

Multiple Anaphase-promoting Complex/Cyclosome Degrons Mediate the Degradation of Human Sgo1*

Received for publication, September 11, 2008, and in revised form, November 17, 2008. Published, JBC Papers in Press, November 17, 2008, DOI 10.1074/jbc.M807083200

Zemfira Karamysheva, Laura A. Diaz-Martinez, Sara E. Crow, Bing Li, and Hongtao Yu¹

From the Department of Pharmacology, Howard Hughes Medical Institute, University of Texas Southwestern Medical Center at Dallas, Dallas, Texas, 75390

Shugoshin 1 (Sgo1) protects centromeric sister-chromatid cohesion in early mitosis and, thus, prevents premature sister-chromatid separation. The protein level of Sgo1 is regulated during the cell cycle; it peaks in mitosis and is down-regulated in G₁/S. Here we show that Sgo1 is degraded during the exit from mitosis, and its degradation depends on the anaphase-promoting complex/cyclosome (APC/C). Overexpression of Cdh1 reduces the protein levels of ectopically expressed Sgo1 in human cells. Sgo1 is ubiquitinated by APC/C bound to Cdh1 (APC/C^{Cdh1}) *in vitro*. We have further identified two functional degradation motifs in Sgo1; that is, a KEN (Lys-Glu-Asn) box and a destruction box (D box). Although removal of either motif is not sufficient to stabilize Sgo1, Sgo1 with both KEN box and D box deleted is stable in cells. Surprisingly, mitosis progresses normally in the presence of non-degradable Sgo1, indicating that degradation of Sgo1 is not required for sister-chromatid separation or mitotic exit. Finally, we show that the spindle checkpoint kinase Bub1 contributes to the maintenance of Sgo1 steady-state protein levels in an APC/C-independent mechanism.

Loss of sister-chromatid cohesion triggers chromosome segregation in mitosis and occurs in two steps in vertebrate cells (1–3). In prophase, cohesin is phosphorylated by mitotic kinases including Plk1 and removed from chromosome arms (1, 4). Then, cleavage of centromeric cohesin by separase takes place at the metaphase-to-anaphase transition to allow sister-chromatid separation (5). The shugoshin (Sgo) family of proteins plays an important role in the protection of centromeric cohesion (6, 7). Human cells depleted of Sgo1 by RNAi undergo massive chromosome missegregation (8–11). In cells with compromised Sgo1 function, centromeric cohesin is improperly phosphorylated and removed (4, 11), resulting in premature sister-chromatid separation. It has been shown recently that Sgo1 collaborates with PP2A to counteract the action of Plk1 and other mitotic kinases and to protect centromeric cohesin from premature removal (12–14). In

addition, Sgo1 has also been shown to promote stable kinetochore-microtubule attachment and sense tension across sister kinetochores (8, 15). Thus, Sgo1 is crucial for mitotic progression and chromosome segregation.

Orderly progression through mitosis is regulated by the anaphase-promoting complex/cyclosome (APC/C),² a large multiprotein ubiquitin ligase that targets key mitotic regulators for destruction by the proteasome (16). APC/C selects substrates for ubiquitination by using the Cdc20 or Cdh1 activator proteins to recognize specific sequences called APC/C degrons within target proteins (17). Several APC/C degrons have been characterized, including the destruction box (D box) and the Lys-Glu-Asn box (KEN box) (18, 19). The D box, with the consensus amino acid sequence of RXXLXXXN (X indicates any amino acid), are found in many APC/C substrates, including mitotic cyclins and are essential for their ubiquitin-mediated destruction. The KEN box, which contains a consensus KEN motif, is also found in several APC/C substrates and is preferentially but not exclusively recognized by APC/C^{Cdh1}. When APC/C is active, it directs progression through and exit from mitosis by catalyzing the ubiquitination and timely destruction of mitotic regulators, including cyclin A, cyclin B, and the separase inhibitor securin (16). The APC/C activity needs to be tightly controlled to prevent unscheduled substrate degradation. An important mechanism for APC/C regulation is the spindle checkpoint, which prevents the activation of APC/C and destruction of its substrates in response to kinetochores that have not properly attached to the mitotic spindle (20).

Recent evidence shows that Sgo1 is a substrate of APC/C, and its protein levels oscillate during the cell cycle (8, 9). In this article we study the degradation of Sgo1 in human cells. We show that Sgo1 is degraded during mitotic exit, and this degradation depends on APC/C^{Cdh1}. We further show that both KEN and D boxes are required for Sgo1 degradation *in vivo* and ubiquitination *in vitro*. Removal of these motifs stabilizes Sgo1 *in vivo*. The prolonged presence of stable Sgo1 protein in human cells does not change the kinetics of chromosome segregation and mitotic exit. Therefore, a timely scheduled degradation of Sgo1 takes place but is not required for mitotic exit. Finally, we show that Bub1 regulates Sgo1 protein levels through a mechanism that does not involve APC/C-mediated degradation.

* This work was supported, in whole or in part, by National Institutes of Health Grant GM76481. This work was also supported by the March of Dimes Foundation, the Welch Foundation, and the Leukemia and Lymphoma Society. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Author's Choice—Final version full access.

¹ An Investigator at the Howard Hughes Medical Institute. To whom correspondence should be addressed: Dept. of Pharmacology, UT Southwestern Medical Center, 6001 Forest Park Rd, Dallas, TX 75390-9041. Tel.: 214-645-6161; Fax: 214-645-6156; E-mail: hongtao.yu@utsouthwestern.edu.

² The abbreviations used are: APC/C, anaphase-promoting complex/cyclosome; APC/C^{Cdh1}, the complex between APC/C and Cdh1; D box, destruction box; KEN box, lysine-glutamate-asparagine box; RNAi, RNA interference; HA, hemagglutinin.

EXPERIMENTAL PROCEDURES

Antibodies and Immunoblotting—The production of α -Sgo1 and α -APC2 antibodies was described previously (9, 21). The following antibodies were purchased from commercial sources: CREST (ImmunoVision), α -cyclin B1 (Santa Cruz Biotechnology), α -HA and α -Myc (Roche Applied Science). For immunoblotting, the antibodies were used at 1:2000 dilution for crude sera or 1 μ g/ml for purified IgG.

Mammalian Cell Culture, Drug Treatments, and Transfection—HeLa Tet-On (Clontech) cells were grown in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% fetal bovine serum and 10 mM L-glutamine. To arrest cells at G₁/S, cells were incubated in growth medium containing 2 mM thymidine (Sigma) for 18 h. To arrest cells in mitosis, cells were treated with 300 nM nocodazole (Sigma) for 16–18 h or as indicated. Cycloheximide was added to the medium when needed at a concentration of 50 μ M.

Plasmid transfection was performed when cells reached 50% confluency using the Effectene reagent (Qiagen) according to the manufacturer's protocols. The plasmids were derived from pCS2-Myc or pCS2-HA. For RNAi experiments, HeLa cells were transfected using Lipofectamine RNAiMax (Invitrogen) according to manufacturer's protocols and analyzed 36–48 h after transfection. For RNAi experiments, the siRNA were chemically synthesized at Dharmacon. The sequences of the siRNAs used in this study are Bub1-targeting siRNA oligonucleotide (GAGUGAUCACGAUUUCUAUTT) and Sgo1 siRNA (GAGGGGACCCUUUACAGATT).

To establish stable cell lines, HeLa Tet-On cells were transfected with pTRE2-Myc based plasmids encoding Myc-Sgo1^{WT} and Myc-Sgo1 ^{Δ KEN/ Δ DB2}, and selection of stable clones was performed in the presence of 300 μ g/ml hygromycin. The surviving clones were screened for the induced expression of corresponding Myc-tagged proteins in the absence or presence of 1 μ g/ml doxycycline (Clontech).

Immunofluorescence and Metaphase Chromosome Spreads—Mitotic cells were harvested by shake-off and swelled in a pre-warmed hypotonic solution containing 55 mM KCl for 15 min at 37 °C. Cells were spun onto microscope slides at 1500 rpm for 4 min using a Shandon Cytospin centrifuge. Chromosomes were fixed and stained as described (12). Images were acquired and processed with the Slidebook software (Intelligent Imaging) and pseudocolored in Photoshop. A series of z-stack images were captured at 0.2–0.3- μ m intervals and deconvolved using the nearest neighbor algorithm. The maximum z-projection is then created for the deconvolved images. For the analysis of chromosome morphology, a Giemsa staining protocol was used as described (22).

In Vitro Translation and Ubiquitination Assays—The *in vitro* transcription and translation system was purchased from Promega, and the reactions were performed according to the manufacturer's instructions. Briefly, 80 ng of plasmid DNA, nuclease-free water, 0.2 μ l of [³⁵S]methionine (10 μ Ci/ μ l), and 4 μ l of rabbit reticulocyte lysate were mixed to give a final volume of 5 μ l. The reaction mixture was incubated at 30 °C for 90 min.

The interphase APC/C was purified from interphase *Xenopus* egg extracts using anti-APC3/Cdc27 antibody coupled to

protein A support as described (23, 24). The expression and purification of human Cdc20 and Cdh1 proteins from Sf9 cells were described previously (23, 25). Each ubiquitination assay contained a 5- μ l mixture of an energy-regenerating system, 150 μ M ubiquitin, 5 μ M recombinant ubiquitin-activating enzyme, 2 μ M recombinant UbcH10, 1 μ l of *in vitro* transcribed and translated substrates, and 3 μ l of the APC/C beads.

Live-cell Imaging—For live-cell imaging, eGFP-H2B-expressing cells were plated in 4-well chambered coverslips (LabTek) and synchronized in early S-phase by thymidine arrest for 18 h and released into fresh medium just before filming. Doxycycline was added to induce Sgo1 expression 24 h before filming. Cells were imaged in CO₂-independent medium (Invitrogen) at 37 °C using a Deltavision microscope. 3 z-stacks were acquired every 2 min for 24 h. Image manipulations were performed in ImageJ (contrast enhancement, cropping, and conversion to Quicktime).

RESULTS

Sgo1 Is Degraded during Mitotic Exit, and Its Degradation Depends on Cdh1—Previous studies have shown that the protein level of Sgo1 in nocodazole-arrested mitotic cells is higher than that of Sgo1 in cells enriched in G₁/S phase by thymidine arrest (9), suggesting that Sgo1 levels are regulated during the cell cycle. To confirm this finding, we examined Sgo1 steady-state levels in synchronized cell cultures. HeLa cells were arrested in mitosis by nocodazole treatment and released into fresh medium. Then cells were harvested at different time points, and Sgo1 protein levels were analyzed by Western blotting. The Sgo1 protein level was high in mitosis but dropped sharply upon nocodazole release (Fig. 1A). In contrast, the protein level of Apc2 remained unchanged, serving as a loading control. The Sgo1 profile during this experiment was similar to that of cyclin B1. Many proteins, including cyclin B1, undergo rapid degradation during mitotic exit to coordinate exit from mitosis. A sharp decrease in Sgo1 protein level suggests that this protein is degraded during mitotic exit.

In addition to the band at about 80 kDa that corresponded to the full-length Sgo1, we observed two additional bands of smaller sizes (Fig. 1A). It is well documented that Sgo1 has several isoforms produced by alternative splicing (11). These two smaller protein bands had sizes corresponding to the proteins produced from two major Sgo1 splice variants, suggesting that they very likely were Sgo1 isoforms (26, 27). Their protein levels were reduced upon release from nocodazole in the same fashion as the full-length Sgo1, suggesting that they too were degraded during mitotic exit.

Many proteins degraded during mitotic exit, such as cyclin B1, Bub1, or Plk1, are substrates for APC/C^{Cdh1}. The *Xenopus* Sgo1 protein has been shown to be a substrate of APC/C (8). We next tested that human Sgo1 was a substrate of APC/C^{Cdh1}. We transfected HeLa cells with Myc-Sgo1 in the absence or presence of HA-Cdh1. Overexpression of Cdh1 greatly reduced the protein levels of Myc-Sgo1, supporting that Sgo1 was a substrate for APC/C^{Cdh1} (Fig. 1B, lanes 1 and 2).

APC substrates generally contain cis-elements, such as D boxes or KEN boxes, required for their ubiquitination. We asked whether the Sgo1 protein also had those elements. We

Sgo1 Contains Multiple APC/C Degrons

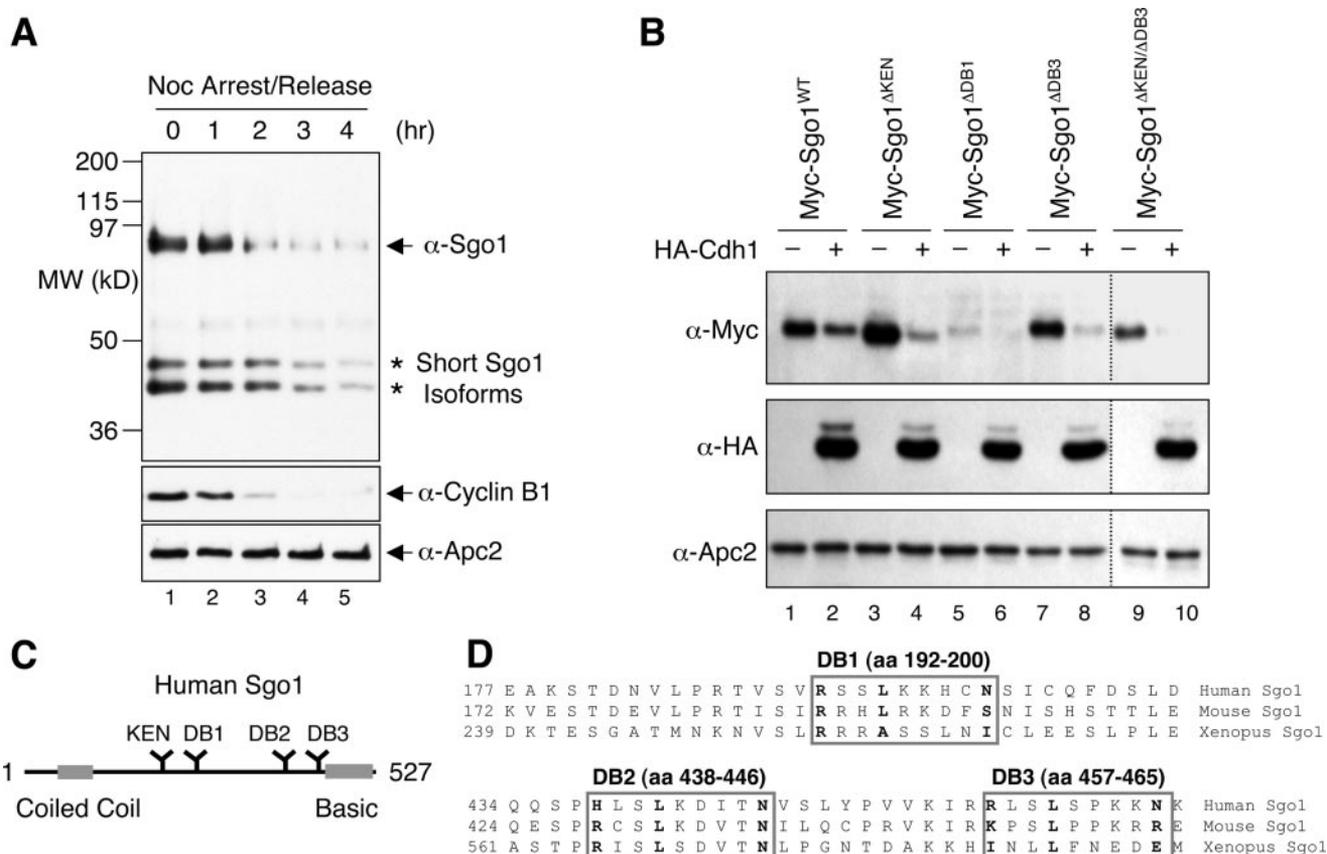


FIGURE 1. The protein levels of Sgo1 decrease rapidly during mitotic exit. *A*, HeLa cells were arrested in mitosis by nocodazole (*Noc*), released into fresh growth medium, and collected at the indicated time points. The cell lysates were immunoprecipitated with anti-Sgo1, and the anti-Sgo1 immunoprecipitates were blotted with anti-Sgo1. The cell lysates were also blotted with anti-cyclin B1 and anti-Apc2 (loading control). Asterisks indicate putative Sgo1 isoforms. *B*, HeLa cells were transfected with the indicated Sgo1 plasmids with or without the HA-Cdh1 construct. The cell lysates were blotted with the indicated antibodies. *C*, domain structure of human Sgo1. The putative APC/C degrens, the coiled coil motif, and the basic domain are indicated. *D*, sequence alignment of the C-terminal regions of human, mouse, and *Xenopus* Sgo1 proteins, showing the conservation of the putative D box 2 (DB2) but not DB1 or DB3.

carefully inspected the sequence of Sgo1 and identified a putative KEN box corresponding to residues 310–312 of human Sgo1. This motif is not conserved in mouse and *Xenopus* Sgo1. We also found two putative D boxes with a consensus motif of RXXLXXXN in human Sgo1 at residues 192–200 and 457–465 (Fig. 1, *C* and *D*). Both mouse and *Xenopus* Sgo1 proteins contain an RXXLXXXN motif that corresponds to residues 438–446 in human Sgo1, except that human Sgo1 contains a histidine instead of an arginine at position 438. We decided to consider residues 438–446 as a putative D box in human Sgo1. We named it D box 2 (DB2), whereas two others were named D box 1 (DB1) and D box 3 (DB3) accordingly (Fig. 1*D*). DB2 and DB3 are located next to each other in the C-terminal portion of Sgo1. DB1 is in the middle portion of the protein. DB1 and DB3 are not conserved in mouse and *Xenopus* Sgo1, whereas DB2 is conserved with the exception of the first arginine (Fig. 1*D*).

To determine whether these motifs were important for Sgo1 degradation, we first made mutants with either DB1 or DB3 deleted (Δ DB1 or Δ DB3), as they both had a perfect consensus motif. We also made a mutant with the KEN box deleted (Δ KEN) and a double mutant with both DB3 and the KEN box deleted (Δ KEN/ Δ DB3). HeLa cells were transfected with these Myc-Sgo1 mutant constructs with or without the HA-Cdh1 expression vector, and the protein levels were examined by Western blotting. The protein levels of all Sgo1 mutants were

still reduced by Cdh1 overexpression (Fig. 1*B*). These data suggest that either those motifs are not functional, or deletion of each motif alone may not be sufficient to stabilize Sgo1. It is also possible that DB2 or other degradation motifs may mediate Sgo1 degradation.

Degradation of Sgo1 Requires Both KEN- and D-box Motifs—To systematically identify what regions of Sgo1 were important for its degradation, we made three constructs containing N-terminal (Sgo1N), middle (Sgo1M), or C-terminal (Sgo1C) fragments of Sgo1 (Fig. 2*A*). Sgo1N does not contain putative degradation motifs. Sgo1M has the putative KEN and DB1 motifs (Fig. 1*D*). Sgo1C has the putative DB2 and DB3 motifs. These constructs were expressed in HeLa cells in the absence or presence of Cdh1 overexpression. Myc-Sgo1N was stable even with overexpression of Cdh1 (Fig. 2*B*, lanes 1 and 2), consistent with the lack of putative degradation motifs in this region. By contrast, the protein levels of both Myc-Sgo1M and Myc-Sgo1C were reduced by Cdh1 overexpression, suggesting that both fragments might be substrates of APC/C^{Cdh1} and that Sgo1 might have multiple degradation motifs (Fig. 2*B*, lanes 3, 4, 9, and 10).

To determine which degradation motifs in Sgo1M were functional, we made two Sgo1M constructs lacking either KEN box or DB1 and examined their protein levels in HeLa cells (Fig. 2*B*, lanes 5–8). Deletion of the KEN box, but not DB1, stabilized

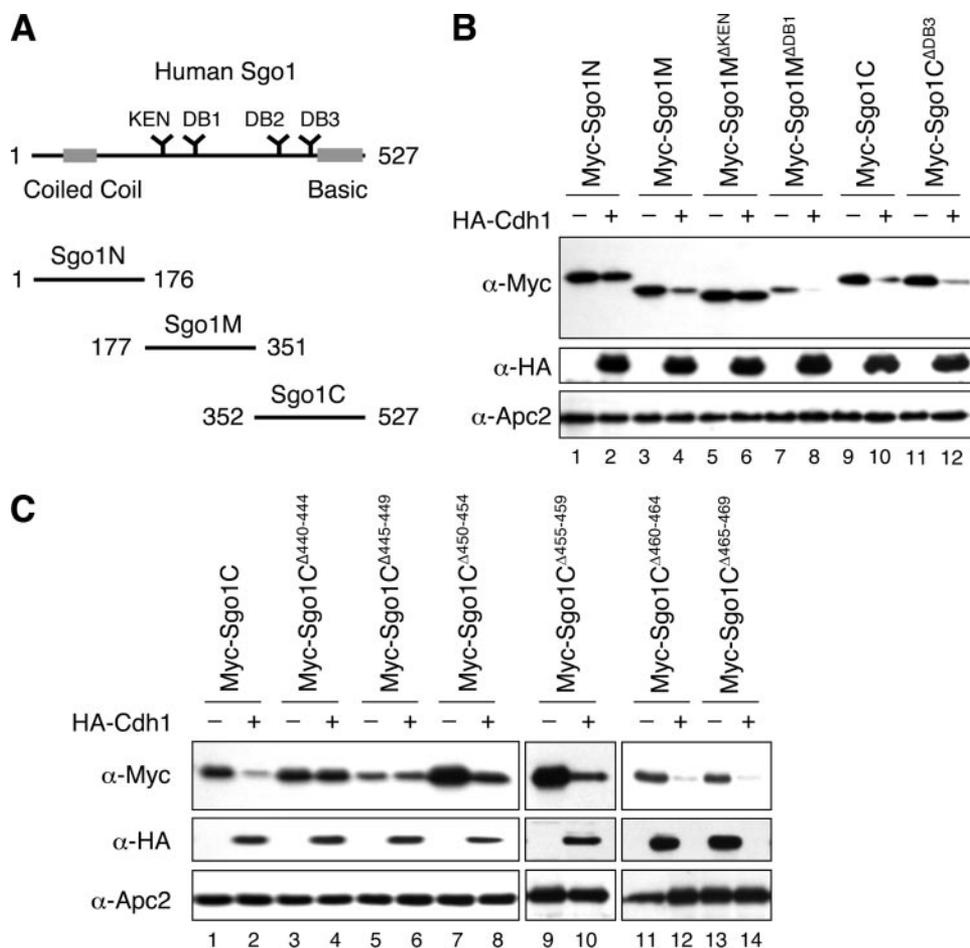


FIGURE 2. Sgo1 degradation induced by Cdh1 overexpression depends on both its KEN and D boxes. *A*, schematic diagram of the boundaries of the N-terminal (Sgo1N), middle (Sgo1M), and C-terminal (Sgo1C) fragments of Sgo1. *B*, HeLa cells were transfected with the indicated Sgo1 plasmids with or without the HA-Cdh1 construct. The cell lysates were blotted with the indicated antibodies. *C*, HeLa cells were transfected with the indicated Sgo1 plasmids with or without the HA-Cdh1 construct. The cell lysates were blotted with the indicated antibodies.

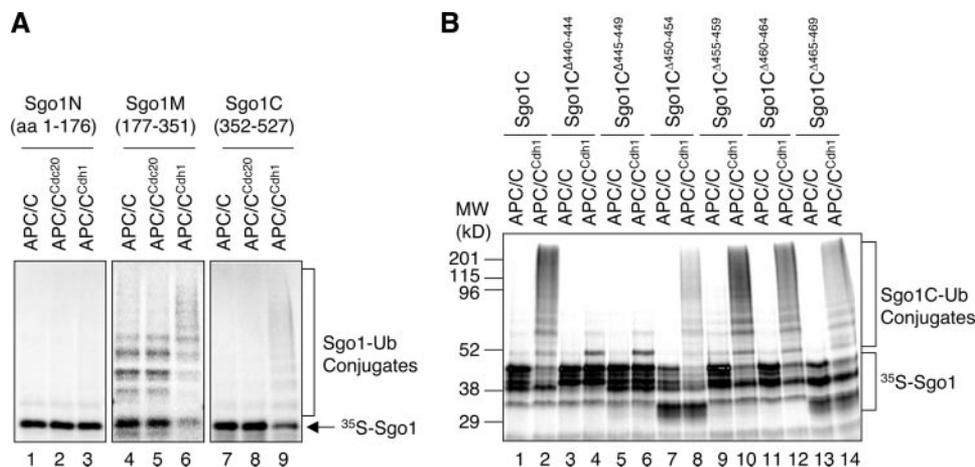


FIGURE 3. Ubiquitination of Sgo1 by APC/C^{Cdh1} *in vitro* depends on its KEN and D boxes. *A*, APC/C was immunoprecipitated from *Xenopus* egg extracts using anti-APC3/Cdc27 antibody beads and supplemented with recombinant Cdc20 or Cdh1. Sgo1N, Sgo1M, and Sgo1C were *in vitro* translated in the presence of [³⁵S]methionine and used as substrates in the ubiquitination reactions. The reaction mixtures were separated by SDS-PAGE and analyzed using phosphorimaging. *B*, same as in *A* except that Sgo1C deletion mutants were used as substrates for ubiquitination with or without Cdh1.

Sgo1M in the presence of Cdh1 overexpression. Thus, degradation of Sgo1M depends on the KEN box. The KEN box is a functional APC/C degro capable of driving efficient degrada-

tion of Sgo1M. Removal of this motif alone, however, was insufficient to stabilize full-length Sgo1 protein in the presence of overexpressed Cdh1 (Fig. 1*B*, lanes 3 and 4), suggesting that Sgo1 might have several degradation motifs. Consistently, Sgo1C underwent Cdh1-dependent degradation (Fig. 2*B*, lanes 9 and 10), suggesting that an additional degradation motif(s) was present in this region. Deletion of the putative DB3 did not stabilize Sgo1C in the presence of Cdh1 overexpression (Fig. 2*B*, lanes 11 and 12), indicating that this motif was not functional. To determine whether DB2 was critical for the degradation of Sgo1C, we made several constructs of Sgo1C with consecutive deletions of 5 residues in the region containing residues 440–469 in Sgo1. Overexpression of Cdh1 did not reduce protein levels of Sgo1C^{Δ440–444} and Sgo1C^{Δ445–449} (Fig. 2*C*, lanes 3–6). DB2 was partially deleted in both of these mutants, indicating that DB2 was required for Cdh1-dependent degradation of Sgo1C. By contrast, other Sgo1C mutants with residues neighboring DB2 deleted were not stabilized. Consistent with the putative DB3 not being functional, two Sgo1C constructs with DB3 partially deleted, Sgo1C^{Δ455–459} and Sgo1C^{Δ460–464}, were still degraded by Cdh1 overexpression. Thus, degradation of Sgo1 in the presence of an excess amount of Cdh1 depends on both its KEN-box and DB2, suggesting that Sgo1 might be an APC/C^{Cdh1} substrate with multiple degro.

Sgo1 Is Ubiquitinated by APC/C *In Vitro*—To confirm that human Sgo1 was a substrate for APC/C, we tested whether immunoprecipitated APC/C^{Cdh1} or APC/C^{Cdc20} could catalyze ubiquitination of Sgo1 *in vitro*. The three Sgo1 fragments (Sgo1N, Sgo1M, and Sgo1C) were translated *in vitro* in the presence of [³⁵S]methionine and used as substrates in ubiquitination assays.

Sgo1N was not ubiquitinated (Fig. 3*A*, lanes 1–3), consistent with its inability to undergo Cdh1-mediated degradation in cells. Both Sgo1M and Sgo1C were efficiently ubiquitinated by

Sgo1 Contains Multiple APC/C Degrons

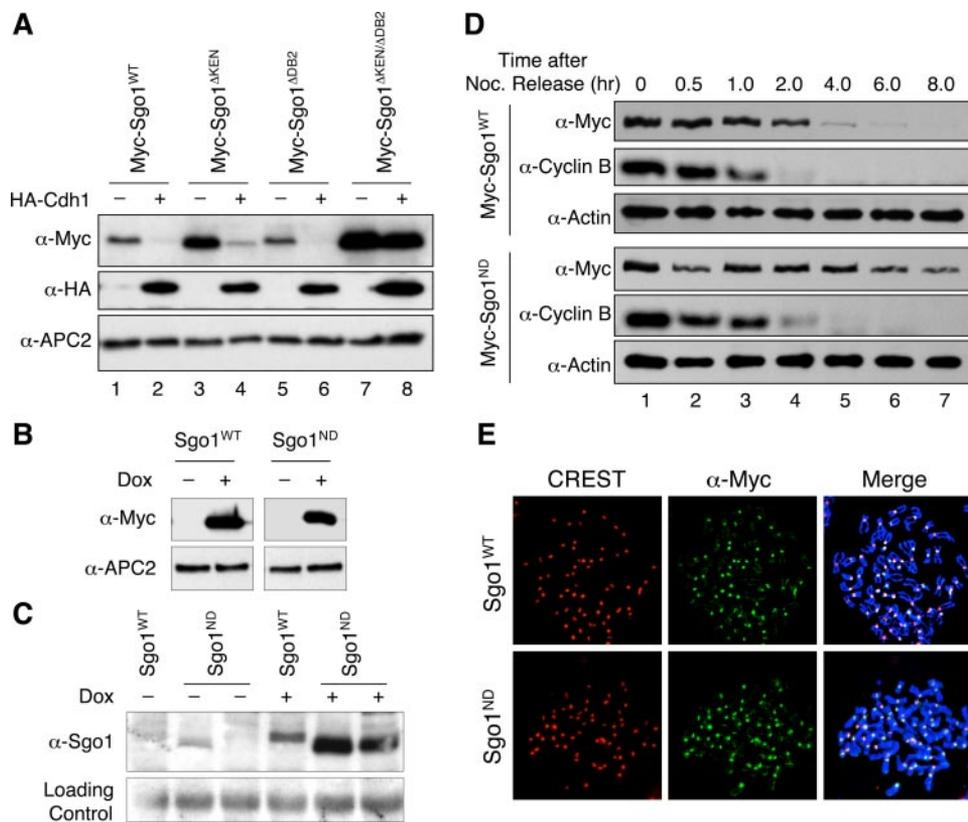


FIGURE 4. Sgo1 with its KEN and D boxes deleted is stable during mitotic exit. *A*, HeLa cells were transfected with the indicated Sgo1 plasmids with or without the HA-Cdh1 construct. The cell lysates were blotted with the indicated antibodies. *B*, HeLa Tet-on cells stably transfected with Myc-Sgo1^{WT} or Sgo1^{ΔKEN/ΔDB2} (referred to as non-degradable Sgo1 or Sgo1ND) were incubated for 24 h in the presence or absence of doxycycline (*Dox*). Cell lysates were blotted with the indicated antibodies. *C*, cell lysates in *B* and lysates of an additional Sgo1ND clone were blotted with anti-Sgo1. A cross-reacting band was used as the loading control. *D*, HeLa cells induced with doxycycline for Sgo1^{WT} or Sgo1ND expression were treated with nocodazole, released into a fresh medium containing cycloheximide, and collected at different time points. Cell lysates were blotted with the indicated antibodies. *E*, mitotic chromosome spreads of HeLa Tet-on cells stably expressing Myc-Sgo1^{WT} or Myc-Sgo1ND in the presence of doxycycline. 4,6-Diamidino-2-phenylindole staining is shown in *blue*. CREST staining is shown in *red*. Myc staining is shown in *green*.

APC/C^{Cdh1} but not by APC/C^{Cdc20} (Fig. 3*A*, lanes 4–9). Sgo1M also underwent Cdc20- and Cdh1-independent ubiquitination (Fig. 3*A*, lanes 4 and 5). The significance of this ubiquitination event was unclear and was not further pursued. Sgo1M^{ΔKEN} was not ubiquitinated (data not shown), indicating that ubiquitination of this region occurs via the KEN box. Consistent with results in Fig. 2*C*, Sgo1C^{Δ440–444} and Sgo1C^{Δ445–449} that contained deletions of DB2 were not efficiently ubiquitinated by APC/C^{Cdh1} (Fig. 3*B*, lanes 3–6). Deletions of DB3 and other residues near DB2 did not prevent the ubiquitination of Sgo1C (Fig. 3*B*, lanes 7–14). Our results indicate that Sgo1 is a substrate of APC/C^{Cdh1} *in vitro* and that it contains two functional APC/C degrens, the KEN box and DB2.

Sgo1 with the KEN Box and DB2 Deleted Is Stabilized during Mitotic Exit—We next tested whether deletion of both the KEN box and DB2 stabilized full-length Sgo1 in HeLa cells overexpressing Cdh1. As expected, deletion of either KEN or DB2 alone was not sufficient to stabilize Sgo1 in the presence of Cdh1 overexpression (Fig. 4*A*, lanes 3–6). Deletion of both motifs, however, greatly stabilized Sgo1 in cells overexpressing Cdh1 (Fig. 4*A*, lanes 7 and 8). Thus, either the KEN box or DB2 of Sgo1 is sufficient to drive its degradation in

cells with elevated levels of Cdh1. Deletion of both motifs is required to stabilize Sgo1 in cells. We will hereafter refer to the Sgo1 mutant with both KEN and DB2 deleted as non-degradable Sgo1 (Sgo1ND). We then established cell lines that stably expressed full-length Myc-tagged wild-type Sgo1 (Sgo1^{WT}) or non-degradable Sgo1 (Sgo1ND) driven by a tetracycline-inducible promoter. We verified that Sgo1 expression was induced by doxycycline in these lines (Fig. 4*B*). Blotting of the cell lysates with anti-Sgo1 readily detected Myc-Sgo1^{WT} and Myc-Sgo1ND in the presence of doxycycline but failed to detect the endogenous Sgo1 protein (Fig. 4*C*). Therefore, the expression levels of Myc-Sgo1^{WT} and Myc-Sgo1ND were higher than that of the endogenous Sgo1. The signal for endogenous Sgo1 was below the detectable level under these blotting conditions.

Next, we examined the kinetics of degradation of Myc-Sgo1^{WT} and Myc-Sgo1ND in these cells after the release from nocodazole-mediated mitotic arrest by blotting the total cell lysates with anti-Myc. As expected, Myc-Sgo1^{WT} was degraded rapidly during mitotic exit, and its degradation profile was similar to that of cyclin B1 and the

endogenous Sgo1 (Fig. 4*D*, upper panels). By contrast, Myc-Sgo1ND was degraded with a much slower kinetics, indicating that deletion of KEN and DB2 greatly impeded the degradation of Sgo1 during mitotic exit. The kinetics of cyclin B1 degradation, however, was unchanged in cells expressing Sgo1ND (Fig. 4*D*, lower panels), suggesting that timely Sgo1 degradation was not required for proper mitotic exit.

To ascertain that Sgo1ND was still functional and that the mutations did not grossly affect the functions of Sgo1, we first examined the kinetochore localization of Sgo1ND. Both Myc-Sgo1^{WT} and Myc-Sgo1ND localized normally to centromeres (Fig. 4*E*). Thus, Sgo1ND retains the ability to localize to centromeres. We next tested whether expression of Sgo1ND rescued the mitotic arrest phenotype of Sgo1 RNAi cells (Fig. 5). For this purpose, we measured the mitotic indices of Sgo1 RNAi cells transfected with Myc-Sgo1^{WT} or Myc-Sgo1ND using flow cytometry. Mitotic cells had 4N DNA content and positive MPM2 staining. As expected, depletion of Sgo1 by RNAi resulted in mitotic arrest (Fig. 5, *A* and *B*). Expression of either Sgo1^{WT} (Fig. 5*D*) or Sgo1ND (Fig. 5*F*) rescued this mitotic arrest phenotype, indicating that Myc-Sgo1ND was functional.

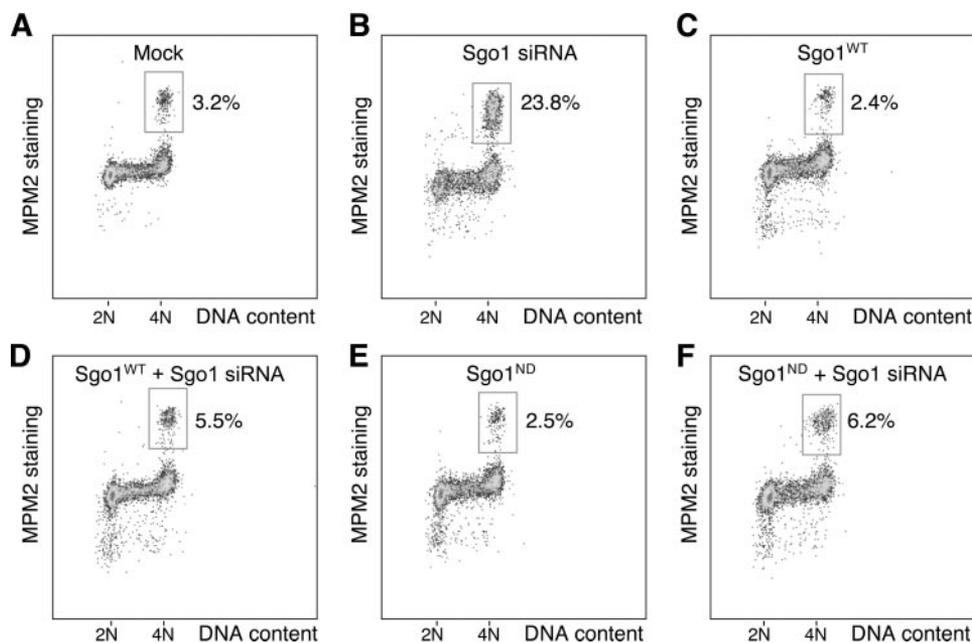


FIGURE 5. Expression of Sgo1ND rescues the mitotic arrest phenotype of Sgo1 RNAi cells. Log phase HeLa cells (A), Sgo1 RNAi cells (B), cells transfected with Myc-Sgo1^{WT} (C), cells transfected with both Sgo1^{WT} and Sgo1 siRNA (D), cells transfected with Myc-Sgo1ND (E), and cells transfected with both Sgo1ND and Sgo1 siRNA (F) were fixed and stained with the MPM2 antibody and propidium iodide and subjected to flow cytometry analysis. The mitotic cells were boxed. The mitotic indices are indicated.

Ectopic Expression of Sgo1ND Does Not Delay Chromosome Segregation—Because human Sgo1 is known to play a role in the protection of centromeric cohesion during mitosis (8–10), we asked whether Sgo1 degradation is required for proper chromosome segregation. Briefly, HeLa cells expressing Sgo1^{WT} or Sgo1ND were synchronized in mitosis by a single thymidine block followed by a 4-h incubation in nocodazole (Fig. 6A). Mitotic cells were collected by mitotic shake-off and released from the mitotic block. Samples were taken at different times. These samples were fixed with Carnoy's fixative, processed for chromosome spreads, and stained with Giemsa. Cells expressing human Sgo1^{WT} or Sgo1ND had chromosomes normally cohered at their centromeres, similar to their non-induced controls (Fig. 6B). Furthermore, the timing of sister-chromatid separation and chromosome decondensation in the induced cells was indistinguishable from their non-induced controls (Fig. 6, B and C). These data indicate that Sgo1 degradation is not required for proper sister-chromatid separation or chromosome decondensation after release from a mitotic block.

To confirm these findings, we examined the chromosome morphology and dynamics in cells overexpressing Sgo1^{WT} or Sgo1ND during unperturbed mitoses. Cells expressing Sgo1^{WT} or Sgo1ND were synchronized in early S phase by a double thymidine block and released into fresh medium (Fig. 7A). Doxycycline was added to both cell lines during the second thymidine block. Samples were taken when cells reached their peak mitotic index and analyzed as described above. Chromosome cohesion and morphology in the induced cultures were indistinguishable from their non-induced controls (Fig. 7B). All phases of mitosis were present at the expected ratios, indicating that the inability to degrade Sgo1

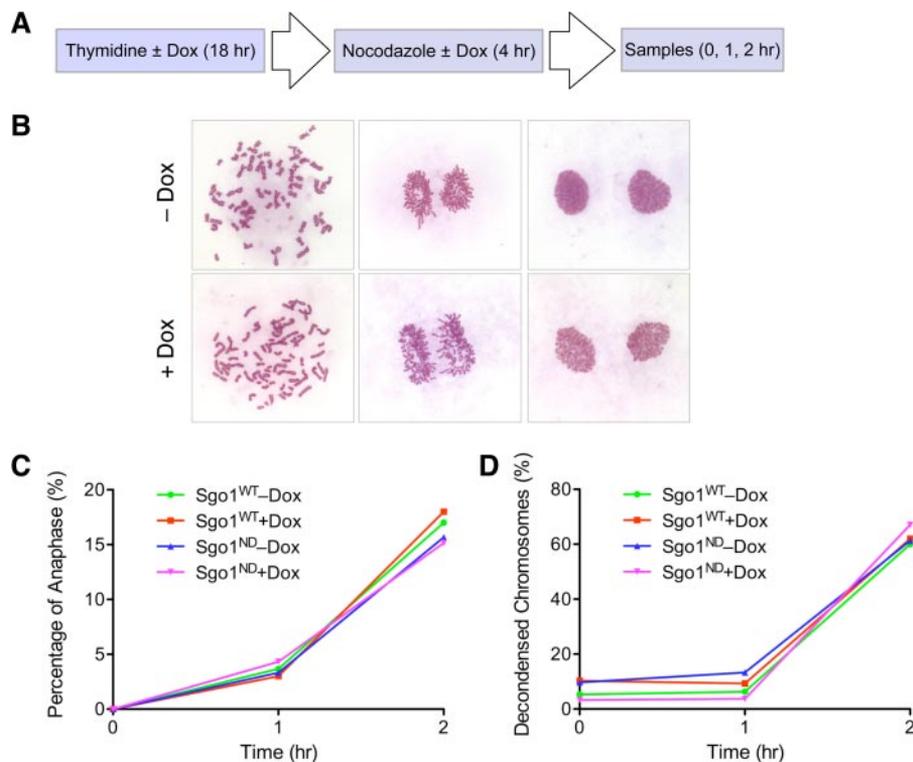


FIGURE 6. Expression of non-degradable Sgo1 (Sgo1ND) does not delay chromosome segregation after the release from nocodazole-mediated mitotic arrest. A, schematic diagram of drug treatments for experiments shown in B and C. B, three major categories of chromosome morphology observed in chromosome spreads of HeLa Tet-on cells stably transfected with Myc-Sgo1ND in the absence or presence of doxycycline (Dox) after the release from nocodazole-mediated mitotic arrest. C, kinetics of chromosome segregation in cells expressing Myc-Sgo1^{WT} or Myc-Sgo1ND in the presence or absence of doxycycline after the release from nocodazole arrest. A total of 100 chromosome spreads were analyzed for each sample. D, kinetics of chromosome decondensation in cells expressing Myc-Sgo1^{WT} or Myc-Sgo1ND in the presence or absence of doxycycline after the release from nocodazole arrest. A total of 100 chromosome spreads were analyzed for each sample.

Sgo1 Contains Multiple APC/C Degrons

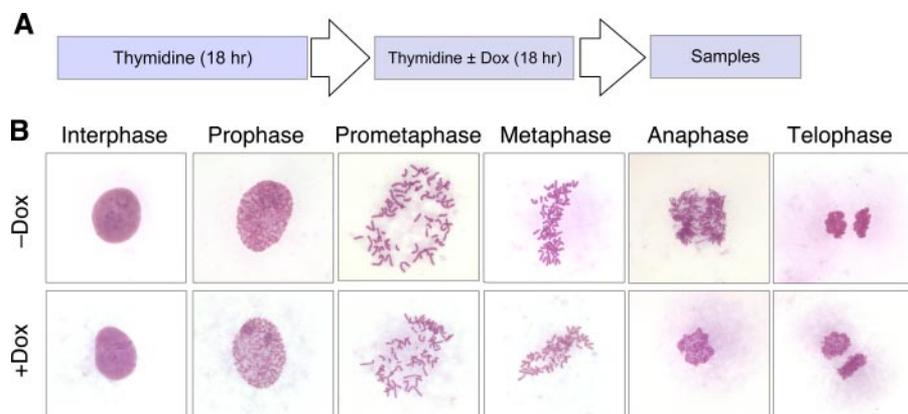


FIGURE 7. Expression of non-degradable Sgo1 ($Sgo1^{ND}$) does not delay chromosome segregation during unperturbed mitosis. *A*, schematic diagram of the time course of experiments in *B*. *B*, chromosome spreads of HeLa Tet-on cells expressing $Sgo1^{ND}$ with or without doxycycline (*Dox*) made 10 h after the release from the second thymidine block. Major categories of morphology were shown.

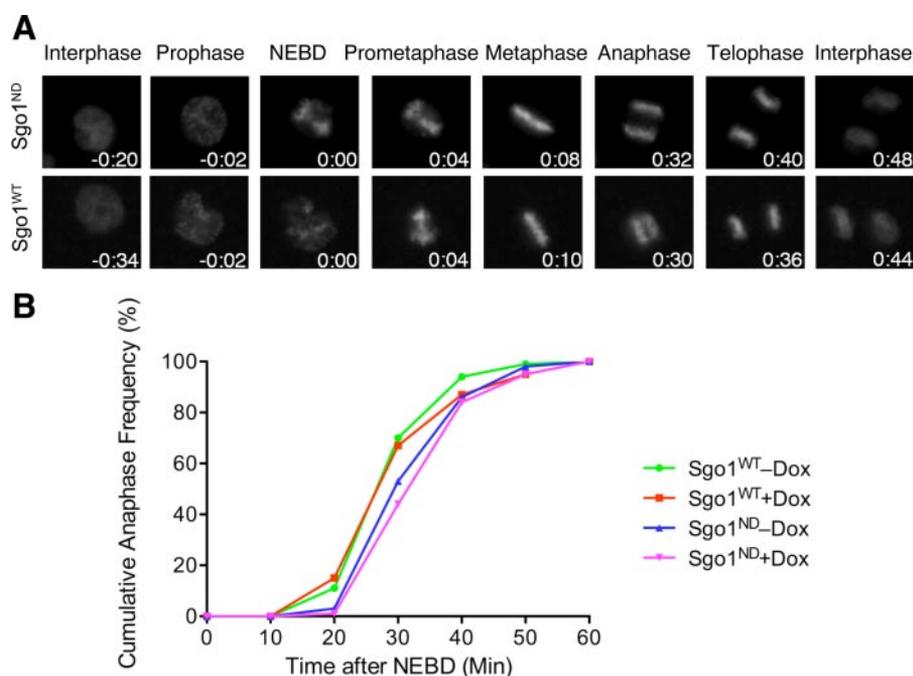


FIGURE 8. Live cell time-lapse analysis of cells stably expressing non-degradable Sgo1. *A*, cells stably expressing non-degradable Sgo1 ($Sgo1^{ND}$) in the absence or presence of doxycycline (*Dox*) were filmed for 20 h after release from thymidine arrest. Selected frames of cells in interphase and different stages of mitosis are shown. Time (h:min) is shown at the lower right corner of each image. Nuclear envelope breakdown (*NEBD*) is defined as time 0. *B*, cumulative frequency of cells undergoing anaphase within the indicated time windows after nuclear envelope breakdown. Anaphase timing in HeLa Tet-on cells expressing $Sgo1^{WT}$ and $Sgo1^{ND}$ was determined from time-lapse movies.

does not induce changes in chromosome morphology or mitotic arrest.

Last, a detailed analysis of chromosome dynamics and anaphase timing was performed in live cells by time-lapse microscopy (Fig. 8*A*). Both cells expressing $Sgo1^{WT}$ or $Sgo1^{ND}$ condensed their chromosomes, formed a metaphase plate, and underwent anaphase with similar timings (Fig. 8, *A* and *B*). Taken together, these results indicate that delayed Sgo1 degradation does not prevent chromosome segregation and that Sgo1 degradation is not required for later mitotic processes, such as chromosome decondensation and cytokinesis.

Bub1 Regulates the Steady-state Level of Sgo1 through an APC/C-independent Mechanism

It has been shown previously that the protein levels of Sgo1 are reduced in Bub1 RNAi cells (9), indicating that Bub1 is required for the maintenance of the steady-state levels of Sgo1. Bub1 also phosphorylates Sgo1 protein *in vitro* at several sites, including Ser-436 and Ser-440, which are in and around DB2 (data not shown). We, thus, tested whether Bub1 regulated APC/C-dependent degradation of Sgo1. We depleted Bub1 by RNAi in cells stably expressing Myc-Sgo1^{WT} or Sgo1ND and blotted cell lysates with anti-Myc. As expected, the levels of Myc-Sgo1^{WT} were regulated during the cell cycle and were lower in log phase cells than in thymidine- or nocodazole-arrested cells (Fig. 9, lanes 4–6). Depletion of Bub1 further reduced the levels of Sgo1^{WT} under all three conditions (Fig. 9, compare lanes 1–3 with lanes 4–6). The levels of Sgo1ND in log-phase cells were similar to that in thymidine- or nocodazole-arrested cells (Fig. 9, lanes 10–12), consistent with it not being regulated by APC/C. We expected that, if Bub1 regulated Sgo1 levels through preventing APC/C-dependent ubiquitination of Sgo1, then Sgo1ND should not display a reduction in protein levels in Bub1 RNAi cells. In contrast to our expectations, we found that the levels of Sgo1ND were still reduced in Bub1 RNAi cells (Fig. 9, compare lanes 7–9 with lanes 10–12). Thus, our data confirm that Bub1 regulates Sgo1 protein levels but suggest that it does so through a mechanism independent of APC/C.

DISCUSSION

Sgo1 Contains Multiple APC/C Degrons—Sister-chromatid cohesion is crucial for faithful chromosome segregation in mitosis and meiosis (3). Sgo1 is required for the protection of centromeric cohesion from prophase to the metaphase-to-anaphase transition until all kinetochores are properly captured by the spindle microtubules (7). In this study, we have shown that human Sgo1 is degraded in an APC/C-dependent manner during mitotic exit. Overexpression of Cdh1 causes a significant reduction in Sgo1 protein levels. Consistent

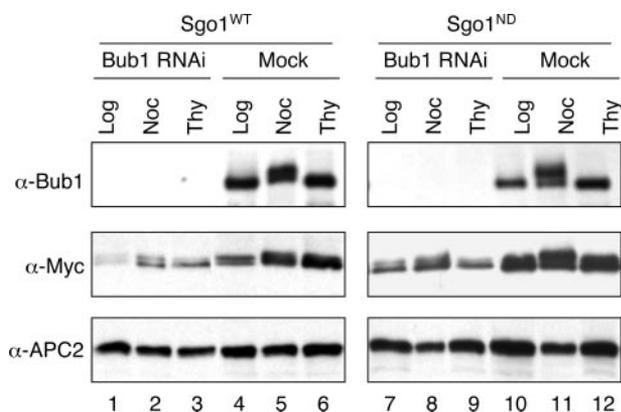


FIGURE 9. Depletion of Bub1 by RNAi decreases steady-state levels of Sgo1^{WT} and Sgo1ND. Log phase, nocodazole (Noc)-treated, or thymidine (Thy)-treated HeLa Tet-on cells stably expressing Myc-Sgo1^{WT} (left panel) or Myc-Sgo1ND (right panel) were transfected with Bub1 siRNA for 24 h and analyzed by Western blotting with the indicated antibodies.

with our data, *Xenopus* Sgo1 is also a substrate of APC/C^{Cdh1} (8). We have further identified two functional APC/C degrons, a KEN box and a D box, in Sgo1. Thus, similar to other APC/C substrates, such as securin (28), Cdc6 (29), and Nek2A (30), Sgo1 contains multiple APC/C degrons, which ensure its robust degradation during mitotic exit.

Sgo1 contains three putative D boxes, but only one D box (DB2) is functional. In another study, DB3 of human Sgo1 was identified as a functional APC/C degron (31). Our results directly contradict those data. We have carefully examined the potential role of DB3 in Sgo1 degradation. In our experiments, removal of DB3 alone or together with the KEN box does not stabilize full-length Sgo1 in the presence of Cdh1 overexpression (Fig. 1B). Similar results were obtained when DB3 was deleted in a C-terminal fragment of Sgo1 (Fig. 2B). A systematic deletional analysis of the C-terminal region of Sgo1 further confirms that DB2 is the only functional APC/C degron in this region, and removal of this motif is solely responsible for the stabilization of Sgo1C in the presence of Cdh1 overexpression (Fig. 2C). Finally, Sgo1C mutants with DB3 partially deleted can still be efficiently ubiquitinated by APC/C in an *in vitro* reconstituted ubiquitination assay (Fig. 3B). Thus, DB3 is not a functional APC/C degron.

Degradation of Sgo1 Is Not Required for Mitotic Progression—APC/C regulates orderly progression through mitosis by targeting key mitotic regulators for ubiquitination and degradation (16). Sgo1 localizes to kinetochores, protects centromeric cohesion during prophase, and prevents premature sister-chromatid separation in mitosis (7). In meiosis I, the shugoshin family of proteins also protects centromeric cohesion from cleavage by separase. We had initially envisioned that degradation of Sgo1 might be required for its inactivation, thus allowing the timely cleavage of centromeric cohesion by separase at the metaphase-to-anaphase transition. However, expression of a non-degradable mutant of Sgo1 in HeLa cells did not cause a delay in chromosome segregation or mitotic exit. We also did not observe changes in chromosome morphology at different stages of mitosis. These results suggest that degradation of Sgo1 itself is not crucial

for proper progression through mitosis. Additional mechanisms may exist to inactivate Sgo1 at the metaphase-to-anaphase transition. Alternatively, Sgo1 does not prevent the cleavage of centromeric cohesion by separase in mitosis.

Bub1 Regulates Sgo1 Protein Levels in an APC/C-independent Mechanism—The spindle checkpoint kinase Bub1 is required for the maintenance of Sgo1 protein levels (9). Bub1 phosphorylates Sgo1 in the vicinity of its APC/C degrons *in vitro*. Recently, it has been shown that cyclin-dependent kinases phosphorylate Cdc6 in the vicinity of its APC/C degrons, thus rendering these motifs inaccessible for APC/C recognition and ubiquitination (29). We had hypothesized that Bub1 might regulate Sgo1 through a similar mechanism. This does not appear to be the case, as the levels of the non-degradable Sgo1 mutant are still reduced in Bub1 RNAi cells. Our results, thus, suggest that Bub1 regulates Sgo1 levels through an APC/C-independent mechanism. The centromeric localization of Sgo1 depends on Bub1 (9, 10). It is possible that Sgo1 becomes unstable in Bub1-depleted cells because it loses its centromeric localization. On the other hand, Bub1 targets PP2A to centromeres, which in turn maintains Sgo1 at centromeres by counteracting Plk1-mediated chromosome removal of Sgo1 (12). Although Sgo1 also loses its centromeric localization in PP2A RNAi cells, its protein levels are not reduced in these cells (12). Therefore, the loss of its centromeric localization *per se* does not necessarily lead to Sgo1 degradation. Future experiments are needed to uncover the mechanisms by which Bub1 regulates the steady-state levels of Sgo1.

Acknowledgments—We thank the members of the Yu laboratory for helpful discussions.

REFERENCES

- Waizenegger, I. C., Hauf, S., Meinke, A., and Peters, J. M. (2000) *Cell* **103**, 399–410
- Losada, A., Hirano, M., and Hirano, T. (2002) *Genes Dev.* **16**, 3004–3016
- Onn, I., Heidinger-Pauli, J. M., Guacci, V., Unal, E., and Koshland, D. E. (2008) *Annu. Rev. Cell Dev. Biol.* **24**, 105–129
- Hauf, S., Roitinger, E., Koch, B., Dittrich, C. M., Mechtler, K., and Peters, J. M. (2005) *PLoS Biol.* **3**, e69
- Hauf, S., Waizenegger, I. C., and Peters, J. M. (2001) *Science* **293**, 1320–1323
- Kitajima, T. S., Kawashima, S. A., and Watanabe, Y. (2004) *Nature* **427**, 510–517
- Watanabe, Y. (2005) *Curr. Opin. Cell Biol.* **17**, 590–595
- Salic, A., Waters, J. C., and Mitchison, T. J. (2004) *Cell* **118**, 567–578
- Tang, Z., Sun, Y., Harley, S. E., Zou, H., and Yu, H. (2004) *Proc. Natl. Acad. Sci. U. S. A.* **101**, 18012–18017
- Kitajima, T. S., Hauf, S., Ohsugi, M., Yamamoto, T., and Watanabe, Y. (2005) *Curr. Biol.* **15**, 353–359
- McGuinness, B. E., Hirota, T., Kudo, N. R., Peters, J. M., and Nasmyth, K. (2005) *PLoS Biol.* **3**, e86
- Tang, Z., Shu, H., Qi, W., Mahmood, N. A., Mumby, M. C., and Yu, H. (2006) *Dev. Cell* **10**, 575–585
- Kitajima, T. S., Sakuno, T., Ishiguro, K., Iemura, S., Natsume, T., Kawashima, S. A., and Watanabe, Y. (2006) *Nature* **441**, 46–52
- Riedel, C. G., Katis, V. L., Katou, Y., Mori, S., Itoh, T., Helmhart, W., Galova, M., Petronczki, M., Gregan, J., Cetin, B., Mudrak, I., Ogris, E., Mechtler, K., Pelletier, L., Buchholz, F., Shirahige, K., and Nasmyth, K. (2006) *Nature* **441**, 53–61

Sgo1 Contains Multiple APC/C Degrons

15. Indjeian, V. B., Stern, B. M., and Murray, A. W. (2005) *Science* **307**, 130–133
16. Peters, J. M. (2006) *Nat. Rev. Mol. Cell Biol.* **7**, 644–656
17. Yu, H. (2007) *Mol. Cell* **27**, 3–16
18. Glotzer, M., Murray, A. W., and Kirschner, M. W. (1991) *Nature* **349**, 132–138
19. Pfleger, C. M., and Kirschner, M. W. (2000) *Genes Dev.* **14**, 655–665
20. Yu, H. (2002) *Curr. Opin. Cell Biol.* **14**, 706–714
21. Fang, G., Yu, H., and Kirschner, M. W. (1998) *Mol. Cell* **2**, 163–171
22. Gimenez-Abian, J. F., Diaz-Martinez, L. A., Wirth, K. G., Andrews, C. A., Gimenez-Martin, G., and Clarke, D. J. (2005) *Cell Cycle* **4**, 1561–1575
23. Tang, Z., and Yu, H. (2004) *Methods Mol. Biol.* **281**, 227–242
24. Qi, W., and Yu, H. (2007) *J. Biol. Chem.* **282**, 3672–3679
25. Tang, Z., Bharadwaj, R., Li, B., and Yu, H. (2001) *Dev. Cell* **1**, 227–237
26. Wang, X., Yang, Y., and Dai, W. (2006) *Cell Cycle* **5**, 635–640
27. Suzuki, H., Akiyama, N., Tsuji, M., Ohashi, T., Saito, S., and Eto, Y. (2006) *Cell Cycle* **5**, 1094–1101
28. Zur, A., and Brandeis, M. (2001) *EMBO J.* **20**, 792–801
29. Mailand, N., and Diffley, J. F. (2005) *Cell* **122**, 915–926
30. Hayes, M. J., Kimata, Y., Wattam, S. L., Lindon, C., Mao, G., Yamano, H., and Fry, A. M. (2006) *Nat. Cell Biol.* **8**, 607–614
31. Fu, G., Hua, S., Ward, T., Ding, X., Yang, Y., Guo, Z., and Yao, X. (2007) *Biochem. Biophys. Res. Commun.* **357**, 672–678