The E2-C Vihar Is Required for the Correct Spatiotemporal Proteolysis of Cyclin B and Itself Undergoes Cyclical Degradation

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Supplemental Experimental Procedures

Drosophila Genetics

The *vihar*^{1} mutation was first identified in a collection of third chromosome P-*lacw* insertion mutants [1]. Complementation tests were carried out between the *vihar* stock and the deficiency stocks *Df(3L)iro-2*, *Df(3L)F10*, and *Df(3L)E44*, which were obtained from the Bloomington *Drosophila* Stock Center. Hemizygous females of *vih*^{1}/*Df(3L)iro-2*, *vih*^{2}/*Df(3L)iro-2*, *vih*^{1}/*Df(3L)F10*, and *vih*^{2}/*Df(3L)F10* are referred to as vih^{1}/− and vih^{2}/−. The complementation tests were performed with the deficiency chromosome supplied by either the male or female parent. In order to revert the *vih*^{1} mutation, the P-lacW element was remobilized under dysgenic conditions. About 250 jump starter males of w^{1118}/Y; *vih*^{1}/*TM3,Sb,ry,[Δ2−3, ry*$^{+}$*]* or w^{1118}/Y;*vih*^{1}/*Sb[Δ2−3]* genotypes were crossed individually to w^{1118}/w^{1118}; *TM3,Sb,Ser/ TM6b,Tb* virgins. From their progeny, the *vih*^{1}/*TM3* and *vih*^{1}/*TM6b* flies were scored for *w*$^{−}$ phenotype. For each jump starter male, only one fly was selected showing the *w*$^{−}$ or the modified *w*$^{+}$ phenotype, and lines were established for these revertants balanced over *TM6b,Tb*. Forty-two revertants were isolated that were viable and female fertile when homozygous and had *w*$^{−}$ eyes in a *w*$^{−}$ background, indicating the complete reversion of the *vih*^{1}-associated mutant phenotype and the precise excision of the P-lacW element. These complete revertants crossed back to the original *vih*^{1} allele, or an uncovering deficiency, *Df(3L)iro-2*, gave viable and fertile offspring, suggesting that the P-lacW insertion is responsible for the *vih*^{1}- associated phenotype. Females of the partial revertant, *vih*^{2}, were fully sterile when homozygous, hemizygous, or transheterozygous with *vih*^{1}; in all cases the syncytial embryos showed similar mitotic defects. The *TM3,Sb,ry,[Δ2−3, ry*$^{+}$*]/Df(3R)C7,ry*^{507} stock was given by János Gausz. The homozygous and hemizygous *vih* mutant phenotypes were determined on a *TM6b,Tb* or *TM6c,Tb,Sb* background as described by Deák et al. [1]. Individual sterility tests were performed for homozygous, hemizygous, and transheterozygous *vih* mutant females by crossing them to wild-type Canton-S males.

Cloning and Sequencing

Plasmid rescue of inserted *P-lacW* elements and in situ hybridization to salivary gland polythene chromosomes were carried out as described [1]. We screened the European *Drosophila* Genome Project cosmid library using the P-lacW flanking chromosomal sequences and identified cosmids carrying the *vih* gene, from which we selected a 2.8 kb BamHI-EcoRI restriction fragment for insertion into the pSK(+) vector (Stratagene). The clone had been sequenced from both directions using synthetic internal primers. DNA sequencing was carried out using the ABI Prism machine with the Big Dye terminator cycle sequencing chemistry in the sequencing facility of the Department of Genetics, University of Cambridge. Nucleotide sequence analysis and amino acid comparisons were performed using the DNASTAR (Lasergene) and the BLAST programs accessed through NIH or Flybase. The rescued chromosomal sequences flanking *P-lacW* inserts were used to screen for cDNAs. We were able to isolate three identical cDNA clones containing this open reading frame: one from an embryonic library (the gift of David Huen), one from a testis library (kindly provided by Brian Oliver and Justen R. Andrews), and one identified by the deposited EST, AI946542. The GenBank accession number of the full-length *vih* cDNA is AF410850. The full-length *vihar* cDNA was cloned into the pCASPER4 germline transformation vector, and several independent transgenic lines were isolated.

S2 Cell Culture

S2 cells were purchased from Invitrogen and grown in Schneider's *Drosophila* medium (GIBCO-BRL), supplemented with 10% heat-inactivated fetal calf serum containing 50 μg/ml streptomycin and penicillin at 25°C. For FACS analysis, the cells were washed three times with PBS and fixed with 90% ice-cold ethanol. The cells were incubated at 37°C for 30 min in PBS containing 50 μg/ml DNase-free RNaseA and 1 μg/ml propidium iodide before analysis. Nocodazole arrest was achieved by incubating S2 cells in serum-supplemented medium containing 16 μM nocodazole for 24 hr. After being washed twice in serum-medium, the cells were cultured in nocodazole-free medium, and samples were collected at 1 hr intervals for preparation of total protein extracts.

Conditions for *vih* RNAi in Cultured S2 Cells

RNAi was carried out using the method of Clemens et al. [2] as modified by Giet and Glover [3]. Cells were plated out in 6-well plates the day before transfection at a density of 0.5×10^{6} per well. For transfection, the cells were washed three times in serum-free media and transfected with Transfast lipid reagent (Promega) according to the manufacturer's instructions. A mixture of 20 μg *vih* dsRNA/15 μl Transfast was used to transfect each well plate. Cells were incubated for 3 days, but due to the rapid turnover of Vihar, the effects of the RNAi on the cells could be seen on the first day after treatment. Samples were taken at various times after transfection and analyzed by FACS, Western blot, and immunostaining.

Western Blot Analysis of *Drosophila* Extracts

Equal amounts of total protein extracts were loaded onto 7.5% or 15% SDS-PAGE gels and after electrophoresis were blotted onto Hybond ECL nitrocellulose membranes (Amersham). α-tubulin was used as a loading control. The anti-α-tubulin was from Amersham (clone N513), and HRP-labeled anti-rabbit secondary antibody was purchased from Jackson Immunoresearch Laboratories Inc. The blots were developed using the ECL kit (Amersham). Semiquantitative analysis was achieved by establishing a standard curve of the response using bacterially expressed protein.

HeLa Cell Extract Preparation

HeLa cells were grown at 37°C in the presence of 5% CO_2 in DMEM supplemented with 10% FCS, 0.3 μg/ml L-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin. Cells were arrested in mitosis by the addition of 100 ng/ml nocodazole for 18 hr. HeLa cell extracts were prepared as described [4].

Immunoprecipitation of APC/C and In Vitro Ubiquitination Assay

Affinity-purified anti-Cdc27 antibodies coupled to protein-A-Affiprep beads (BioRad) were used to immunoprecipitate APC/C from

Table S1. Quantitation of Mitotic Phenotypes after *vihar* RNAi

	MI	Percent Mitosis	Percent Cytokinesis
Control	5.5	39	61
First day RNAi	15	78	24
Second day RNAi	25	90	10
Third day RNAi	40	99.9	0.1

The mitotic index (MI) represents percent of mitotic/total cells. Other values represent proportion of the dividing cell population at the indicated stages. Five microscope slides were studied per five RNAi experiments.

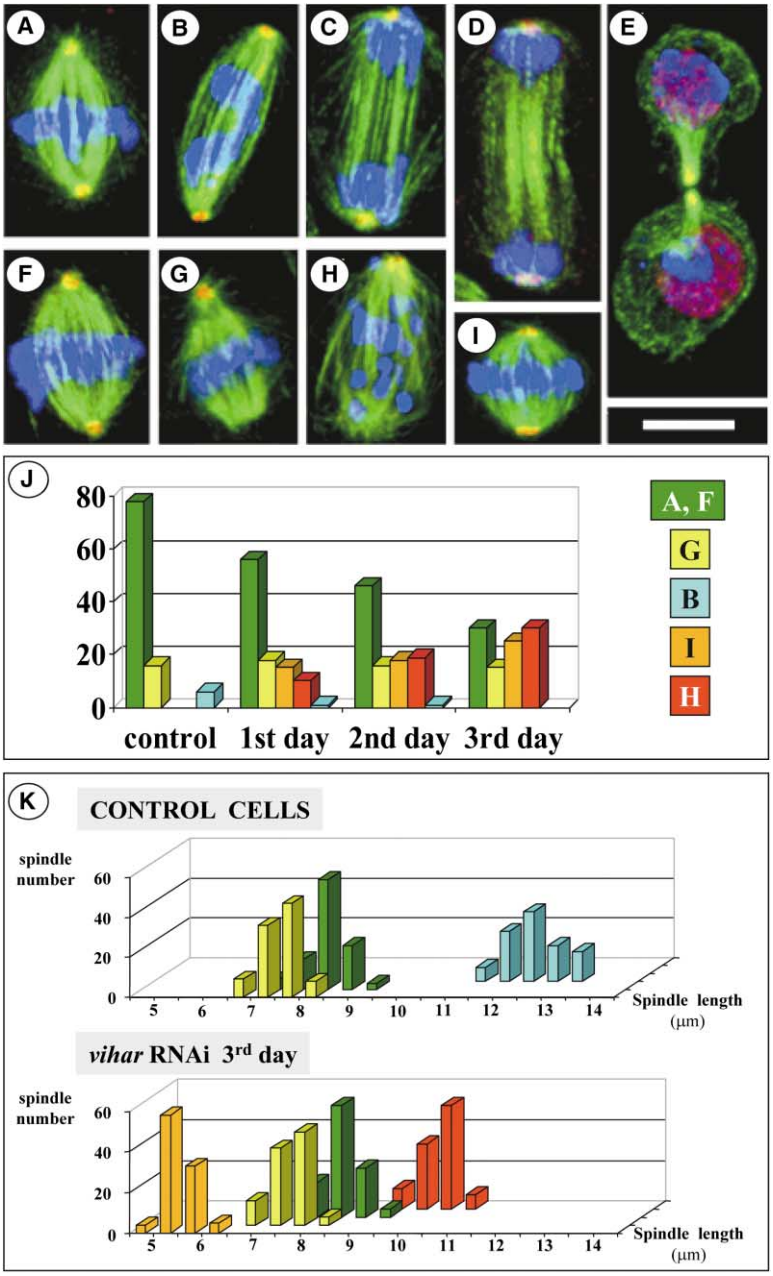

Figure S1. Mitotic Spindle Defects in Cultured *Drosophila* Cells after *vih* RNAi

(A–E) Mitotic spindles from untreated S2 cells at metaphase (A), early (B) and late (C) anaphases, telophase (D), and cytokinesis (E) were stained to reveal tubulin (green), CP190 (red), and DNA (blue).

(F–I) S2 cells following transfection with double-stranded *vih* RNA and similarly stained to reveal metaphase spindles (F), bipolar monocentrosomal spindles with chromosomes aligned in the equatorial region (G), elongated bipolar monocentrosomal spindles with scattered chromosomes (H), and diminutive spindles with aligned chromosomes (I). The scale bar represents 5 μm.

(J) A graph showing the proportions of mitotic spindle categories during the successive days of *vih* RNAi experiment.

(K) Distribution of spindle length for 100 spindles in the phenotypic classes indicated in (A)–(I). Proportions of cell number for each of these classes are also given in Table S2 of metaphase and metaphase-like spindles in control and *vih* dsRNA-treated cells. The spindle shows prominent lengthening as anaphase develops in wild-type cells. Those spindles with scattered chromosomes did show an increase in spindle length (by about 12%) beyond that of a metaphase spindle in untreated cells. However, this was much less than the 50% increase in length that is seen as these cells progress through a normal anaphase (Table S2).

HeLa cell extracts. Ten volumes of cell extracts diluted in IP buffer (20 mM Tris [pH 7.5], 150 mM NaCl, 20 mM β-glycerophosphate, 1 μM okadaic acid, 5 mM EDTA) to 15 mg/ml was incubated with one volume of antibody beads for 1–2 hr at 4°C. The beads were washed three times with low-salt buffer (BL: 20 mM Tris [pH 7.5], 150 mM NaCl, 0.02% Tween 20).

For the in vitro ubiquitination assays, 5 μl of immunopurified APC/C on beads was used in 5 μl BL containing purified E1 (80 μg/ml), UBC4 and UBCx or Vihar E2-C proteins (50 μg/ml), ubiquitin (1.25 mg/ml), ATP regenerating system (7.5 mM creatine phosphate, 1 mM ATP, 1 mM $MgCl_2$, 0.1 mM EGTA, 30 units/ml rabbit creatine phosphokinase type I [Sigma-Aldrich]), and 20 μg/ml myc-tagged Cyclin B fragment (amino acids 13–110) in a total volume of 10 μl. The reaction mix was incubated for 30 min at 37°C on a thermoshaker and stopped by the addition of SDS-PAGE sample buffer. The samples were resolved by SDS-PAGE and analyzed by immunoblotting with the anti-myc antibody 9E10 (Sigma).

Immunocytochemistry and Confocal Microscopy

Aceto-orcein cytological preparations of third instar larval brains were made according to Sunkel and Glover [5]. The fixation and immunostaining of 0–2 hr old embryos were carried out with either formaldehyde or methanol. Bleach-dechorionized embryos were fixed with a formaldehyde fixative (500 μl heptane, 400 μl 37% formaldehyde, 100 μl 100 mM EGTA [pH7.5]), followed by a methanol fixative (500 μl heptane, 500 μl methanol), both for 5 min. In the case of the methanol-fixed embryos, the formaldehyde fixation was left out. The embryos were then rehydrated in PBT (PBS, 0.1% Triton X-100), and with the primary antibodies overnight incubations were carried out at 4°C, while the secondary antibodies were incubated for 2–4 hr at room temperature. For the colchicine treatment, prior to formaldehyde fixation the embryos had been incubated in a mixture of 500 μl heptane, 500 μl buffer B (100 Mm KH_2PO_4/K_2HPO_4 [pH 6.8], 150 mM NaCl, 450 mM KCl, 20 mM $MgCl_2 \times 6H_2O$), containing 50 μg/ml colchicine for 10 min. Taxol treatment was carried out in

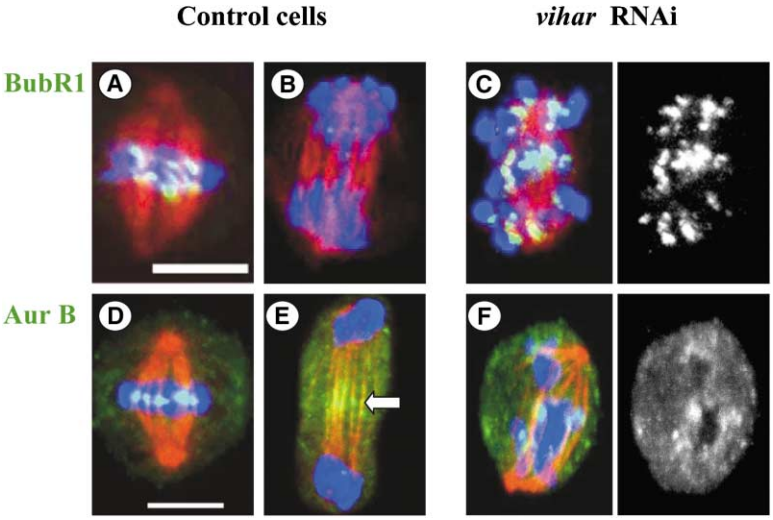

Figure S2. Centromeric Proteins on Scattered Chromosomes in Cultured *Drosophila* Cells after *vih* RNAi

Mitotic cells from untreated (left panel) and double-stranded *vih* RNA-treated (right panel) S2 cells were stained to reveal either BubR1 or Aurora B (green), tubulin (red), and DNA (blue). The BubR1 protein is present at kinetochores of normal metaphase (A) cells and absent in late anaphase (B). In *vih* RNAi cells, BubR1 is present on all kinetochores, including those of the scattered chromosomes, suggesting that they are attempting congression (C). In untreated cells, Aurora B is also seen on metaphase kinetochores (D) and reaccumulates in the equatorial region of the central spindle (arrow) after anaphase (E). In spindles with scattered chromosomes, some Aurora B protein is still associated with chromosomes (F).

similar mixtures that contained 0.25 μl of 10 mM taxol. Lactacystin (Affiniti Research Products Ltd.) treatment of wild-type embryos was carried out for 2–5 min by adding 1 μl of 0.1 mg/ml lactacystin immediately before fixation. The cultured S2 cells were grown on glass coverslips and fixed with formaldehyde according to Lemos et al. [6]. The microtubules were detected with YL1/2 rat monoclonal anti-α-tubulin antibody (Sera Lab, Inc.), while the mouse monoclonal anti-γ-tubulin (clone number GTU88; Sigma), Rb188 rabbit anti-*Drosophila* CP190, and rabbit anti-*Drosophila* CNN antibodies were used to stain the centrosomes. The Rb 271 rabbit anti-*Drosophila* Cyc B antibody was used to study the subcellular distribution of Cyclin B. The DNA was counterstained with the TOTO3 dye (Molecular Probes). The goat Alexa 488 anti-rabbit, Alexa 488 anti-rat, Alexa 594 anti-rat, and Alexa 594 anti-rabbit secondary antibodies were obtained from Molecular Probes. The Zenon Rabbit IgG labeling kit (Molecular Probes) was used to produce conjugates of Alexa 594-rabbit anti-His antibody (Amersham Biosciences) to detect the double D box mutant Vihar E2-C protein in embryos. Digital images of optical sections were collected with a Bio-Rad 1024 confocal microscope.

Supplemental References

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Table S2. Proportions of Mitotic Figures with Characteristic Spindle Morphologies in Untreated and *vihar* dsRNA-Treated Cells

Spindle Phenotype	Percent Untreated	Percent *vih* RNAi		
		First day	Second day	Third day
Bicentrosomal, normal length spindle with aligned chromosomes	78	56	46	30
Monocentrosomal spindle with aligned chromosomes	16	18	16	15
Bicentrosomal, small spindles with aligned chromosomes	0	15	18	25
Monocentrosomal spindle with scattered chromosomes	0	10	19	30
Wild-type ana-telophase	6	1	1	0

The values represent percent of the total mitotic figures. Three microscopic slides were studied per five independent *vihar* RNAi experiments.

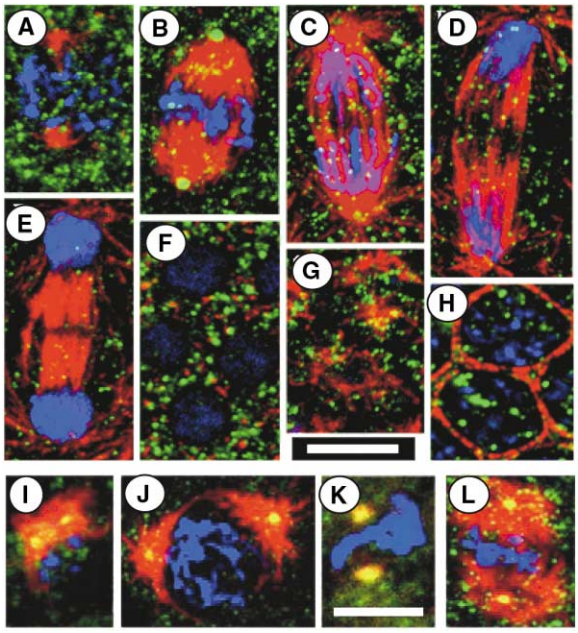

Figure S3. Cyclical Degradation of Vihar E2-C in Embryos

Mitotic spindles from wild-type syncytial embryos were stained to reveal Vihar E2-C (green), α-tubulin (red), and DNA (blue). The one exception is (K), in which γ-tubulin is stained red.

(A–H) Subcellular localization of Vihar E2-C in syncytial embryos fixed with formaldehyde at prophase (A). As mitosis was initiated, Vihar E2-C accumulated on the centrosomes, its staining being strongest at metaphase (B). However, as anaphase commenced, the staining diminished dramatically such that only traces of Vihar E2-C were visible at the centrosomes (C and D), and by the end of telophase it appeared to have completely disappeared from the poles (E). Vihar protein first appears in the cytoplasm during interphase (F). It accumulates on the apical surface of the cleavage nuclei, where the centrosome-nucleated microtubule asters are visible (G), and in the cleavage nuclei while the chromosomes start to condense (H). Images (G) and (H) are of the same cleavage nuclei but on different focal planes.

(I and J) Prophase nuclei from embryos fixed with methanol that appeared to show earlier accumulation of Vihar E2-C on centrosomes.

(K) Mitotic figure in an embryo treated with colchicine prior to formaldehyde fixation and stained with Vihar E2-C (green), γ-tubulin (red), and DNA (blue). Colocalization of Vihar E2-C and γ-tubulin can be observed on the centrosomes, indicating that its centrosomal accumulation is independent of microtubules.

(L) A metaphase spindle in an embryo treated with taxol prior to formaldehyde fixation shows Vihar E2-C associated with centrosomes and astral microtubules.