A Network of Multi-Tasking Proteins at the DNA Replication Fork Preserves Genome Stability

Martin E. Budd1, Amy Hin Yan Tong2,3, Piotr Polaczek1, Xiao Peng1, Charles Boone2,3, Judith L. Campbell1

1 Braun Laboratories, California Institute of Technology, Pasadena, California, United States of America, 2 Banting and Best Department of Medical Research, University of Toronto, Toronto, Ontario, Canada, 3 Department of Medical Genetics and Microbiology, University of Toronto, Toronto, Ontario, Canada

To elucidate the network that maintains high fidelity genome replication, we have introduced two conditional mutant alleles of DNA2, an essential DNA replication gene, into each of the approximately 4,700 viable yeast deletion mutants and determined the fitness of the double mutants. Fifty-six DNA2-interacting genes were identified. Clustering analysis of genomic synthetic lethality profiles of each of the DNA2-interacting genes defines a network (consisting of 322 genes and 876 interactions) whose topology provides clues as to how replication proteins coordinate regulation and repair to protect genome integrity. The results also shed new light on the functions of the query gene DNA2, which, despite many years of study, remain controversial, especially its proposed role in Okazaki fragment processing and the nature of its in vivo substrates. Because of the multifunctional nature of virtually all proteins at the replication fork, the meaning of any single genetic interaction is inherently ambiguous. The multiplexing nature of the current studies, however, combined with follow-up supporting experiments, reveals most if not all of the unique pathways requiring Dna2p. These include not only Okazaki fragment processing and DNA repair but also chromatin dynamics.

Citation: Budd ME, Tong AHY, Polaczek P, Peng X, Boone C, et al. (2005) A network of multi-tasking proteins at the DNA replication fork preserves genome stability. PLoS Genet 1(6): e61.

Introduction

In order to preserve the fidelity of genome duplication during DNA replication, cells with complex genomes have evolved a network of pathways composed of the DNA replication apparatus, DNA repair proteins, and regulatory activities. Despite years of general characterization, knowledge of the specific mechanisms by which these pathways are integrated to protect the genome is still incomplete because of the complexity of underlying replication fork processes and their regulation. The challenge in understanding high fidelity genome transmission has progressed from identification and characterization of the individual DNA replication components to investigation of how they combine to form pathways orchestrating repair and regulation.

The first line of defense against genome instability resides with the enzymes of the DNA replication apparatus itself. While the most familiar example is the proofreading activity found in the DNA polymerases, other proteins of the replisome have also evolved substrate specificities to address errors made during replication fork progression. One of these proteins is Dna2p, a helicase/nuclease. The dna2–1 mutation was identified in a screen for yeast mutants defective in DNA replication based on an assay using permeabilized cells [1]. dna2–1 strains were then shown to accumulate subgenomic-size DNA fragments when incubated at the restrictive temperature [2]. Dna2p has both DNA helicase and single-stranded nuclease activities [3,4]. Biochemical and genetic characterization has revealed that Dna2p is involved in the processing of some, but not all, Okazaki fragments. Specifically, it has been proposed that Dna2p acts with FEN1 to remove RNA primers from Okazaki fragments whose 5’ RNA/DNA termini have been extensively displaced by DNA polymerase (pol) δ. Four lines of evidence support a role for Dna2p in Okazaki fragment processing (OFP). First, Dna2p co-purifies with FEN1, which is a structure-specific nuclease required for OFP in the SV40 in vitro replication system [5,6]. Second, overexpression of the Saccharomyces cerevisiae FEN1 gene, RAD27, suppresses the temperature-sensitive (ts) growth defect of a dna2–1 strain, and furthermore, the dna2–1 rad27Δ double mutant is synthetically lethal [7,8]. Third, biochemical reconstitution experiments have shown that excessive strand displacement by pol δ creates long 5’ flaps that are cleaved inefficiently by FEN1, and that initial cleavage of these flaps by Dna2p potentiates more efficient subsequent cleavage by FEN1 [9–13]. Finally, Dna2p prefers to act on flaps with secondary structures in vitro, i.e., hairpins or fold-backs containing CTG repeats, which is probably where helicase functionality becomes necessary [12]. Thus, the Dna2p nuclease has evolved the ideal mechanism for highly specific action at a replication fork, requiring the presence of an unpaired 5’ terminus for....
activity, and displaying a complete lack of activity for single-stranded gaps in duplex DNA, which would result in recombinogenic double-strand breaks (DSBs). Nevertheless, there is a real question of whether the excessively displaced flaps used to study Dna2p in vitro ever occur in vivo, and some biochemical evidence suggests this may not be the case. Therefore, although biochemical data indicate that Dna2p is required for OFF when FEN1 or pol δ activity is impaired, identification of Dna2p’s in vivo substrates requires more appropriate genetic and physiological assays than have been applied to date [14].

The second and third lines of defense for preventing genome instability during DNA replication are the DNA repair pathways and regulatory pathways, such as cell cycle checkpoints. Increasing evidence suggests that these pathways are integrated into the replication pathway through their use of certain replication proteins [15]. Dna2p seems to be one of these multitasking proteins. Besides its function in OFF, our evidence strongly suggests that Dna2p provides a link between DNA replication and DNA repair, since dna2 mutants are sensitive to methyl methane sulfonate (MMS), X-rays, bleomycin, and hydroxyurea (HU) [8,16,17]. To provide a comprehensive view of the roles of Dna2p at the replication fork and in other genome maintenance pathways, we conducted a large-scale synthetic lethal screen by synthetic genetic array (SGA) analysis using DNA2 as a query [18,19]. Two genes are synthetically lethal if single mutants, defective in either gene, are viable, while double mutants, defective in both genes, are inviable. Two mutants are synthetically sick if the double mutant grows significantly slower than either single mutant. Synthetic lethality is useful for identifying redundancy and complementarity, e.g., pathways that compensate for functional deficiencies in each other or genes encoding products that are both required to efficiently process a common substrate, which is often the case for DNA replication and repair proteins. Synthetic lethal screens not only reveal previously unknown genetic interactions with queried mutants but also how gene products and their corresponding pathways functionally associate.

Our results provide a catalogue of most, if not all, pathways that are interdependent with or require Dna2p, thus revealing both the extent and limits of its multitasking character. The work not only confirms a role in OFF, but also identifies functions in (1) a replication/repair helicase subnet, (2) DSB repair and mismatch repair, (3) the replication stress checkpoint, (4) sister chromatid cohesion, (5) chromatin dynamics, (6) histone modification, and (7) osmotic and oxidative stress responses. In a more general sense, the interactions link a specific network of DNA repair and regulatory pathways to a specific network of replication genes that together maintain high fidelity lagging-strand DNA replication.

Results

SGA Screens

Since DNA2 is an essential gene, either a conditional or hypomorphic allele is required for a synthetic lethal screen. We chose two alleles, dna2–1, a ts mutant sensitive to a variety of DNA damaging agents, and dna2–2, a mutant that grows at 25 °C and 37 °C, but is sensitive to MMS, bleomycin, and X-rays [8,20]. dna2–1 contains a P504S substitution in a region of the protein N-terminal to both the nuclease and helicase domains [3]. All enzymatic activities of the Dna2–1 protein are reduced relative to wild-type (WT)—DNA-stimulated ATPase, DNA helicase, and single-stranded DNA nuclease activity [21]. The dna2–2 mutation changes arginine at position 1235, an invariant residue in helicase region IV, to glutamine [8]. A crystal structure of Pcr helicase shows that residues in region IV bind the adenine base of ATP [22]. The dna2–1 strain grows very slowly, even at the permissive temperature, so synthetic sickness is sometimes difficult to unambiguously assign for this mutant. The dna2–2 mutant grows faster than the dna2–1 mutant, and synthetic sickness, characterized by slow growth, can be assigned with greater confidence using dna2–2 strains. The use of each dna2 allele in a separate SGA screen expands the range of detectable synthetic lethal interactions.

Table 1 lists the validated synthetic lethal and synthetic sick interactions of dna2–1 and dna2–2 strains obtained from the SGA screen (see Materials and Methods). Validation was performed by preparing a new heterozygous diploid between the respective dna2 allele and the candidate gene, followed by sporulation and tetrad dissection. The dna2–2 allele specificity of some of the interactions may turn out to be significant, as the mutations differentially affect helicase and nuclease activity. Mutants that appeared to show growth defects in the primary screen but that did not meet the stringent requirements (see Materials and Methods) for interaction imposed by the secondary tetrad analysis are found in Table S1. Mutants synthetically lethal or synthetically sick with either dna2–1 or dna2–2 fall into the following categories: genes involved in OFF (rad27A, exo1A, yen1A, rnh202A, pol3–01, rpa1, elg1Δ, pol1, and pri1), nonessential helicases involved in maintaining chromosome stability (srs2Δ, mms1Δ, and rrm3Δ), genes involved in repair (rad52A, mre11A, rad50A, xrs2A, swf2Δ, mms1Δ, mms22A, stx5, and stx8), genes involved in the DNA replication checkpoint (mre1Δ, csm3, and tof1Δ), genes involved in chromosomal cohesion (cfl1A and cfl18A), genes involved in chromatin disassembly/assembly and nucleosome modification and remodeling (spt16, pob3, rad6Δ, bre1Δ, swd1Δ, swd3Δ, hst3Δ, rpd3Δ, pho23Δ, rtf1Δ, and the
Okazaki Fragment Processing

Although Dna2p is commonly considered to be required for OFP, this role is far from established. We were therefore interested to find that the SGA experiments identified interactions between DNA2 and additional structure-specific nucleases that, like FEN1, are thought to be involved in overlapping OFP pathways. First, dna2–1 was found to be synthetically lethal with mutations in genes encoding two subunits, rnh35Δ and rnh202Δ, of the main RNaseH in yeast [25], suggesting that Dna2p may be involved in an OFP pathway redundant with RNaseH2.

Second, dna2–2 was synthetically lethal with mutations in genes encoding two exo-endonucleases structurally related to RAD27, exo1Δ and yen1Δ. EXO1 is thought to provide backup function for FEN1 in OFP, since rad27Δ exo1Δ is synthetically lethal (in some genetic backgrounds) and since overexpression of EXO1 suppresses the ts growth of rad27Δ mutants [20–28]. Overexpression of EXO1 also suppressed the temperature sensitivity of dna2–1 at the restrictive temperature of 30 °C (Figure 3), though not at 37 °C [21]. Exo1p nuclease acts on 5’ flap-containing structures, and these genetic interactions suggest that such flaps may be the common in vivo substrate of both Dna2p and Exo1p [29].

We also found that dna2–2 is synthetically sick with yen1Δ at 30 °C and lethal, i.e., ts, at 37 °C. Yen1p shows 23% identity with Rad27p, 33% identity with Rad2p (founding member of this nuclease family and involved in nucleotide excision repair), and 24% identity with Din7p (Rad2p–like endonuclease proposed to be involved in mitochondrial mismatch repair). yen1Δ mutants do not appear to have DNA damage or growth defects, and a yen1Δ exo1Δ dna2–1 mutant has no growth defect at either 30 °C or 37 °C [26]. Synthetic lethality with dna2 is the first informative phenotype reported for yen1Δ, although yen1 is synthetically lethal with another gene that is in turn synthetically lethal with several replication mutants (see Protocol S1).

Elg1p, like Dna2p, is proposed to be involved in OFP, as well as in telomere silencing and length regulation [17,30,31]. elg1Δ dna2–1 mutants are synthetically lethal (Table 1). Elg1p is homologous to Rfc1p (the large subunit of the replication factor C clamp loader), to Rad24p (a Rfc1p homolog required for DNA damage checkpoints), and to Ctf18p (another Rfc1p homolog involved in chromosomal cohesion). dna2–2 is also synthetically lethal with ctf18Δ [8], but not with rad24Δ (this work). S phase progression in elg1Δ mutants is slower than in WT strains, suggesting that Elg1p is involved in replication fork translocation [30–32].

Interaction of dna2 with Pol δ

It has been proposed, based on in vitro biochemical reconstitution in vitro, that not all Okazaki fragments require Dna2p for processing. Instead, the in vivo substrates of Dna2p are only those Okazaki fragments on which pol 6 produces 5’ flaps longer than 30 nucleotides [10,12,14,33]. We wished to devise a genetic test of this hypothesis. One way to do so is by generating excessive strand displacement in vivo and assessing whether there is then an increased requirement for DNA2. This was accomplished by testing the viability of a strain containing both the dna2–1 mutation and pol3–01, a mutation in pol 6 known to increase strand displacement in vitro [33]. As shown in Figure 4, dna2–1 pol3–01 is synthetically lethal. We propose that excessive strand displacement in vivo in the

| dna2–1 | Synthetic Lethal | Synthetic Sick |
|--------|------------------|----------------|
| dna2–2 | Synthetic Lethal | Synthetic Sick |

| asf1 | cla4 | cal20 | asf1 (ts) |
|------|------|-------|-----------|
| ctf4 | csm3 | csm3 | bse1 |
| egf1 | hrb3 | eso1 | clb4 |
| hog1 | rad6 | lys7 | cm1 |
| lys7 | snr2 | mms1 | elg1 |
| mms1 | swd1 | mms22 | rad6 |
| mrc1 | swd3 | mrc1 | rad1 |
| mre11 | mei1 | mei1 | rad10 |
| pho23 | rad27 | sxe5 | ubc4 |
| rad50 | snr2 | mms1 | yen1 (30 °C) |
| rad52 | sns2 | rad6 |
| rnh35 | sns2 | rad6 |
| rad50 | sns2 | rad6 |
| rad52 | sns2 | rad6 |

Table 1. Genes Identified as Putative DNA2 Interactors from the SGA Screen with dna2–1 and dna2–2 Queries and Verified by Tetrad Dissection

DOI: 10.1371/journal.pgen.0010061.t001

histone chaperone asf1Δ, genes involved in the oxidative stress response (lys7Δ and sod1Δ), a gene in the osmotic stress response (bog1Δ), and genes involved in degradation of short-lived proteins (ube1Δ), in polarized cell growth (克拉A), and in mRNA processing (strf4A and rtt103A). Previous studies showed dna2 to be synthetically lethal with several essential genes: mcm10–1, involved in initiation of replication [23]; cdc9, a DNA ligase ([24], although see also [8]); rpa1, a single-stranded DNA binding protein [13]; and sp416 and pol3, two genes involved in chromosome remodeling [8].

The total number of genes that interact with dna2 at date is 56, a connectivity similar to that found in previous screens [18]. Figure 1 shows two-dimensional hierarchical clustering of the data from the current screen and that of previous screens using 43 genes that interact with DNA2 as queries [18]. Figure 2 provides a graphical representation of the network (consisting of 322 genes and 876 interactions) in which DNA2, RAD27, SGS1, SRS2 (HPR2), RRM3, and POL32 form the six major hubs (largest number of connections). We were surprised that none of these genes showed interactions with cell cycle genes, ctf4Δ, however, is synthetically lethal with all six of these hub genes and many of the other nodes as well (Figure 2), is synthetically lethal with clb2Δ, clb3Δ, clb5Δ, mad1Δ, mad2Δ, mad3Δ, bub1Δ, bub2Δ, and bub3Δ [18]. Thus, CTF4 may provide a missing link between hub genes and cell cycle and kinetochore functions. The same information is shown in tabular form in Table S2, which contains the interacting genes organized by functional categories defined in the MIPS database (http://mips.gsf.de/genre/proj/yeast/index.jsp). Mutations (identified in studies reported below) that suppress dna2 alleles are also included. We propose that the genes showing the greatest number of interactions encode proteins that share at least one substrate or have at least one overlapping function.
pol3–01 dna2–1 strain causes the lethality, supporting the predictions from purely biochemical reconstitution.

Another polδ subunit mutant, pol32Δ, is synthetically lethal with rad27Δ, and the network of synthetic lethal interactions of pol32Δ is similar to that of rad27Δ, rrm3Δ, sgs1Δ, and srs2Δ, which are also synthetically lethal with dna2 (See Figure 2). We were surprised that pol32Δ did not show up in our SGA screen, and directly tested dna2–1 pol32Δ for synthetic lethality. Rather than showing synthetic lethality, pol32Δ suppressed the slow growth phenotype of the dna2–1 strain at 23°C and suppressed the ts growth phenotype of the dna2–1 strain at 30°C (Figure 5A). The pol32Δ mutation did not suppress the lethality of the dna2–1 strain at 37°C (Figure 5A) or the lethality of a DNA2 deletion (not shown). The pol32Δ deletion mutation also suppressed the DNA damage sensitivity of the dna2–1 and dna2–2 mutants (Figure 5B and 5C, respectively). pol32Δ mutants are sensitive to different concentrations of MMS than are dna2 mutants. dna2–1 and dna2–2 mutants are sensitive to 0.005% MMS, while pol32Δ strains are resistant to 0.005% MMS but are sensitive to 0.01%. MMS. Both dna2–1 pol32Δ and dna2–2 pol32Δ mutants showed MMS sensitivity similar to pol32Δ strains rather than to dna2–1 or dna2–2 strains (Figure 5B and 5C). Pol32p is a nonessential subunit of the pol δ holoenzyme and is required for full processivity of pol δ [34]. Therefore, a less processive pol δ suppresses the growth and repair defects of both nuclease- and helicase-deficient mutants of dna2, suggesting that Dna2p is acting on flaps arising from excessive strand displacement by pol δ during DNA repair as well as during OFP. The synthetic lethality of dna2 and pol3–01 and the suppression of dna2–1 by pol32Δ provide strong genetic evidence that the Dna2p substrates shown to be optimal in vitro are also substrates in vivo (see Discussion).

The Helicase Network

We have reported previously that dna2–2 is synthetically lethal with sgs1Δ, srs2Δ, and rrm3Δ [35–37], and our SGA screen also detected these genes. More significantly, there is extensive overlap between genes that are synthetically lethal with dna2 and those that are synthetically lethal with sgs1Δ and srs2Δ, as shown in Figures 1 and 2.

To assess the functional relationship between Dna2p and these helicases, we have tried to further determine whether the synthetic lethality between dna2 and the helicase genes
shown in Table 2 stems from defects in DNA replication per se or from the aberrant DNA structures that arise during repair of DNA replication errors. Towards this end, we first checked whether the lethality was suppressed by mutations in genes considered necessary for resolving potentially lethal intermediates that form when the original lesions enter the recombination pathway for repair [38]. The results of our current analysis of dna2–2 sgs1Δ, dna2–2 srs2Δ, and dna2–2 rrm3Δ are summarized in Table 2. dna2–2 sgs1Δ and dna2–2 srs2Δ were synthetically lethal and were either inefficiently or efficiently suppressed, respectively, by rad51Δ, as we reported previously ([35,37]; see Discussion). New here is the finding that dna2–2 rrm3Δ lethality was not suppressed by mutations in the recombination pathway (Table 2). This indicates that cell death in dna2–2 rrm3Δ does not result from the accumulation of nonresolvable recombination intermediates but from the formation of early replication intermediates, or possibly blocked DNA replication forks.

Gene pairs encoding three different heterodimeric complexes (MMS4/SLX3, SLX1/4, and SLD5/8), although they are not DNA helicases, are required for viability in the absence of...
SGS1 and, thus, are part of the helicase network [39]. We find that only one of these complexes, defined by slx5Δ and slx8Δ, shows synthetic lethal interactions with dna2. Since the function of the Slx5/8p complex is unknown, we cannot yet predict the nature of the substrates that might be shared with Dna2p.

DNA Repair
We have previously shown that dna2 and rad52Δ mutants are synthetically sick [16]. rad52Δ also appeared synthetically sick with the dna2 alleles in the SGA screen. We were surprised that other genes required for recombinational repair did not appear synthetically lethal with dna2 in the SGA screen, since they are synthetically lethal with rad27Δ. To ensure that we had not missed such genes, dna2–1 rad51Δ and dna2–1 rad53Δ, as well as dna2–2 rad51Δ and dna2–2 rad59Δ, mutants were constructed. Consistent with the global screen, these double mutants did not appear to be synthetically lethal or sick. We also found that dna2–2 rad51Δ rad59Δ triple mutants were viable (not shown). Our results indicate that the Rad51p/Rad59p portion of the Rad52p pathway does not buffer Dna2p function. MMS1 and MMS2 are two additional presumed DNA repair genes that are also required for normal S phase progression and that show synthetic lethality with dna2 mutants in the SGA screen, as previously described [40]. The Mms1p/Mms22p system may correspond to the pathway that results in dna2–2 rad52Δ growth defects.

Previously, we reported that dna2–2 and rad50–5 double mutants were viable and epistatic for repair [16]. Therefore, one of our most unexpected findings was that dna2 mre11Δ and dna2 rad50Δ strains are synthetically lethal (see Table 1). rad50–5 is a point mutation of RAD50 that is as sensitive to irradiation as either a rad50Δ or a rad52Δ strain. Clearly, some function must remain in the point mutant as compared to the deletion mutant, however, since the dna2–2 rad50–5 strain is viable while the dna2–2 rad50Δ strain is inviable. MRE11, RAD50, and XR52 encode members of the Mre11p complex, which is required for the intra-S phase checkpoint, for homologous recombination, for non-homologous end joining, and for telomere maintenance ([41,42] and references therein). Although xrs2Δ was not found in our SGA screen, we have since shown that dna2–2 xrs2Δ is synthetically lethal (this work).

\[ \text{DNA Repair} \]

We have previously shown that dna2 and rad52Δ mutants are synthetically sick [16]. rad52Δ also appeared synthetically sick with the dna2 alleles in the SGA screen. We were surprised that other genes required for recombinational repair did not appear synthetically lethal with dna2 in the SGA screen, since they are synthetically lethal with rad27Δ. To ensure that we had not missed such genes, dna2–1 rad51Δ and dna2–1 rad53Δ, as well as dna2–2 rad51Δ and dna2–2 rad59Δ, mutants were constructed. Consistent with the global screen, these double mutants did not appear to be synthetically lethal or sick. We also found that dna2–2 rad51Δ rad59Δ triple mutants were viable (not shown). Our results indicate that the Rad51p/Rad59p portion of the Rad52p pathway does not buffer Dna2p function. MMS1 and MMS2 are two additional presumed DNA repair genes that are also required for normal S phase progression and that show synthetic lethality with dna2 mutants in the SGA screen, as previously described [40]. The Mms1p/Mms22p system may correspond to the pathway that results in dna2–2 rad52Δ growth defects.

Previously, we reported that dna2–2 and rad50–5 double mutants were viable and epistatic for repair [16]. Therefore, one of our most unexpected findings was that dna2 mre11Δ and dna2 rad50Δ strains are synthetically lethal (see Table 1). rad50–5 is a point mutation of RAD50 that is as sensitive to irradiation as either a rad50Δ or a rad52Δ strain. Clearly, some function must remain in the point mutant as compared to the deletion mutant, however, since the dna2–2 rad50–5 strain is viable while the dna2–2 rad50Δ strain is inviable. MRE11, RAD50, and XR52 encode members of the Mre11p complex, which is required for the intra-S phase checkpoint, for homologous recombination, for non-homologous end joining, and for telomere maintenance ([41,42] and references therein). Although xrs2Δ was not found in our SGA screen, we have since shown that dna2–2 xrs2Δ is synthetically lethal (this work).

\[ \text{DNA Repair} \]

\[ \text{DNA Repair} \]

\[ \text{DNA Repair} \]

\[ \text{DNA Repair} \]

\[ \text{DNA Repair} \]

\[ \text{DNA Repair} \]

\[ \text{DNA Repair} \]

\[ \text{DNA Repair} \]

\[ \text{DNA Repair} \]

\[ \text{DNA Repair} \]

\[ \text{DNA Repair} \]

\[ \text{DNA Repair} \]

\[ \text{DNA Repair} \]

\[ \text{DNA Repair} \]

\[ \text{DNA Repair} \]

\[ \text{DNA Repair} \]

\[ \text{DNA Repair} \]

\[ \text{DNA Repair} \]

\[ \text{DNA Repair} \]

\[ \text{DNA Repair} \]

\[ \text{DNA Repair} \]

\[ \text{DNA Repair} \]

\[ \text{DNA Repair} \]

\[ \text{DNA Repair} \]

\[ \text{DNA Repair} \]

\[ \text{DNA Repair} \]

\[ \text{DNA Repair} \]

\[ \text{DNA Repair} \]

\[ \text{DNA Repair} \]

\[ \text{DNA Repair} \]

\[ \text{DNA Repair} \]

\[ \text{DNA Repair} \]

\[ \text{DNA Repair} \]

\[ \text{DNA Repair} \]

\[ \text{DNA Repair} \]

\[ \text{DNA Repair} \]

\[ \text{DNA Repair} \]

\[ \text{DNA Repair} \]

\[ \text{DNA Repair} \]

\[ \text{DNA Repair} \]

\[ \text{DNA Repair} \]

\[ \text{DNA Repair} \]

\[ \text{DNA Repair} \]

\[ \text{DNA Repair} \]

\[ \text{DNA Repair} \]

\[ \text{DNA Repair} \]

\[ \text{DNA Repair} \]

\[ \text{DNA Repair} \]

\[ \text{DNA Repair} \]

\[ \text{DNA Repair} \]

\[ \text{DNA Repair} \]

\[ \text{DNA Repair} \]

\[ \text{DNA Repair} \]

\[ \text{DNA Repair} \]

\[ \text{DNA Repair} \]

\[ \text{DNA Repair} \]

\[ \text{DNA Repair} \]

\[ \text{DNA Repair} \]

\[ \text{DNA Repair} \]

\[ \text{DNA Repair} \]

\[ \text{DNA Repair} \]

\[ \text{DNA Repair} \]

\[ \text{DNA Repair} \]

\[ \text{DNA Repair} \]

\[ \text{DNA Repair} \]

\[ \text{DNA Repair} \]

\[ \text{DNA Repair} \]

\[ \text{DNA Repair} \]

\[ \text{DNA Repair} \]

\[ \text{DNA Repair} \]

\[ \text{DNA Repair} \]

\[ \text{DNA Repair} \]

\[ \text{DNA Repair} \]

\[ \text{DNA Repair} \]

\[ \text{DNA Repair} \]

\[ \text{DNA Repair} \]

\[ \text{DNA Repair} \]

\[ \text{DNA Repair} \]

\[ \text{DNA Repair} \]

\[ \text{DNA Repair} \]

\[ \text{DNA Repair} \]

\[ \text{DNA Repair} \]

\[ \text{DNA Repair} \]

\[ \text{DNA Repair} \]

\[ \text{DNA Repair} \]

\[ \text{DNA Repair} \]

\[ \text{DNA Repair} \]

\[ \text{DNA Repair} \]

\[ \text{DNA Repair} \]

\[ \text{DNA Repair} \]

\[ \text{DNA Repair} \]

\[ \text{DNA Repair} \]

\[ \text{DNA Repair} \]

\[ \text{DNA Repair} \]
other dna2 alleles actually show improved survival in the absence of the checkpoint ([43]; see below), and suggested that such synthetic lethality might be due to another function of MRC1. MRC1 and TOF1 appear to play a direct role in yeast DNA replication, as well as in the checkpoint. mrc1D strains also show a slow S phase, and Mrc1p and Tof1p have been localized to moving replication forks [46]. Osborn and Elledge [45] constructed a separation-of-function mrc1 mutant that has all 17 TQ and SQ Mec1p target sites mutated to non-phosphorylatable AQ. This mutant, mrc1AQ, like mrc1D, is checkpoint defective, as evidenced by the fact that Rad53p phosphorylation is blocked in mrc1AQ rad9D mutants upon treatment with HU or MMS. However, mrc1AQ mutants are replication proficient. The mrc1AQ mutant allowed us to ask whether the replication defect of mrc1D was responsible for dna2 mrc1D synthetic lethality. dna2–1 mrc1AQ and dna2–2 mrc1AQ strains were constructed and were viable and did not appear synthetically sick, indicating that the checkpoint function of Mrc1p is not required for viability in dna2 mutant backgrounds (Figure 6). In order to be sure that RAD9 was not substituting for MRC1 in the dna2–2 mrc1AQ mutant, dna2–2 mrc1AQ rad9D mutants, which lack both the DNA damage and replication stress checkpoints, were constructed (Figure 6). The viability of dna2–2 mrc1AQ rad9D strains suggests that it is

Figure 5. Suppression of Slow Growth and MMS Sensitivity of dna2 Mutants by pol32Δ
(A) WT, pol32Δ, dna2–1 pol32Δ, and dna2–1 strains were grown to log phase, serially diluted, and plated on YPD plates and incubated at 23 °C, 30 °C, and 37 °C for 5 d.
(B) WT, pol32Δ, dna2–2, and pol32Δ dna2–2 strains were grown to log phase, serially diluted, and incubated on MMS-containing YPD plates for 3 d at 30 °C.
(C) WT, pol32Δ, dna2–1 pol32Δ, and dna2–1 strains were grown to log phase, serially diluted, and grown on MMS-containing YPD plates for 5 d at 37 °C. All strains are isogenic with strain 4741 (Table S4). (dna2–1 grows slowly even at 23 °C, and plates at 23 °C were photographed before they were fully grown so that the other strains would not be overgrown.)
DOI: 10.1371/journal.pgen.0010061.g005

Table 2. Genetic Interactions of DNA2 with Other Helicases

| Mutant     | Phenotype | Suppressed by rad51Δ | Suppressed by fob1Δ | Suppressed by Sorbitol |
|------------|-----------|----------------------|---------------------|------------------------|
| dna2Δ      | Lethal    | Partial (weak)       | Yes                 | Yes                    |
| dna2Δ      | Lethal    | Yes                  | Not determined      | No                     |
| dna2Δ      | Lethal    | No                   | No                  | No                     |

DOI: 10.1371/journal.pgen.0010061.s002
inactivation of the replication function of MRC1 that is responsible for synthetic lethality in the dna2 mrc1Δ mutants.

mrc1Δ and rrm3Δ mutants are also synthetically lethal [47] and, because RRM3 and DNA2 interact (see Table 2), we asked whether the mrc1Δ rrm3Δ synthetic lethality is caused by a checkpoint or replication defect. The mrc1AQ rrm3Δ double mutants showed the same viability as the single mutants (Figure 6). Since dna2Δ and rrm3Δ are also synthetically lethal, this suggests that DNA2, RRM3, and MRC1 functions may be interdependent in DNA replication.

To further test the idea that S phase checkpoint signaling is dispensable for dna2 mutant viability, the interaction of dna2 with mec1Δ and tel1Δ, mutations in genes upstream of MRC1 and RAD9 in the checkpoint, were investigated. A dna2–2/ dna2Δ tel1Δ/mec1Δ/mec1Δ/sml1Δ/sml1Δ heterozygote was sporulated, and Table S3 lists the genotypes obtained among the tetrads. (sml1Δ allows for mec1Δ viability.) The mec1Δ mutation partially suppressed the slow growth phenotype of dna2–2 strains. Thus, as previously observed for dna2–20 [43], the Mec1p-mediated checkpoint is deleterious in the dna2–2 mutant (Figure 7). The tel1Δ mutation shows negative synergy but not lethality with dna2–2 at 37 °C (Figure 7). The negative synergy between dna2–2 and tel1Δ is evidence that Dna2p and Tel1p may function together at DSBs and/or at telomeres, along with the Mre11p complex [48]. dna2–2 tel1Δ mec1Δ triple mutants were recovered. As shown in Figure 7, however, the dna2–2 tel1Δ mec1Δ mutant grew more slowly than any of the single or double mutants. The telomere defects of the mec1Δ tel1Δ strain caused it to senesce as rapidly as est2Δ (telomerase catalytic subunit deleted) strains [49].
The dna2–2 mutation may cause additional defects in telomere replication. Thus, enhanced telomeric senescence might account for the slow growth of the dna2–2 tel1Δ mec1Δ mutant, just as we have shown that dna2–2 est2Δ is synthetically lethal due to accelerated senescence [17].

Finally, we tested the interaction of DNA2 with the checkpoint effector kinase RAD53. We crossed dna2–2 and dna2–2 to an isogenic rad53Δ sml1Δ strain. dna2–2 rad53Δ sml1Δ and dna2–2 rad53Δ sml1Δ mutants were fully viable. The viability of dna2 rad53 strengthens the conclusion that the DNA damage arising in a dna2 mutant is not sufficient to require the S phase checkpoint for viability. dna2–1 mutants, however, do induce an amount of damage above the threshold for checkpoint activation at restrictive temperatures, since they arrest at the metaphase to anaphase transition in a MECL-dependent manner [M. E. B. and J. L. C., unpublished data].

Sister Chromatid Cohesion and Repair of DSBs in the rDNA
Ctf4p is a pol α-binding protein [50], and ctf4Δ strains are defective in sister chromatid cohesion [20]. dna2–2 was identified as a mutant synthetically lethal with ctf4Δ, but we have shown that dna2 mutants are not defective in cohesion [51]. We previously reported that the dna2–2 mutation gave rise to an increased frequency of DSBs at the replication fork barrier (RFB) in the rDNA and that deleting POB1, which is required for pausing at the RFB, suppressed DSB formation. A reasonable explanation for all of these observations is that the DSB damage sustained by dna2–2 mutants at the RFB might require Ctf4p-mediated sister chromatid cohesion for repair. If so, then one would expect pob1Δ to suppress dna2–2 ctf4Δ synthetic lethality. We dissected 55 tetrads from a dna2–2 ctf4Δ pob1Δ heterozygote and incubated the spores at 30 °C. Viable dna2–2 ctf4Δ pob1Δ mutants were obtained in the expected numbers, demonstrating suppression. Although the dna2 ctf4Δ pob1Δ triple mutants grew at 23 °C and at 30 °C, they did not grow at 37 °C and were highly sensitive to X-rays (Figure 8). The behavior of the triple mutant indicates that defects in the rDNA locus are critical for some of the phenotypes of dna2–2, but that defects elsewhere throughout the chromosome must still occur, giving rise to ts growth and DNA damage sensitivity.

Nucleosome Remodeling: Dna2p Interacts with Pol α and Primase
Pob3p and Spt16p/Cdc68p form a heterodimer that is a component of the ATP-independent chromatin remodeling activity yFACT [52]. dna2–2 is synthetically lethal with a non- ts allele of POB3, pob3–21 [52], and various alleles of spt16 are synthetically lethal or sick with dna2–2 [53]. Since yFACT may participate in both DNA replication and transcription, to investigate a potential link with the role of Dna2p in replication and/or repair, we took advantage of the observation that yFACT interacts both genetically and physically with pol α [54,55]. This suggested dna2 might be synthetically lethal with a mutant containing a pol α protein that fails to interact with Pob3p/Sp16p, poi1–1 (with glycine at position 493) [56]. We established the synthetic lethality of dna2–2 and poi1–1 (18 tetrads, 46 viable spores, no double dna2–2 poi1–1 mutants). The dna2–2 poi1–1 lethality is allele specific, since dna2–1 is not synthetically lethal with poi1–17, a catalytic site mutant [57] (see Discussion). Although yFACT may also affect transcription and the synthetic effects between dna2 and yFACT components could be due to reduced transcription of dna2–2 or of other replication genes in the double mutants, the synthetic lethality of dna2–2 poi1–1 argues that Dna2p and yFACT may interact during DNA replication.

We went on to investigate genetic interactions between dna2 and genes encoding other pol α subunits. We found that dna2–2 is synthetically lethal with a primase subunit mutant, pri1–M4 (18 tetrads, 51 viable spores, no double mutants). This result further implicates Dna2p in lagging-strand DNA replication, as the pri1–M4 mutant is defective in elongation [58].

Chromatin Remodeling and Histone Modification
In addition to clarifying the relationship between dna2 and the yFACT complex by identifying the dna2/poi1–1 interac-

Figure 7. Synthetic Lethality of mec1Δ tel1Δ with dna2–2 Mutations
Strains used in these experiments are listed in Table S4, and were isogenic or congenic with W303 RADS5. Segregants of a MECL/mec1Δ TEL1/tel1Δ DNA2/dna2–2 SML1/sml1Δ diploid were placed on a YPD plate incubated at 30 °C (A) or 37 °C (B).
DOI: 10.1371/journal.pgen.0010061.g007
mutants are defective in OFP, dna2–2 ctf4–8 and dna2–2 swd3–14 (Table 1). Bre1p-mediated histone H2B ubiquitylation is necessary for histone H3 lysine 4 methylation by Swd1p and Swd3p. These results may indicate that dna2 rad6Δ growth defects do not result from defective post-replication repair, but rather from defects in histone modification.

Other Interactions

Additional nodes in the network (oxidative stress genes, osmotic stress genes, and genes involved in RNA modification/catabolism [mutants tef4Δ and rtt103A]) that are synthetically lethal with dna2 are described and discussed in Protocol S1. Genes involved in transcription elongation (CAF20, for example) have not yet been further analyzed because they may reduce transcription of Dna2p or another replication protein and thus indirectly cause lethality.

Discussion

We find that 56 genes interact genetically with DNA2. Comparison of our results with those of previous synthetic lethal screens using 45 of the DNA2-interacting genes defines a set of pathways, all of which are interdependent with DNA2 and that form a network for preserving genome stability (see Figure 2). The six major hubs shown in Figure 2 link DNA replication, DNA repair, chromosome dynamics, checkpoints, chromosome structure/chromatin, osmotic stress, oxidative stress, and RNA metabolism. A major link to the cell cycle and
the kinetochore occurs through a single gene, CTF4. Analysis of mutants that give rise to gross chromosomal rearrange-
ments, the type of damage considered to be the most likely result of replication apparatus failure, identifies the same pathways [69–75]. The comprehensive nature of the SGA 
screen, however, allows greater insight into the structure of the network that coordinates these events. It is striking that this topology can be superimposed on the prokaryotic DNA replication interactome recently identified using protein–protein interactions [76]. The bacterial genome maintenance network consists of many of the E. coli orthologs (i.e., pol III holoenzyme, SSB, RecQ, RecG, SbeB, and RecJ) of the yeast replicative polymerase, its subunits, and the helicases and nucleases that form hubs and major nodes in our genetic network [76]. The common denominator in the diverse approaches was the use of a replication gene or protein as the bait. The parallels between the organisms point to evolutionary conservation in the coordination of processes that protect the genome. We suggest that the complexity of such processes was required for the evolution of large genomes, where the fidelity of the replication apparatus itself could not guarantee a sufficiently high level of accuracy and stability in genome transmission.

Current methods of scoring interactions do not result in identification, in the initial SGA screen, of every interacting gene (see discussions in [18]). To approach completeness, following identification of a single gene in a pathway, e.g., MRE11, in the SGA screen, we pursued “traditional” investigation, on a gene-by-gene basis, of other genes in the putative pathway, such as XR52. Similarly, the identification of the Bre1p ubiquitylation pathway was interpreted only after testing downstream genes in the histone H2B modification pathway. This type of comprehensive genetic analysis is a powerful new tool for rapidly characterizing the full complement of processes requiring replication genes that might be coming under analysis for the first time, as well as rendering a coherent picture of years of genetic analysis of other genes. The genetic screen then enables one to rationally design experiments to determine, in molecular terms, the contribution of the replication protein to these processes. The outcome of such secondary analyses of DNA2 is discussed and interpreted below.

Stronger Links between Dna2p and OFP during Lagging-Strand Replication: RNH32, RNH202, EXO1, YEN1, POL3, POL32, POL1, and PR11

Structure-specific nucleases. Although it has been known for some time that dna2 and rad27Δ are synthetically lethal, convincing genetic interactions between dna2 and other lagging-strand activities have not been previously identified. As shown by our demonstration in this work of synthetic lethality of dna2 and rtf1Δ and the viability of dna2 and paf1Δ (see Results), synthetic lethality with one gene in a pathway does not prove interaction with other genes in that pathway. Therefore, the identification of so many additional lagging-strand genes in the current study is a matter of some note. The synthetic lethality of dna2 with the genes encoding structure-specific nucleases (RNH32, RNH202, EXO1, and YEN1) provides the first evidence, to our knowledge, that RNA may be a substrate of Dna2p in vivo and strengthens evidence, as predicted from our in vitro studies [14], that Dna2p acts primarily if not exclusively on flaps in vivo. It has been known for some time that rad27Δ rnh35Δ is synthetically sick, but not lethal [77]. Since FEN1 is generally considered the major OFP nuclease, the more significant synthetic lethality of dna2 rnh35Δ and dna2 rnh202A was somewhat unexpected, and suggests that Dna2p also acts in an OFP pathway redundant with RNaseH2. Since Rnh35p does not act on flap-containing structures, the common substrate for RNaseH2 and Dna2p is probably RNA. Alternative to a role in OFP, the dna2 rnh35Δ synthetic lethality might reflect a redundant role for RNaseH2 and Dna2p in mRNA processing, an additional function proposed for RNaseH2 [78]. Dna2p might be coming under analysis for the first time, as well as rendering a coherent picture of years of genetic analysis of other genes. The genetic screen then enables one to rationally design experiments to determine, in molecular terms, the contribution of the replication protein to these processes. The outcome of such secondary analyses of DNA2 is discussed and interpreted below.
on Okazaki fragments. pol δ-DV is another exonuclease-deficient allele of pol δ, and rad27-D is a proliferating cell nuclease antigen noninteracting mutant of rad27. Overproduction of Dna2p suppresses the lethality of a pol3-DV rad27-p rad51A strain [11], supporting our conclusions.

dna2–1 and dna2–2 mutants are suppressed by deleting the nonessential POL32-encoded subunit of pol δ that is required for optimum strand displacement. Pol32p is required for efficient in vitro DNA replication with pol δ in the presence of replication factor C, proliferating cell nuclease antigen, and a primed template. The ability to displace 5’ ends is drastically decreased for pol δ lacking Pol32p [35,43], and the pol δ complex is expected to be defective in strand displacement synthesis in pol32Δ strains, thus reducing the need for Dna2p. Pol32p has also been shown to interact with pol α, and the same mutant that is synthetically lethal with dna2–2, pol1–1 (G493R), is synthetically sick with pol32Δ [82]. This might hint at coordination between Okazaki fragment initiation and elongation, although there is no reported phenotype associated with a mutation (pol32Δ–8) that disrupts the Pol32p–pol δ interaction. While this manuscript was being prepared, it was reported that a mutation in cdc27Δ, the Schizosaccharomyces pombe ortholog of POL32, suppresses one allele of S. pombe dna2 [83], so this suppression is conserved. Why is pol32Δ rad27α lethal [18] while pol32Δ dna2 grows more robustly than dna2 mutants? Loss of pol32Δ may shift the course of OFP from a flap removal pathway to one employing RnasH. In the RnasH pathway, FEN1 exonuclease may become essential to remove the last ribonucleotide, an activity that Dna2p does not appear to possess.

Interaction with Pol α and Primase (and Mcm10p). Our demonstration here of synthetic lethal interactions of dna2 with pol1–1 and with pri1-M4, components of pol α-primase, may also fortify the argument that Dna2p participates in OFP. Although pri1-m4 has an S phase checkpoint defect in addition to a DNA replication defect, the replication defect is more likely to be responsible for the synthetic lethality with dna2, given our data that dna2 is not synthetically lethal with any of the mutations in major checkpoint genes, including mec1Δ smt1Δ. Certain alleles of dna2 are also synthetically lethal with mcn10–1 [23]. Recently, McM10 has been implicated in elongation and in stabilizing pol α in vivo as well as in stimulating pol α in vitro [84,85]. The interdependent functions of DNA2 and McM10 may reflect an interaction in lagging-strand replication. Alternatively, Dna2p might play a role in repair of mcn10–1-generated damage. The combined new data on interactions between Dna2p, pol Δ, pol α, and primase may be evidence for a previously unexpected coupling of primer synthesis, polymerase switching, and primer removal.

Differences between the Genetic Interactions of DNA2 and Those of RAD27

Comparison of the data presented here (see Figure 2; Table S2) and the results of a similar thoroughly validated SGA screen using rad27A as a query gene [86] reveals a wide (and unanticipated) divergence between genes that are synthetically lethal with dna2 and those that are synthetically lethal with rad27A. This divergence implies that the two enzymes may have slightly different sets of substrates. As pointed out in Results, the synthetic lethality of rad27A [69,87], but not dna2, with checkpoint mutants and with recombination mutants suggests that rad27A mutants probably accumulate more single-stranded DNA (the proposed signal for checkpoint activation) and more DSBs (repaired by recombination) [87], than dna2 mutants do. If dna2 mutants accumulate less damage than rad27A mutants, this in turn might suggest that FEN1 is the major OFP nuclease and that Dna2p is required at fewer (or different) sites than FEN1 [88]. This conclusion is consistent with other previously published evidence (despite the potential conundrum that deleting DNA2 is lethal and deleting RAD27 is not). First, pol δ, proliferating cell nuclease antigen, and FEN1 appear to act in a highly concerted fashion on templates that are optimal for pol δ efficiency in vitro, with little evidence that flaps longer than a few nucleotides are ever produced [10,33]. Second, dna2 mutants are weak mutants, while rad27A mutants are strong mutants, as measured by point mutations or stability of di- or trinucleotide repeats, or even larger repeats [16,87,89–91]. Dna2p may be specialized to function in OFP in genomic locations where the DNA sequence poses problems for pol δ, creating flaps that are not good substrates for FEN1. These regions are likely to include the rDNA and telomeres, since we have shown significant replication defects in these loci in dna2 mutants [17,33–37]. The role of Dna2p is not likely to be limited to these regions, however, as our previous immunofluorescence and chromatin immunoprecipitation analyses show Dna2p to be located at many other genomic regions during S phase [17]. Possible sites are replication slow zones or the inverted Ty repeats that give rise to genomic instability.

If FEN1 is the major flap nuclease, either Dna2p might help FEN1 on some flaps or Dna2p might recognize discrete subsets of flaps and process them independently. The genetic differences observed, combined with the quantitative biochemical data that show that Dna2p is very inefficient at stimulating FEN1, even on long flaps, direct future attention to potentially independent roles for Dna2p and FEN1.

The Helicase Network for Preserving Genomic Stability

Previous screening of the nonessential gene knock-out collection with sgs1Δ and srs2Δ as queries identified a so-called helicase network defined by a set of common interactions [92]. Genes in this network are implicated by dozens of recent studies in the repair of damaged replication forks through sister chromatid recombination and replication restart mechanisms, but coupling of the network to DNA replication remains poorly understood (e.g., [93]). By reversing the screening process and using an essential lagging-strand replication gene as query, we have found that dna2 is synthetically lethal with mutations in all of these helicases and in the genes with which they interact. The interactions shown in Figures 1 and 2 and our subsequent work (see Table 2) suggest that Dna2p may be one of the major replication proteins that coordinate this helicase network and replication.

The synthetic lethality of dna2–2 sgs1Δ and its lack of suppression by rad51Δ (Table 2) suggests that Sgs1p participates directly in DNA replication by aiding Dna2p in stimulating flap cleavage during OFP under some circumstances. This interpretation is attractive since the human Sgs1p orthologs, BLM and WRN, interact physically with Dna2p and suppress the replication defects in dna2–2 mutants when overexpressed in yeast [94,95]. (The suppression could not be investigated with yeast SGS1, since its overproduction is toxic [A. Morgan, personal communication; unpublished...
data.) The reproducible effect of deleting RAD51 in restoring defective growth to dna2–2 sgs1A mutants, however, is consistent with SGS1 playing an additional role in a late stage of recombinational repair of dna2-induced lesions, such as in the resolution of Holliday junction intermediates to viable products, a reaction that requires Sgs1p in conjunction with Top3p [96]. Dna2p might be required to remove 5’ ends of nascent DNA in reversed forks, while Sgs1p serves as a helicase to resolve the forks, like RecJ and RecQ in bacteria [97]. Lesions due to DNA2 deficiency do have the potential to lead to lethal recombinational intermediates, because we find that dna2 is synthetically lethal with srs2A and that this lethality is efficiently suppressed by deleting RAD51. SRS2, given its role in inhibiting an early step in recombination [98–100], probably prevents dna2–2 sgs1A-derived lesions from entering the recombinational repair pathway. Thus, the interaction between Sgs1p and Dna2p is multipotential.

Rrm3p promotes DNA replication through non-nucleosomal protein–DNA complexes, including the rDNA RFB, Rap1p-binding sites in the telomere, inactive ARS sites, and centromere DNA [101–104]. Rrm3p may act on the same replication intermediates as Dna2p rather than on downstream toxic intermediates formed during repair of faulty replication, since rad51 mutations do not suppress the dna2–2 rrm3A synthetic lethality. Recently, rrm3A has been tested for synthetic lethality against a number of candidate gene deletions. Unlike dna2–2 rrm3A, most of the synthetically lethal combinations, such as rrm3A srs2A and rrm3A sgs1A, were suppressed by recombination mutants [47,75]. Thus, early replication intermediates cause cell lethality in dna2 rrm3A mutants, while recombination intermediates cause cell lethality in rrm3A sgs1A mutants. These intermediates may not involve FEN1, since rad27A rrm3A is not synthetically lethal [47]. Although both dna2 and rrm3A mutants show significant pausing in the rDNA, a fob1A mutation did not restore viability to the dna2–2 rrm3A mutant (Table 2), so there are additional sequences replicated by these genes. We have recently demonstrated that Dna2p can stimulate FEN1 cleavage of long flaps with secondary structure, but that the reaction is inefficient [12,105]. Since Rrm3p is a 5’ to 3’ helicase, Rrm3p is a candidate for a helicase that may aid Dna2p in flap processing.

DNA Repair

It does not appear that dna2 interacts with genes involved in nucleotide excision repair, consistent with the relative resistance of dna2 mutants to UV irradiation [16]. Base excision repair (long patch) involves all of the proteins also involved in OFP, and therefore a role for Dna2p in base excision repair would be supported by the interactions found in this work and the MMS sensitivity of dna2 mutants [8]. A role for Dna2p in an unidentified Rad52p-dependent pathway was discussed above.

The synthetic lethality of dna2 with each member of the Mre11p complex may contribute to emerging evidence that the Mre11p complex functions at the replication fork. First, the seven genes whose interactions overlapped most significantly with dna2 (see Figure 2) are also synthetically lethal with the genes of the Mre11p complex. Second, the Mre11p complex associates with chromatid primarily during S phase, and this association does not appear to require DSBs [106]. It has been suggested that the Mre11p complex assists sister chromatid association [106], and that this association is required for recombinational repair of DSBs during DNA replication. Another view derives from the fact that the Mre11p complex and Exo1p are both required for activation of the Rad53p checkpoint kinase after inhibition of replication by HU. This leads to the inference that the Mre11p complex and Exo1p may convert DSBs arising at stalled replication forks into single-stranded DNA, a signal for subsequent repair. Since replication forks stall in dna2 mutants, the synthetic lethality with mutations in Mre11p complex components or Exo1p could be explained by a failure to produce the single-stranded DNA signal. It is possible that DSBs that arise during normal DNA replication are repaired in Mre11p complex mutants, but are lethal in cells lacking both Mre11p and Dna2p [7]. Finally, or alternatively, the synthetic lethality of dna2 mre11A may indicate that DNA2 is involved in a telomere defect, as has been shown in S. pombe [48,107]. We cannot eliminate the possibility that the Dna2p/Mre11p interaction is involved in repair, but we note that dna2–2 and rad50–5, which is as deficient in repair as rad52A, are epistatic with respect to repair.

Sister Chromatid Cohesion

Replication forks in dna2 mutants pause at the RFB in the rDNA, where DSBs result [17,35,37]. Our current finding that deletion of FOB1 suppresses the synthetic lethality of dna2–2 ctf4A, damage could be explained if Ctf4p-mediated sister chromatid cohesion is necessary to repair damage at the RFB due to defective Dna2p. This requirement for cohesion in DNA2 mutants is not limited to the rDNA, since the dna2–2 ctf4A fob1A strain is ts and radiation sensitive. Thus, the Dna2p deficiency must give rise to damage requiring cohesion for repair elsewhere in the chromosome as well. A role for cohesion in maintaining the replication fork during stalling or collapse is attractive since cohesion is required for efficient DSB repair [108]; a role for sister chromatid cohesion in preventing excessive sister chromatid exchange due to breaks at the RFB has been directly demonstrated [109]. We note that our analysis of replication in dna2–2 strains by two-dimensional gel electrophoresis indicates a high incidence of stalled replication forks at sequences throughout the rDNA, not limited to the RFB, suggesting general replication fork stalling in dna2 mutants and providing physical evidence of a more delocalized requirement for sister chromatid cohesion, perhaps throughout the chromosome [35,37].

Chromatin Remodeling, Disassembly, and Reassembly

During DNA replication, the chromatin in front of the replication fork is disassembled and then reassembled behind the fork. Our new findings add to recent findings from many sources that are providing the first insight into the molecular links between the replication machinery and chromatin dynamics. Dna2p interacts with both Asf1p and yFACT. The Asf1p/Dna2p interaction in chromatin assembly/remodeling is too ill-defined for further inferences at the moment. However, the allele-specific synthetic lethality between dna2 and pol1–1 suggests that Dna2p participates in the recently demonstrated interplay between Spt16p (a component of the FACT-like nucleosome reorganization factor), Ctf4p, and pol α [56]. The pol1–1 mutant protein fails to interact with Spt16p and shows altered temporal interaction with Ctf4p. The compromised association between Spt16p and pol α in the
pol1–1 mutant is accompanied by a delay both in pol α recruitment to late origins and its release, leading to a slow S phase [56]. By adding a link between Dna2p and this particular aspect of pol α function, our results support the model of the Formosa lab that the yeast Spt16p complex is likely to be directly involved in DNA replication [52–54,110], as has also been suggested for the frog FACT complex [111]. The Spt16p remodeling complex may facilitate the movement of pol α and Dna2p through nucleosomes, as proposed for human FACT in transcription [112,113]. The caveat that yFACT defects might result in reduced transcription of gene(s) that interact with DNA2 was mentioned above.

Other DNA2-interacting genes encode specific sets of histone modification enzymes that catalyze histone ubiquitylation, methylation, and deacetylation. We discovered here that the synthetic sickness of dna2 and rad6 is related to Rad1p/Bre1p-mediated ubiquitylation of histone H2B, which in turn leads to methylation of H3 at the lysine at position 4 by the SET1 complex, containing Set1p, Swh1p, and Swd3p [114]. set1 mutants are sensitive to HU and may accumulate DNA damage during S phase. set1 was not recovered in the SGA screen because it is not in the deletion collection. Ubiquitylation and methylation alter silencing and chromatin structure at the rDNA and at telomeres, which may suggest a mechanism for interaction with dna2 [114,115].

The interaction between Dna2p and Hst3p and the Rpd3p/Pho23p histone deacetylases is interesting because mutations in ORC, the replication initiator, as well as rad27A, pol32A, and sgs1Δ also show synthetic interaction with hst3A [18,116]. HST3 performs a redundant function in DNA replication but hst3 hst4 cells have phenotypes indicative of replication defects, such as increased rates of chromosome loss and mitotic recombination, decreased telomere silencing, and hypersensitivity to UV treatment [117]. DNA2 is the only DNA replication gene thus far found to interact with RPD3. Histone deacetylation by the Rpd3p/Pho23p complex has been previously implicated in the temporal regulation of origin activation, but not elongation, in DNA replication [67,68]. The observation that hst1Δ, hst3Δ, rpd3A, bre1Δ, spt16A, cot2A, andvid21A are all linked to the network shown in Figure 2 suggests that the existing nucleosome structure has been optimized for high fidelity DNA replication (see Protocol S1).

Conclusions

We propose that the genome maintenance network is coordinated by physical interaction of the replication proteins with the complexes that carry out regulation and repair. A good deal of evidence supports this. Originally we found that Dna2p co-purified with Fen1 and later demonstrated the genetic interaction. We discovered a genetic interaction between Dna2p and BLM and WRN helicases—the human orthologs of Sgs1p—and then found that this genetic interaction also represented a direct physical interaction between Dna2p and those helicases [94]. Others found genetic interactions between DNA2 and RFA1, encoding the single-stranded DNA binding protein RPA, and similarly documented a physical interaction between the proteins Dna2p and RPA [13,118]. Indeed, most DNA replication proteins multitask and are components of many complexes. Pol δ and ε are involved in DNA repair complexes as well as replication complexes. Pol ε is, in addition, involved in the S phase checkpoint. Fen1 itself is involved in multiple reactions, including long patch base excision repair [15]. Though circumstantial, the resemblance between the basic topology of the genetic network described here and the proteomic network described in bacteria [76] further suggests the model that replication proteins physically coordinate repair and regulatory genome maintenance complexes. We anticipate that this model will be verified in detail when proteomic approaches in yeast yield the kind of comprehensive data that can already be obtained from genetic screens such as SGA.

Materials and Methods

Strains. All strains used in the study are found in Table S4. The strains used in the SGA screen and subsequent verification were as follows: 4741 MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0; 4741 MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0; strain 4741dna2Δ1–6D (MB100) MATa dna2Δ1 his3Δ1 leu2Δ0 met15Δ0 ura3Δ0; strain 4741dna2Δ2–11D (MB101) MATa dna2Δ2–2::LEU2 his3Δ1 leu2Δ0 met15Δ0 ura3Δ0; Y3656 can1Δ::NDA1p::HIS3-Mps1::LEU2 his3Δ1 leu2Δ0 met15Δ0 ura3Δ0; Y3656dna2Δ1–1; Y356 dna2Δ2–1::URA3; Y356dna2Δ2–2; Y3556 dna2Δ2–2::URA3; and W30303 MATa ade2Δ1 can1–100 his3–11,15 leu2Δ0 C. Any strain failing to generate viable double mutants was deemed synthetically lethal. A strain was tested using 4741dna2Δ1 or Y3656dna2Δ2 as indicated.

SGA screen. The SGA screen was performed as previously described [19]. Y3656dna2Δ1–1 and Y3656dna2Δ2–2 were constructed for this work and used as query strains. SGA analysis was performed for each of the dna2 alleles. Genes that showed synthetic lethality or synthetic sickness in the primary screen were tested by standard tetrad dissection [119]. For the secondary tetrad analysis, new heterozygous diploids were constructed between MB100 (4741 dna2Δ1–1) and MB101 (4741 dna2Δ2–2) and each of the candidate deletion mutants in strain 4741 (Invitrogen, Carlsbad, California, United States). Thus, each synthetic interaction that is reported here was tested using two independent diploids, one in the original screen and one in the secondary screen. The dna2–2 dissections were incubated at 30°C and dna2–1 dissections at 23°C. Any strain failing to generate viable double mutants was deemed synthetically lethal. A strain was considered synthetically sick if tetrads gave fewer double mutants than expected and double mutants grew slower than either single mutant. At least ten tetrads were dissected for each double mutant, and usually more were dissected.

X-ray and MMS sensitivity. The X-ray source was Pantak (East Haven, Connecticut, United States) Mk II 70 kev 20 ma. The source was calibrated and experiments were carried out as previously described [16]. For the MMS sensitivity assay, 0.005% and 0.01% MMS was added to yeast-jepeptide-dextrose (YPD) plates after autoclaving, and plates were used the same day.

Supporting Information

Protocol S1. Other

Found at DOI: 10.1371/journal.pgen.0010061.s001 (53 KB DOC).

Table S1. Genes That Show Little, If Any, Interaction with dna2Δ1 or dna2Δ2 in Secondary Screen

Found at DOI: 10.1371/journal.pgen.0010061.s001 (107 KB PDF).

Table S2. Synthetic Lethal Interactions Used to Prepare Figure 2

Found at DOI: 10.1371/journal.pgen.0010061.s002 (111 KB PDF).

Table S3. Number of Spores after Dissection of dna2Δ2–2 me1 tel1 slm1 Heterozygote

Found at DOI: 10.1371/journal.pgen.0010061.s003 (95 KB PDF).

Table S4. Strains Used

Found at DOI: 10.1371/journal.pgen.0010061.s004 (103 KB PDF).

Acknowledgments

This work was supported in part by United States Public Health Service grant GM55508 to JLC and by a National Science and Engineering Research Council of Canada grant, a grant from the Canadian Institutes of Health Research, and funds from Genome
Saccharomyces
dna2
CTF4
DNA2
to 5
Saccharomyces cerevisiae
EXO1, a
Saccharomyces cerevisiae
exonuclease activities of Pol delta in the creation
Saccharomyces cerevisiae
MMS1
possesses a single-stranded DNA-specific endonuclease activity
rrm3
cells requires the intra-S-phase
encodes a DNA helicase
are required for sister chromatid cohesion. Mol Cell Biol
DNA polymerase epsilon and polymerase sigma interact physi-
hybrids mediate transcription elongation impairment and transcription-
mediated replication fork arrest. Mol Cell Biol 23: 2773–2784.
89. Askre SH, Yehuda T, Smolikos S, Gurevich R, Hawk J, et al. (2004) A genome-wide screen for Saccharomyces cerevisiae deletion mutants that affect telomere length. Proc Natl Acad Sci U S A 101: 8658–8663.
90. Luong M, Morgan DO (2005) Specificity in the phosphorylation of cyclin-dependent kinase substrates. Nature 434: 104.
91. Jin YH, Obreht B, Burgers PMJ, Kunkel TA, Resnick MA, et al. (2001) The 5'-3' exonuclease of DNA polymerase delta can substitute for the 3' flap endonuclease Rad27/Fen1 in processing Okazaki fragments and preventing genome instability. Proc Natl Acad Sci U S A 98: 5122–5127.
92. Huang ME, de Calignon A, Nicolas A, Gallbort F (2000) POL32, a subunit of the Saccharomyces cerevisiae DNA polymerase delta, defines a link between DNA replication and the mutagenic bypass repair pathway.Curr Genet 38: 178–187.
93. Tuncak H, Ryu GH, Seo YS, MacNeill SA (2004) Genetics of lagging strand DNA synthesis and maturation in fission yeast: Suppression analysis links the Dna2-2Cdc42 complex to DNA polymerase delta. Nucleic Acids Res 32: 6367–6377.
94. Riecke RJ, Bielesky AK (2004) Mcm10 regulates the stability and chromatin association of DNA polymerase-alpha. Mol Cell 16: 173–185.
95. Fien K, Cho YS, Lee JK, Raychaudhuri S, Tappin I, et al. (2004) Primer utilization by DNA polymerase alpha is influenced by its interaction with Mcm10p. J Biol Chem 279: 16144–16153.
96. Loeillet S, Palancade B, Cartron M, Thierry A, Fichard GF, et al. (2003) Mutagenic network interactions among replication repair and nuclear pore deficiencies in yeast. DNA Repair (Amst) 4: 459–468.
97. Tischkoff DF, Filosi N, Gaida GM, Kolodner RD (1997) A novel mutation avoidance mechanism dependent on Saccharomyces cerevisiae RAD27 is distinct from DNA mismatch repair. Mol Cell 1: 253–262.
98. Liu Y, Kao HI, Bamba RA (2004) Flap endonuclease 1: A central component of DNA metabolism. Annu Rev Biochem 73: 589–615.
99. Johnson RE, Gopala KK, Prakash L, Prakash S (1995) Requirement for the yeast BRF1 5' to 3' exonuclease for the stability of repetitive DNA. Science 269: 238–240.
100. Callahan JL, Andrews KJ, Zikan VA, Frendrich GE (2003) Mutations in yeast replication proteins that increase CACC/GCTG expansions also increase repeat fragility. Mol Cell Biol 23: 7849–7860.
101. Lopes J, Debrabrandere H, Buurd J, Nicolas A (2002) Instability of the human minichromosome C8R1 in rad27A and dna1-2 replication-deficient yeast cells. EMBO J 21: 3201–3211.
102. Ooi SL, Shoemaker DD, Boeke JD (2005) DNA helicase gene interaction network defined using synthetic lethality analysis by microarray. Nat Genet 35: 277–286.
103. Ghetti G, Maffioletti G, Lucca C, Cioli O, Raveryshnikova A, et al. (2005) Rad51-dependent DNA structures accumulate at damaged replication forks in sgi1 mutants defective in the yeast ortholog of BLM RecQ helicase. Genes Dev 19: 339–350.
104. Imamura O, Campbell JL (2003) The human Bloom syndrome gene suppresses the DNA replication and repair defects of yeast dna2 mutants. Proc Natl Acad Sci U S A 100: 8193–8198.
105. Sharma S, Sommers JA, Brosh RM Jr (2004) In vivo function of the conserved non-catalytic domain of Saccharomyces cerevisiae Srs2 helicase in DNA replication. Hum Mol Genet 13: 2247–2261.
106. Oakley TJ, Hickson ID (2002) Defending genome integrity during S-phase: Putative roles for RecQ helicases and topoisomerases. DNA Repair (Amst) 1: 235–247.
107. Courcelle J, Hanawalt PC (1999) RecQ and RecJ process block replication forks prior to resumption of replication in UV-irradiated Escherichia coli. Mol Gen Genet 262: 543–551.
108. Schiestl RH, Prakash S, Prakash L (1990) The Srn2 helicase prevents recombination by disruption Rad51 nuclear protein filaments. Nature 423: 309–312.
109. Krejci L, Van Rommen S, Li Y, Vellapa S, Reddy MS, et al. (2003) DNA helicase Srs2 disrupts the Rad51 presynaptic filament. Nature 423: 305–309.
110. Ivesa AS, Zikan VA (2002) To fire or not to fire: Origin activation in Saccharomyces cerevisiae ribosomal DNA. Genes Dev 16: 2459–2464.
111. Ivesa AS, Zhou QJ, Schulz VP, Monson EK, Zakian VA (2002) Saccharomyces Rrm3p, a 3’ to 5’ DNA helicase that promotes replication fork progression through telomeric and subtelomeric DNA. Genes Dev 16: 1383–1396.
112. Ivesa AS, Lenzmeier BA, Beslter B, Goudsouzian LK, Schnakenberg MR, et al. (2003) The Saccharomyces cerevisiae Rrm3p helicase is required for DNA replication past nonhistone protein-DNA complexes. Mol Cell 12: 1525–1536.
113. Maker A, Ihlewez A, Blackburn EH (2004) Anatomy and dynamics of DNA replication fork movement in yeast telomeric regions. Mol Cell Biol 24: 4015–4031.
114. Kao HI, Hendriksen LA, Liu Y, Bamba RA (2002) Cleavage specificity of Saccharomyces cerevisiae flap endonuclease 1 suggests a double-flap structure as the cellular substrate. J Biol Chem 277: 14379–14389.
106. Mirzoeva OK, Petrini JHJ (2003) DNA replication-dependent nuclear dynamics of the Mre11 complex. Mol Cancer Res 1: 207–218.
107. Tomita K, Kibe T, Kang HY, Seo YS, Uritani M, et al. (2004) Fission yeast Dna2 is required for generation of the telomeric single-strand overhang. Mol Cell Biol 24: 9557–9567.
108. Sjogren C, Nasmyth K (2001) Sister chromatid cohesion is required for postreplicative double-strand break repair in Saccharomyces cerevisiae. Curr Biol 11: 991–995.
109. Kobayashi T, Horinouchi T, Tongalnkar P, Vu L, Nomura M (2004) SIR2 regulates recombination between different rDNA repeats, but not recombination within individual rRNA genes in yeast. Cell 117: 441–455.
110. Wittmeyer J, Formosa T (1997) The Saccharomyces cerevisiae DNA polymerase alpha catalytic subunit interacts with Cdc68/Spt16 and with Pob3, a protein similar to an HMG1-like protein. Mol Cell Biol 17: 4178–4190.
111. Okuhara K, Ohta K, Seo H, Shioda M, Yamada T, et al. (1999) A DNA unwinding factor involved in DNA replication in cell-free extracts of Xenopus eggs. Curr Biol 9: 341–350.
112. Orphanides G, Wu WH, Lane WS, Hampsey M, Reinberg D (1999) The chromatin-specific transcription elongation factor FACT comprises human ST16 and SSRP1 proteins. Nature 400: 284–288.
113. Orphanides G, LeRoy G, Chang CH, Luse DS, Reinberg D (1998) FACT, a factor that facilitates transcript elongation through nucleosomes. Cell 92: 105–116.
114. Nagy PL, Griesenbeck J, Kornberg RD, Cleary ML (2002) A trithorax-group complex purified from Saccharomyces cerevisiae is required for methylation of histone H3. Proc Natl Acad Sci U S A 99: 90–94.
115. Krokan NJ, Dover J, Khorrami S, Greenblatt JF, Schneider J, et al. (2002) COMPASS, a histone H3 (lysine 4) methyltransferase required for telomeric silencing of gene expression. J Biol Chem 277: 10753–10755.
116. Suter B, Tong A, Chang M, Yu L, Brown GW, et al. (2004) The origin recognition complex links replication, sister chromatid cohesion and transcriptional silencing in Saccharomyces cerevisiae. Genetics 167: 579–591.
117. Brachmann CR, Sherman JM, Devine SL, Cameron EE, Pillus L, et al. (1993) The SIR2 gene family, conserved from bacteria to humans, functions in silencing, cell cycle progression, and chromosome stability. Genes Dev 9: 2988–2992.
118. Bae KH, Kim HS, Bae SH, Kang HY, Brill S, et al. (2003) Bimodal interaction between replication-protein A and Dna2 is critical for Dna2 function both in vivo and in vitro. Nucleic Acids Res 31: 3006–3015.
119. Guthrie C, Fink G, editors (1991) Guide to yeast genetics and molecular biology. New York: Academic Press. 933 p.
120. Morrison A, Sugino A (1992) Roles of POL3, POL2 and PMS1 genes in maintaining accurate DNA replication. Chromosoma 102: S147–S149.
121. Toyn JH, Gunyuzlu PL, White WH, Thompson LA, Hollis GF (2000) A counterselection for the tryptophan pathway in yeast: 5-fluoroanthranilic acid resistance. Yeast 16: 553–560.