The Anaphase-Promoting Complex/Cyclosome Controls Repair and Recombination by Ubiquitylating Rhp54 in Fission Yeast[▽]

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Homologous recombination (HR) is important for maintaining genome integrity and for the process of meiotic chromosome segregation and the generation of variation. HR is regulated throughout the cell cycle, being prevalent in the S and G₂ phases and suppressed in the G₁ phase. Here we show that the anaphase-promoting complex/cyclosome (APC/C) regulates homologous recombination in the fission yeast *Schizosaccharomyces pombe* by ubiquitylating Rhp54 (an ortholog of Rad54). We show that Rhp54 is a novel APC/C substrate that is destroyed in G₁ phase in a KEN-box- and Ste9/Fizzy-related manner. The biological consequences of failing to temporally regulate HR via Rhp54 degradation are seen in haploid cells only in the absence of antirecombinase Srs2 function and are more extensive in diploid cells, which become sensitive to a range of DNA-damaging agents, including hydroxyurea, methyl methanesulfonate, bleomycin, and UV. During meiosis, expression of nondegradable Rhp54 inhibits interhomolog recombination and stimulates sister chromatid recombination. We thus propose that it is critical to control levels of Rhp54 in G₁ to suppress HR repair of double-strand breaks and during meiosis to coordinate interhomolog recombination.

Successful progression through the cell cycle relies on programmed degradation of key proteins via the ubiquitin-proteasome system, which is based on an ATP-dependent attachment of ubiquitin moieties onto target proteins (ubiquitylation) and subsequent degradation by the 26S proteasome. Ubiquitylation requires three enzymes, a ubiquitin-activating enzyme (E1), a ubiquitin-conjugating enzyme (E2), and a ubiquitin ligase (E3), and this enzymatic cascade will result in chains of four or more ubiquitin molecules on the substrates, which will then be recognized and degraded by the proteasome (14).

The anaphase-promoting complex/cyclosome (APC/C), a large (20S) multisubunit E3 ligase, catalyzes the final step of ubiquitylation of cell cycle proteins in mitosis and the G₁ phase (28, 36, 47). Although the critical targets are securin/Pds1/Cut2 and cyclin B/Clb2/Cdc13, which regulate sister chromatid separation and mitotic exit, respectively, a number of substrates have been identified since its discovery. These include proteins associated with the control of spindle function, Xkid1, Ase1, Kip1, Cin8, and Aurora A, as well as geminin and Cdc6, which regulate origin licensing for DNA replication (12, 32, 37), which verifies that APC/C is a key regulator of the cell cycle.

APC/C function requires the binding of the WD40-containing Fizzy family of activator proteins Fizzy/Cdc20/Slp1 and Fizzy-related (FZR)/Cdh1/Ste9 as well as the mitosis-specific phosphorylation of several subunits (28, 36, 47). Fizzy/Cdc20 associates with the mitotic APC/C from prophase to early anaphase, whereas FZR/Cdh1 associates with the APC/C from late anaphase into G₁ and G₀ to activate APC/C. This sequential and exclusive binding of APC/C with activators partly explains why substrates are destroyed at different times during

mitotic progression/exit and interphase. In addition, the timing of APC/C substrate degradation is dependent upon short destruction motifs in their primary sequences, such as the destruction box (D-box) and the KEN-box (12, 36, 47). The D-box, first identified in the N terminus of cyclin B (11), is present in many APC/C substrates destroyed at anaphase, and can be recognized by both Fizzy/Cdc20- and FZR/Cdh1-activated APC/C. In contrast, an alternative destruction motif, the KEN-box (40), is more often, although not exclusively, present in substrates recognized by FZR/Cdh1-APC/C. Hence, substrates containing the D-box are degraded before those containing KEN-box because Fizzy/Cdc20-APC/C is active before FZR/Cdh1-APC/C.

Double-strand breaks (DSBs) are dangerous lesions that can lead to genomic instability if the damage is left unrepaired or is misrepaired. Eukaryotic cells repair DSBs either by nonhomologous end joining (NHEJ) or by homologous recombination (HR), which are regulated through the cell cycle (17, 49). In yeasts, error-prone NHEJ functions in G₁ phase when the sister chromatid is not available, whereas error-free HR is the major pathway for repair of DSBs from late S phase through G₂ phase, when the sister chromatid is available as a template. The HR pathway relies upon processing of the DSB by 5'-to-3' exonucleases. The resulting single-strand DNA ends first are bound by the single-strand DNA binding protein RPA, which is subsequently replaced by the Rad51 recombinase. This switch from RPA to the helical Rad51 nucleoprotein filament requires additional factors, including Rad52, Rad54, Rad55, and Rad57. Upon formation, the filament can then search for homologous duplex donor DNA, which it invades to form a displacement loop (D-loop). The invading strand is subsequently extended by DNA polymerase, and either the D-loop can capture the second end to form a double Holliday junction (HJ) or the newly synthesized strand is displaced to undergo synthesis-dependent strand annealing with the free DNA end (21, 35, 45, 53).

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FIG. 1. Rhp54 is destroyed in *Xenopus* egg extracts in an FZR-APC/C-dependent manner. (A) Schematic of a cell-free FZR/Cdh1-APC/C-dependent destruction assay reconstituted in *Xenopus* egg extracts. Addition of CaCl_2 releases CSF arrest into interphase, whereas addition of *Xenopus* FZR/Cdh1 (FZR) activates the interphase APC/C. Substrates (Sub) are added at time zero, and samples are taken at the indicated time. Cycloheximide (CHX) is added to prevent protein synthesis. (B) A bona fide FZR substrate, Fizzy/Cdc20, and cyclin B/Cdc13 are destroyed in an FZR-dependent destruction assay. ^{35}S -labeled in vitro-translated Cdc13 together with a version of Cdc13 lacking the N-terminal 67 residues (Δ 67, stable control) (lanes 1 to 8) and Fizzy/Cdc20 (lanes 9 to 16) were used as substrates. (C) Same as panel B, but ^{35}S -labeled Rhp54 was used as a substrate (lanes 1 to 8). The N-terminal 70 residues of Cdc13 (N70; lanes 9 to 12) or MG132 (lanes 13 to 16) were added to the reaction mixtures prior to the addition of Rhp54. Since N70 contains a D-box, the excess amounts specifically inhibit APC/C. (D) Fizzy/Cdc20-APC/C-dependent destruction assay reconstituted in *Xenopus* egg extracts. CaCl_2 was added to activate Fizzy/Cdc20-APC/C. Rhp54 was stable (lanes 1 to 8), whereas Cdc13 was destroyed upon the addition of CaCl_2 (lanes 9 to 16). (E) Rhp54 destruction is dependent upon APC/C. (Left panel) Interphase egg extract (lane 1), APC/C-depleted (dep) interphase extracts (lane 2) using specific Apc3/Cdc27 antibody, or mock-depleted extract (lane 3) was immunoblotted with the indicated antibodies (αApc3 and $\alpha\text{PSTAIRE}$). (Right panel) Same as panel C, but APC/C-depleted egg extracts were also used for the destruction assay (lanes 9 to 12) and mock depleted (lanes 13 to 16).

In meiosis, the HR pathway establishes the HJs that physically connect the maternal and paternal chromosomes. These connections are required for accurate separation of the homologous chromosome pairs in the first meiotic division (MI). Subsequent HJ resolution results in gene conversion with or without reciprocal exchange of chromosome arms, contributing to the generation of genetic diversity (33, 38).

Using a cell-free APC/C-dependent destruction assay, we have identified the *Schizosaccharomyces pombe* homolog of Rad54, Rhp54, as a new APC/C substrate. We show that Rhp54 destruction only occurs once per cell cycle, in G_1 phase, and requires the APC/C activator FZR/Ste9 and an amino-terminal KEN-box. Regulation of Rhp54 destruction by APC/C is physiologically important for DSB repair and homologous recombination since a stable version of Rhp54 results in hypersensitivity to DNA-damaging agents as well as suppression of interhomolog recombination in meiosis.

MATERIALS AND METHODS

***Xenopus* egg extracts and destruction assay.** *Xenopus* cytotostatic factor-arrested egg extracts (CSF extracts) were prepared as described previously (31). In these extracts, cyclin B destruction, inactivation of maturation promoting factor, and inactivation of CSF can be triggered by the addition of 0.4 mM CaCl_2 . Fizzy/Cdc20-APC/C-dependent destruction assays were performed as described previously (55). A cell-free FZR/Cdh1-APC/C-dependent destruction assay was reconstituted by adding *Xenopus* FZR/Cdh1 to interphase egg extracts (see Fig. 1A).

Yeast general methods. Methods of handling *Schizosaccharomyces pombe* were described previously (27). Strains used in this study are listed in Table 1. Thiamine (2 μM) was added to the media to repress the *nmt1* promoter. For the sensitivity assay against DNA-damaging agents, 10 μl of 10-fold serial dilutions were spotted out on YE media plus a five-amino-acid supplement (YE + 5S) or YE + 5S containing hydroxyurea (HU), camptothecin (CPT), methyl methane-sulfonate (MMS), and bleomycin and incubated at 30°C for 3 to 4 days. NHEJ and HR assays were performed as described previously (10, 24, 34). Meiotic recombination assays were carried out as described previously (5).

Plasmid construction and mutagenesis. The coding region of *rhp54*⁺ was amplified from an *S. pombe* cDNA library and subcloned into suitable vectors to

TABLE 1. Strains used in this study

Strain	Genotype	Source or reference
HY24	<i>h⁻ ade6-704 leu1-32 ura4-D18</i>	Our laboratory stock
FY2122	<i>h⁺ rhp54::ura4⁺ ade6-M210 leu1-32 ura4-D18</i>	29
HY177	<i>h⁻ myc-rhp54Km::KanMX ade6-704 leu1-32 ura4-D18</i>	This study
HY95	<i>h⁺ cdc10-V50 leu1-32 ura4-D18</i>	Our laboratory stock
HY606	<i>cdc10-V50 myc-rhp54Km::KanMX leu1-32 ura4-D18</i>	This study
HY8	<i>h⁺ cdc25-22 leu1-32</i>	Our laboratory stock
HY146	<i>h⁻ wee1-50 leu1-32 ura4-D18</i>	Our laboratory stock
HY184	<i>cdc10-V50 cut9-665 leu1-32</i>	Our laboratory stock
HY164	<i>h⁻ mts2-1 leu1-32 ura4-D18</i>	Our laboratory stock
HY41	<i>h⁺ nda3-KM311 leu1-32</i>	Our laboratory stock
KJ218-2B	<i>h90 cdc10-V50 ste9::ura4⁺ 3HA-cig2⁺ leu1-32 ura4-D18</i>	Kenji Kitamura
AMC2077	<i>h⁺ pku70::his3 leu1-32 ura4-D18 ade6-M216 his3-D1</i>	Antony Carr
FY1251	<i>h⁺ ade6-M26 his4-239</i>	5
FY1252	<i>h⁻ ade6-52 lys4-95</i>	5
FY1898	<i>h⁻ rdh54::ura4⁺ ade6-L469/pUC8/his3⁺/ade6-M375 leu1-32 ura4-D18 his3-D1</i>	5
FY1901	<i>h⁻ smt-0 rhp54::ura4⁺ ade6-L469/pUC8/his3⁺/ade6-M375 leu1-32 ura4-D18 his3-D1</i>	5
FY1921	<i>h⁻ rdh54::ura4⁺ ade6-M26 ura4-D18 his4-239</i>	5
FY1941	<i>h⁺ rdh54::ura4⁺ ade6-52 ura4-D18 lys4-95</i>	5
FY1942	<i>h⁺ rdh54::ura4⁺ ade6-M375-M210 leu1-32 ura4-D18 his3-D1</i>	5
FY1944	<i>h⁺ rhp54::ura4⁺ ade6-M375-M210 leu1-32 ura4-D18 his3-D1</i>	5
FY2133	<i>h⁻ ade6-M375-M210 leu1-32 his3-D1</i>	5
HY490	<i>h⁺ myc-rhp54Km::KanMX ade6-M26 his4-239</i>	This study
HY492	<i>h⁻ myc-rhp54Km::KanMX ade6-52 lys4-95</i>	This study
HY504	<i>h⁺ myc-rhp54::KanMX ade6-M26 his4-239</i>	This study
HY506	<i>h⁻ myc-rhp54::KanMX ade6-52 lys4-95</i>	This study
HY508	<i>h⁻ myc-rhp54::KanMX ade6-M375-M210 leu1-32 his3-D1</i>	This study
HY608	<i>h⁻ myc-rhp54::KanMX ade6-L469/pUC8/his3⁺/ade6-M375 leu1-32 ura4-D18 his3-D1</i>	This study
HY545	<i>h⁻ N70-myc-rhp54Km::KanMX ade6-M375-M210 leu1-32 his3-D1</i>	This study
HY543	<i>h⁻ N70-myc-rhp54Km::KanMX ade6-52 lys4-95</i>	This study
HY541	<i>h⁻ N70-myc-rhp54Km::KanMX ade6-M26 his4-239</i>	This study
HY607	<i>h⁻ myc-rhp54Km::KanMX ade6-L469/pUC8/his3⁺/ade6-M375 leu1-32 ura4-D18 his3-D1</i>	This study
HY539	<i>h⁻ N70-myc-rhp54Km::KanMX ade6-L469/pUC8/his3⁺/ade6-M375 leu1-32 ura4-D18 his3-D1</i>	This study
MCW 23	<i>h⁺ ade6-L469/pUC8/his3⁺/ade6-M375 leu1-32 ura4-D18 his3-D1</i>	34
MCW 547	<i>h⁺ srs2::ura4⁺ ade6-L469/pUC8/his3⁺/ade6-M375 leu1-32 ura4-D18 his3-D1</i>	9
TISP130	<i>h⁺ srs2::KanMX leu1-32 ura4-D18 his3-D1 arg3-D1</i>	Jacob Dalgaard
HY609	<i>h⁺ myc-rhp54Km::KanMX srs2::ura4⁺ ade6-L469/pUC8/his3⁺/ade6-M375 leu1-32 ura4-D18 his3-D1</i>	This study
HY610	<i>h⁺ myc-rhp54Km::KanMX srs2::KanMX ade6-704 leu1-32 ura4-D18</i>	This study
HY611	<i>h⁺ N70-myc-rhp54Km::KanMX srs2::ura4⁺ ade6-704 leu1-32 ura4-D18</i>	This study
HY612	<i>h⁺ myc-rhp54Km::KanMX srs2::KanMX rhp51::ura4⁺ ade6-704 leu1-32 ura4-D18</i>	This study
ES262	<i>h⁺ rhp51::ura4⁺ ade6-M210 leu1-32 ura4-D18</i>	Albert Pastink

express in bacteria or in a coupled in vitro transcription-translation system (TNT; Promega, United Kingdom). *rhp54* constructs with mutations were generated by PCR-based mutagenesis. The cDNAs of different HR family members were amplified from the *S. pombe* cDNA library, whereas *Saccharomyces cerevisiae* *RAD54* and human homologs (Rad54A and Rad54B) were amplified from appropriate cDNA libraries and subcloned into an expression vector for TNT. Constructs were confirmed by DNA sequencing (Cogenics, United Kingdom).

Synchronous cultures. The *wee1-50* strain was grown overnight at 25°C in YE media plus peptone and adenine and uracil (YEP + AU). Cells were synchronized at 25°C using a JE-5.0 elutriation system (Beckman Instruments, Inc.) and then shifted to 36°C. Samples were taken every 20 min for protein extraction and for flow cytometry analysis for G₁ DNA content. To induce synchronous meiosis, homozygous diploid (*h⁻/h⁻*) cells were grown in Edinburgh minimal media 2 (EMM2) to mid-log phase, washed with EMM2 minus NH₄Cl (EMM2-N), and grown in EMM2-N at 25°C for 15 h. Then, the culture was shifted to 34°C to induce meiosis. Cells were collected every 20 min and analyzed by microscopy and immunoblotting.

Antibodies. For preparation of rabbit polyclonal antibodies against Rhp54, the His₆-tagged N-terminal 90 residues of Rhp54 (Rhp54N90) were expressed in *Escherichia coli* and purified using Ni-nitrilotriacetic acid (NTA) beads under denaturing conditions as described by the supplier (Qiagen). The Rhp54N90 protein was further gel purified and used to immunize rabbits (BioGenex; Germany). The following antibodies were used in this study: anti-Apc3/Cdc27 (1:200; BD Biosciences, United Kingdom), anti-myc (4A6, 1:500; Upstate, NY), anti-Cdc2 (monoclonal antibody Y100, 1:3,000), anti-Cdc13 (1:200), anti-Rhp54 (1:200), anti-human Rad51 (1:200; Santa Cruz Biotechnology), anti-PSTAIR (1:

10,000; a gift from Y. Nagahama, Japan), and anti-HA (12CA5, 1:3,000; Roche). To deplete APC/C, monoclonal antibody AF3.1 (anti-Apc3) was used (54).

RESULTS

Identification of Rhp54 as an APC/C substrate. To identify novel substrates of the APC/C, we set up an FZR/Cdh1-dependent destruction assay in *Xenopus* egg extracts (Fig. 1A). Since the activity of Fizzy/Cdc20-APC/C is absent or very low at 2 h after addition of CaCl₂ to CSF extracts, addition of recombinant FZR into the interphase extracts results in activation of FZR-dependent APC/C. It should be noted that endogenous FZR appears to be absent in *Xenopus* egg extracts (23). As shown in Fig. 1B, upon addition of FZR, both Cdc13 (cyclin B in fission yeast [the D-box-containing protein]) and Fizzy (the KEN-box-containing protein) were destroyed. To look for novel KEN-box-containing substrates in *S. pombe*, we scanned the entire *S. pombe* genome for all proteins containing a KENxP motif (where x is any amino acid) using the Sanger Centre Motif Scan program (GeneDB) and identified 25 possible candidates for the APC/C substrate. Then, radiolabeled

FIG. 2. Rhp54 destruction is dependent upon an amino-terminal KEN-box. (A) Schematic diagram of Rhp54 and mutant Rhp54 used in this study. Wild-type Rhp54 has two KEN box-like and four D-box-like sequences, as indicated. The first KEN-box (KEN26) matches the KENxP motif we used for the initial screening. Km is the first KEN-box mutant (KEN to AAA; indicated by the filled box). ΔN94 is truncation of the N-terminal 94 residues removing one KEN-box and two D-box-like sequences. L22A is a version of Rhp54 with a random mutation adjacent to the KEN-box (leucine 22 to alanine). *S. cerevisiae* Rad54 (ScRad54) does not have KEN box sequences. Rhp54N50-ScRad54 illustrates the fusion of the N-terminal 50 residues of Rhp54, including KEN26, onto *S. cerevisiae* Rad54 to make a chimeric construct. (B) Mutation of the KEN-box (Km) and truncation of the N-terminal 94 residues (ΔN94) stabilizes Rhp54 in the presence of FZR in a cell-free destruction assay, but the random mutation L22A does not. ³⁵S-labeled Km, ΔN94, or L22A as well as Rhp54 were used as substrates. (C) The N-terminal 50 residues of Rhp54 (Rhp54N50) can work as a transfer-

able destruction module. ³⁵S-labeled *S. cerevisiae* Rad54 or Rhp54N50-ScRad54 was used for an FZR-APC/C-dependent destruction assay. (D) The Myc-tagged wild type (lanes 1 and 2) or Km mutant (lanes 3 and 4) of Rhp54 and His₆-ubiquitin (Ub) (lanes 1 and 3) or empty vector (lanes 2 and 4) were coexpressed using pREP41 and the pREP2 *nmt1* promoter, respectively in the temperature-sensitive *mts2-1* mutant at permissive temperature prior to shifting to restrictive temperature (36°C) for 4 h. In lane 5, His₆-ubiquitin only was expressed in the *mts2-1* mutant. Ubiquitylated proteins were isolated under denaturing condition using Ni-NTA beads followed by immunoblotting with anti-myc (αMyc) antibody. Whole cell extracts (WCE) were also analyzed by immunoblotting with anti-myc and anti-Cdc2 (αCdc2) antibodies.

substrates were prepared by coupled transcription and translation in the presence of [³⁵S]methionine and subjected to the above FZR-dependent destruction assay as well as Fizzy/Cdc20-dependent destruction assay (55). We found that the HR protein Rhp54 (29), an ortholog of Rad54, was destroyed in these interphase extracts in an FZR-dependent manner, but not in the Fizzy/Cdc20-dependent destruction assay. In addition, destruction of Rhp54 was blocked in the presence of the N-terminal 70 residues of cyclin B/Cdc13 (N70) or the 26S proteasome inhibitor, MG132 (Fig. 1C and D). In agreement with this finding, when APC/C was depleted from *Xenopus* egg extracts by preincubation with Apc3 antibody, Rhp54 was hardly degraded even upon addition of FZR (Fig. 1E). Therefore, Rhp54 is degraded in an FZR-APC/C- and proteasome-dependent manner in vitro. Other members of the HR family, including Rhp51, Rad22, Rhp55, Rhp57, and Rdh54, as well as *S. cerevisiae* Rad54 and human Rad54 homologs (Rad54A and Rad54B), were also examined, but none were destroyed in the FZR- or Fizzy/Cdc20-dependent destruction assay (data not shown). Sequence analysis indicated that only human Rad54B contained a KEN-box, which was embedded within the sequence and not at the N-terminal portion as with Rhp54.

The KEN-box is required for Rhp54 ubiquitylation and destruction. Inspection of the Rhp54 sequence revealed not only the KENxP motif but also one more KEN-box-like motif and four D-box-like motifs (RXXL, where X is any amino acid) (Fig. 2A). We next investigated which motif was responsible for Rhp54 destruction. Mutation of the KENXP motif (residues 26 to 30) KEN26 to AAA (Rhp54Km) stabilized Rhp54 (Fig. 2B). Consistently, truncation of the amino-terminal 94 residues (Rhp54ΔN94), removing two D-box-like motifs as well as the KENXP motif but leaving two D-box like motifs and an additional KEN-box-like motif in the C-terminal portion of the protein, also stabilized the protein, whereas mutation of a residue adjacent to the KEN-box (lysine 22 to alanine; Rhp54L22A), did not affect degradation (Fig. 2B). Thus, only the KEN-box (residues 26 to 28) is apparently required for FZR-dependent destruction of Rhp54 in vitro. Also, we investigated whether this destruction module can be transferable, so we fused the amino-terminal 50 residues of Rhp54 containing the KEN-box onto the nondegradable *S. cerevisiae* Rad54 (Rhp54N50-ScRad54). Remarkably, addition of the KEN box was sufficient to catalyze the degradation of Rhp54N50-ScRad54 in an FZR-dependent manner (Fig. 2C). Furthermore, we examined whether Rhp54 was ubiquitylated in vivo.

Myc-tagged wild-type or KEN-box mutant Rhp54 and His₆-tagged ubiquitin were coexpressed in fission yeast cells in which ubiquitylated proteins were stabilized by a conditional mutation in the S4 (*mts2*) subunit of the 26S proteasome, and the ubiquitylated proteins were isolated under denaturing conditions using Ni-NTA beads and analyzed (Fig. 2D). Ubiquitylated Rhp54 bands were observed only when His₆-tagged ubiquitin was coexpressed (lanes 1 and 2), whereas such bands were reduced three- to approximately fourfold in the Rhp54Km protein (lanes 3 and 4). Taken together, these results indicate that Rhp54 is a novel substrate of the FZR-APC/C, whose polyubiquitylation is dependent upon an amino-terminal KEN-box.

Rhp54 is destroyed in G₁ in an Ste9-dependent manner in vivo. To see Rhp54 destruction in more detail in *S. pombe*, we first investigated the half-life of Rhp54. We expressed a non-tagged version of *rhp54*⁺ from the derepressed *nmt81* promoter in cells blocked at various stages of the cell cycle utilizing temperature-sensitive mutants of *cdc10* (G₁ phase), *cdc25* (G₂/M phase), and *nda3* (prometaphase) as well as the drug HU (S phase), prior to the addition of cycloheximide and thiamine. As shown in Fig. 3A, the half-life of Rhp54 was very short only in G₁, but not in G₂, M, or S phase. Levels of endogenous Cdc13 and Cdc2 serve as cell cycle stage and loading controls, respectively. Next, we made a *cdc10 ste9Δ* strain and checked the half-life of Rhp54 since the *S. pombe* FZR homolog is *ste9*⁺ (also known as *srw1*⁺) (18). Rhp54 remained stable if *ste9*⁺ was deleted (lanes 11 to 15, Fig. 3B), indicating that Rhp54 destruction in vivo is dependent upon Ste9, mimicking our in vitro data. As a control, endogenous Cdc13 was also stabilized. To show that this stability was dependent upon APC/C, a temperature-sensitive allele of the *S. pombe* Apc6 subunit, *cut9-665*, was combined with *cdc10*, which again resulted in the stabilization of Rhp54 as well as Cdc13 (lanes 6 to 10, Fig. 3B). We also investigated the half-life of the KEN-box mutant of Rhp54 (Rhp54Km) in *cdc10*-arrested cells and found that Rhp54Km was stable even in G₁ phase when Ste9-APC/C is active (Fig. 3C). In addition, synchronization in G₁ via nitrogen starvation further highlighted these observations with low Rhp54 levels in comparison with asynchronous cells. Rhp54Km levels remained high (Fig. 3D). Thus, we conclude that Rhp54 destruction in *S. pombe* is dependent upon an amino-terminal KEN-box.

Moreover, to investigate whether endogenous Rhp54 levels indeed fluctuate in a cell cycle-dependent manner, a synchronous culture was prepared by centrifugal elutriation and analyzed. Initially, we used wild-type cells; however, no Rhp54 oscillation was observed, although Cdc13 levels clearly oscillated in the synchronous culture (data not shown). We reasoned that a very short G₁ phase in wild-type *S. pombe* may prevent detection of Rhp54 destruction, thus, we switched to *wee1-50* cells, which have an extended G₁ phase at the restrictive temperature (36°C), to meet the minimal cell size requirement to pass Start. These were grown at 25°C prior to the elutriation process and shifted to 36°C and followed for two cell cycles (2). This time, Rhp54 oscillation was successfully observed and Rhp54 levels were very low transiently when cells enter G₁ phase, but just before Cig2 levels peak (S phase) (Fig. 3E). It is noteworthy that unlike Cdc13, Rhp54 destruction was never observed even in highly synchronous culture by *cdc25*

block/release, suggesting that Rhp54 is destroyed only in G₁ phase, not anaphase. Altogether, these data indicate that Rhp54 is degraded in G₁ in an FZR/Ste9-dependent manner in vivo.

Effects of stabilization of Rhp54 in haploid cells and in diploid cells. We next sought to investigate the biological importance of Rhp54 destruction. To this end, we integrated a *c-myc* epitope-tagged version of *rhp54Km* as well as wild-type *rhp54*⁺ into the *rhp54* locus, allowing expression from the native promoter. However, the *rhp54Km* mutation did not have a profound effect on vegetative growth; e.g., cell length and doubling time reflected the wild type at various temperatures (data not shown). Also, the *rhp54Km* cells did not show any sensitivity to DNA-damaging agents compared with the wild type (Fig. 4A), whereas the *rhp54Δ* mutant was extremely sensitive to HU, CPT, bleomycin, MMS, and UV (Fig. 4A), as reported previously (30). In agreement with this, expression of *rhp54*⁺ from an *nmt1* promoter did not show any discernible phenotype. Since G₁ cells use the NHEJ pathway rather than the HR pathway to repair DSBs, we next asked whether the presence of HR pathway protein Rhp54 in G₁ affects NHEJ efficiency by using a plasmid-based circularization assay (24). The NHEJ assay exploits transformation of yeast cells with a replication origin-containing plasmid that is linearized within sequences that lack homology to the yeast genome. Transformation efficiency is dependent upon plasmid circularization via NHEJ. An uncut plasmid is transformed in parallel to normalize for differences in transformation efficiency. As shown in Fig. 4B, cells expressing nondegradable Rhp54 (*rhp54Km*) showed NHEJ activity almost as efficient as that of wild-type cells, regardless of whether cells were asynchronously growing or arrested in G₁, where NHEJ levels are high as expected (10). These results suggest that stabilization of Rhp54 has no apparent effects on NHEJ. Not surprisingly, deletion of *rhp54* (*rhp54Δ*) also did not affect NHEJ activity, whereas *ku70Δ* cells showed NHEJ deficiency.

We then looked into the effects of stabilization of Rhp54 in diploid cells, where homologous chromosomes are always present. We speculated that cells might require tighter control of HR repair for DSBs under such a circumstance. Intriguingly, in contrast to the *rhp54Km* haploid cells, the *rhp54Km/rhp54Km* diploid cells were extremely sensitive to HU and bleomycin and slightly sensitive to MMS and UV, but not to CPT (Fig. 4C and D). Therefore, we hypothesize that Rhp54 destruction might be necessary to prevent deleterious or uncontrolled recombination.

APC/C-dependent destruction of Rhp54 is important for the repair of DSBs in the absence of Srs2. To understand better the importance of Rhp54 destruction, epistasis analysis was performed. In support of our hypothesis, we found that an *srs2Δ rhp54Km* double mutant was hypersensitive to HU and CPT and slightly sensitive to bleomycin (Fig. 5A) but not to UV (data not shown). It is already known that an *srs2Δ rhp54Δ* double mutant is synthetically lethal and that this lethality can be rescued by the additional deletion of *rhp51*, suggesting that the lethality is caused by uncontrolled Rad51 activity (9). Similarly, to determine whether the sensitivity is dependent on Rhp51, we made an *srs2Δ rhp54Km rhp51Δ* triple mutant, which was no more sensitive to DNA-damaging agents than the *rhp51Δ* single mutant (Fig. 5A). Since Srs2 has been re-

FIG. 3. Rhp54 is destroyed in the G₁ phase of the *S. pombe* cell cycle. (A) Half-life analysis of Rhp54 at various cell cycle stages. Rhp54 was expressed from the *nmt1* (rep81) promoter in the wild type (Asyn), *cdc10-V50*-blocked G₁ phase, HU-arrested S phase, *cdc25-22*-blocked G₂ phase, or *nda3-KM311*-arrested prometaphase, and then cycloheximide (a protein synthesis inhibitor) and thiamine (a repressor of the *nmt1* promoter) were added at time zero. Samples were taken at the indicated times. The arrow indicates the Rhp54 band, whereas the asterisk denotes nonspecific bands detected by the anti-Rhp54 antibodies. ("α" represents "anti-" for each of the antibodies indicated.) As controls, endogenous Cdc13 and Cdc2 were used. (B) Rhp54 destruction in vivo depends on the APC/C and FZR/Ste9. Panel B is the same as panel A, but Rhp54 was expressed in the *cdc10-V50*-, *cdc10-V50 cut9-665*-, or *cdc10-V50 ste9Δ*-blocked G₁ phase, and the half-lives were analyzed. (C) Endogenous Rhp54 is destroyed in G₁-arrested cells in a KEN-box-dependent manner. Myc-tagged wild type and the KEN-box mutant (Km) of Rhp54 were integrated into the *S. pombe* genome under the native promoter. *cdc10-V50 myc-rhp54* and *cdc10-V50 myc-rhp54Km* cells were grown at 26°C and shifted to 36°C for 4 h, and cycloheximide was added to analyze the half-lives. (D) *myc-rhp54*⁺ and *myc-rhp54Km* cells were grown in EMM2 overnight (asynchronous [Asyn]; lanes 1 and 3), and then the cells were washed in EMM2-N and cultured for 20 h (lanes 2 and 4); the steady-state levels were analyzed by immunoblotting. (E) Rhp54 is destroyed during G₁ in cycling cells. (Upper panel) *wee1-50* cells were synchronized in G₂ by centrifugal elutriation and then shifted to 36°C to inactivate Wee1. Samples were taken every 20 min and analyzed by immunoblotting, fluorescence-activated cell sorting, and DAPI (4',6'-diamidino-2-phenylindole) staining. To detect *S. pombe* Rhp51, anti-human Rad51 antibody was used. (Lower panel) The G₁ index of each time point was measured by fluorescence-activated cell sorting analysis and DNA staining.

FIG. 4. The *rhp54Km/rhp54Km* diploid cells, but not haploid cells, exhibit DNA damage sensitivity. (A) Asynchronous wild-type, *rhp54Δ*, *rhp54Km*, *rdh54Δ*, and *ku70Δ* cells were spotted with decreasing cell numbers onto rich media containing various DNA-damaging agents and incubated for 4 days. (B) NHEJ is not affected by Rhp54Km. NHEJ was measured by circularization of a transformed linearized plasmid. Asynchronous and G₁-arrested cells (*cdc10-150* or nitrogen starvation) were transformed with uncut or linearized plasmids, and the transformation efficiency was scored as cut/uncut ratios. NHEJ was up-regulated in G₁-arrested cells. Error bars show standard deviation values derived from at least three transformation assays. (C) Same as panel A, but asynchronous homozygous diploid (*h⁺/h⁺*) cells and wild-type (wt/wt) and *rhp54Km* (*Km/Km*) cells were spotted. (D) Survival following irradiation with UV light of cells from a diploid wild type (open circle) and from a diploid *rhp54Km* strain (closed circle).

ported to prevent or limit potentially deleterious recombination by disassembly of the nucleofilament as antirecombinase (20, 50), it is possible that in the absence of *srs2*, nondegradable Rhp54 (Rhp54Km) whose levels are enhanced pushes inappropriate HR in a Rad51-dependent manner. To reinforce this notion, the amino-terminal 70 residues of *S. pombe* cyclin B (N70) were fused to Rhp54Km and integrated into the *rhp54* locus, allowing APC/C-dependent destruction of the fusion protein, N70-Rhp54Km, in G₁ phase (Fig. 5B). Strikingly, addition of N70 to Rhp54Km rescued the synergistic effect with *srs2Δ* (Fig. 5B and C), underscoring the importance of APC/C-dependent Rhp54 destruction for the proper repair of DSBs in the absence of Srs2 protein.

We furthermore considered the mechanism by which the *srs2Δ rhp54Km* double mutant shows synergistic effects using an HR assay (34). This involves the recovery of Ade⁺ recombinants from strains containing intrachromosomal recombination substrate consisting of a tandem direct repeat of *ade6* heteroalleles separated by a *his3⁺* gene. The frequency of Ade⁺ recombinants depends on HR, and the inclusion of the *his3⁺* gene allowed us to differentiate between deletion (*His⁻*) and conversion (*His⁺*) types of recombination. We found in the *rhp54Km* strain the Ade⁺ recombinant frequency was similar to that in wild type, whereas the frequency of Ade⁺ recombinants in the *srs2Δ* mutant was approximately twofold higher than that in the wild type, and ~60% of these recom-

binants were conversion types (Fig. 5D). The latter result is in agreement with previous reports (9). Notably, when *rhp54Km* was combined with the *srs2Δ* mutant, the frequency of Ade⁺ recombinants in the *srs2Δ rhp54Km* double mutant became more than fourfold higher than that in the wild type, and ~65% of these were conversion types. These results suggest that the increased sensitivity of the *srs2Δ rhp54Km* mutant to HU, CPT, and bleomycin probably reflects an increase in the occurrence of deleterious recombination. Presumably, in the single *rhp54Km* strain, the antirecombinase function of Srs2 is able to suppress most of such uncontrolled recombination.

Rhp54 destruction regulates meiotic recombination. In the haploid vegetative cell cycle, the sister chromatid is the preferred template for HR, whereas in meiosis, it is the homologous chromosome. To support this favored mode of interhomolog recombination, there is one more Rad54 homolog in *S. pombe*, Rdh54 (5). We asked whether stabilization of Rhp54 plays a role in preventing unwanted recombination between sister chromatids in meiosis. To assess interhomolog recombination, we utilized strains with two sets of interhomolog alleles (*his4-239 ade6-M26* × *lys4-95 ade6-52*) in chromosomes 2 and 3, respectively (Fig. 6A). Interhomolog recombination was measured by the rate of Lys⁺ His⁺ prototroph as well as Ade⁺ prototroph formation. Sister chromatid recombination was measured by the recombination rate within a tandem direct repeat of *ade6* heteroalleles separated by a *his3⁺* gene (Fig.

FIG. 5. Rhp54 destruction and Srs2 ensure proper HR repair. (A) Nondegradable Rhp54 (*rhp54Km*) increases DNA damage sensitivity of the *srs2Δ* mutant. Asynchronous haploid cells were spotted in decreasing cell number onto rich media containing various DNA-damaging agents and incubated for 3 to 4 days. (B) (Left panel) Schematic diagram of the constructs N70-Rhp54Km as well as Rhp54 and Rhp54Km. The KEN-box is indicated by unfilled box, whereas the KEN-box mutant is indicated by the filled box. The Cdc13 N-terminal 70 residues (N70) contain the D-box, which works as an APC/C-dependent destruction module for other proteins. (Right panel) Addition of N70 causes Rhp54Km destruction in G_1 . N70 fused to *rhp54Km* (*N70-rhp54Km*) was integrated in the genome under the native promoter. *cdc10-V50 myc-rhp54* (lanes 1 and 2), *cdc10-V50 myc-rhp54Km* (lanes 3 and 4), and *cdc10-V50 myc-N70-rhp54Km* (lanes 5 and 6) cells were grown at 26°C (Asyn) and shifted to 36°C for 4 h (G_1), and steady-state levels were analyzed by immunoblotting. (C) Destruction of Rhp54Km via APC/C rescues the toxicity of *srs2Δ rhp54Km*. Spot assays were carried out as described for panel A. (D) *srs2Δ rhp54Km* has elevated levels of spontaneous HR. Strains containing a tandem direct repeat of *ade6* heteroalleles *ade6-L469* and *ade6-M375* flanking a functional *his3* gene were constructed in the indicated genetic background. Spontaneous Ade⁺ recombinant frequencies were measured. Error bars show standard deviation values derived from at least three experiments.

6C) (5, 34). In this system, only when sister chromatid recombination occurs do Ade⁺ prototrophs recover from the *ade*⁻/*his*⁺/*ade*⁻ heteroallele. We investigated these recombination rates in the presence of nondegradable Rhp54 (*rhp54Km*) or in *rdh54Δ* as well as wild-type cells (Fig. 6). Intriguingly, *rhp54Km* suppressed interhomolog recombination approximately two-fold (Fig. 6B). Conversely, *rhp54Km* promotes sister chromatid recombination two- to threefold compared with the wild type (Fig. 6D). These results suggest that Rhp54 destruction prevents unwanted sister chromatid recombination. Moreover, these characteristic traits of *rhp54Km* are very similar to those of *rdh54Δ*. Since Rdh54, but not Rhp54, has been proposed to promote interhomolog recombination during meiosis (5), destruction of Rhp54 is likely to be required for proper Rdh54 function in meiosis. Altogether, our results suggest that Rhp54 destruction is physiologically important for accurate HR repair and meiotic recombination in *S. pombe*.

Rhp54 protein levels oscillate during meiosis. To evaluate the influence of nondegradable Rhp54 (Rhp54Km) on meiotic recombination, we finally investigated Rhp54 levels through meiotic progression. Meiosis was synchronously induced by thermal inactivation of the Pat1 kinase in prestarved homozygous (*h*⁻/*h*⁻) diploid cells. The progression of meiosis was monitored by counting the number of nuclei in these cells (Fig. 7A). Rhp54 levels were absent or very low in prestarved G_1 phase (Fig. 7B, left panel, time zero), and Rhp54 appeared as cells progressed through premeiotic S phase (~2 h). Intriguingly, Rhp54 levels slightly degraded just before anaphase I and mostly at anaphase II in meiosis, which is similar to other APC/C substrates such as Cdc13/cyclin B and Cut2/securin, suggesting that Rhp54 is degraded by APC/C in meiosis as well (Fig. 7B). In contrast, Rhp54Km levels were relatively high even in prestarved G_1 cells (Fig. 7B, right panel, time zero), increased around premeiotic S phase, and remained high.

FIG. 6. Nondegradable Rhp54 affects meiotic recombination. (A) Schematic for a meiotic interhomolog recombination assay. Recombination frequency between *lys4-95* and *his4-239* and between *ade6-M26* and *ade6-52* was measured. Pairs of sister chromatids following meiotic S phase are shown on the left. The interhomolog recombination will result in Lys⁺ His⁺ or Ade⁺ progeny. (B) Nondegradable Rhp54 (*rhp54Km*) inhibits interhomolog recombination. Wild-type, *myc-rhp54⁺*, *myc-rhp54Km*, or *rdh54Δ* haploid strains with *lys4-95 ade6-52* and *his4-239 ade6-M26* were mated, and the rate of Lys⁺ His⁺ or Ade⁺ progeny was measured. Means from at least three experiments with standard deviations are shown. Relative ratios against the wild type are also listed. *myc-rhp54⁺* and wild-type cells were examined to confirm that *myc* tagging does not affect Rhp54 function. (C) Schematic for a meiotic sister chromatid recombination assay. Haploid strains containing a tandem direct repeat of *ade6* heteroalleles, *ade6-L469* and *ade6-M375*, flanking a functional *his3* gene were mated, and the rate of Ade⁺ progeny was measured. Pairs of sister chromatids following meiotic S phase are shown on left. When a DSB occurs in one of the *ade6/his⁺/ade6* sisters (marked with a star), only repair via intra- or intersister chromatid recombination results in Ade⁺ progeny. (D) *rhp54Km* promotes sister chromatid recombination like *rdh54Δ*. Means of at least three experiments with standard deviations are shown. Relative ratios against the wild type are also listed.

These elevated levels of Rhp54Km did not affect meiotic progression or APC/C activity since the destruction profiles of Cdc13 and Cut2 are indistinguishable from those in cells expressing wild-type Rhp54. These results suggest that defects of nondegradable Rhp54 in meiotic recombination are a consequence of entering meiosis and/or progressing through meiosis with uncontrolled high levels of Rhp54. It may be noteworthy that a component of the HR pathway, Rhp51, was relatively

constant throughout meiosis in diploid cells expressing Rhp54 or Rhp54Km.

DISCUSSION

We aimed to isolate new APC/C substrates, in particular KEN-box-containing FZR/Ste9 substrates from *S. pombe*. FZR/Ste9 is believed to act between late anaphase and the end

FIG. 7. Rhp54 levels are regulated during meiotic progression. (A) *myc-rhp54⁺* or *myc-rhp54Km* was integrated into the *rhp54* locus in homozygous (*h⁺/h⁺*) diploid cells, the cells were grown overnight in rich media and then cultured in EMM2 overnight (asynchronous [Asyn]), and then the cells were washed in EMM2-N and cultured for 15 h, all at 25°C. The cells were shifted to 34°C to inactivate Pat1 kinase and induce meiosis. Samples were taken every 20 min and examined microscopically for the number of nuclei in a cell to monitor progression through meiosis. (B) Samples from panel A were analyzed by immunoblotting.

of G₁ phase, during which time cells exit mitosis and prepare for DNA replication (28, 36, 47). Because fission yeast have a very short G₁ phase and it is difficult to observe protein turnover of substrates specifically degraded in G₁, we established a cell-free FZR/Cdh1-dependent destruction assay in *Xenopus* egg extracts. Combining this with the Motif Search (Sanger Centre, United Kingdom), we identified Rhp54 as an APC/C substrate. We show that Rhp54 has a functional N-terminal KEN-box and is ubiquitinated and destroyed in an FZR/Ste9-APC/C-dependent manner. Consistently, Rhp54 is destroyed every cell cycle during the G₁ phase.

Rhp54 is a member of the SNF2/SWI2 superfamily (46) and functions in HR for the repair of DSBs. HR relies on the presence of homolog/sister chromatid to act as a substrate for error-free repair and thus operates from late S through to the end of G₂. In contrast, NHEJ is an error-prone process involving the direct sealing of broken ends. In yeast, NHEJ functions for repair only when DSBs arise during G₁, when the sister chromatid is not available. In mammalian cells, NHEJ is the main pathway for repair in both G₁ and G₂ cells, although HR is suppressed in G₁ cells as it is in yeast. The suppression of HR during the G₁ period of the cell cycle is likely to reflect cyclin-Cdk levels. It has been shown that cyclin B regulates HR (1, 4, 13, 16) and that HR and NHEJ are reciprocally regulated during the mitotic cell cycle (10, 44). Cdk activity in *S. cerevisiae* has been shown to directly regulate DNA processing (1, 16).

In *S. pombe*, the CtIP homolog Ctp1, which is essential for Mre11-Rad50-Nbs1 (MRN)-dependent DNA processing that initiates HR, has recently been shown to be regulated by Cdc10-dependent periodical transcription at G₁/S combined with ubiquitin-dependent proteolysis (22). Cdc10-dependent

transcription is initiated by an increase in Cdk activity and ensures that Ctp1 is predominantly present in the S and G₂ phases. Thus, low levels of Ctp1 during G₁ have been proposed to restrict the initiation of HR during the G₁ phase. Here we show that another HR protein, Rhp54, is independently regulated by APC/C, which is itself stimulated by high Cdk and which then negatively regulates Cdk1/Cdc2 kinase. Thus, it is likely that APC/C is involved in the switching between NHEJ and HR via ubiquitinating HR proteins or positive regulators of HR.

We would speculate that stabilizing Rhp54 protein in G₁ might increase the use of inappropriate HR during this stage of the cell cycle, possibly resulting in increased cell death in response to genotoxic stress. However, the *rhp54Km* mutant did not increase sensitivity of cells to DNA-damaging agents, unless recombination was further deregulated by concomitant deletion of *srs2Δ*. Double mutants showed hypersensitivity to HU and CPT (Fig. 5). Srs2 is an SF1 (superfamily 1) DNA helicase, which prevents recombination by dissociating Rhp51/Rad51 nucleoprotein filaments and channels DNA lesions to the postreplication repair pathway (20, 50). Since *rhp54Km* stimulates sister chromatid recombination, the hypersensitivity of the *srs2Δ rhp54Km* mutant is presumably due to deleterious recombination, which may occur prematurely during DNA replication. Most importantly, since addition of cyclin B D-box (N70) to *rhp54Km* clearly rescues the sensitivity to DNA damage (Fig. 5C), APC/C must regulate DSB repair by ubiquitinating Rhp54 in G₁.

Unlike the *rhp54Δ* mutant, in a haploid state, the *rhp54Km* strain does not show any profound phenotype or sensitivity to genotoxic reagents. However, when in a diploid state, the *rhp54Km* strain shows sensitivity to a variety of DNA-damaging agents, HU, bleomycin, MMS, and UV (Fig. 4). These results resemble that of the *S. cerevisiae* Rad54 homolog in meiosis, Rdh54/Tid1. The *rdh54Δ* diploid cells, but not the *rdh54Δ* haploid cells, show MMS sensitivity (19). In addition, Rdh54 functions in meiotic recombination between homologous chromosomes (5, 19). Thus, *rdh54Δ* in meiosis shows a decrease in interhomolog recombination as well as an increase in the more mitotic sister-chromatid recombination. When Rhp54 destruction is blocked, *rhp54Km* showed a similar effect on meiotic recombination (Fig. 6). Rdh54 functions with the Rad51 homolog in meiosis, Dmc1, which is dependent upon Rad51 for function (42). The reflection of the *rdh54Δ* phenotype in *rhp54Km* suggests that Rhp54 could be hindering Rdh54-Dmc1 function by binding and sequestering Rhp51, thereby preventing Dmc1 focus formation. This possibility is consistent with roles of Rad54 at a presynaptic phase of HR, promoting/facilitating Rad51 nucleofilament formation (26, 53), as well as in later phases, aiding strand invasion and homologous DNA pairing (39, 43, 56).

In conclusion, we have uncovered a novel role for APC/C; APC/C is a regulator of HR, destroying a key recombinase ancillary factor, Rhp54, in G₁ to control HR for the repair of DSBs as well as proper meiotic recombination. Neither *S. cerevisiae* Rad54 nor its human homologs (Rad54A and Rad54B) were destroyed in our cell-free destruction system, highlighting that alternative systems exist to control HR. It should be noted that there are differences between the organisms with regard to meiosis and meiotic recombination. Unlike

S. cerevisiae and humans, *S. pombe* strains do not form a synaptonemal complex during meiosis for the pairing of homologous chromosomes, and instead, telomere-led chromosome movement for some hours in the elongated (known as “horse-tail”) nucleus facilitates the alignment of homologous chromosomes and promotes their pairing and recombination (6–8, 41, 51). These differences may in part explain why we could only find the *S. pombe* Rad54 to be a substrate of APC/C. Recently, *S. cerevisiae* Hed1 has been reported to inhibit the formation of Rad51-Rad54 complex, thereby facilitating Dmc1-Rdh54-dependent interhomolog recombination (3, 48). Since meiosis is initiated when Rhp54 levels are absent (Fig. 7), it is tempting to speculate that Rhp54 destruction in *G*₁ might play a role in switching to the Rdh54-dependent pathway in *S. pombe* (Fig. 6), although it still remains possible that a Hed1 homolog (equivalent) exists and functions in a similar way to *S. cerevisiae* Hed1. Furthermore, in humans, Rad51AP1 (Rad51-associated protein 1) has been shown to enhance Rad51 recombinase activity and a knockdown impaired D-loop formation and genomic integrity (52), which are similar properties to Rad54 (15). The importance of controlling HR is underscored by the observation that in *BRCA1*-deficient breast tumors, not only Rad51 but also Rad54 and Rad51AP1 are overexpressed to promote cancer development (25). Therefore, it is conceivable that programmed proteolysis of HR factors may add one more layer to precisely control the pathway.

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