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Interaction of Skp1 with CENP-E at the midbody is essential for cytokinesis

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Abstract

Centromere-associated protein E (CENP-E) is a kinesin-related microtubule motor protein that is essential for chromosome congression during mitosis. Our previous studies show that microtubule motor CENP-E represents a link between attachment of spindle microtubules and the mitotic checkpoint signaling cascade. However, the molecular function of CENP-E at the midbody had remained elusive. Here we show that CENP-E interacts with Skp1 at the midbody and participates in cytokinesis. CENP-E interacts with Skp1 in vitro and in vivo via its coiled-coil domain. Our yeast two-hybrid assays mapped the binding interfaces to the central stalk region of CENP-E (955–1571 aa) and the C-terminal 33 amino acids of Skp1, respectively. Our immunocytochemical studies revealed that CENP-E targets to the midbody prior to Skp1 and the midbody localization of CENP-E becomes diminished as Skp1 arrives at the midbody. Suppression of Skp1 in mitotic HeLa cells by siRNA resulted in accumulation of telophase cells with elongated inter-cell bridges and with midbodies stretched 2–3 times longer than that of normal cells. These Skp1-eliminated or -suppressed cells accumulate higher level of CENP-E, suggesting that spatiotemporal regulation of CENP-E degradation at the midbody is essential for cytokinesis. Over-expression of Skp1 lacking the CENP-E-binding domain confirmed that Skp1–CENP-E interaction is essential for faithful cytokinesis. We hypothesize that CENP-E degradation is essential for faithful mitotic exit and the proteolysis of CENP-E is mediated by SCF via a direct Skp1 link.

Keywords: CENP-E; Skp1; Midbody; Cytokinesis; Kinesin; Chromosome; Mitosis

During cell division, chromosome segregation is orchestrated by the interaction of spindle microtubules with the centromere (e.g., [1]). A dramatic reorganization of interpolar microtubules into a highly organized central spindle between the separating chromatids is required for the initiation and execution of cytokinesis.

CENP-E is a 312 kDa mitotic kinesin motor that binds to kinetochore through early mitosis. Both immunofluorescence and immuno-electron microscopic studies indicated that CENP-E is restricted to bundles of microtubules in the midzone during anaphase and is ultimately concentrated in the developing midbody at the end of cytokinesis [2,3]. It is a cell cycle regulated protein with a continuous accumulation just before mitosis and a specific degradation after anaphase onset [4,5]. Previous research has discovered some important functions of this huge molecule at microtubule–kinetochore connection [3,6,7], chromosome congression [8,9], and even production of "wait anaphase" signal [10,11]. It has also been proposed that CENP-E destruction defines a fourth point in a mitotic cascade of timed proteolysis just after that of cyclin A, cyclin B, and unidentified components at the metaphase/anaphase transition, and

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probably degrades in a D-box dependent, ubiquitin-mediated pathway like cyclins [5]. However, there is no experimental evidence validating such notion.

There is mounting evidence that spatio-temporally regulated ubiquitin-mediated proteolysis occurs during mitosis. This degradation is mainly achieved via two E3 ligases such as SCF (skp1-cullin-F-box) and APC (anaphase promoting complex)/cyclosome (e.g., [12]). It has been shown that ubiquitin-mediated mitotic degradation is essential for perfect cell division (e.g., [12]). Skp1, a core component of SCF localized to the yeast kinetochore, is essential for the formation of CBF3 complex [13,14]. It connects cell cycle regulators to the ubiquitin proteolysis machinery through a specific motif, the F-box [15], which links Skp1 to F-box proteins possessing the substrate recognizing center of SCF complexes.

To illustrate the molecular mechanisms underlying CENP-E proteolysis, we carried out yeast two-hybrid assays to search for CENP-E-binding partners using the coiled-coil domain of CENP-E as bait. Among twenty-two positive clones isolated, Skp1 is a strong positive. Biochemical characterization validates the interaction between CENP-E and Skp1 while functional analyses demonstrate the importance of Skp1 for CENP-E degradation. Thus, we hypothesize that SCF governs CENP-E destruction at the end of mitosis, which is essential for faithful cell separation.

Materials and methods

Cell culture and synchronization. HeLa and 293T cells, from American Type Culture Collection (Rockville, MD), were maintained as subconfluent monolayers in DMEM (Invitrogen; Carlsbad, CA) with 10% FBS (Hyclone, Logan, UT) and 100 U/ml penicillin plus 100 μg/ml streptomycin (Invitrogen; Carlsbad, CA) at 37 °C with 10% CO_2. Cells were synchronized at G1/S with 5 mM thymidine for 16 h, then washed with PBS three times and cultured in thymidine-free medium for 12 h to release. After another round of thymidine treatment for 12 h, cells were released for 11.5–12 h to initiate cytokinesis. In some cases, nacodazole was added to increase the synchronization efficiency of prometaphase cells.

Antibodies. Affinity purification of CENP-E rabbit antibody was described previously [3]. Mouse monoclonal antibody to Skp1 was purchased from BD Biosciences (Palo Alto, CA) and diluted at 1:1000 for Western blot. Mouse monoclonal antibody to GST was purchased from Cell Signaling (Beverly, MA) and used at 1:1000 for Western blot. Mouse monoclonal antibody to GFP was obtained from BD Biosciences (Palo Alto, CA).

Plasmids. The following plasmids were used for in vitro and in vivo studies: human GST-Skp1 in pGEX-2T, GFP-Skp1 in pEGFP-C1, and GFP-Skp1Δ in pEGFP-C1 (deleted Skp1 C-terminal 130–163 aa), a series of pGBKT-7 human Skp1 deletion plasmids, all these plasmids were subcloned from pACT-2 human Skp1 that was screened out from HeLa cDNA library by yeast two-hybrid assay. Histidine-tagged CENP-E fragment of HpX was built in pET28a (Novagen Inc.).

Yeast two-hybrid screen and Skp1 interacting domain mapping. A yeast two-hybrid interaction screen was performed as described [16]. Briefly, the CENP-E bait containing amino acids 955–1571 (HpX) was inserted into the *Bam*HI–*Eco*RI sites of pGBKT-7 to create a HPX fusion with amino acids 1–147 of the gal4 DNA binding domain. The resultant pGBKT-7 HpX was transformed into AH109 along with the GAL4 reporter plasmid PCL and a negative control plasmid pGBKT-7 Lam. Protein expression was validated by Western blot using GAL4 and HpX antibody. Transformants did not activate the his3 reporter gene and then were transformed with the HeLa cDNA library. Transformants were selected on Leu^-, Trp^-, His^- SD plates and then positive clones were streaked to Leu^-, Trp^-, His^-, Ade^-, X-α-Gal^+ SD plates. When they grew up, we screened the blue colonies with two times of Leu^-, Trp^- SD plates' incubation to help them grow stronger and to get rid of false multi-copy plasmids. Fifty-six colonies were picked out by last Leu^-, Trp^-, His^-, Ade^-, X-α-Gal^+ SD plates' screening. In some cases, the cDNA was directly amplified from the yeast mini-prep plasmids by PCR using primers flanking the cloning site: 5′-CTA TTC GAT GAT GAA GAT ACC CCA CCA AAC CC; 3′-TT CAC TTG AAC GCC CCA AAA AGT CAT AGA TGC. Mini-prep plasmids were isolated from *Escherichia coli* DH5α, and PCR products were digested with several restriction enzymes and grouped according to their digestion patterns. Specificity of the interaction was independently verified by retransforming the candidate cDNAs back into AH109 along with pGBKT-7 HpX. Those cDNAs that can make colonies grow up from Leu^-, Trp^-, His^-, Ade^- SD plates were sent for sequencing using GAL4 AD primer: T ACC ACT ACA ATG GAT G.

From the candidate positive clones, we selected Skp1 to perform further research and inserted a series of Skp1 deletion into pGADT-7 vector. pGBKT-7 HpX and these pGADT-7 Skp1 deletion plasmids were co-transformed into AH109 yeast strain to map out minimum interacting segment of Skp1 with CENP-E.

Expression and purification of recombinant proteins. The histidine-tagged HpX and GST-skp1 fusion protein were expressed in *E. coli* strain BL21 (DE3) pLysS and purified with using Ni^{2+}–NTA beads and glutathione beads, respectively, as previously described [2,17].

Far-Western assay. HeLa cells were lysed directly in 1× Laemmli sample buffer and resolved in 8% SDS–PAGE, and proteins were transferred to nitrocellulose membrane. After blocking in TBST buffer containing 2% milk powder for 1 h, the membrane was washed with TBST buffer for 3 times and incubated with in vitro expressed GST-tagged Skp1 protein for 1 h. After washing 3 times with TBST, Skp1 monoclonal antibody and HRP-conjugated goat anti-mouse antibody were used for immuno-detection of Skp1.

Pull-down assay. In vitro pull-down assay was performed with purified His-tagged HpX and GST-tagged Skp1 protein bound to glutathione-agarose beads, in a buffer containing 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, and 0.20% Tween 20 (PBST) for 4 h at 4 °C. The beads were washed 3 times with ice-cold PBST and 6× Laemmli sample buffer was added and boiled for 5 min. Proteins were resolved by SDS–PAGE and quantified by FluorImaging Western blot detection system (Amersham Bioscience).

Immunoprecipitation. 293T cells were transfected with GFP-Skp1 and GFP plasmids individually and synchronized as described. The cells were then lysed using lysis buffer (0.5% NP-40, 50 mM Tris–Cl, pH 7.4, 150 mM NaCl, 5 mM EDTA, 0.02% sodium azide, and PMSF was added prior to use). HpX polyclonal antibody was incubated for 4 h with protein A/G beads and washed for 3 times with wash buffer (0.1% NP-40, 50 mM Tris–Cl, pH 7.4, 150 mM NaCl, 5 mM EDTA, 0.02% sodium azide, and PMSF was added just before use). These antibody-bound A/G beads were added to cell lysate and incubated for 4 h at 4 °C. After incubation, beads were washed 4 times with wash buffer and once with PBS. Samples were resolved by 10% SDS–PAGE and proteins were transferred to nitrocellulose membrane to perform Western blot using GFP monoclonal antibody and HPX polyclonal antibody individually.

Small inhibitory RNA (SiRNA) and time-lapse image. For the siRNA studies, a smart pool of double-stranded siRNA against Skp1 was obtained from Dharmacon Research Inc. (Lafayette, CO). A series of concentration and time course assays was used to search for the most efficient work conditions of this oligo duplex and 50 nM for 48 h was finally selected.

To directly describe Skp1 function in living HeLa cells, we performed time-lapse imaging. Forty-eight hours after oligo transfection, images were acquired using an Axiovert200 inverted microscope with Axiovision 3.0 software (Carl Zeiss, Germany). Cells were incubated in a series 20 chamber (Model RC-21B, Warner Instruments, Inc.) and temperature was

maintained at 37 °C by using Dual Channel Heater Controller (model TC-344B, Warner Instruments, Inc.) combined with an In-line Solution Heater (SH-27B, Warner Instruments, Inc.).

Transfection and Immunofluorescence. Cells were transfected with siRNA and GFP fusion plasmids in 24-well plate by Oligofectamine Reagent (Invitrogen, CA) and Lipofectamine 2000 (Invitrogen, CA), respectively, according to the manufacturer's manuals.

For immunofluorescence, HeLa cells were seeded onto sterile, acid-treated 12-mm coverslips in 24-well plates (Corning, New York). Double thymidine blocked and released cells were transfected with siRNA of Skp1 and GFP fusion plasmids as described above. Fixation and staining were done as described [10]. Antibodies against HpX and GFP were used at the dilution of 1:500 and 1:300, respectively. Images were acquired using an Axiovert200 inverted Microscope with Axiovision 3.0 software.

Results

Skp1 is a potential CENP-E binding partner

Our previous studies demonstrate that suppression of CENP-E protein expression by antisense oligonucleotide yielded chronic arrest in prometaphase, suggesting the role of CENP-E in spindle checkpoint [10]. In addition, our studies indicate that the C-terminus of CENP-E binds to BubR1 and CENP-F, and is responsible for its kinetochore localization (Fig 1A; ref. [18]). Despite our recent demonstration of the stimulatory effect of CENP-E on BubR1 activity [19], it remains to be established how CENP-E interacts with other kinetochore proteins and relocates to the midbody after sister chromatides separate.

As a constituent of corona fibers, CENP-E coiled-coil domain extends at least 50 nm from the kinetochore outer plate along microtubules [3]. We reasoned the coiled-coil domain would allow for binding to other kinetochore components. To delineate the molecular function of CENP-E during mitosis, its rod domain (amino acids 955–1571, designated as HpX, illustrated in Fig. 1A) was chosen as bait for yeast two-hybrid screen. From 22 positive clones, Skp1 was chosen for further analysis given the fact that budding yeast Skp1 is an important kinetochore protein [13]. Co-transformation experiment confirms the interaction between CENP-E and Skp1 (Fig. 1B).

To validate this interaction and map the CENP-E binding interface on Skp1, a series of Skp1 deletion constructs was built and transformed into yeast strains pre-transformed by CENP-E fragment HpX. As shown in Fig. 1B, co-transformation of the C-terminal 33 aa of Skp1 (130–163 aa) with HpX fragment of CENP-E resulted in blue colonies, indicating that Skp1 interacts with CENP-E via its C-terminal 33 amino acids. Thus, we conclude that Skp1 binds to CENP-E via its C-terminal 33 amino acids.

Skp1 binds to CENP-E in vitro and in vivo

To examine whether Skp1 binds to coiled-coil CENP-E structure or monomeric CENP-E, we carried out far-Western assay on immunoprecipitated CENP-E from mitotic HeLa cells. CENP-E immunoprecipitates were resolved by SDS–PAGE, and then proteins were transferred to nitrocellulose membrane. Western blotting was first incubated with recombinant Skp1 from bacteria followed by incubation with Skp1 monoclonal antibody. As shown in Fig. 1C, a 320 kDa protein band was recognized by Skp1 antibody. Re-probing the same blot, after stripping, with CENP-E rabbit antibody verified this band is CENP-E (data not shown). From this result, we speculated that Skp1–CENP-E interaction depends at least in part on some specific amino acids but not conformation of the protein. To assess whether the three-dimensional structure will affect this interaction, we mixed GST and GST-Skp1 with His-tagged HpX, respectively, and incubated for 4 h followed by analyses in SDS–PAGE. It becomes readily apparent that recombinant HpX polypeptide was found in GST-Skp1 pull-down but not GST precipitates (Fig. 1D). Thus, we conclude that Skp1 directly binds to the HpX fragment of CENP-E.

To validate a direct interaction between Skp1 and CENP-E in vivo, an immunoprecipitation (IP) experiment was carried out using 293T cells transfected with GFP-Skp1 and GFP plasmids, respectively. Transfected cells were first synchronized with double thymidine followed by a 12-h release to allow the majority of cells enter late mitosis before cell lysates were prepared. HpX polyclonal antibody was used to isolate CENP-E protein complex followed by separation on a 10% SDS–PAGE gel. Precipitation of GFP-Skp1 by CENP-E antibody was evident by a GFP-reacting band around 41 kDa, indicating that CENP-E brings down GFP-Skp1 protein. The specificity of Skp1–CENP-E interaction was validated as GFP tag alone was never pulled down by CENP-E (Fig. 1E). Thus, we conclude that Skp1 interacts with CENP-E in vitro and in vivo.

Skp1 is co-localized with CENP-E to the midbody

Previous studies revealed that Skp1 is a kinetochore protein in budding yeast [13]. To test whether Skp1 is co-distributed with CENP-E, we carried out immunofluorescence experiment first in GFP-Skp1 transfected HeLa cells. The subcellular distribution of Skp1 and CENP-E was visualized using FITC and rhodamine-conjugated secondary antibody, respectively, followed by a mouse GFP antibody and rabbit CENP-E antibody. Skp1 was evident as a double-dot between two telophase cells, which is reminiscence of midbody localization of CENP-E (e.g., [2]). The co-localization of CENP-E with Skp1 to the midbody is readily apparent when the two images were merged (Fig. 2E and F, merge in upper panel).

Our immunofluorescence study showed that Skp1 and CENP-E migrate toward the midzone in anaphase B cells (Fig. 2C), and became concentrated and co-localized to the midboby in telophase cells prior to cytokinesis (Fig. 2D). Thus, our studies provide first line of evidence in which Skp1 is a midbody component interacting with CENP-E in telophase cells.

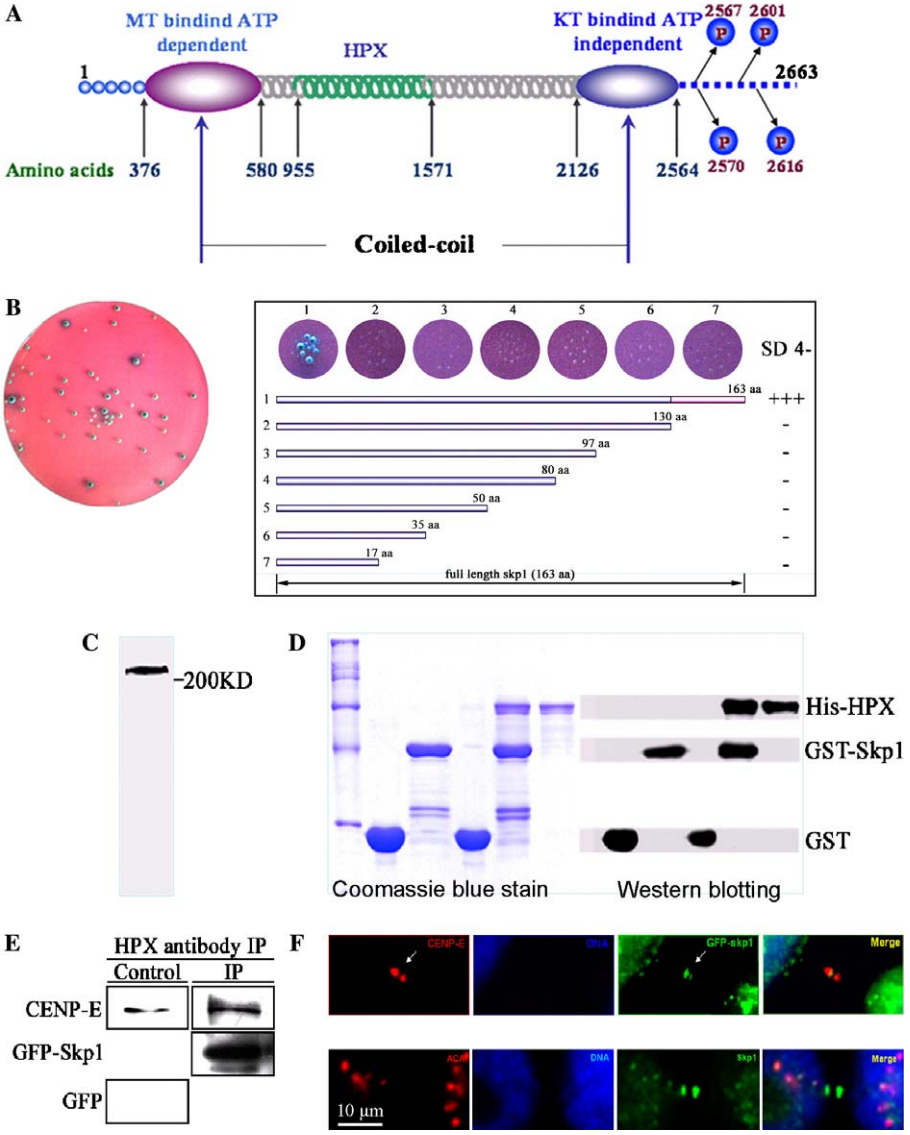

Fig. 1. Identification and characterization of CENP-E–Skp1 interaction. (A) A molecular illustration of CENP-E. HpX is a portion of an extended coiled-coil domain of CENP-E, which was used as bait in our yeast two-hybrid screening assay. (B) Yeast cells were co-transformed with an HpX bait construct and the indicated prey constructs. An example of such an experiment in which cells were selected on supplemented minimal plates lacking uracil, tryptophan, leucine, and histidine. This experiment demonstrated that HpX interacts directly with $Skp1^{130–163aa}$. (C) Direct CENP-E–Skp1 interaction verified by far-Western blotting assay. An aliquot of mitotic HeLa cell lysates was separated by SDS–PAGE and transferred onto a nitrocellulose membrane. The membrane was then blocked and incubated with GST-Skp1 protein for 2 h as described in Materials and methods. After washing, the blot was then incubated with a monoclonal antibody to GST and developed using a ECL kit. A 325 kDa band reacting with GST antibody was verified as CENP-E, based on re-probing same blot with CENP-E antibody. (D) GST pull down assay confirms Skp1–CENP-E interaction. Recombinant His-tagged HpX was mixed with GST-Skp1 and GST pre-bound glutathione-agarose beads, respectively. Proteins from starting material, GST-Skp1 and GST pulled down, were analyzed by SDS–PAGE (left) and immunoblotting (right) using HpX antibody, Skp1 antibody and GST antibody, respectively. (E) Immunoprecipitation of Skp1 isolated CENP-E. 293T cells were transfected with GFP-Skp1 and GFP plasmids, respectively, and then synchronized with double thymidine block as described in Materials and methods. Twelve hours after the release, cells were extracted in lysis buffer as described in Materials and methods. Clarified lysates were incubated with a CENP-E antibody pre-bound protein A/G beads while immunoprecipitates were resolved by SDS–PAGE and immunoblotting with HpX antibody, GFP antibody, respectively. Western blotting verifies that CENP-E immunoprecipitates contain GFP-Skp1. No GFP was detected in CENP-E precipitates, validating the specificity of the immunoprecipitation. (F) Both endogenous Skp1 and exogenously expressed GFP-Skp1 proteins target to the midbody of telophase cells. HeLa cells were synchronized with double thymidine block and released for 11.5 h. Upper panel shows that HeLa cells transfected with GFP-Skp1 triply stained for GFP-Skp1 (green), DAPI (blue), CENP-E (red), and their merged images. Lower panel shows untransfected HeLa cells triply labeled with Skp1 (green), DAPI (blue), centromere antibody ACA (red), and their merged images. Both GFP-Skp1 and endogenous Skp1 are localized to the midbody during cytokinesis. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.)

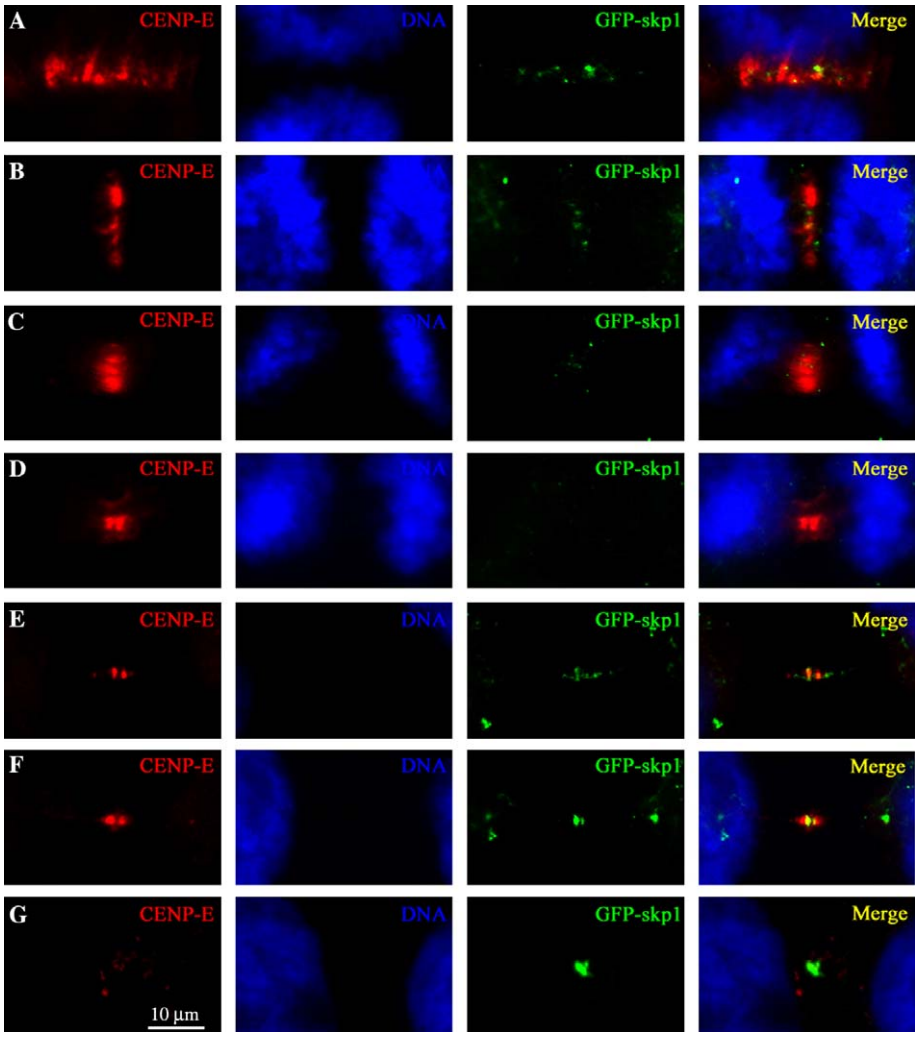

Fig. 2. Cell cycle regulated localization of skp1. This set of montage represents optical images collected from a group of HeLa cells, transited from interphase to late telophase, triply stained for rabbit anti-CENP-E antibody (CENP-E, red), DAPI (DNA, blue), mouse GFP antibody (GFP-Skp1, green), and their merged images. CENP-E antibody stained midbody of telophase HeLa cells as a pair of resolved double dots (D). GFP-Skp1 staining also appears as a pair of clearly resolved double dots (green; D). A merge image shows a superimposition of Skp1 to that of CENP-E staining. In addition, this set of montage shows the amount of CENP-E was gradually reduced concomitant with the progression of cell cycle. Bars, 10 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.)

With the progression of cytokinesis, CENP-E signal was gradually reduced (Fig. 2D–G) and disappeared untill two daughter cells separate completely which indicates CENP-E was degraded efficiently during this process.

Disruption of Skp1–CENP-E interaction yields telophase cells with prolonged midbody

The appearance of Skp1 on the midbody with concomitant loss of CENP-E led us to hypothesize that Skp1 may be linked to CENP-E degradation machinery. To evaluate our hypothesis, we transfected HeLa cells with Skp1 deletion mutant lacked CENP-E-binding domain (Skp1Δ) and assayed for their respective localization profiles. Our immunofluorescence studies show that Skp1 remained its midbody localization in the absence of C-terminal 33 amino acids (Fig. 3A–D), suggesting that its interaction with CENP-E is not essential for localization of Skp1 to the midbody. However, in these Skp1 deletion mutant-expressing cells, the labeling intensity of CENP-E was markedly increased (Fig. 3A–D). Quantitation of normalized pixel intensities shows that midbody-bound CENP-E levels in Skp1Δ-expressing cells are increased to 4.3 ± 0.5-fold compared to those of wild type Skp1-expressing cells. CENP-E labeling with a C-terminal antibody gave similar value, validating that increased midbody labeling of CENP-E in Skp1Δ-expressing cells is not related to increased accessibility of antibody labeling. Therefore, we conclude that uncoupling CENP-E–Skp1 interaction stabilized midbody-bound CENP-E.

Careful examination of those Skp1Δ-expressing cells also revealed that a high proportion of elongated midbodies

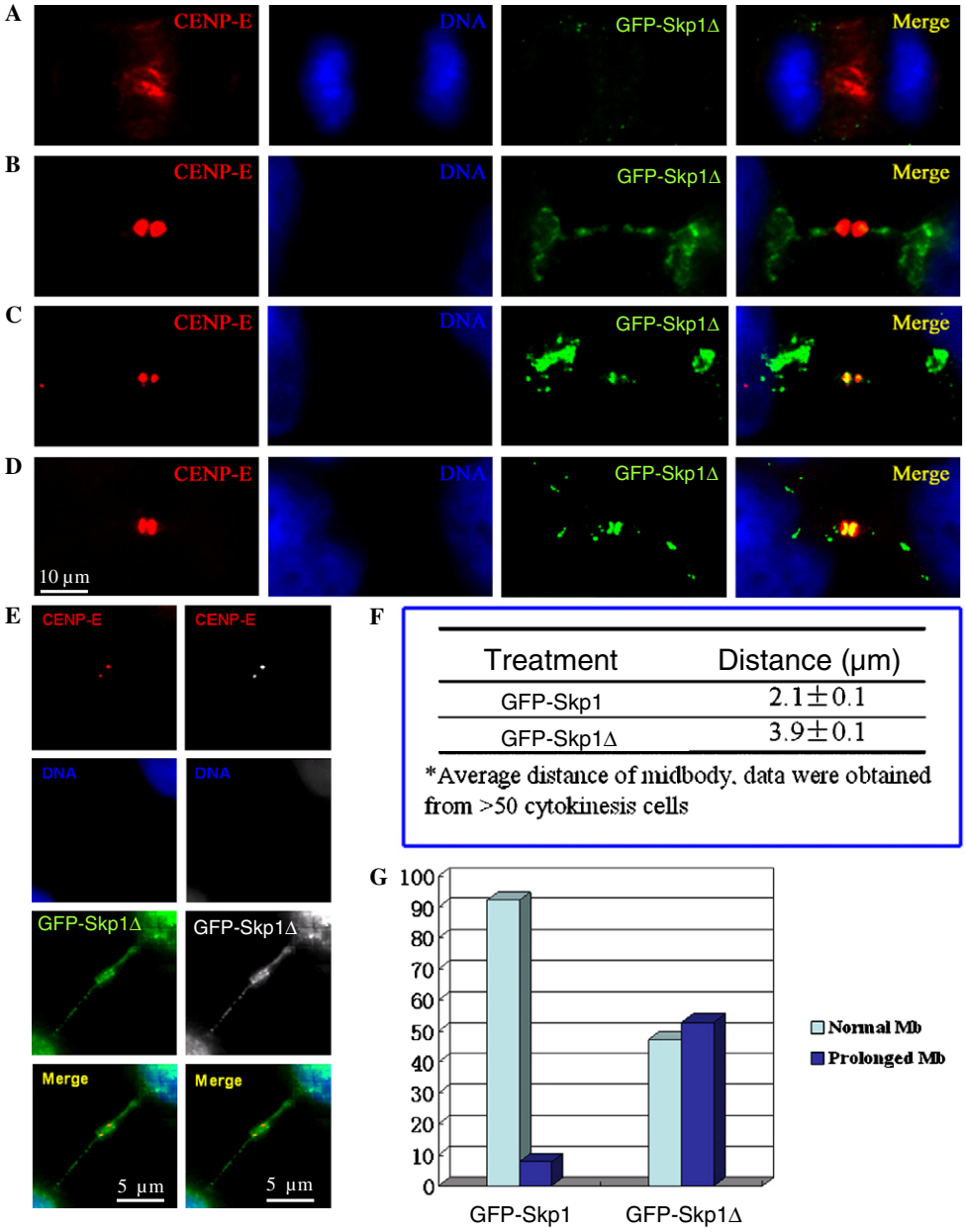

Fig. 3. Disruption of Skp1–CENP-E interaction stabilizes CENP-E protein at the midbody HeLa cells were transfected with GFP-Skp1Δ plasmids and synchronized as described in Materials and methods. This set of optical images collected from HeLa cells triply stained for GFP-Skp1Δ (green), DAPI (blue), CENP-E (red), and their merged images. Midbody-bound CENP-E accumulation in Skp1Δ-expressing cells caused extension of midbody bridge 2–3 times longer than that of normal cells. Midbody bridge was calculated based on the distance between the two inter-connecting sister cells. Quantitative data were obtained from 50 randomly selected cells in late telophase transfected with wild type GFP-Skp1 and GFP-Skp1Δ, respectively. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.)

extending 2–3 times to that of normal cells with markedly increased CENP-E labeling in the middle (Fig. 3E and G), indicating that stabilization of midbody-bound CENP-E is concomitant with cytokinesis delay. Measurement of midbody length shows that Skp1Δ-expressing cells have an extended length of cell bridge (3.9 ± 0.3 μm) compared to those of wild type Skp1-expressing cells (2.1 ± 0.2 μm) based on counting more than 50 GFP-Skp1 positively transfected cells. Thus, we conclude that Skp1 is essential for destabilizing midbody-bound CENP-E.

Suppression of Skp1 level led to CENP-E accumulation and aberrant cytokinesis

To validate the function of Skp1 in cytokinesis, siRNA oligonucleotide was transfected in HeLa cells. Trial experiments revealed an optimal concentration of 50 nM for suppressing Skp1 protein accumulation. Western blot analysis of Skp1 protein level revealed a time-dependent suppression of Skp1 protein accumulation with a maximal inhibition reached at 36 h post-transfection (Fig. 4B;

Skp1). Significantly, reduction of Skp1 protein level is concomitant with an increase in CENP-E protein accumulation (Fig. 4B; CENP-E) while the tubulin level remained unchanged in the siRNA-treated sample, suggesting that Skp1 is responsible for CENP-E degradation. Immunocytostaining of HeLa cells treated with Skp1 siRNA oligonucleotide for 36 h revealed no apparent Skp1 labeling (Fig. 4A; lower panel) while scramble oligonucleotide effected no alteration of midbody-bound Skp1 distribution, indicating the efficacy of Skp1 protein suppression mediated by siRNA treatment.

Careful examination of those Skp1-depleted cells revealed failure in mitotic exit. Those Skp1-suppressed cells failed to undergo cytokinesis and resulted in two rounds of nuclear division without cytoplasmic division (Fig. 4C). These chronically arrested cells underwent apoptosis (Liu et al., unpublished observation), suggesting that faithful separation of cells is essential for cell health. Our immunofluorescence studies indicated that these Skp1-depleted cells underwent nuclear division in the presence of cross-bridge between the two daughter cells (Fig. 4D; upper inset). Interestingly, two daughter cells become asynchronized in these Skp1-depleted cells. We, therefore, concluded that Skp1 is essential for a faithful cytokinesis.

Disruption of Skp1–CENP-E interaction led to abnormal cytokinesis

To validate whether Skp1–CENP-E interaction is essential for cytokinesis, we transfected GFP-Skp1Δ plasmid into HeLa cells and assayed for any phenotypic changes associated with disruption of Skp1–CENP-E interaction. Trial experiments show that GFP-Skp1Δ was unable to pull down CENP-E, confirming the disruption of Skp1–CENP-E interaction. As shown in Fig. 4E, GFP-Skp1Δ-expressing cells display a phenotype similar to that seen in the Skp1-suppressed cells. For example, the daughter cells enter next cycle of mitosis while the tie between two daughter cells remains. Immunofluorescence staining revealed

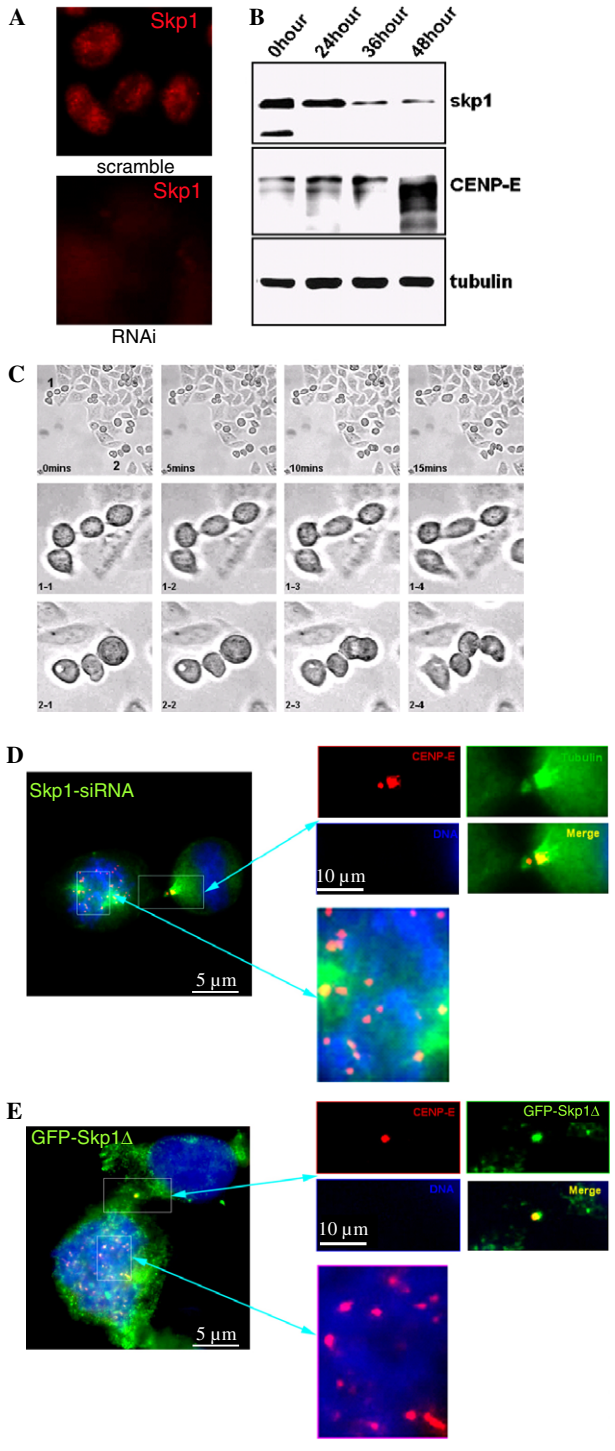

Fig. 4. Suppression of Skp1 effects accumulation of midbody-bound CENP-E and aberrant cytokinesis. (A) Indirect immunofluorescence with Skp1 monoclonal antibody (red) and DAPI (blue) staining clearly indicates Skp1 level has been markedly reduced after treatment with Skp1 siRNA oligonucleotide for 48 h. A scramble oligonucleotide was used as a control. Bar, 10 μm. (B) Time course of RNA interference of Skp1. HeLa cells were transfected with 50 nM Skp1 siRNA oligonucleotide for different intervals and subjected to SDS–PAGE and immunoblotting. Upper panel, immunoblot against Skp1; middle panel, immunoblot of CENP-E; lower panel, immunoblot against tubulin. A scramble oligonucleotide was used as a control. Note the reduction of Skp1 is concomitant with an increase in CENP-E accumulation. (C) Suppression of Skp1 level by siRNA oligonucleotide led to aberrant nuclear division without cytoplasmic separation. Forty hours after treatment of Skp1 siRNAoligonucleotide, real-time images of HeLa cell division were acquired every 5 min (movies in supplemented data clearly indicate suppression of skp1 level leads to abnormal cytokinesis). (D) HeLa cells were treated with Skp1 siRNA oligonucleotide and assayed for phenotypic change associated with Skp1 suppression. This set of optical images collected from HeLa cells triply stained for tubulin (green), DAPI (blue), CENP-E (red), and their merges. The Skp1-depleted cells underwent nuclear division in the presence of cross-bridge between the two daughter cells. The resulting two interconnected daughter cells become asynchronized in these Skp1-depleted cells. Bar, 5 μm. (E) HeLa cells were transfected with GFP-Skp1Δ plasmids and stained as described in Fig. 3. This set of optical images collected from HeLa cells triply stained for GFP-Skp1Δ (green), DAPI (blue), CENP-E (red), and their merged images. The Skp1Δ-expressing cell underwent nuclear division in the presence of cross-bridge between the two daughter cells. Bar, 5 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.)

that CENP-E was accumulated at the elongated midbodies, which is consistent with the siRNA experiments. Thus, Skp1–CENP-E interaction is necessary for CENP-E degradation that is a prerequisite for faithful cytokinesis.

Discussion

It has been previously speculated that CENP-E will be degraded via ubiquitin-mediated pathway [5], however, the molecular machinery, in particular the E3 ligase, has not been defined to date. Our effort to identify interacting partners for CENP-E discovered Skp1, a yeast kinetochore protein. Interestingly, our studies show that human Skp1 is mainly located to the midbody of mitotic cells, which is co-distributed with CENP-E in telophase. We further provided functional evidence that Skp1–CENP-E interaction is essential for a faithful cytokinesis as aberrant accumulation of CENP-E at the midbody disrupts cell separation. Our studies support the notion that cell cycle-regulated proteolysis of CENP-E is likely involved in SCF.

Previous research has indicated "DSGXXS" is very important for β-Trcp to recognize its substrate [19]. Examination of CENP-E amino acid sequence revealed "DSRSKS" motif located to the C-terminal tail of CENP-E (Fig. 5), which is similar to the recognition motif of β-Trcp. Based on this "DSRSKS" motif in CENP-E, we generated a working model accounting for the association of CENP-E to Skp1 and another unknown F-box protein (Fig. 5) for proteolysis at the midbody.

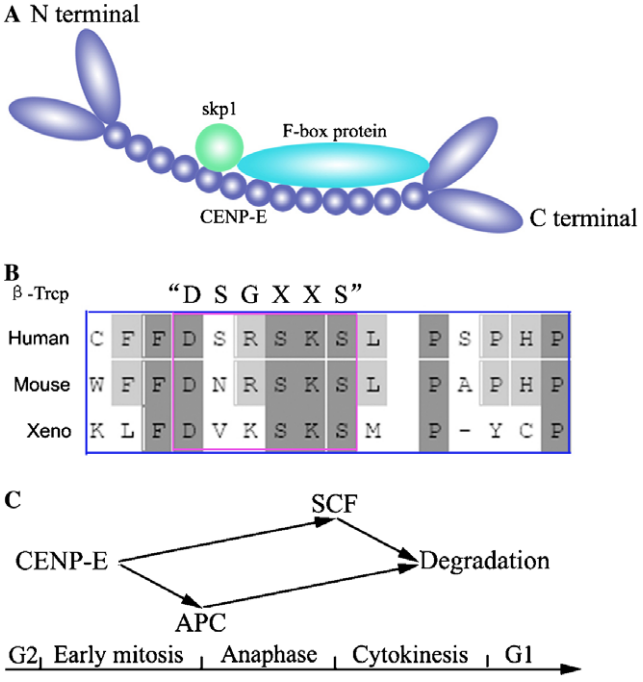

Fig. 5. Working model accounting for Skp1–CENP-E interaction at the midbody. (A) The hypothetic model illustrates Skp1–F-box complex accessory to CENP-E molecule. (B) The "DSRSKS" region (human 2608–2613 aa) is highly similar to the β-Trcp "DSGXXS" motif, which suggests that the C terminus of CENP-E may link SCF complex for CENP-E proteolysis in late telophase. (C) Potential mechanisms underlying CENP-E degradation. APC/C is likely responsible for CENP-E degradation at anaphase to telophase, whereas SCF governs the proteolysis of CENP-E from telophase to cytokinesis. Accumulated CENP-E due to the loss of Skp1 stimulates microtubule bundling and midbody compaction. In normal cells, Skp1's accumulation at midbody triggers CENP-E proteolysis via SCF complex, which promotes the dissociation of microtubule bundles for disposition of midbody prior to cytokinesis.

It was showed that CDC25A, an important CDK regulator, is destroyed via two pathways in a temporally regulated manner. At the end of mitosis and early G1, CDC25A is degraded via an APC/C-mediated pathway, whereas SCF controls CDC25A proteolysis in interphase [20,21]. CENP-E degradation could possibly be regulated by two pathways in a similar temporal manner. During metaphase–anaphase transition, APC/C is activated for releasing the tie between sister chromatids when CENP-E levels decline [5,22]. Since CENP-E contains D-box, it is possible that APC/C binds to the D-box and subsequently catalyzes CENP-E degradation via an ubiquitin-mediated pathway during anaphase. Proteolysis of CENP-E may adapt to SCF-mediated proteolysis during cytokinesis as the APC/Cdc20 level declines (e.g., Fig. 5C; ref. [22]). It has been predicted that Skp1 binds to Skp2 via its COOH terminal 66 aa (97–163 aa) based on crystal structure data of Skp1–Skp2 complex [23], while GST pull-down experiment demonstrated the importance of Skp1 N-terminus for a Skp1–Skp2 association [24]. Our experiment in which expression of Skp1 deletion mutant did not disrupt the Skp1–Skp2 interaction and effect any cell cycle arrest indicated that the most C-terminal 33 amino acids are not involved in Skp1–Skp2 interaction. It would be of great interest to evaluate precisely how SCF complex governs CENP-E proteolysis at the midbody.

One interesting phenotype revealed in this study is the failure in midbody elimination in the presence of accumulated CENP-E. Our early electron microscopic study suggested functional importance of CENP-E in midzonal microtubule bundling [2], which may stabilize the midbody structure. Several lines of evidence support the roles of other proteins such as Plk1, Mklp2, and Aurora-B in cytokinesis [25,26]. The midbody is composed of highly packed anti-parallel microtubules associated with large collection of accessory proteins, the molecular mechanism underlying midbody dynamics and regulation remains elusive despite a recent proteomic dissection of human midbody complex [27]. Additional functional analyses will be required to delineate the molecular dynamics of midbody in mitosis. Our present study demonstrates functional importance of midbody-bound CENP-E–Skp1 interaction in faithful cell division.

Taken together, our finding of the interaction of CENP-E with Skp1 demonstrates a critical role of Skp1 in midbody dynamics. The fact that elimination of Skp1 disrupts proteolysis of CENP-E at the midbody, abrogates cytokinetic checkpoint arrest, and induces prolonged midbody cross-bridges in HeLa cells prematurely exited from cytokinesis demonstrates the importance of Skp1 in faithful cell division.

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Appendix A. Supplementary data

The movie clearly indicates Skp1 depleted cells exhibit abnormal cytokinesis. Two daughter cells have not been separated completely, but entered next mitosis with a connection to each other. Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2006.04.062.

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