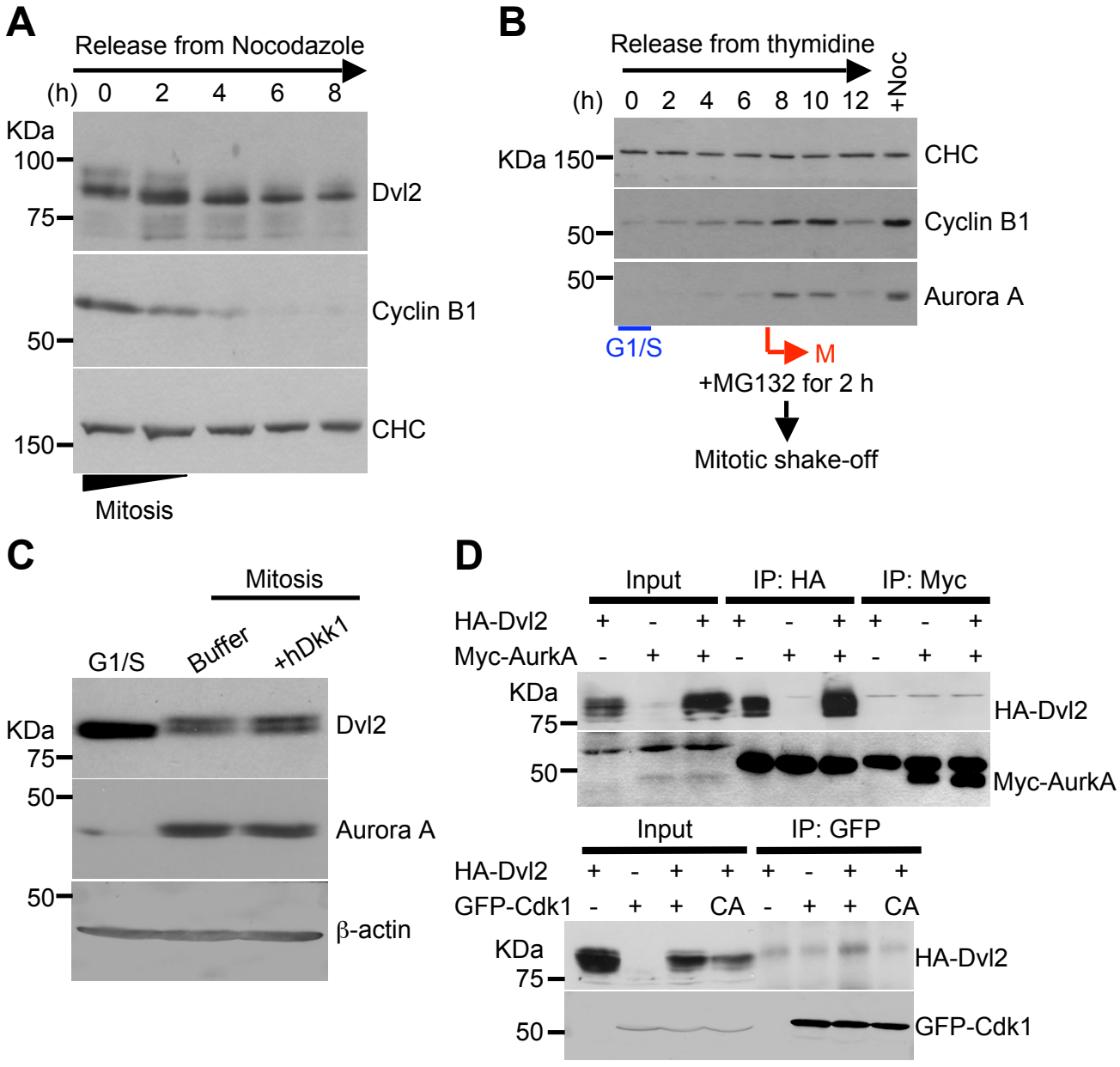

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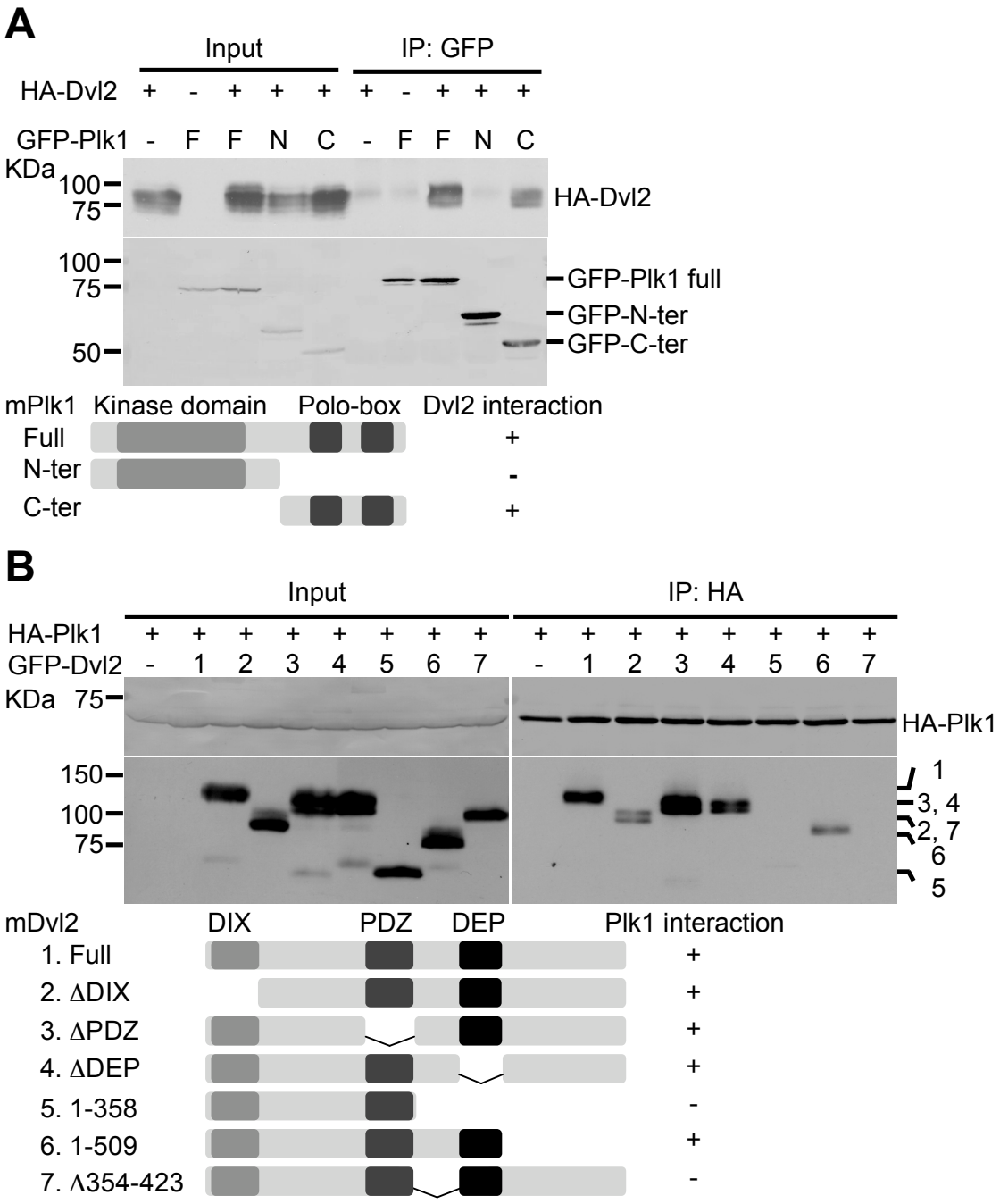

Kikuchi_Fig. S4

A

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PtDvl2   199 ELESTSLGDSDEEDTMSRFSSSTEQSSASRLLKRH-RRRRKQRPPRLERTSSFSSVTDSTMSLN 261
CfDvl2   202 ELESTSLGDSDEDDTMSRFSSSTEQSSASRLLKRH-RRRRKQRPPRLERASSFSSVTDSTMSLN 264
MmDvl2   202 ELESTSLGDSDEDDTMSRFSSSTEQSSASRLLKRH-RRRRKQRPPRMERTSSFSSVTDSTMSLN 264
GdDvl2   305 ELETTSFFDSDEDDSTSRFSSSTEQSSASRLMRRHKRRRRKQKAPRIERSSSFSSITDSTMSLN 368
DrDvl2   189 ELDTTSFCDSDDDDTMSRFSSSTEQSTASRLLKRH-RRRRKQRP-RLERASSFSSVTDSTMSLN 250
XDsh     190 EIETS-ICDSEEDDTMSRFSSSTEQSSASRLLKRH-RRRRKQRPPRLERTSSFSSVTDSTMSLN 251
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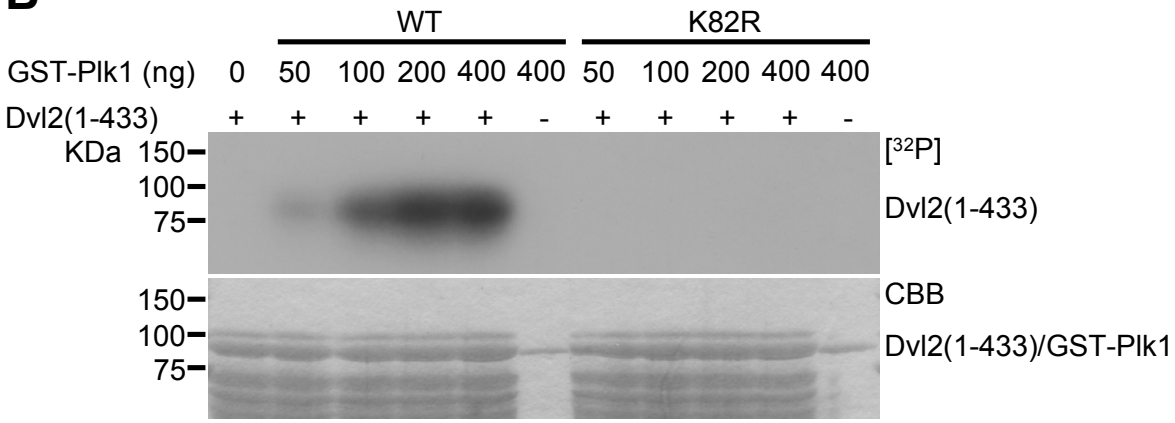
B



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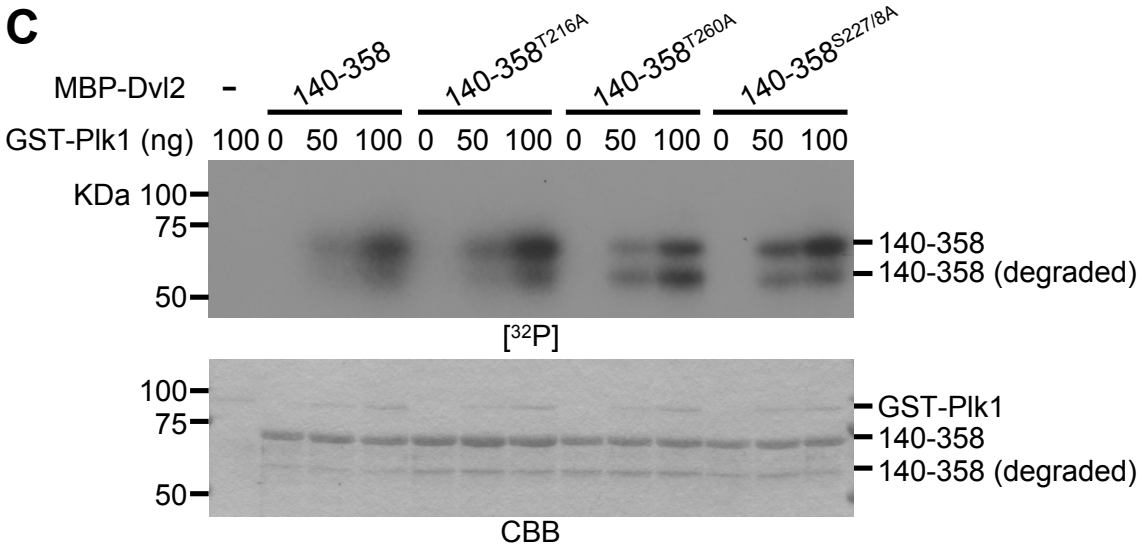

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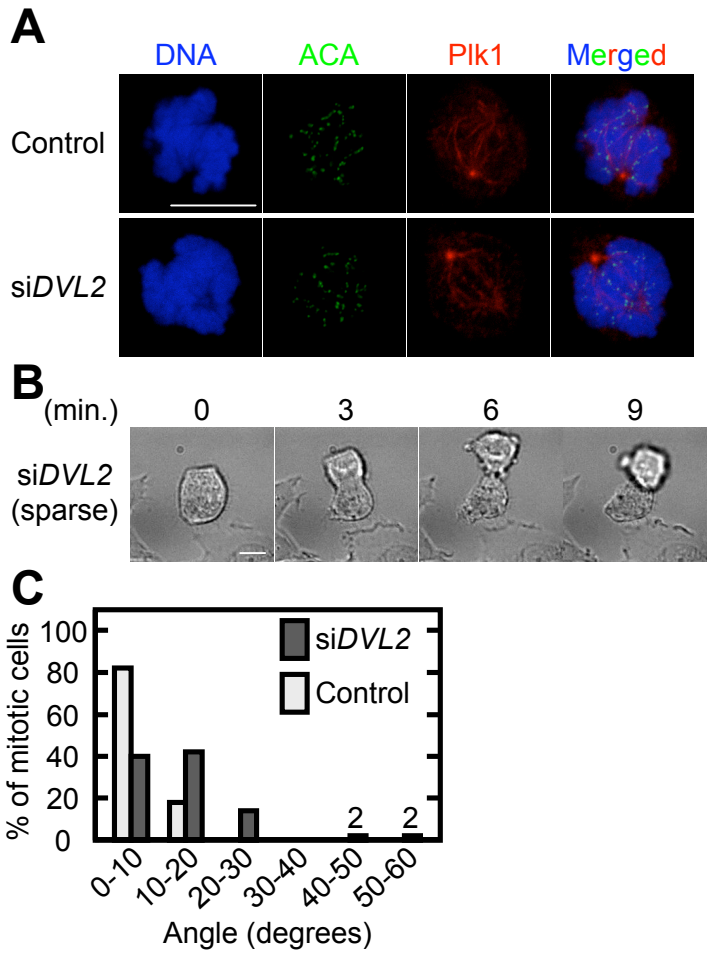

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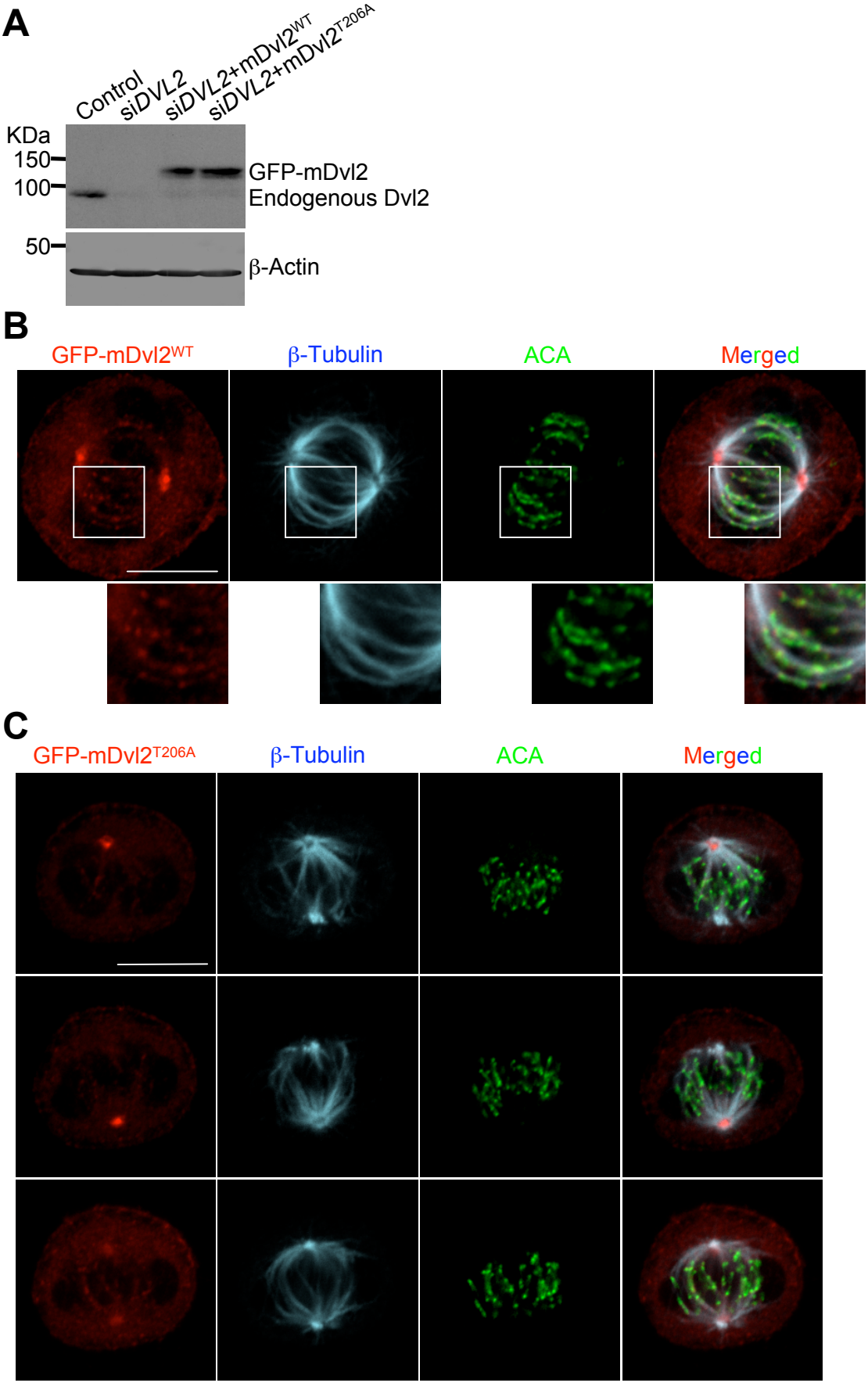

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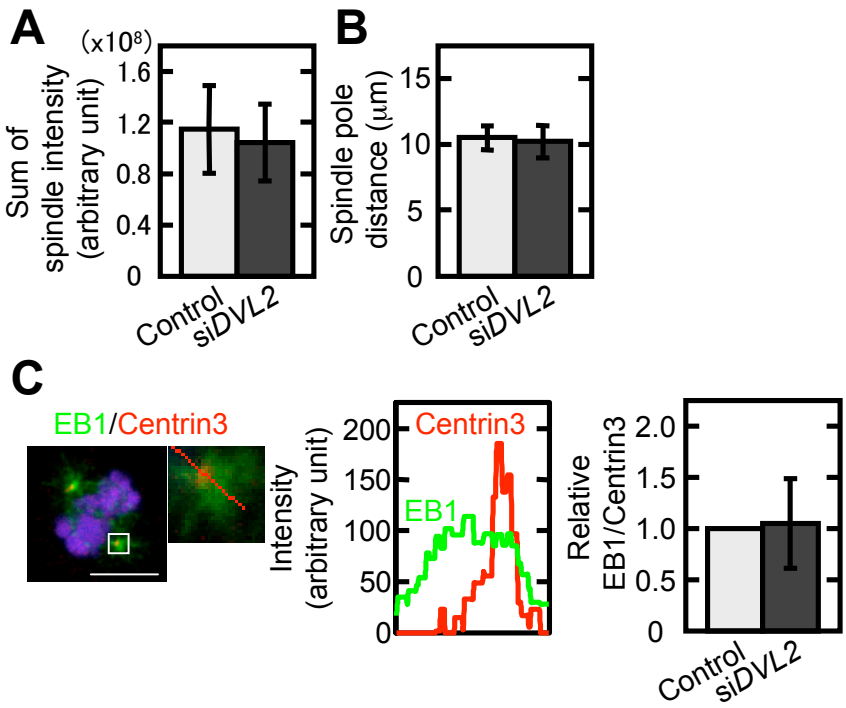

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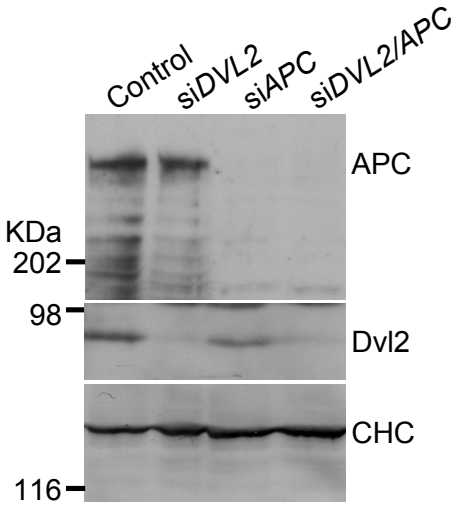

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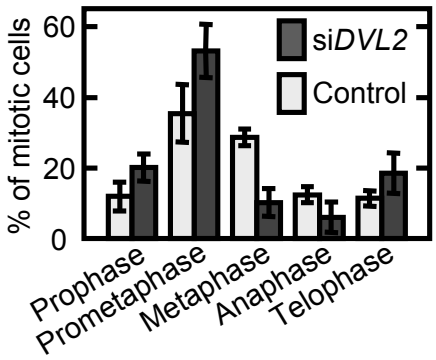

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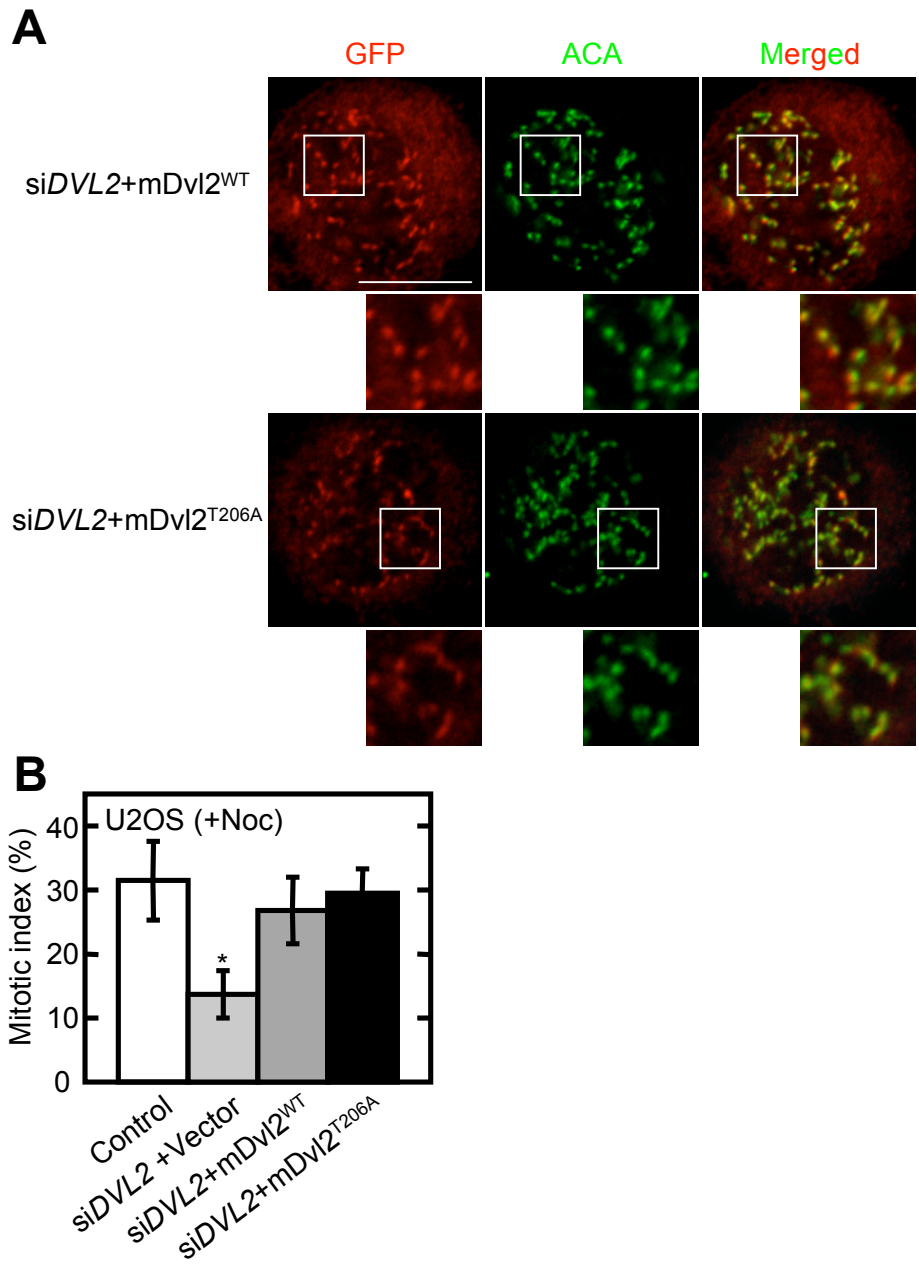

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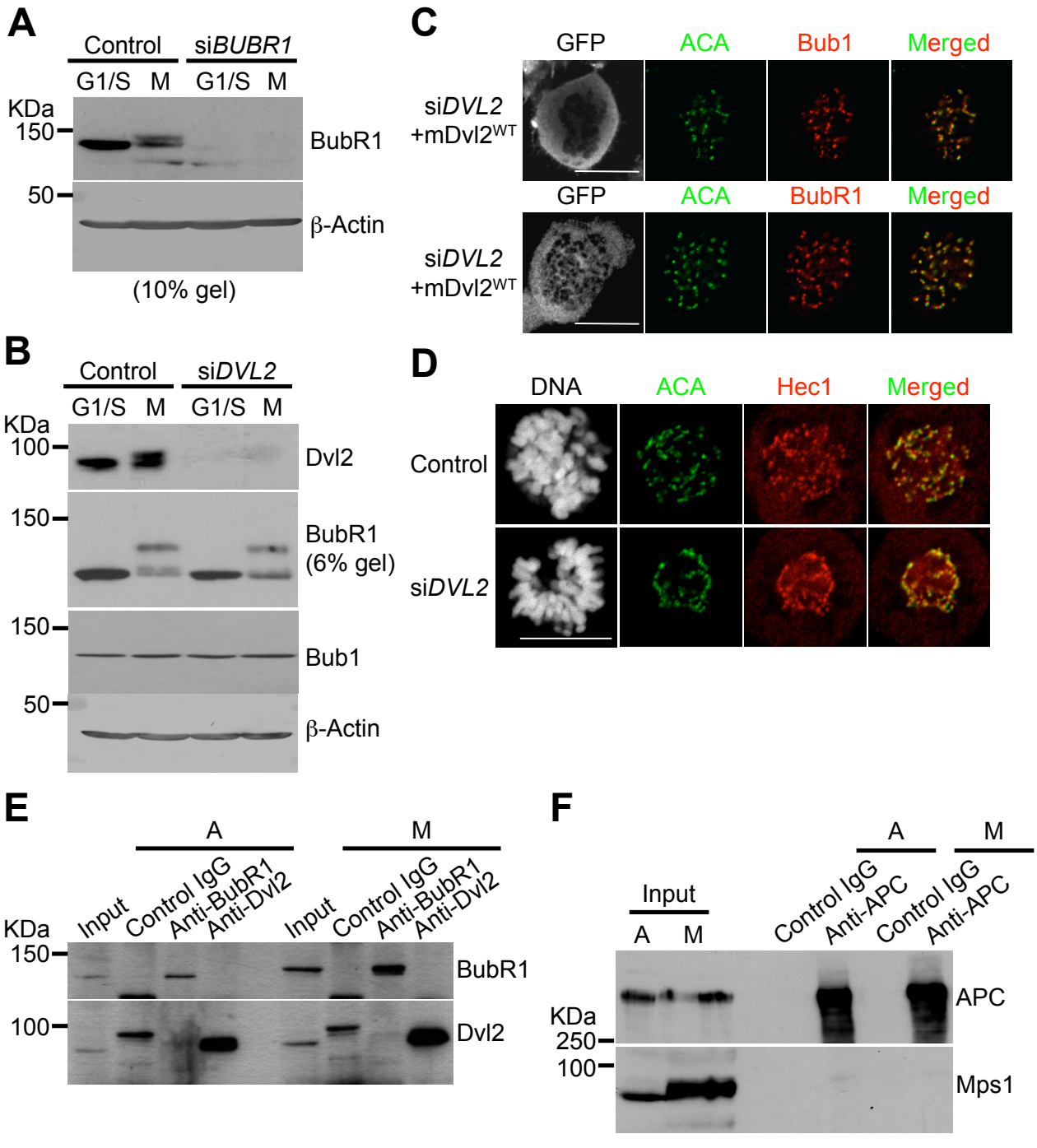

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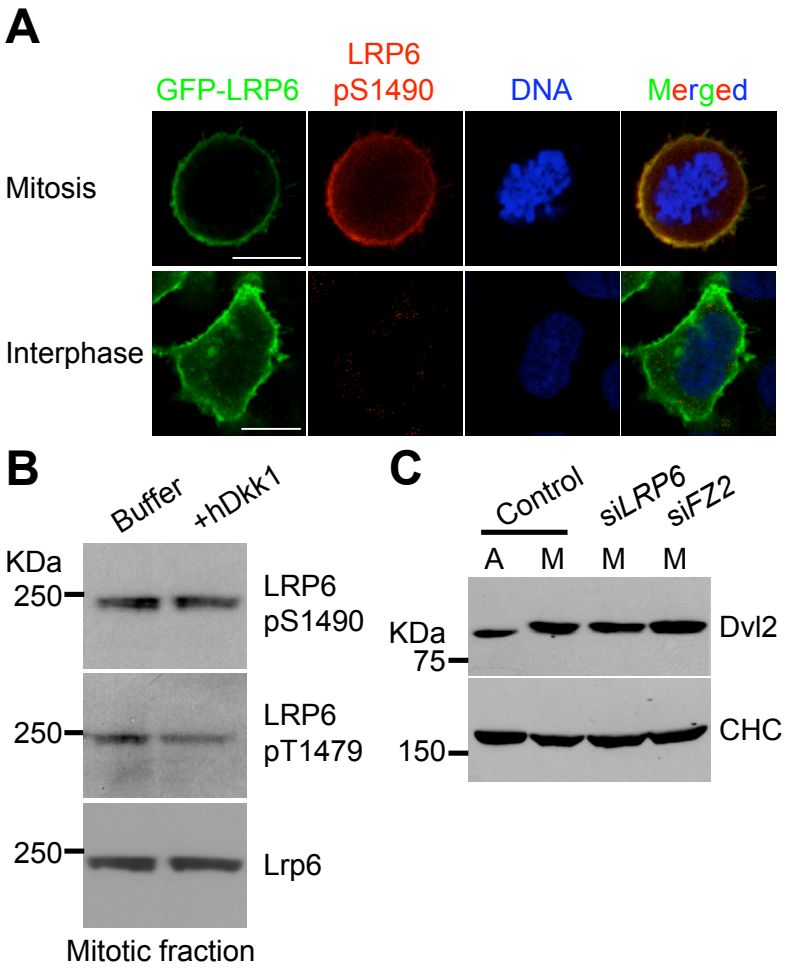

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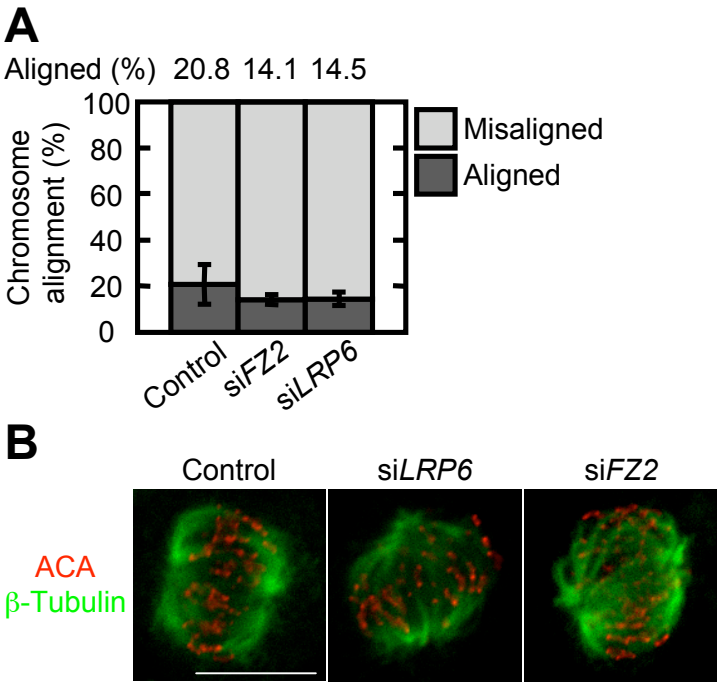

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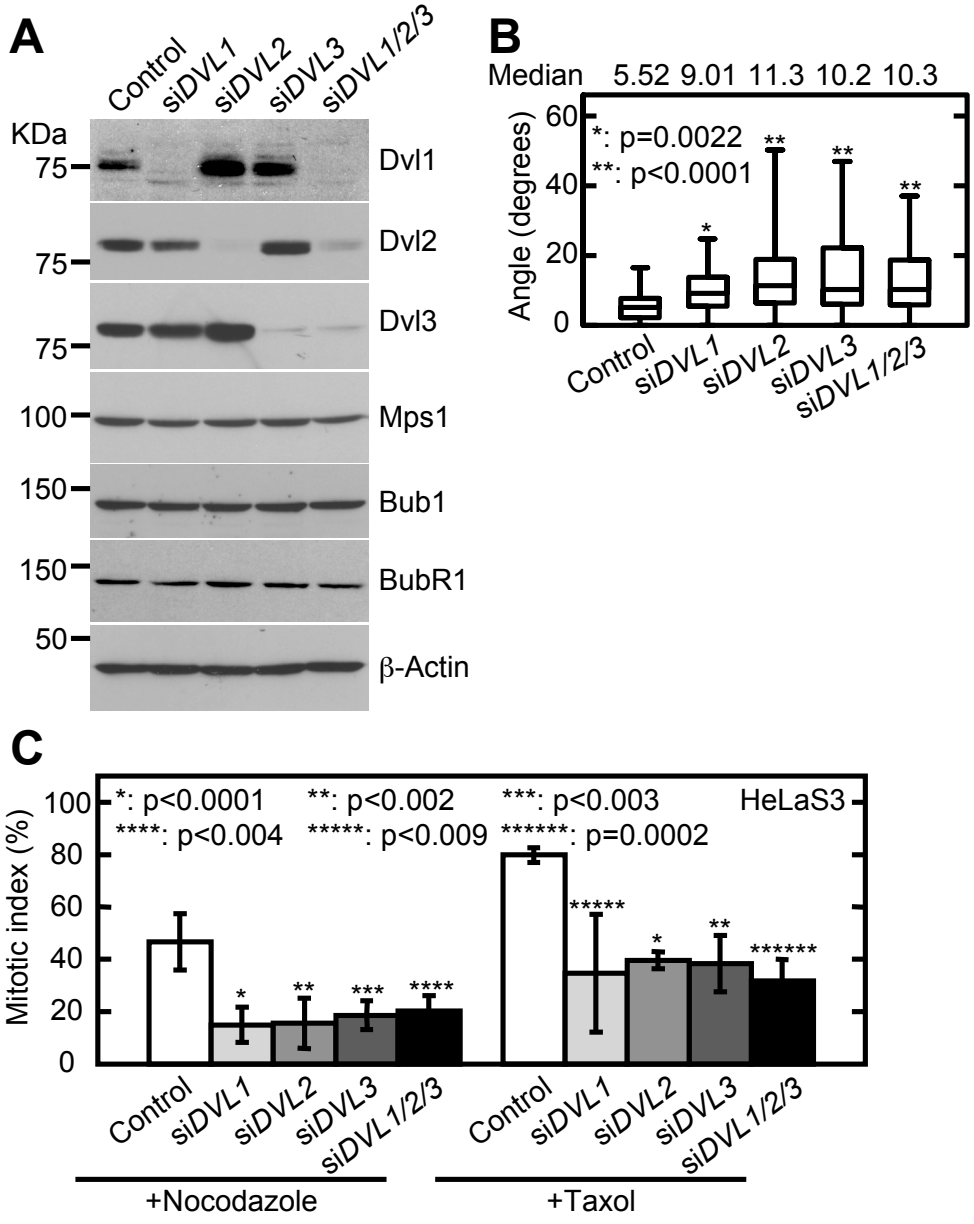

Supplementary Table S1 Primary antibodies used in this study.

Company	Name	Used for
Abcam	Rabbit anti-Plk1	IB, IF
BD Biosciences	Mouse anti-EB1	IF
	Mouse anti-Clathrin heavy chain	IB
Cell Signaling Technology	Mouse anti-Cyclin B1	IB
	Rabbit anti-Dvl2	IB
	Rabbit anti-Lrp6	IB
	Rabbit anti-Lrp6 pS1490	IB, IF
Chemicom	Mouse anti-Bub1	IF
	Mouse anti-BubR1	IF
Cortex Biochem	Anti-centromere (ACA)	IF
Covance	Mouse anti-HA 16B12	IB, IP
MBL	Rabbit anti-HA	IB
	Chicken anti-GFP	IF
Molecular Probes	Rabbit anti-GFP	IB, IF, IP

(Continued)

Company	Name	Used for
Novus Biologicals	Mouse anti-Hec1	IF
Santa Cruz Biotechnology	Mouse anti-Dvl1	IB
	Mouse anti-Dvl2	IB, IP
	Mouse anti-Dvl3	IB
	Mouse anti-γ-tubulin	IF
	Mouse anti-GFP	IB
Sigma	Rabbit anti-Flag	IF
	Mouse anti-β-actin	IB
	Mouse anti-β-tubulin	IB, IF
	Rabbit anti-γ-tubulin	IF
Transgenic	Rabbit anti-Aurora A	IB
Upstate	Mouse anti-Mpm2	IF
Zymed Laboratories	Mouse anti-Mps1	IB, IP
	Mouse anti-Plk1	IB

(Continued)

Company	Name	Used for
Gifted from M. Bornens	Rabbit anti-Centrin3	IF
Gifted from T. Hirota	Rabbit anti-Cenp-A pS7	IF
Gifted from Y. Mimori-Kiyosue	Rabbit anti-APC	IB, IP
	Rat anti-APC	IB
Gifted from C. Niehrs	Rabbit anti-Lrp6 pT1479	IB
Made in house	Mouse anti-Myc 9E10	IB, IP
	Rabbit anti-DIX(Kishida *et al*, 1999)	IP

IB, Immunoblotting; IF, Immunofluorescence; IP, Immunoprecipitation

Supplementary Table S2 siRNAs used in this study.

Name	Sequence (sense)
Randomized control	5'-CAGUCGCGUUUGCGACUGGTT-3'
DVL2(Matsumoto *et al*, 2010)	5'-GGAAGAAAUUUCAGAUGACTT-3'
APC(Matsumoto *et al*, 2010)	5'-GAGCGGCAGAAUGAAGGUCAA-3'
LRP6-1(Sato *et al*, 2010)	5'-AUUGCCCAUCCUGAUGGUATT-3'
LRP6-2(Sato *et al*, 2010)	5'-CCAAAGUCCAAGCUCGAAUTT-3'
FZ2(Sato *et al*, 2010)	5'-CGGUCUACAUGAUCAAAUATT-3'
ROR2(Sato *et al*, 2010)	5'-GCAACCUUUCCAACUACAATT-3'
WNT5A-1(Matsumoto *et al*, 2010)	5'-GUUCAGAUGUCAGAAGUAUTT-3'
WNT5A-2(Matsumoto *et al*, 2010)	5'-GUGGAUAACACCUCUGUUUTT-3'
BUBR1-1(Elowe et al., 2007)	5'-GGAGAUCCUCUACAAAGGGTT-3'
BUBR1-2(Elowe et al., 2007)	5'-GUCUCACAGAUUGCUGCCUTT-3'
PLK1(Matsumura et al., 2007)	5'-GGGCGGCUUUGCCAAGUGCTT-3'