GSK-3 β Homolog Rim11 and the Histone Deacetylase Complex Ume6-Sin3-Rpd3 Are Involved in Replication Stress Response Caused by Defects in Dna2

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ABSTRACT Lagging strand synthesis is mechanistically far more complicated than leading strand synthesis because it involves multistep processes and requires considerably more enzymes and protein factors. Due to this complexity, multiple fail-safe factors are required to ensure successful replication of the lagging strand DNA. We attempted to identify novel factors that are required in the absence of the helicase activity of *Dna2*, an essential enzyme in Okazaki-fragment maturation. In this article, we identified *Rim11*, a GSK-3 β -kinase homolog, as a multicopy suppressor of *dna2* helicase-dead mutant (*dna2-K1080E*). Subsequent epistasis analysis revealed that *Ume6* (a DNA binding protein, a downstream substrate of *Rim11*) also acted as a multicopy suppressor of the *dna2* allele. We found that the interaction of *Ume6* with the conserved histone deacetylase complex *Sin3-Rpd3* and the catalytic activity of *Rpd3* were indispensable for the observed suppression of the *dna2* mutant. Moreover, multicopy suppression by *Rim11/Ume6* requires the presence of sister-chromatid recombination mediated by *Rad52/Rad59* proteins, but not vice versa. Interestingly, the overexpression of *Rim11* or *Ume6* also suppressed the MMS sensitivity of *rad59 Δ* . We also showed that the lethality of *dna2* helicase-dead mutant was attributed to checkpoint activation and that decreased levels of deoxynucleotide triphosphates (dNTPs) by overexpressing *Sml1* (an inhibitor of ribonucleotide reductase) rescued the *dna2* mutant. We also present evidence that indicates *Rim11/Ume6* works independently but in parallel with that of checkpoint inhibition, dNTP regulation, and sister-chromatid recombination. In conclusion, our results establish *Rim11*, *Ume6*, the histone deacetylase complex *Sin3-Rpd3* and *Sml1* as new factors important in the events of faulty lagging strand synthesis.

KEYWORDS GSK-3b; genome instability; checkpoint; Dna2; HDAC

DNA replication is an important source of intrinsic DNA damage due to a number of barriers that need to be overcome, which include erroneously incorporated nucleotides, lesions, aberrant DNA structures in the template, tightly bound proteins, and clashes between replication and transcription machineries (for a review on eukaryotic replication machinery see Leman and Noguchi 2013). The lagging strand synthesis is a highly complex process and thus is prone to flawed DNA synthesis. Unlike leading strands, lagging

strands are synthesized as discontinuous short fragments, called Okazaki fragments, which are later ligated to form continuous duplex DNA. Polymerase (Pol) α -primase synthesizes ~ 10 nt of RNA, followed by the extension of short (~ 20 – 30 nt) DNA. Replication factor C recognizes the RNA–DNA primer, dissociates the Pol α -primase, and in turn loads the proliferating nuclear antigen (PCNA). PCNA subsequently recruits Pol δ , which extends the primer RNA–DNA to form an Okazaki fragment. When Pol δ encounters a downstream Okazaki fragment, it may displace the 5'-end region of the downstream fragment, creating a single-stranded DNA (ssDNA) flap structure. This flap DNA is then removed by the combined actions of *Rad27* (yeast *Fen1*) and *Dna2* (Bae *et al.* 2001a), resulting in the formation of nicks that are readily sealed by *Cdc9* (DNA ligase I). The flaps generated during Okazaki fragment synthesis, if not properly processed, can be

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doi: <https://doi.org/10.1534/genetics.116.198671>

Manuscript received November 26, 2016; accepted for publication April 18, 2017; published Early Online May 2, 2017.

Supplemental material is available online at www.genetics.org/lookup/suppl/doi:10.1534/genetics.116.198671/-/DC1.

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an intrinsic source of replication stress, due to a variety of possible structures depending on the sizes and nucleotide sequences of the flaps. It was shown that the helicase activity of *Dna2* can play a critical role by removing the secondary structure in flaps, facilitating the subsequent cleavage catalyzed by its own endonuclease activity. In the absence of *Dna2* helicase, RPA plays a critical role in assisting *Dna2* nuclease (Bae *et al.* 2001a, 2002). Long flaps rapidly bind RPA, forming RPA-coated flaps free of secondary structures that are readily cleaved by *Dna2*. The survival of the *dna2* helicase mutant was shown to require the DNA-annealing activity of *Rad52* and its ability to interact with *Rad59* and sister-chromatid cohesion (Lee *et al.* 2014).

Although the nuclease functions of *Dna2* have been studied intensively, its helicase function has not been clearly defined. To better understand the role of *Dna2* helicase, a genetic screen for multicopy suppressors of *dna2-KE* (K1080E, an ATPase/helicase-negative mutant) was performed. We isolated *Rim11*, a GSK-3 β homolog, and subsequently its substrate *Ume6* as multicopy suppressors of *dna2-KE*. *Rim11* was initially discovered as a suppressor of a meiosis-inducible transcription factor, *Ime1*, toxicity in starved haploid cells, hence the name Regulator of Inducer of Mitosis 11 (*RIM11*) (Mitchell and Bowdish 1992). Since *Ime1* activates transcription of early meiotic genes, its overexpression is toxic to haploid cells under starvation conditions. This toxicity is exacerbated by a mutation in *RAD52*. Recessive mutations in *RIM11* suppress *Ime1* toxicity in starved *rad52* haploid mutant cells. In media with acetate as the sole carbon source, *Rim11* phosphorylates *Ime1* and *Ume6*, a ubiquitously expressed transcription factor, consequently promoting the formation of a *Ume6*-*Ime1* complex (Bowdish *et al.* 1994; Malathi *et al.* 1997), which plays a critical role in regulating meiosis-specific genes (Rubin-Bejerano *et al.* 1996).

Ume6 is also known for its other functions in transcription that include regulation of phospholipid biosynthesis (Jackson and Lopes 1996), arginine metabolism (Strich *et al.* 1994), peroxisomal function (Einerhand *et al.* 1995), autophagy (Bartholomew *et al.* 2012), and DNA repair genes (Sweet *et al.* 1997). *Ume6*, along with other genes like *RAD9*, *SRS2*, and *TOF1*, was identified in a screen for gene deletions that suppress an allele of *MCM2* lacking DDK phosphorylation sites (*mcm2_{AA}*) (Stead *et al.* 2012). Moreover, another screen found *UME6* as an essential gene for cell viability when *Srs2* or its mutants are overexpressed (Leon Ortiz *et al.* 2011). Furthermore, *UME6* deletion enhances the camptothecin (CPT) sensitivity of *rad55 Δ* , *rad51-K191R*, and *rad52 Δ -327* cells (Valencia-Burton *et al.* 2006). The *Ume6* null mutant also display higher chromosomal loss and duplication compared to wild type (Fazzio *et al.* 2001). Although these reports implicate *Ume6* in joint action with several genes in DNA replication and repair, its role has not been examined thoroughly.

Ume6 binds to Upstream Repressing Sequence 1 (*URS1*) in a Zn²⁺-dependent manner via the Zn₂Cys₆ binuclear cluster domain located at its C terminus (Anderson *et al.* 1995).

Ume6 recruits two complexes, *Sin3-Rpd3* (Kadosh and Struhl 1997) and *Isw2* (Goldmark *et al.* 2000), to *URS1*. Interestingly, a synthetic-sickness interaction was observed between *rp3* and *dna2* (Budd *et al.* 2005), suggesting that the *Sin3-Rpd3* complex is important when *Dna2* is defective. Furthermore, deletion of *SIN3* or *RPD3* suppresses *rad9 Δ* and *mec1-21* UV sensitivity and restores the G₂/M delay induced by DNA damage in these checkpoint mutants (Scott and Plon 2003). Another study showed that deletion of *RPD3* causes early activation of late origins and that in the presence of hydroxyurea (HU), *rp3 Δ* cells elude checkpoint inhibition (Aparicio *et al.* 2004), implying a role for *Sin3-Rpd3* in regulating replication timing and the intra-S checkpoint.

In this study, we identified a *Rim11*-initiated pathway consisting of *Ume6* and the *Sin3-Rpd3* deacetylase complex that requires the presence of recombination proteins *Rad52* and *Rad59*. Additionally, we showed that second site mutations in checkpoint genes *RAD9* or *RAD53* and downregulation of deoxynucleotide triphosphate (dNTP) levels allow *dna2-KE* cells to be viable.

Materials and Methods

Strains and plasmids

The genotypes of *Saccharomyces cerevisiae* strains and plasmids used in this study were summarized in Table 1 and Table 2, respectively. All strains are haploid and are derived from YPH499 or YPH500. Deletion strains were prepared by standard gene replacement techniques (Longtine *et al.* 1998) and genetic crosses. In YJA1BJK65, the chromosomal copy of *DNA2* was deleted and complemented with wild-type *DNA2* in a low-copy plasmid, pRS316-*Dna2*. YJA1BJK65 was designed to also carry pRS314-*Dna2*-K1080E, a low-copy plasmid with a helicase-negative *DNA2* allele. The *RIM11*, *UME6*, *IME1*, *SIN3*, *RPD3*, *RAD52*, *RAD59*, *RAD9*, *RAD53*, and *SML1* genes were individually deleted in YJA1BJK65. Overexpression experiments were carried out using high-copy (2 μ -based) plasmid, pRS325, under the control of the indicated genes' native promoter. For complementation of mutations, the low-copy (*CEN*-based) plasmid, pRS315, was used instead.

dna2-KE suppression assay

YJA18JK65 (*dna2 Δ ::HIS3*, pRS316-*Dna2*, and pRS314-*Dna2*-K1080E) was transformed with the pRS315-*Dna2* plasmid expressing wild-type *Dna2* (positive control), empty pRS325 (negative control), or other 2 μ -based plasmids (Table 2) as indicated in each figure. Transformants were inoculated in synthetic dropout (SD) liquid medium (3 ml) and grown at 30° until saturation. Each saturated culture was spotted in 10-fold serial dilutions on SD plates without (control, *DNA2*) or with (5-FOA, *dna2-KE*) 5-FOA (1 g/l) to allow the removal of pRS316-*Dna2*, containing the wild-type *DNA2*, followed by incubation for 4–5 days at 30°. For Figure 4D, Figure 6B, and Figure 7B, ~300 cells were spread onto

Table 1 Strains used in this study

Strain	Genotype	References
YPH499	<i>MATa ade2-101 ura3-52 lys2-801 trp1-63 his3-200 leu2-Δ1 GAL+</i>	Laboratory collection
YPH500	<i>MATα ade2-101 ura3-52 lys2-801 trp1-63 his3-200 leu2-Δ1 GAL+</i>	Laboratory collection
YJA1BJK65	<i>MATα ade2-101 ura3-52 lys2-801 trp1-63 his3-200 leu2-Δ1 GAL+ dna2Δ::HIS3 [pRS316-DNA2, pRS314-dna2-K1080E]</i>	Kang et al. (2009)
YAD1	<i>YJA1BJK65 rim11Δ::KanMX4</i>	This study
YAD2	<i>YJA1BJK65 ume6Δ::KanMX4</i>	This study
YAD3	<i>YJA1BJK65 ime1Δ::KanMX4</i>	This study
YAD4	<i>YJA1BJK65 sin3Δ::KanMX4</i>	This study
YAD5	<i>YJA1BJK65 rpd3Δ::KanMX4</i>	This study
YAD6	<i>YJA1BJK65 rad52Δ::KanMX4</i>	Lee et al. (2014)
YAD7	<i>YJA1BJK65 rad59Δ::KanMX4</i>	This study
YAD8	<i>YJA1BJK65 rad9Δ::KanMX4</i>	This study
YAD9	<i>YJA1BJK65 rad53-R605A::KanMX4</i>	This study
YAD10	<i>YJA1BJK65rad53Δ::LEU2 sml1Δ::KanMX4</i>	This study
YAD11	<i>YPH499, ume6Δ::HphMX</i>	This study
YAD12	<i>YPH499, rad51Δ::KanMX4</i>	This study
YAD13	<i>YPH499, rad52Δ::KanMX4</i>	This study
YAD14	<i>YPH499, rad59Δ::KanMX4</i>	This study
YAD15	<i>YPH499, rad51Δ::KanMX4 ume6Δ::HphMX</i>	This study
YAD16	<i>YPH499, rad52Δ::KanMX4 ume6Δ::HphMX</i>	This study
YAD17	<i>YPH499, rad59Δ::KanMX4 ume6Δ::HphMX</i>	This study
JKM139	<i>MATa ho hml::ADE1 hmr::ADE1 ade1-100 leu2-3,112 lys5 trp1::hisG ura3-52 ade3::GAL10::HO</i>	Laboratory collection
YAD18	<i>JKM139, UME6::UME6-13myc-KanMX4</i>	This study
YAD19	<i>YPH500, DNA2::dna2-K1080E-HIS3</i>	This study

SD media without 5-FOA to determine the total number of cells and ~1000 cells were spread onto SD media containing 5-FOA to determine the number of *dna2-KE* survivors.

DNA damage sensitivity assay

Asynchronously growing cells (~200–300 cells) were spread onto YPD medium with or without the indicated DNA damaging agents MMS, HU, 4-nitroquinoline 1-oxide (4-NQO), or CPT. Plates were incubated at 30° and the surviving colonies were counted after ~3–6 days of incubation. For Figure 5A, cells densities were adjusted to OD 1, and 10-fold serial dilutions of each strain were spotted onto SD medium with or without the indicated DNA damaging agents.

Analysis of protein levels

Total proteins were extracted by the trichloroacetic acid (TCA) precipitation method. Briefly, harvested cells were suspended in lysis buffer (1.85 M NaOH, 7.5% β-mercaptoethanol, 150 μl) and incubated for 10 min on ice. TCA (20% w/v, 150 μl) was added and incubated for another 10 min. Samples were centrifuged for 10 min at 13,000 rpm. Supernatants were discarded and the pellets were suspended in standard SDS sample buffer and boiled for 10 min. The TCA-precipitated proteins were separated by SDS-PAGE and blotted onto nitrocellulose membrane. The expression levels of proteins were determined using M2 FLAG antibody (Sigma Chemical, St. Louis, MO). This antibody constantly produces a nonspecific band (~100 kDa) and therefore was routinely used as an internal loading control for Western blot analysis.

Cell-cycle progression analysis

Yeast cells in log-phase growth were synchronized in G₁-phase with α-factor (2.5 μg/ml). After 1 hr of treatment, a fresh dose of α-factor was added to a final concentration of 5 μg/ml, followed by incubation until >95% cells became unbudded as determined by microscopic analysis. The release from α-factor arrest was performed by centrifugation and resuspension of cells in fresh YPD media. G₁-arrested cells were released into YPD and samples were collected every 15 min. Harvested cells (1 ml) were then suspended in 70% ethanol and fixed overnight at 4°. Cells were washed and suspended in 0.5 ml of 50 mM sodium/citrate buffer. RNase A was added to cells to a final concentration of 0.25 mg/ml, and the mixture was left overnight at 37°. Cells were collected by centrifugation and suspended in 1 ml of 50 mM sodium/citrate-containing propidium iodide (16 μg/ml). To disrupt cell aggregates, suspended cells were subjected to brief sonication prior to flow cytometric analysis.

Reverse-transcription and real-time PCR

Total RNA was extracted using the QIAGEN (Valencia, CA) RNeasy Kit according to the manufacturer's instructions, and subsequently treated with DNA-free DNase treatment and removal reagents (Ambion). Quantitative PCR (qPCR) and RT-PCR were carried out using TOPreal qPCR 2X PreMIX (SYBR Green with low ROX) (Enzynomics) and TOPscript cDNA Synthesis Kit (Enzynomics), respectively. The expression of *DMC1* was detected using primer pairs (forward 5' GAAGTCTGGTGGGATATACACAG 3'; and reverse

Table 2 Plasmids used in this study

Plasmid	Reference
316-Native-DNA2	Kang <i>et al.</i> (2009)
315-Native-DNA2	This study
314-Native-dna2-K1080E	Kang <i>et al.</i> (2009)
313-Native-dna2-K1080E	This study
315-Native-dna2-K1080E	This study
303-Native-dna2-K1080E	This study
325-Native-RIM11-FLAG	This study
325-Native-rim11-K68A-FLAG	This study
325-Native-lme1-FLAG	This study
325-Native-UME6-FLAG	This study
325-Native-ume6-6C6A-FLAG	This study
325-Native-ume6-M530T-FLAG	This study
325-Native-ume6-K635E-FLAG	This study
325-Native-SIN3-RPD3-FLAG	This study
325-Native-UME6-RPD3-FLAG	This study
325-Native-UME6-rpd3-H150A-H151A-FLAG	This study
325-ADH1-RAD52	This study
325-Native-RAD52	This study
325-Native-RAD52-UME6	This study
325-Native-SML1	This study
325-Native SML1-UME6	This study
325-ADH1-RAD59	This study
315-Native-RAD52	This study
315-Native-RAD51	This study

5' GGGATAAATCCCACCTGTATGA 3'). The relative expression between samples was analyzed using the $2^{-\Delta\Delta CT}$ method.

Data availability

Strains and reagents are available upon request. All data that we used for our investigations are included in the manuscript or in the Supplemental Material in [File S1](#).

Results

While the function of the endonuclease activity of *Dna2* has been extensively studied, its helicase functions remain largely unclear. To understand the role of *Dna2* helicase, a genetic screen was conducted to identify novel factors important in cells with defective *Dna2* helicase activity. A point mutation in the ATP binding motif (K1080E) of *Dna2* led to inactivation of its ATPase and helicase activities, rendering the mutant inviable (Budd *et al.* 1995).

GSK-3 β homolog, Rim11, and its substrate Ume6 are multicopy suppressors of dna2-KE

In this screen, we identified *Rim11* as a multicopy suppressor of the *dna2-KE* (*dna2-K1080E*) mutant. Overexpression of kinase-dead *Rim11* (*Rim11-KA*, K68A) did not suppress the inviability of *dna2-KE*, suggesting that *Rim11* kinase activity is essential for the suppression observed (Figure 1A). Western blot analysis of both wild-type and kinase-defective *Rim11* revealed comparable expression levels, indicating that the lack of expression was not the cause of *Rim11-KA* failure to rescue *dna2* (Figure 1B). Expression of *Rim11* using multicopy (2 μ -based) plasmid

rescued the *dna2-KE* mutant, but *Rim11* expression using low-copy (*CEN*-based) plasmid failed to do so (Figure S1 in [File S1](#)).

Since *Rim11* is a kinase, we asked whether one of its known physiological substrates is responsible for this suppression. It was shown that *Rim11* has two proteins, *Ume6* and *Ime1*, as its kinase substrate (Malathi *et al.* 1997). To determine which one of the two proteins is responsible for the suppression, each of *Ume6* and *Ime1* was overexpressed under their respective native promoters in the *dna2-KE* mutant, and the resulting cell growth was examined on 5-FOA plates. We found that only *Ume6* rescued the inviability of *dna2-KE* upon overexpression (Figure 1C). The failure of *Ime1* to suppress the *dna2-KE* lethality is not due to lack of expression (Figure 1D). These results indicate that *Ume6*, not *Ime1*, mediates the *Rim11*-dependent suppression of the *dna2-KE* lethality as a substrate of *Rim11*.

Next, to confirm that both *Rim11* and *Ume6* are working in an epistatic manner, *dna2-KE* mutants devoid of *RIM11*, *UME6*, or *IME1* were constructed as described in *Materials and Methods*. Each of these double mutants was transformed with 2 μ plasmids expressing *RIM11*, *UME6*, or *IME1* under the control of their respective native promoters. Overexpression of *Rim11* in *ume6 Δ dna2-KE* failed to rescue the lethal phenotype of *dna2-KE*; however, when *Ume6* was overexpressed in *rim11 Δ dna2-KE*, the cells were viable (Figure 1E). Deletion of *IME1* did not affect the suppression ability of either *Rim11* or *Ume6*. This result is consistent with the results above shown in Figure 1, A and C, supporting that *Ume6* functions downstream of *Rim11* as a substrate and that this event is independent of *Ime1*.

Ume6 exhibits a Zn²⁺-dependent binding to the *URS1* DNA sequence that is dependent on the cysteine-rich region on its C terminus (Anderson *et al.* 1995). To determine whether this zinc-cluster domain is important for *Ume6* to rescue the *dna2-KE* mutant, the six cysteine residues at the C terminus were mutated to alanine (*Ume6-6C6A*). The result is that the overexpression of *Ume6-6C6A* did not suppress the *dna2-KE* mutant (Figure 1F), indicating that the ability of *Ume6* to bind DNA via its zinc-cluster domain is crucial for rescuing the *dna2-KE* mutant. To exclude the possibility that the introduction of multiple point mutations was affecting *Ume6-6C6A* expression, the expression level of *Ume6-6C6A* was examined and it was found that its expression was not impaired in *dna2-KE* cells (Figure 1G).

Ume6 has a role in DNA replication and repair

Since *Ume6* suppressed the helicase defect of *dna2*, an essential enzyme in DNA replication, we examined the possibility of whether deletion of *UME6* could affect the S-phase progression of cells. To this end, we arrested wild-type and *ume6 Δ* cells in G₁ phase by α -factor treatment and released cells into YPD medium. We found that wild-type cells completed S phase in 45 min after release from G₁ phase. In contrast, it took a longer time (90 min) for *ume6 Δ* cells to finish S phase (Figure 2A). The observed delay in S-phase

Figure 1 Rim11 and its substrate Ume6 are multicopy suppressors of *dna2-KE*. (A) Overexpression of Rim11 suppressed *dna2-KE* and it is dependent on its kinase activity. Briefly, YJA18JK65 (*dna2 Δ ::HIS3*, pRS316-Dna2, and pRS314-Dna2-K1080E) was transformed with pRS315-Dna2 (positive control), vector only (none, negative control), pRS325-Rim11, or pRS325-Rim11-KA (K68A) under the control of each native promoter. Transformants were inoculated in SD liquid medium (3 ml) and grown until saturation. Each saturated culture was spotted in 10-fold serial dilutions on SD plates without (control) or with 5-FOA (0.1%) followed by incubation for 4–5 days at 30°. (B) Expression of Rim11 and Rim11-KA proteins were examined by Western blot using anti-FLAG antibody. Nonspecific band (~100 kDa) that always appears with anti-FLAG antibody was used as internal loading control. (C) Overexpression of Ume6, not Ime1, suppressed *dna2-KE* mutant. (D) Expression of Ime1 from a multicopy plasmid was confirmed by Western blot using anti-FLAG antibody, while a nonspecific band serves as a loading control. (E) Ume6 is working downstream of Rim11 in the rescue of *dna2-KE* mutant defect. (F) Zn²⁺-dependent DNA binding activity of Ume6 is indispensable for the suppression of *dna2-KE*. (G) Ume6-6C6A protein level was examined by Western blotting using anti-FLAG antibody.

progression raises the possibility that Ume6 plays a role in facilitating DNA replication.

Rim11 protein abundance increases in response to DNA replication stress (Tkach *et al.* 2012). We asked whether the same is true for Ume6. Logarithmically growing cells with endogenously myc-tagged Ume6 were grown in YPD broth with or without the presence of 0.033% MMS for 1.5 hr and subsequently subjected to total protein precipitation and Western blotting. The levels of Ume6 protein were compared, and the result was that in the presence of MMS, a significant reduction in the level of Ume6 was observed (Figure 2B). This observation suggests that the presence of DNA damage affects the stability of Ume6 protein *in vivo*.

ume6 Δ cells were resistant to CPT, but when combined with the *rad51* or *rad55* mutation, they became more sensitive to CPT than *rad51* and *rad55* single mutants (Valencia-Burton *et al.* 2006). High-throughput studies revealed that deletion of UME6 renders cells highly sensitive to MMS and HU (<http://yeastgenome.org>). We decided to reexamine the resistance of the *ume6 Δ* mutant to DNA damaging agents including MMS, HU, CPT, and 4-NQO. We found that *ume6 Δ* cells have increased sensitivity to all agents tested (Figure 2C), although they were only mildly sensitive to CPT which induces double-strand breaks (DSBs). It is noteworthy that the concentration of CPT used in our experiment was higher (7 μ g/ml) compared to that in Valencia-Burton *et al.* (2006),

where a CPT concentration of 0.6 μ g/ml was used. The sensitivity phenotype of *rad52*, *rad55*, and *rad51* mutants are apparent even in a low concentration of CPT (0.6 μ g/ml) (data not shown) (Valencia-Burton *et al.* 2006).

The interaction of Ume6 with the Sin3-Rpd3 complex is essential for *dna2-KE* survival

Ume6 forms a complex with the highly conserved histone deacetylase complex Sin3-Rpd3 to regulate expression of a number of genes. It was shown that Ume6 helps recruit Sin3-Rpd3 to DNA via direct and specific protein–protein interactions between Ume6 and Sin3; the Sin3-Rpd3 complex itself lacks detectable DNA binding activity (Washburn and Esposito 2001). Next, we addressed the question whether the transcription function of Ume6 and its direct interaction with Sin3 are important for the suppression of the *dna2-KE* growth defect or not. For this purpose, we tested two previously characterized mutant proteins of Ume6, Ume6-MT (M530T) and Ume6-KE (K635E). While both are defective in the transcription function, Ume6-KE retains its ability to interact with Sin3, but Ume6-MT does not. The point mutation M530T abolished the ability of Ume6 to suppress the *dna2-KE* mutant (Ume6-MT, Figure 3A). On the contrary, the mutation K635E showed wild-type-like suppression (Ume6-KE, Figure 3A). Western blot data confirmed that this is not due to differences in expression levels since each

Figure 2 A role for Ume6 in DNA replication and repair. (A) Cells lacking Ume6 are impeded in S-phase progression. (B) Ume6 protein level diminished when exposed to DNA damaging agent, MMS. Logarithmically growing cells with endogenously tagged (myc) Ume6 were grown in YPD liquid media with or without the presence of 0.033% MMS for 1.5 hr. Total proteins were extracted and subjected to Western blot analysis. (C) Ume6 is required in cells resistance against DNA damage. Cells (200–300 cells) of the indicated strains were spread onto YPD media with or without the indicated concentrations of CPT, HU, MMS, and 4-NQO. Plates were grown at 30° for ~3–6 days and the number of surviving colonies were counted. Data are representative of three independent experiments. WT, wild type.

mutant showed comparable levels of expressed proteins (Figure 3B). Moreover, overexpression of *Rim11* or *Ume6* in the absence of *SIN3* or *RPD3* did not allow *dna2-KE* to grow (Figure 3C). These results indicate that *Ume6* has to interact with *Sin3-Rpd3* to rescue the lethal phenotype of *dna2-KE*.

Since we found that both *Rim11* and *Ume6* act as multicopy suppressors of *dna2-KE*, we asked whether the *Sin3-Rpd3* complex itself could rescue *dna2-KE* when overexpressed using 2μ-based plasmid. Although the ability of *Ume6* to interact with the *Sin3-Rpd3* complex is crucial for suppression (Figure 3A), the multicopy expression of *Sin3* and *Rpd3* failed to suppress the *dna2-KE* mutant (Figure 3D). One possible explanation for this observation is that *Ume6* is the limiting factor and, thus, increases in the level of *Sin3* and *Rpd3* may not be sufficient to rescue *dna2-KE*.

Since the ability of *Ume6* to interact with *Sin3-Rpd3* is indispensable, we asked whether the deacetylase activity of

Rpd3 is essential for the *Ume6*-mediated suppression of the *dna2* helicase-dead mutant. *Ume6* was coexpressed with either wild-type *Rpd3* or deacetylase-negative *Rpd3*-HHAA (H150, 151A) in *rp3Δ dna2-KE*. We found that the *Rpd3*-HHAA mutation abolished the ability of *Ume6* to suppress *dna2-KE* (Figure 3E). Western blot analysis revealed that both *rp3* mutant alleles were expressed at a comparable level (Figure 3F). This result suggests that the deacetylase activity of *Rpd3* is essential for suppression of the *dna2-KE* cells.

Rim11/Ume6-mediated suppression of dna2-KE requires recombination proteins Rad52 and Rad59

We previously isolated *Rad52* as a multicopy suppressor of *dna2-KE* (Lee et al. 2014). We then asked whether there is a connection between *Rim11/Ume6* and *Rad52*. The *Rad52*-mediated suppression of *dna2-KE* depends on *Rad59* but not

Figure 3 Rim11/Ume6 suppressed the lethality of *dna2-KE* mutant in a manner dependent on the histone deacetylase complex, Sin3-Rpd3. (A) Ume6 interaction with Sin3 is essential and not its transcription regulation activity *per se*. (B) The expression level of Ume6 mutants were examined by Western blot using anti-FLAG antibody. (C) Ume6-dependent suppression of *dna2-KE* requires the presence of both Sin3 and Rpd3. *dna2-KE* mutant devoid of either *SIN3* or *RPD3* was transformed with 2 μ -based plasmid expressing either Rim11 or Ume6. (D) Overexpression of Sin3-Rpd3 did not rescue *dna2-KE*. (E) Rpd3 deacetylase activity is essential for the Ume6-dependent suppression of *dna2-KE*. (F) The expression levels of Rpd3 wild-type and deacetylase-negative proteins were examined by Western blot.

Rad51. Therefore, we constructed the double mutants *rad52Δ dna2-KE* and *rad59Δ dna2-KE* to test whether *Rim11* or *Ume6* retains its ability to rescue the growth defect of *dna2* in cells devoid of *RAD52* or *RAD59*. Our result shows that in the absence of *Rad52* or *Rad59*, *Rim11* or *Ume6* failed to rescue the *dna2* mutant phenotype (Figure 4A). In addition, the *Rim11/Ume6*-mediated rescue of *dna2-KE* was independent of *Rad51*, as indicated by the ability of *Rim11/Ume6* to rescue *dna2-KE* even in the absence of *RAD51* (data not shown). However, the loss of *RIM11*, *UME6*, *SIN3*, or *RPD3* did not affect the ability of *Rad52* to suppress growth defects of *dna2* (Figure 4, B and C). These results give rise to two possibilities: (1) *Rim11/Ume6* may have a role upstream of *Rad52/Rad59*; or (2) *Rim11/Ume6* and *Rad52/Rad59* are working on two parallel pathways, in which *Rad52/Rad59* is the major pathway, therefore deletion of *RAD52* or *RAD59* makes the *dna2-KE* cells more “sick” and the *Rim11/Ume6* overexpression simply does not overcome this additional stress. We decided to examine the suppression level of *dna2-KE* when both *Ume6* and *Rad52* were cooverexpressed. We found that the cooverexpression of *Rad52* and *Ume6* showed better suppression compared to *Ume6* or *Rad52* only (Figure 4D). It is also apparent that *Rad52* is a stronger suppressor than *Ume6*. Collectively, our results suggest that the *Rad52/Rad59*-mediated recombination is essential even in the presence of multicopy expression of *Rim11/Ume6*, but not vice versa.

UME6 interacts genetically with recombination genes

Since we found that in the absence of *Rad52* or *Rad59*, *Rim11* or *Ume6* failed to rescue *dna2*, we decided to further investigate this interaction. The null mutants *rad52Δ*, *rad59Δ*, and *rad51Δ* are sensitive to MMS (see Figure 5A). In SD plates, all three mutants above could not grow in the presence of

0.005% (data not shown; *rad59Δ*, Figure 5A). In the presence of reduced levels (0.001%) of MMS, they could grow in the SD plates (*rad52Δ* and *rad51Δ*, Figure 5A). Under this condition, we tested whether multicopy expression of either *Rim11* or *Ume6* has any influence on the MMS sensitivities of the *rad* mutants. Surprisingly, overexpression of *Rim11* or *Ume6* rendered *rad59Δ* mutant cells resistant to 0.005% MMS (top panel, Figure 5A). The growth of *rad52Δ* and *rad51Δ* mutants, however, were not affected by the multicopy expression of *Rim11* or *Ume6* when they were grown on SD plates containing 0.001% MMS (middle and bottom panel, respectively; Figure 5A).

To further examine this interaction, double mutants of *ume6Δ* in combination with *rad52Δ*, *rad59Δ*, or *rad51Δ* were constructed via mating and tetrad dissection. Based on tetrad analysis, we confirmed that these double mutants were viable but showed synthetic sickness (data not shown). DNA-damage-sensitivity assays with these double mutants revealed an additive/synergistic effect in terms of MMS or HU sensitivity (Figure 5B). The double mutants were more sensitive to MMS and HU compared to single mutants alone. These results suggest a role for *Ume6* in DNA repair when recombination factors *Rad51*, *Rad52*, and *Rad59* are absent (Figure 5C).

dna2-KE is rescued by mutation in checkpoint genes and reduced levels of dNTP

A new role of *Dna2* in checkpoint control was previously reported (Kumar and Burgers 2013). Other mutations in the helicase domain of *Dna2* such as *dna2-1* and *dna2-2* led to increased phosphorylation of *Rad53* (ATM) (Budd *et al.* 2006), and deletion of *RAD9*, a gene coding for a checkpoint protein, alleviated the growth defects of *dna2-2* (Olmezer

Figure 4 Ume6 is working independently of Rad52/Rad59. (A) Multicopy suppression by Rim11/Ume6 requires the presence of Rad52 and Rad59. The Rad52/Rad59-dependent suppression of *dna2-KE* does not require (B) Rim11, Ume6, (C) Sin3, or Rpd3. (D) Ume6 and Rad52 showed synergistic interaction in the suppression of *dna2-KE*. Data are representative of three independent experiments.

et al. 2016). Moreover, *Sin3-Rpd3* itself was implicated in the checkpoint (Scott and Plon 2003; Aparicio *et al.* 2004). We, therefore, asked whether the checkpoint has a role in the inviability of *dna2-KE* mutant. We combined the mutation *dna2-KE* with *rad9Δ*, *rad53-R605A* (with reduced kinase activity) (Pike *et al.* 2003), or *rad53Δ sml1Δ* and tested the viability of the resulting multiple mutants (*dna2-KE rad9Δ*, *dna2-KE rad53-R605A*, *dna2-KE rad53Δ sml1Δ*) on 5-FOA plates (Figure 6A). We did not test *rad53Δ* alone, since *rad53Δ* cells are inviable (Zhao *et al.* 1998). All three mutations in the checkpoint made the *dna2-KE* cells viable.

Deletion of *SIN3* or *RPD3* restores the DNA damage-induced G₂/M delay in *rad9Δ* (Scott and Plon 2003), suggesting the involvement of *Sin3* or *Rpd3* in relieving cell-cycle arrest. However, *Ume6* has not been tested in this regard. Since checkpoint activation is the likely cause of the *dna2-KE* growth defect as shown above, we asked whether *Ume6* works in checkpoint inhibition. The combination of *Ume6* overexpression and *RAD9* deletion was tested in *dna2-KE*. We found that the combination of *Ume6* overexpression and *rad9Δ* resulted in a synergistic increase in cell viability; the combined action of increased levels of *Ume6* and *rad9Δ* gave rise to a greater number of viable cells than the sum of *Ume6* overexpression and *rad9Δ* each did alone (Figure 6B). This observation implies that *Ume6* is working in a different process independent from, but in parallel with, checkpoint inhibition in the suppression of *dna2-KE*.

One prominent physiological process during checkpoint activation is the degradation of *Sml1*, an inhibitor of ribonucleotide reductase (RNR); *Sml1* is acutely degraded in response to checkpoint activation, and deletion of *MEC1* or *RAD53* allows *Sml1* to accumulate (Zhao *et al.* 2001; Barlow *et al.* 2008). We asked whether overexpression of *Sml1* could allow *dna2-KE* cells to grow. For this purpose, *Sml1* was overexpressed using 2μ-based plasmid in the *dna2-KE* mutant, and the resulting cells were spotted on 5-FOA plate. We found that *Sml1* overexpression suppressed

the inviability of *dna2-KE* cells (Figure 7A) and is dependent on its ability to inhibit RNR (data not shown). Next, we asked whether the *Sml1*-mediated rescue of *dna2-KE* is dependent on *Rad52/Rad59*, *Rim11/Ume6*, or *Yen1*. To test this possibility, *Sml1* was overexpressed in the double mutants *rad59Δ dna2-KE*, *ume6Δ dna2-KE*, and *yen1Δ dna2-KE*. The result was that *Sml1*-dependent suppression of *dna2-KE* was intact, although noticeably decreased in the absence of *Rad59*, *Ume6*, or *Yen1* (Figure 7A). These observations indicate that *Sml1* works in parallel with *Rad52/Rad59*, *Rim11/Ume6*, and *Yen1*. In support of this, coexpression of *Ume6* and *Sml1* gave rise to a larger number of survivors of *dna2-KE* cells (Figure 7B). Also, *Sml1* showed stronger suppression compared to *Ume6*. This, together with the results above, suggests that *dna2-KE* inviability is largely attributed to checkpoint activation and that reduced levels of dNTPs are beneficial for cells with defects in *Dna2* helicase activity.

DNA2 copy number matters

Recently, it was shown that the presumed helicase-dead *dna2* mutant (*dna2-2*, R1253Q; Formosa and Nittis 1999) lacks any detectable DNA-dependent ATPase and DNA helicase activities (Olmezer *et al.* 2016), and this mutant strain is viable. This discrepancy could be attributed to the gene copy number of *dna2*. In our experiments and in those of others (Budd *et al.* 1995), plasmids harboring the *dna2* helicase-dead mutant allele (*dna2-KE*, K1080E) were used to express the mutant *Dna2* proteins. In contrast, both groups (Formosa and Nittis 1999; Olmezer *et al.* 2016) used genomic integration as the means to express the mutant allele of *dna2*. We decided to examine the influence of *dna2-KE* mutation copy number in cell growth. We compared two types of mutant: (1) the *dna2Δ* null mutant complemented with *dna2-KE* in a *CEN*-based plasmid (pRS313), and (2) the *dna2-KE* mutation integrated at the original locus of *DNA2* by using yeast-integrating plasmid (pRS303). We found that a single copy of the *dna2-KE* mutation rendered cells growth deficient but

Figure 5 *RIM11* and *UME6* genetically interact with homologous recombination genes. (A) Overexpression of Rim11 or Ume6 suppressed the MMS sensitivity of *rad59Δ*. Briefly, *rad51Δ*, *rad52Δ*, and *rad59Δ* cells were transformed with plasmids as indicated in the figure. Transformants were inoculated in SD medium and incubated at 30° until saturation. Serial dilutions (10-fold) of the indicated transformants were spotted onto SD medium with or without the indicated concentrations of MMS and incubated at 30° for 5–6 days. (B) Double mutants *rad51Δ ume6Δ*, *rad52Δ ume6Δ*, and *rad59Δ ume6Δ* showed increased sensitivity to MMS and HU. Cells (200–300 cells) of the indicated strains were spread onto YPD media with or without the indicated concentrations of MMS and HU. Plates were grown at 30° for ~3–6 days and the number of surviving colonies were counted. Data are representative of three independent experiments. (C) A summary of the genetic interactions observed between *RIM11/UME6* and the recombination genes, *RAD51*, *RAD52*, and *RAD59*. The Rim11/Ume6 pathway appears to compensate specifically for the Rad59-dependent role of Rad52 (bold font). WT, wild type.

viable, whereas the *dna2-KE* mutation carried by the low-copy plasmid are inviable (Figure 8A). This result suggests that the difference in copy number determine the extent of cells' growth defect. To further confirm this, we transformed an additional copy of the *dna2-KE* allele (in pRS315) in cells with singly integrated *dna2-KE*. Indeed, our result confirmed that the additional copy number of *dna2-KE* caused cell inviability (Figure 8B). Judging from our observations and those of others (Parenteau and Wellinger 1999), it appears that the copy number of *Dna2* is important, in keeping with the observation that the overexpression (2μ-based plasmid) of wild-type *Dna2* itself in wild-type cells yield inviable cells.

Discussion

We identified a GSK3-β homolog, *Rim11*, its substrate *Ume6*, as well as an inhibitor of ribonucleotide reductase, *Sml1*, as novel multicopy suppressors of the helicase-deficient *dna2* mutant (*dna2-KE*). However, *Rim11/Ume6*, *Sml1*, and *Rad52* failed to show significant rescue of *dna2-H547A* (Figure S2 in File S1), a mutant allele that possesses ~50% of endonuclease activity compared to wild type (Lee *et al.* 2000). This observation suggests that *Rim11/Ume6*, *Sml1*, and *Rad52* are allele-specific suppressors of the *dna2* mutant deficient in its helicase, but not nuclease function.

Figure 6 Inviability of *dna2-KE* is caused by checkpoint activation. (A) Inviability of *dna2-KE* is suppressed by second site mutations: *rad9Δ*, *rad53-R605A*, and *rad53Δ sml1Δ*. (B) Ume6 mode of suppression is independent of that of *rad9Δ*. Data are representative of three independent experiments.

Roles of Ume6 in DNA repair

Our result suggests that the presence of DNA damage affects the stability of Ume6 protein (Figure 2B). Post-translational modifications such as phosphorylation offer a dynamic way to regulate protein activity, subcellular localization, and stability. In the presence of replication stress, the increase in Rim11 protein abundance may provide a means to regulate the stability of Ume6 protein via phosphorylation. If Rim11-dependent phosphorylation of Ume6 is important for its stability, it is not surprising that the overexpression of Ume6 could bypass the requirement for Rim11-dependent phosphorylation (Figure 1E).

Our finding that Ume6 has a role in the repair of faulty lagging strand synthesis is consistent with the observations that *ume6Δ* cells were hypersensitive to MMS, HU, and 4-NQO; but mildly sensitive to CPT, a DSB-inducing agent (Figure 2C) (Valencia-Burton *et al.* 2006). Taken together, these findings raise the possibility that Ume6 has an important role in cells' recovery from stalled replication forks, but not in the subsequent events including the repair of DSBs. HU is known to exert its effect by depleting dNTPs (Bianchi *et al.* 1986). This drug initially leads to stalled replication forks

Figure 7 Reduced levels of dNTP is beneficial for *dna2-KE* cells. (A) Sml1 is a multicopy suppressor of *dna2-KE* defects and acts in parallel with Rad52/Rad59, Rim11/Ume6, and Yen1 pathways. (B) Sml1 and Ume6 showed synergistic interaction in the suppression of *dna2-KE*. Data are representative of three independent experiments.

that, after prolonged treatment, collapse into DSBs. Fork collapse gives rise to elevated rates of gross chromosomal instability (Tercero and Diffley 2001; Kolodner *et al.* 2002). This is in keeping with the previous report that cells lacking Ume6 show an increased rate of chromosomal loss and duplication (Fazzio *et al.* 2001).

Roles of Ume6 in transcription

The overexpression of the *dna2-KE* mutant allele itself can suppress the growth defect of *dna2-KE* (Lee *et al.* 2010), raising the possibility that Ume6 suppresses *dna2-KE* by increasing the level of Dna2-KE protein since Ume6 is involved in transcriptional control. We explored this possibility by examining the expression level of Dna2-KE protein tagged at the C terminus with 13-Myc in cells overexpressing Rim11 or Ume6. However, we found no alteration in the levels of Dna2-KE protein in these cells (Figure S3 in File S1).

Figure 8 The copy number of *dna2-KE* mutant allele influenced the severity of cell growth defects. (A) A single copy of *dna2-KE* produced viable cells. (B) Introduction of multiple copies of *dna2-KE* allele caused cells inviability. WT, wild type.

It is worthwhile to mention that *Ume6* positively regulates the expression of *PHR1*, a gene encoding a photolyase that repairs pyrimidine dimers (Green and MacQuillan 1980; Sweet *et al.* 1997). To exclude the possibility, although remote, that *Ume6*-mediated suppression of *dna2-KE* involves the upregulation of *Phr1*, we tested whether the overexpression of *Phr1* itself could suppress *dna2-KE* phenotype. The result was that the overexpression of *Phr1* did not rescue *dna2-KE* (Figure S4 in File S1). To examine the direct involvement of transcription functions of *Ume6* in the suppression of *dna2-KE*, two transcription-defective mutants of *Ume6* were tested. We found that only the mutant that retained its ability to interact with *Sin3* could suppress *dna2-KE* as shown in Figure 3A, indicating that the transcription function of *Ume6* *per se* is dispensable.

We also investigated the possible involvement of autophagy that depends on *Ume6* as a recent study showed that *Ume6* acts as a negative regulator of *Atg8*, a core component of the autophagy machinery (Bartholomew *et al.* 2012). In addition, elevated levels of *Tor1*, a negative regulator (Kamada *et al.* 2000) of autophagy, is important for cells with defects in *Dna2* function (Fiorentino and Crabtree 1997). We found that deletion of *ATG8* did not affect the growth defect of *dna2-KE* at all (Figure S5 in File S1), excluding the involvement of autophagy in the suppression of *dna2-KE*. We also showed that the multicopy expression of *Rim11* or *Ume6* has a negligible effect on the expression levels of *RAD52* (Figure S6 in File S1) and *DMC1*, an important factor in meiosis (Table S1 in File S1).

Links of *Rim11/Ume6* to recombination

Our results suggest that *Rim11/Ume6* acts in parallel with the recombination-mediated repair pathway, specifically to compensate for the loss of a *Rad59*-dependent role of *Rad52* (Figure 5A). Although *Rad59* is indispensable in the *Rad52*-mediated multicopy suppression of *dna2-KE*, overexpression

of *Rad59* alone did not rescue the *dna2-KE* defect (Lee *et al.* 2014). Both *Rad52* and *Rad59* proteins possess strand-annealing activity (Wu *et al.* 2006). While *Rad59* alone cannot anneal ssDNA that is complexed with RPA, *Rad59* significantly enhances the *Rad52*-promoted annealing of ssDNA at elevated (>150 mM) concentrations of NaCl. Moreover, the role of *Rad59* protein in the single-strand annealing pathway only become apparent as the length of homologous sequence decreases (Sugawara *et al.* 2000). This could explain why *Rim11/Ume6* only suppressed *rad59Δ* sensitivity to MMS, but not that of *rad52Δ*.

As evidenced by its stronger suppression ability (Figure 4D), the *Rad52/Rad59*-mediated recombination plays a bigger role in dealing with *dna2*-associated defects, therefore its loss cannot be compensated for by multicopy expression of *Rim11/Ume6* (Figure 4A). In conclusion, the combined actions of *Rim11/Ume6* and *Rad52/Rad59* are important for cells coping with replication stress.

Dna2 and checkpoint

We showed that *dna2-KE* is rescued by mutation in checkpoint genes *RAD9* and *RAD53* (Figure 6A). This is consistent with the previous reports that checkpoint mutations rescue several mutants of *dna2* (Fiorentino and Crabtree 1997; Budd *et al.* 2011; Olmezer *et al.* 2016). The downregulation of the checkpoint may work in three ways to the advantage of *dna2-KE* mutant (summarized in Figure 9): (1) it permits the accumulation of *Sml1*, which controls the levels of dNTP in the cells, (2) it allows cell progression into anaphase where Yen1-dependent repair is accessible, and (3) it recruits *Rad52* to sites of damage.

The downregulation of the checkpoint (Figure 6A, *rad9Δ* or *rad53-R605A*) may inhibit the destruction of *Sml1*, which directly influences the levels of dNTP in the cells (Zhao *et al.* 2001; Zhao and Rothstein 2002). Our result indicates that the levels of dNTP plays an important role in the survival of

Multiple pathways to alleviate damages caused by helicase-deficient Dna2

Mechanism	A Sister-chromatid recombination	B Flap cleavage	C Holliday-junction resolution	D Sin3-Rpd3-dependent deacetylation	E Checkpoint inactivation/ slow incorporation of dNTP/ Rad52 recruitment
Type of interaction	Multicopy suppression*	Multicopy suppression*	Synthetic rescue**/ Synthetic sickness***	Multicopy suppression*	Synthetic rescue**/ Multicopy suppression*
Components	Rad52*/ Rad59 Rsc2, Elg1	Rad27*	Rad9** ↓ Yen1***	Rim11* ↓ pp-Ume6* ↓ Sin3-Rpd3	<pre> graph TD Mec1 --> Rad53** Mec1 --> Rad9** Rad53** -- Dun1 Rad9** -- Yen1 Dun1 -- Sml1* Sml1* -- Rnr1 Rnr1 --> dNTP Yen1 -- Rad52* </pre>
Reference	Lee <i>et al.</i> , 2014	Lee <i>et al.</i> , 2014	Olmezer <i>et al.</i> , 2016	This study	

Figure 9 Multiple pathways to alleviate damages caused by helicase-deficient Dna2. (A) Sister-chromatid, recombination-mediated suppression of *dna2* mutant requires the strand-annealing activity of Rad52 and its interaction with Rad59, together with cohesion factors Elg1 and Rsc2. (B) The nuclease activity of Rad27 cleaves DNA flaps, compensating the need for Dna2. (C) Defect in the helicase activity of Dna2 gives rise to toxic recombination intermediates that can be resolved by Yen1 in anaphase. Disruption of G₂/M checkpoint by deletion of *RAD9* allows unrestrained entry of *dna2* cells into anaphase. (D) Rim11-dependent phosphorylation of Ume6 may protect Ume6 against degradation in the presence of DNA damage. The DNA binding activity of Ume6 provides targeted recruitment of the histone deacetylase complex, Sin3-Rpd3. (E) Downregulation of checkpoint activation may (i) permit *dna2-KE* cells to escape G₂/M arrest and proceed to anaphase where Yen1 is activated, (ii) allow unrestrained recruitment of Rad52 to sites of damage, and (iii) prevent the destruction of Sml1 which controls the levels of dNTP. Types of suppression: * indicates Multicopy suppression; ** indicates Synthetic rescue; *** indicates Synthetic sickness.

dna2-KE (Figure 7A). Low levels of dNTPs caused by *Sml1* overexpression could result in slower rates of nucleotide incorporation, which in turn prevents the formation of long flaps. Short flaps can be efficiently removed by Rad27 (FEN1) alone, and do not require the action of Dna2 (Bae *et al.* 2001a). The longer the flaps are, the higher the tendency to form a secondary structure. It is well documented that the removal of these flaps depends critically on the helicase function of Dna2 (Bae and Seo 2000; Bae *et al.* 2001b, 2002). Deletion of a gene encoding a helicase, *PIF1* for example, suppressed the lethality of a *DNA2* null mutant (Budd *et al.* 2006), possibly by decreasing the frequency of long flap formation. Moreover, the *dna2* helicase-dead mutant can be made viable when cells are grown in glycerol-lactate media, but not on media containing glucose which support rapid cell growth (Formosa and Nittis 1999).

However, inhibition of *Sml1* degradation is not the only mechanism behind the observed suppression of *dna2-KE* by checkpoint inactivation, since deletion of both *RAD53* and *SML1* nevertheless gave rise to viable *dna2-KE* cells (Figure 6A, *rad53Δ sml1Δ*). Yen1, a Holliday junction resolvase, is critical to resolve the recombination intermediates that arise in the absence of Dna2 helicase activity (Olmezer *et al.* 2016). Chromosomal DNA replicated in the presence of dysfunctional *dna2* leads to accumulation of cells in the G₂/M phase (Budd *et al.* 1995; Kang *et al.* 2000). In the absence of checkpoint protein, Rad9, *dna2*-helicase-deficient cells are able to escape from G₂/M arrest into anaphase where Yen1 is active (Blanco *et al.* 2014; Olmezer *et al.* 2016).

Checkpoint inhibition is also favorable for *dna2-KE* cells by allowing unrestrained recruitment of Rad52 to sites of damage. The activity of Mec1, an upstream kinase of Rad53,

inhibits the recruitment of Rad52 to both sites of DNA damage and stalled replication forks during the intra-S-phase checkpoint (Barlow and Rothstein 2009). Our results indeed support the notion that *Sml1*, *Yen1*, and *Rad52/Rad59* work in parallel to deal with replication stresses caused by defective Dna2 (Figure 7A). Overall, it appears that checkpoint activation is an unfavorable event for cells with a defect in Dna2 helicase activity.

The physiological function of Dna2 helicase

In our previous report (Bae *et al.* 2002), we showed that cleavage and translocation/unwinding by the endonuclease and helicase activity, respectively, are coupled. In an *in-vitro* experiment using flap-structured DNA substrate, the endonuclease-deficient Dna2-D657A and Dna2-K1080E proteins, when added together, did not yield the same product as the wild-type protein. Furthermore, the coupled reaction appears to be essential *in vivo* since *DNA2*-deleted cells were not viable when two plasmids, one expressing Dna2-K1080E and the other Dna2-D657A, were present in the same cell.

Also, using a flap substrate containing a preformed secondary structure, we demonstrated that in the absence of ATP (inactive helicase), wild-type Dna2 cleaved the terminal ssDNA tail only, and hardly cleaved past the region containing the secondary structure that has a short (10-bp) hairpin (Bae *et al.* 2002; Lee *et al.* 2013). However, cleavage occurred within the secondary structure-containing region when ATP was added (active helicase). In the absence of ATP, addition of RPA, a strand melting protein (Georgaki *et al.* 1992), seems to compensate for the lack of Dna2 helicase activity by allowing cleavage in the secondary structure-containing region. Moreover, multicopy expression of *RFA1*, a subunit of

the RPA complex, rescues the helicase-deficient mutant of *dna2-KE* (Bae *et al.* 2002). Since *Dna2* must enter and thread from the end (Balakrishnan *et al.* 2010), the secondary structure formed in the flap itself may act as a blockage that prevent cleavage by its endonuclease, highlighting an important role for the helicase activity.

Acknowledgments

Funding for open access charge from the National Research Foundation of Korea (NRF-2015R1A2A2A05027960). The authors declare no competing interests.

Author contributions: A.A.D. planned and performed the experiments. A.A.D. and Y.-S.S. wrote the manuscript. A.A.D., M.L., and C.-H.L. contributed reagents.

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Communicating editor: N. Hunter