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Phosphorylation and Cellular Function of the Human Rpa2 N-Terminus in the Budding Yeast *Saccharomyces cerevisiae*

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Abstract

Maintenance of genome integrity is critical for proper cell growth. This occurs through accurate DNA replication and repair of DNA lesions. A key factor involved in both DNA replication and the DNA damage response is the heterotrimeric single-stranded DNA (ssDNA) binding complex Replication Protein A (RPA). Although the RPA complex appears to be structurally conserved throughout eukaryotes, the primary amino acid sequence of each subunit can vary considerably. Examination of sequence differences along with the functional interchangeability of orthologous RPA subunits or regions could provide insight into important regions and their functions. This might also allow for study in simpler systems. We determined that substitution of yeast Replication Factor A (RFA) with human RPA does not support yeast cell viability. Exchange of a single yeast RFA subunit with the corresponding human RPA subunit does not function due to lack of inter-species subunit interactions. Substitution of yeast Rfa2 with domains/regions of human Rpa2 important for Rpa2 function (*i.e.*, the N-terminus and the loop 3–4 region) supports viability in yeast cells, and hybrid proteins containing human Rpa2 N-terminal phospho-mutations result in similar DNA damage phenotypes to analogous yeast Rfa2 N-terminal phospho-mutants. Finally, the human Rpa2 N-terminus (NT) fused to yeast Rfa2 is phosphorylated in a manner similar to human Rpa2 in human cells, indicating that conserved kinases recognize the human domain in yeast. The implication is that budding yeast represents a potential model system for studying not only human Rpa2 N-terminal phosphorylation, but also phosphorylation of Rpa2 N-termini from other eukaryotic organisms.

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Replication Protein A (RPA) is called Replication Factor A (RFA) in budding yeast. Therefore, RFA refers to the yeast complex, and RPA refers to all other eukaryotic forms of the complex. Furthermore, individual human subunits are referred to as Rpa1, Rpa2, and Rpa3, and the homologous yeast subunits are referred to as Rfa1, Rfa2, and Rfa3. Finally, genes are denoted in *italics* and proteins are denoted in sentence case.

Keywords

RPA; N-terminus; phosphorylation; DNA damage response

INTRODUCTION

Understanding the basic mechanisms by which the integrity of genomic DNA is maintained is central to understanding how mutations are prevented. There are numerous ways in which DNA integrity can be compromised, including errors in DNA replication, exposure to environmental stresses, and progression through natural physiological processes. Cells have numerous mechanisms to prevent DNA lesions and to deal with DNA lesions that do arise. Despite this, some DNA lesions can remain unrepaired or be repaired incorrectly, resulting in permanent changes (mutations) in the DNA.

Many DNA processes in the cell are carefully coordinated in an effort to maximize efficiency and minimize errors in the cellular genome. Processes such as DNA replication and DNA repair/recombination result in the formation of a DNA intermediate (single-stranded DNA; ssDNA) that ultimately must be processed to an intact double-stranded DNA (dsDNA) form. At the center of these DNA processing events is the heterotrimeric protein complex Replication Protein A (RPA). The major biochemical activity of RPA is to bind ssDNA with high affinity and low sequence specificity through multiple oligonucleotide/oligosaccharide (OB)-fold domains (*i.e.*, DNA binding domains; DBDs) located within the three subunits (1–3). The largest subunit, Rpa1 (Rpa70; 70 kDa), is the major contributor to high-affinity ssDNA binding (4, 5), while the smallest subunit, Rpa3 (Rpa14; 14 kDa), appears to be important for heterotrimeric complex formation (6). The medium subunit, Rpa2 (Rpa32; 32 kDa) is thought to not only contribute to ssDNA binding (7, 8), but also to regulate function of the RPA complex, especially in response to DNA damage, through multiple post-translational modifications (PTMs) (9–14).

The major emphasis of studies of RPA post-translational modification have focused on phosphorylation of the human Rpa2 N-terminus (NT) on multiple serine/threonine (S/T) residues located within the first 40 amino acids (aa) (15, 16). Human Rpa2 is phosphorylated both *in vitro* and *in vivo* on specific residues by multiple kinases during DNA replication and in response to specific DNA damaging agents. While some of these targets are consensus sequences (S/TQ) for phosphatidylinositol-3 (PI3)-related kinases (ATM and ATR) involved in checkpoint regulation, others are phosphorylation targets of cyclin-dependent kinase (CDK) and DNA-dependent protein kinase (DNA-PK) (17). Many Rpa2 orthologs contain an N-terminal region that is S/T-rich; however, it is not known whether these residues in most orthologs are *1)* actual targets of phosphorylation or *2)* important for RPA cellular function.

Studies of the cellular function(s) of human Rpa2 phosphorylation initially focused on the utilization of “extensive” phospho-mutants, where *all* S/T residues in the Rpa2 NT were mutated to mimic phosphorylation (all aspartic acids; Rpa2-D_x), to prevent phosphorylation (all alanines; Rpa2-A_x), or were removed completely (deletion of first 33 aa; Rpa2-ΔN_x) (9, 18). These mutants, along with mutation of individual or pairs of sites have been

instrumental in implicating this region as important for human RPA function in DNA repair, cell cycle progression, and protein interactions (9–14). For example, it is clear that lack of hyper-phosphorylation of the human Rpa2 NT, either by mutation of serines 4 and 8 (S4/S8) to alanines or by inhibition of DNA-PK activity, leads to defects in the cellular response to replicative stress, including premature replication restart, hyper-recombination, and defective checkpoint arrest (11, 14). Also, ATR-dependent phosphorylation of threonine 21 (T21) and serine 33 (S33) is important for disrupting RPA association with replication centers and preventing replication during replication stress (9, 12, 13). Although none of these effects have been examined beyond a few cell generations due to experimental complexity in human cells, the defective phenotypes would suggest long-term detrimental effects on cells. This is supported by an increase in apoptosis following replicative stress in human Rpa2-T21A/S33A mutant cells (19).

In the budding yeast *Saccharomyces cerevisiae*, it is not as clear what, if any, role phosphorylation of Rfa2 (specifically the Rfa2 N-terminus) has on cells. Phosphorylation of this region by the damage-specific kinase Rad53 during mitosis has been reported, but only when the yeast cells contain a *set1*Δ mutation (20). The Rfa2 N-terminus (NT) is also phosphorylated by the meiosis-specific kinase Ime2 during meiosis (21). However, an unphosphorylatable yeast Rfa2 NT mutant (Rfa2-A_x) has no discernible phenotype in mitotic cell growth or in standard DNA damage assays, indicating that this domain does not have to be phosphorylated for proper function of RFA in response to DNA damage in yeast (22). Furthermore, if mitotic phosphorylation is occurring in this region (in a *SET1* background), it is below the level of detection by western blotting and has not been previously detected by mass spectrometry. Mutation of the Rfa2 NT, either to a constitutively phospho-mimetic form (Rfa2-D_x; analogous to human Rpa2-D_x) or to a form where the N-terminus has been removed (Rfa2-ΔN_x; analogous to human Rpa2-ΔN_x), leads to DNA damage-sensitivity (22). However, removal of the Rfa2 N-terminus has also been reported to partially-suppress the damage-sensitive phenotype observed in *mec3*Δ or *set1*Δ cells, possibly through de-repression of expression of repair genes (20). Taken together, this suggests that this domain is *1)* necessary for the damage response (at least in *SET1* cells) and *2)* if phosphorylated, may need to be dephosphorylated for a proper response to DNA damage (based on the *rfa2-*A_x damage-resistant phenotype). There is precedence for dephosphorylation being important in human cells (and in the yeast *Candida albicans*; 23, 24), as human PP4 phosphatase complex (or the *Candida* equivalent) is necessary to dephosphorylate human Rpa2 and facilitate homologous recombination (25). Both budding yeast (*Saccharomyces cerevisiae*) RFA and human RPA must function correctly to facilitate a proper response to DNA damage, and it is important to determine how each does this despite apparent differences in N-terminal phosphorylation and effects on N-terminal mutants on cellular function.

There have been many studies of RPA to characterize its function either through binding to ssDNA or commonly found DNA intermediates (*e.g.*, ssDNA-dsDNA junctions) (7, 26) or through characterization of protein interactions (15). A number of groups have also studied the interchangeability of RPA subunits. These studies include exchanging yeast RFA subunits individually with the corresponding human RPA subunit (or *vice versa*) (27, 28)

and examination of yeast RFA in an *in vitro* SV40 DNA replication system (29). It is clear that yeast RFA does not function properly in systems that require human RPA, nor do individual human RPA subunits function in yeast cells. Conversely, substitutions of regions of yeast RFA subunits with the equivalent human RPA regions can support cell growth; however, additional phenotypes have not been examined (30). Based on these data, we predicted that an orthologous RPA complex might function in yeast cells, but only if the complete complex were present. To our knowledge, there has not been an examination of whether or not a complete human RPA complex can function in yeast.

We examined the ability of human RPA and yeast RFA subunits to interact and demonstrated that yeast RFA subunit interactions are specific for other yeast subunits, and that human RPA subunit interactions are specific for other human subunits. We postulated that human RPA might function in yeast if it were present as a complete complex; however, neither expression of the canonical RPA complex nor the alternative RPA complex (28, 31, 32) in yeast cells supported viability. However, examination of the human Rpa2 NT in the context of a yeast RFA complex revealed that the human N-terminus can substitute for the yeast Rfa2 NT. Furthermore, human Rpa2 NT phospho-mutant forms display similar phenotypes to the equivalent yeast Rfa2 NT mutant forms. Finally, Rfa2 containing a human Rpa2 NT is phosphorylated at residues that are normally phosphorylated on human Rpa2 in human cells, and phosphorylation of some sites occurs in a damage-specific manner. Our results indicate that experimentally manipulating human (or perhaps any eukaryotic) RPA in yeast cells may yield important insights into RPA complex modification and function.

RESULTS

The human RPA complex cannot substitute for the yeast RFA complex

Yeast Replication Factor A (RFA) cannot substitute completely for human Replication Protein A (RPA) in the *in vitro* SV40 replication system (29), and individual human RPA subunits cannot substitute for individual yeast RFA subunits in yeast cells (27). However, important regions (DBD-A and DBD-B) of human RPA subunits can substitute for equivalent regions of yeast RFA (30). As yeast is a genetically amenable system for studying protein cellular function, it was of interest to investigate if studies of human protein behavior in yeast were feasible, especially if homologous human proteins were able to function in place of the endogenous yeast proteins. However, to our knowledge, substituting the entire human RPA complex for the entire yeast RFA complex in yeast had not been tested. Furthermore, there are two forms of human RPA: canonical RPA (consisting of Rpa1, Rpa2, and Rpa3) that supports DNA replication and alternative RPA (consisting of Rpa1, Rpa4, and Rpa3) that does not support DNA replication (28, 32) in human cells.

A plasmid shuffle assay was used to determine whether or not human RPA (canonical or alternative) can function in yeast cells as the sole form of RPA present. Plasmid vectors (pJM132 derivatives) were generated where the yeast *RFA1*, *RFA2*, and *RFA3* genes were substituted with the homologous human *Rpa1*, *Rpa2* (or *Rpa4*), and *Rpa3* genes, respectively. The expression of each human RPA subunit gene in these constructs was driven by its respective homologous yeast gene endogenous promoter, and the human RPA

subunit gene expression constructs also contained a *ura3*Δ*::kanMX* marker to select for cells containing the plasmid.

Measurement of expression of human RPA subunit mRNA from these constructs showed that all three genes were expressed in yeast cells (Figure 1A). The level of mRNA expression ranged from 41–80% for human *Rpa1*, 47% for human *Rpa2*, and 76% for human *Rpa4* compared to their respective yeast homologs. For human *Rpa3* mRNA expression, the levels ranged from 9–13% compared to yeast *RFA3*. This difference is somewhat exaggerated, because these yeast cells contain both a chromosomal copy and a plasmid copy of the *RFA3* gene; however, the mRNA expression ratio of human *Rpa3* mRNA to yeast *RFA3* mRNA was consistently the lowest. Since reliable antibodies are readily available for human Rpa1 and Rpa2, protein expression for these subunits was also examined. Figure 1B showed that human Rpa1 protein is detected when cells expressed either the canonical RPA plasmid or the alternative RPA plasmid (Figure 1B). Furthermore, human Rpa2 could be detected when the canonical RPA expressing plasmid was present in the cells (Figure 1B).

A spot assay was performed to determine if yeast cells containing only a human RPA-expressing plasmid could survive. Human RPA-expressing cells were grown overnight in YPD+G418, counted, serial diluted, and plated onto YPD+G418 (demonstrates that cells originally contained plasmid expressing human RPA), SD-HLU (required to demonstrate that control cells contained vectors with both WT yeast *RFA1* and *RFA2* genes), and SD+5-FOA (demonstrates the ability of cells to lose the WT yeast *RFA1*, *RFA2*, and *RFA3*-expressing pJM132 plasmid, which all cells contain). Only cells that have a form of RFA or RPA that can support DNA replication in yeast will show growth on 5-FOA. The control cells containing WT *RFA1* and *RFA2* vectors showed growth on 5-FOA-containing plates (Figure 1C; row 1), indicating complementation of chromosomal *rfa1*Δ and *rfa2*Δ. Two independent clones expressing either canonical RPA (Figure 1C; rows 2–3) or alternative RPA (Figure 1C; rows 4–5) did not display observable growth on 5-FOA plates, indicating that expression of the either human RPA complex in yeast cells as the sole source of RPA does not function to support cell viability.

Lack of inter-species interactions among the human RPA and yeast RFA subunits

The inability of individual human RPA subunits to function in place of their respective homologs in yeast suggested that complex formation might not occur properly between inter-species subunits; however, it does not define which subunits do or do not interact. To make this determination, we utilized a yeast two-hybrid assay and examined the ability of the large (70 kDa) subunit of yeast RFA (Rfa1) or human RPA (Rpa1) to interact with their homologous partner subunits. Yeast Rfa1 and human Rpa1 were chosen, because these are the only subunits that do not display auto-activation of the reporter genes (*i.e.*, false-positive interaction). In Figure 2A, four independent yeast Rfa1 constructs fused to the lexA DNA binding domain (BD) were examined for interactions with each of the human RPA subunits fused to the B42 transcriptional activation domain (AD) or an empty AD vector (as a control). The lack of growth on SD-HTUL in the presence of an empty AD vector indicates no auto-activation from any of the BD-Rfa1 fusion constructs. Rfa1-FLAB is a control that

is lacking the C-terminal 208 amino acids (DBD-C) and is incapable of forming a complex with yeast Rfa2 or Rfa3 (33). The remaining three BD-Rfa1 constructs express full-length Rfa1, however, each is slightly different (*i.e.*, expression is driven from a different promoter or the amino acid immediately preceding the first codon of Rfa1 is different). For the BD-Rfa1 (pPM07), expression is driven from a galactose-inducible promoter, and we observe that its overexpression leads to a dominant-negative phenotype for growth (*i.e.*, lack of growth on SG-HTUL and reduced growth on SG-HTU+X-gal). However, this construct normally leads to blue color for the limited growth on SG-HTU+X-gal when in combination with yeast AD-Rfa2 or AD-Rfa3 (33). There is no blue color when this bait construct (BD-Rfa1; 07) is examined with the human RPA subunits. The other two bait constructs, pSJH101 and pENM17, express Rfa1 constitutively from the *ADH1* promoter. There is no blue color observed when combined with any of the human RPA subunits (Figure 2A; SG-HTU+X-gal); however, very slight growth is observed on SG-HTUL plates for AD-Rpa2 and even fainter growth is observed for AD-Rpa3 when combined with BD-Rfa1 expressed from pENM17. This would indicate a very weak interaction is occurring between yeast Rfa1 and human Rpa2 and an even weaker interaction between yeast Rfa1 and human Rpa3. These interactions are very weak compared to those observed for yeast Rfa1 and yeast Rfa2 or Rfa3, where blue color on SG-HTU+X-gal media and strong growth on SG-HTUL for pSJH101 and pENM17 normally occurs (33).

The reciprocal experiment was performed using human Rpa1 as the bait. In Figure 2B (bottom half), no growth was observed for human BD-Rpa1 combined with yeast AD-Rfa1, AD-Rfa2, or AD-Rfa3 on media diagnostic for interaction (SG-HTUL). Furthermore, no blue color was observed for any of these combinations on SG-HTU+X-gal media. This demonstrates that the human Rpa1 does not detectably interact with any of the yeast RFA subunits, and explains why substitution of any individual yeast subunit by the homologous human subunit would not function properly. To demonstrate that the human subunits interact with one another, we also examined human BD-Rpa1 in combination with AD-tagged versions of each human RPA subunit (Figure 2B; top half). Rpa1 only displayed strong interaction (growth on SG-HTUL and blue color on SG-HTU+X-gal) with Rpa2 and Rpa4, but not Rpa3 (Figure 2B; top half).

It was previously shown that recombinant human Rpa2 and Rpa3 form a stable subcomplex that is soluble and can be readily purified (34). It was also shown that siRNA knockdown of human Rpa1 only negatively affects mRNA/protein expression of Rpa1, but that knockdown of human Rpa2 negatively affects detection of cellular Rpa2 and Rpa1 proteins (9, 28, 35, 36). This also supports the idea of an Rpa2-Rpa3 subcomplex in the cell. Our data suggests that human Rpa2 (or Rpa4) is the subunit mediating the interaction of this subcomplex with human Rpa1, as the interaction between human Rpa1 and Rpa2 (or Rpa4) is occurring in yeast cells that are not expressing human Rpa3. To verify this, we expressed human Rpa2 or Rpa4 (pPLG35 or pPLG36; extra plasmid) in addition to BD-Rpa1 and AD-Rpa3 in yeast cells. Interpretation for whether human Rpa2 or Rpa4 mediated interaction with AD-Rpa3 was complicated by the observation of auto-activation (growth on SD-HTUL; Figure 2C; rows 2–3) when either Rpa2 or Rpa4 was expressed from the extra plasmid. However, this observation does lend further support for the interaction of human Rpa2 or Rpa4 with Rpa1,

because BD-Rpa2 or BD-Rpa4 alone are also known to cause auto-activation (31, 33, 37). In this case, we propose that recruitment of Rpa2 or Rpa4 by BD-Rpa1 is having a similar activation effect. Human Rpa2 or Rpa4 were also expressed from plasmids expressing human Rpa3. This appeared to minimize the auto-activation effect observed above (minimal growth on SD-HTUL; Figure 2C; rows 4–5). However, when AD-Rpa3 expression was induced by addition of galactose in these cells, growth on SG-HTUL was noticeably increased (Figure 2C). Here activation is most likely due to recruitment of AD-Rpa3, through an interaction with Rpa2 or Rpa4.

The loop 3–4 region of Rpa2 or Rpa4 does not affect yeast Rfa2 function

Since the human RPA complex cannot substitute for the yeast RFA complex, a domain swap approach was taken to determine the importance of two different regions of human Rpa2 or Rpa4 by examining their effects in yeast cells. The first region was the loop 3–4 (L34) region of human Rpa2 (38) or Rpa4 (28). In the structure of human Rpa2, this loop most likely represents a structurally-disordered flexible region, as a defined structure for this region is only observed in one of the deposited crystal structures for human Rpa2 (38). The L34 region is also of interest, because it is at least partially responsible for the difference in *in vitro* and cellular replication function between human Rpa2 and Rpa4 (28, 32). The major difference between the human Rpa2 and Rpa4 L34 regions lies in the apparent overall charge of the loop. In human Rpa2, this region contains a number of aspartic acid (D) and glutamic acid (E) residues, whereas in the human Rpa4 L34 region, there are more arginine (R) and lysine (K) residues (Figure 3A) (28, 31). In yeast Rfa2, this region contains an additional 14–15 amino acid residues compared to human Rpa2 and Rpa4, respectively (Figure 3A). This region has also been identified by mass spectrometry to contain serines (S) and a tyrosine (Y) that are phosphorylated (39–41), although only one (S122) has been validated and demonstrated to be phosphorylated by Mec1 (the yeast ATR homolog) in response to DNA damage (42, 21) (Figure 3A). Serine 122 currently has no obvious role in the mitotic DNA damage response, but may be important for crossover frequency during meiosis (43).

To determine whether the human Rpa2 or Rpa4 L34 regions could play similar roles in yeast with respect to supporting or preventing cellular DNA replication, respectively, the human Rpa2 or Rpa4 L34 encoding region was cloned into yeast *RFA2* in place of the region encoding the normal Rfa2 L34 region (called *rfa2-h2L34* or *rfa2-h4L34*). Plasmid shuffle was then used to assess the viability of yeast cells expressing the L34 domain-swapped forms. Rfa2 containing either human Rpa2 L34 or Rpa4 L34 supports replication function, as cells were recovered on 5-FOA-containing media. Since cells expressing these mutant Rfa2 forms could be readily recovered, it was important to determine if there is an effect on the response to DNA damage. In Figure 3B, it was observed that the cells expressing Rfa2-h4L34 do not display any obvious DNA damage sensitivity and are indistinguishable from cells containing WT Rfa2 in that respect (Figure 3B and S1A). The cells expressing Rfa2-h2L34 do show a slight DNA damage sensitivity, observed as slightly smaller microcolony growth on media containing hydroxyurea (HU) (Figure 3B) and reduced growth on media containing higher concentrations of camptothecin (CPT) and phleomycin (PHL) (Figure S1A). However, this sensitivity is minor in comparison to that observed for the control cells

expressing yeast Rfa1-t11 (Figure 3B and S1), a mutation previously characterized to have a strong defect in DNA repair (44–46). We conclude that the L34 region is not important for yeast Rfa2 function in DNA replication or repair, and that phosphorylation of previously identified sites in this region is also not important in replication or repair, as they are no longer present in these hybrid proteins. This does not rule out the possibility of phosphorylation of serines (S) or threonines (T) in the human Rpa2 or Rpa4 loops; however, any effect is minimal at best.

The human Rpa2 or Rpa4 N-terminus supports Rfa2 cellular function in replication and repair

The phosphorylation of the N-terminus of human Rpa2 has been well-studied with respect to sites of phosphorylation, kinases involved, and physiological consequences associated with perturbation of phosphorylation (15). The physiological consequences of mutating serines/threonines in this region, especially S4, S8, T21, and S33, to alanines include premature replication restart, defective checkpoint arrest, and hyper-recombination, and mitotic chromosome segregation defects (11–14, 19). Hyper-phosphorylation has also been demonstrated to disrupt RPA interaction with Mre11 (10) and promote increased interaction with Rad51 and Rad52 (47).

We have recently demonstrated that phosphorylation of the yeast Rfa2 N-terminus is unimportant for the cellular response to DNA damage in standard DNA damage assays; however, the presence of this domain is required for a proper response to DNA damage in mitotically-growing yeast cells (22). In fact, phosphorylation of this region is undetectable via western blotting methods, and thus far has not been identified by mass spectrometry in mitotic cells. In meiotic yeast cells, phosphorylation occurs at S27 by the meiosis-specific kinase Ime2 and perhaps at one other location within the yeast Rfa2 N-terminus as determined by mass spectrometry (21). Since phosphorylation of the Rfa2 NT does not appear to be required for replication or repair (as assayed), we postulated that the human Rpa2 or Rpa4 NT would support cell growth (*i.e.*, RFA function) in yeast cells. To test this, we swapped the yeast Rfa2 NT with the N-terminus of human Rpa2 or Rpa4. Shown in Figure S1B is a T-COFFEE (48) alignment of the yeast Rfa2 and human Rpa2 and Rpa4 N-terminal regions. Both of the domains are serine/threonine-rich, and when spaces inserted by T-COFFEE are manually removed from this alignment (Figure 3C), it appears that the serines/threonines are located at similar residue positions. However, the sequence context surrounding these serines/threonines varies between the subunits. Cells expressing chimeric *RFA2* genes encoding the human Rpa2 N-terminus (h2NT) or Rpa4 N-terminus (h4NT), cells are able to survive the loss of pJM132, indicating proper cellular function of the hybrid Rfa2 forms in unstressed conditions. This allowed for the recovery of mutant cells, where the only form Rfa2 being expressed is the hybrid form. The recovered *rfa2-h2NT* and *rfa2-h4NT* cells were then subjected to DNA damaging agents (Figure 3B and S1A). Similar to the results observed for the loop 3–4 domain swaps, both *rfa2-h4NT* and *rfa2-h2NT* cells are nearly indistinguishable from WT *RFA2* cells with respect to resistance to DNA damage. However, both mutants show a very slight sensitivity that is only observed when very high concentrations of damaging agents are used (Figure S1A). This sensitivity is not nearly as

severe as the sensitivity observed for *rfa1-t11* cells, but it is more severe than that observed for the loop domain hybrids.

Human Rpa2 N-terminal chimeric mutants display DNA damage phenotypes similar to analogous Rfa2 N-terminal mutants

One way to study the effects of phosphorylation (or lack thereof) on cellular function of a protein is through the use of phospho-mimetic and unphosphorylatable mutant forms. In human cells, hyper-phosphorylation of human Rpa2 is correlated with induction of DNA damage, and phospho-mutant forms lead to physiological defects in RPA cellular function. This approach has been utilized in human cells not only to elucidate the physiological importance of human Rpa2 modification, but also in yeast cells to elucidate the importance of the analogous domain in yeast Rfa2 (22). It was determined that deletion of the yeast Rfa2 N-terminus (*rfa2-*ΔN_x) results in DNA damage sensitivity, whereas an N-terminal unphosphorylatable form (*rfa2-*A_x) is DNA damage-resistant. This implies that the presence of the domain is what was important, not its modification. However, a constitutive phospho-mimetic N-terminal form of Rfa2 (*rfa2-*D_x) also displays sensitivity to DNA damage, which suggests that if the N-terminus were phosphorylated, its dephosphorylation must also be important in the damage response. Given that phosphorylation of the Rfa2 N-terminus is undetectable and that an unphosphorylatable Rfa2 NT mutant is not damage-sensitive in *S. cerevisiae*, it appears that a highly negatively-charged domain might actually be detrimental to yeast Rfa2 function.

We asked whether similar human Rpa2 N-terminal forms, when attached to yeast Rfa2 (*rfa2-h2*D_x, *rfa2-h2*A_x, and *rfa2-h2*ΔN_x), affect hybrid Rfa2 function in a similar manner to yeast Rfa2 phospho-mutants. The hybrid phospho-mutant forms (Figure 4A) were recovered via plasmid shuffle, indicating that each human N-terminal phospho-mutant form supports RFA replicative function in unstressed cells. The hybrid mutant cells were exposed to DNA damaging agents, and DNA damage-sensitivity was examined. *rfa2-h2*A_x cells displayed a damage resistance that was only very slightly sensitive compared to either *rfa2-*A_x (designated *rfa2-y2*A_x) cells or WT *RFA2* cells (Figure 4B and S2). This was despite the observation that *rfa2-h2*A_x cells grow slower than *rfa2-y2*A_x or WT *RFA2* cells. Cells where the Rfa2 N-terminus was deleted (*rfa2-*ΔN_x; designated *rfa2-y2*ΔN_x) or where the well-studied human N-terminus was deleted (*rfa2-h2*ΔN_x; deletion of first 33 amino acids of human Rpa2 with aa 34–38 attached to yeast Rfa2) both displayed a moderate sensitivity to damaging agents (Figure 4B and S2). Finally, *rfa2-h2*D_x cells displayed the highest sensitivity to DNA damage of all of the hybrid phospho-mutants (Figure 4B and S2). The *rfa2-h2*D_x cells were more damage-sensitive than the corresponding mutation in yeast Rfa2 (*rfa2-*D_x; designated *rfa2-y2*D_x) and nearly as sensitive as *rfa1-t11* cells. Although more severe in damage sensitivity compared to *rfa2-y2*D_x, both are DNA damage sensitive. Overall, the degrees of sensitivity are slightly different, but the pattern of sensitivity is similar between analogous yeast and hybrid mutant forms (Figure 4C), suggesting that the human N-terminal mutant forms function similar to that observed for the yeast N-terminal mutant forms, at least in yeast cells.

The human Rpa2 N-terminus is phosphorylated in yeast cells in a manner similar to that observed in human cells

The human Rpa2 N-terminal domain is phosphorylated on two sites (S23 and S29) during the cell cycle by CDK and is phosphorylated on multiple sites (*e.g.*, S4, S8, S12, T21, S33) by ATR, ATM, and DNA-PK in response to DNA damage (17). It is clear that more than one residue (and potentially all) are phosphorylated concurrently on a single human Rpa2 N-terminus, as indicated by a several kilodalton shift in species. Phosphorylation also appears to be sequential or primed in human cells, as phosphorylation of some sites requires previous phosphorylation of others (12, 17).

Protein expression and phosphorylation were examined for the hybrid Rfa2 proteins. In Figure 5A, it was observed that hybrid Rfa2 containing the human Rpa2 NT is post-translationally modified in unstressed mitotically-growing cells. By comparison, no additional robust species are observed above the predominant Rfa2 species for the hybrid Rfa2 proteins containing the human Rpa2-D_x, Rpa2-A_x, Rpa2-ΔN_x, or Rpa4 N-terminus (Figure 5A). This suggests that post-translational modification(s) occurs in the human Rpa2 N-terminus during the normal cell cycle in yeast, and that it is mapped to the N-terminus of human Rpa2. As there are two sites phosphorylated by CDK in human cells during S-phase of the cell cycle, we propose that these modifications might also be occurring in yeast, perhaps by the homologous kinase Cdc28.

Hybrid Rfa2-expressing cells were also examined for additional phosphorylation upon induction of DNA damage by treatment of cells with methyl methanesulfonate (MMS). As a marker for DNA damage induction and G2/M checkpoint establishment (45), Rad53 phosphorylation was also measured. Upon DNA damage, Rad53 was phosphorylated in cells containing any form (WT or hybrid) of Rfa2. This not only indicates that DNA damage is occurring in these cells, but also that none of the hybrid forms affects the establishment of the G2/M checkpoint upon DNA damage induction (49). Analysis of WT or hybrid Rfa2 reveals additional post-translational modification of Rfa2-h2NT (Figure 5B). Furthermore, we conclude that the additional observed PTM(s) is phosphorylation, as it is not observed for any of the human Rpa2 NT serine/threonine mutant hybrid Rfa2 forms (Figure 5B) in response to DNA damage. We conclude that the human Rpa2 N-terminus can be phosphorylated in the context of the yeast RFA complex by yeast kinases in yeast cells.

We demonstrated that the human Rpa2 NT is phosphorylated in unstressed cells, and that an additional site(s) is phosphorylated upon treatment of cells with MMS. We also demonstrated that Rfa2 NT mutants display sensitivities not only to MMS, but also to other DNA damaging agents, such as CPT, HU, and PHL (Figure 4B), all of which have a different mode of action to generate DNA damage. To address whether or not the human Rpa2 (or Rpa4) NT is phosphorylated under other damage conditions, we examined phosphorylation of Rfa2-h2NT and Rfa2-h4NT hybrid proteins. It was observed that not only is Rfa2-h2NT post-translationally modified in response to MMS, it is also post-translationally modified in response to CPT and PHL (Figure 5C; left half). Apparent post-translational modification was not observed when cells were treated with HU (Figure 5C; left half). Examination of Rfa2-h4NT post-translational modification after DNA damage

indicated that this hybrid protein was also modified in response to CPT and PHL, but not HU (Figure 5C; right half). However, the human Rpa4 N-terminus does not appear to be as strong of a phosphorylation target as the human Rpa2 N-terminus in *S. cerevisiae* (Figure 5C).

The fact that either hybrid protein was not overtly modified in response to HU was intriguing, given that HU in human cells elicits readily observable Rpa2 N-terminal hyper-phosphorylation (50). To determine if we had damaged the DNA sufficiently in cells treated with HU, we measured whether checkpoint activation had occurred by examining phosphorylation of Rad53 (yeast homolog to human Chk2). As demonstrated in Figure 5C, HU-treated cells display significant phosphorylation of Rad53, indicating that there was sufficient DNA damage in the cells. We also measured Rad53 phosphorylation for the other damaging-agent treatments. As observed previously, MMS-treatment resulted in considerable Rad53 phosphorylation; however, CPT treatment did not. Despite the apparent lack of Rad53 activation by CPT, Rfa2-h2NT and Rfa2-h4NT were significantly modified with this treatment (Figure 5C). Finally, PHL-treatment appeared to elicit a strong Rfa2-h2NT modification phenotype, and Rad53 was phosphorylated in these cells. However, Rad53 (either modified or unmodified) was not easily detected in PHL-treated cells, suggesting its modification/stability is different than when cells are treated with other damaging agents. These data demonstrate that there is no correlation between Rad53 and Rfa2-h2NT phosphorylation (Table 1). This is further substantiated by the observation that the damage-treatments elicit the same response with respect to Rad53 phosphorylation, regardless of whether the yeast Rfa2 form is WT, h2NT, or h4NT (Figure 5C).

There are other observations worth noting. First, MMS treatment of cells appears to elicit an additional phenotype – increased expression of Rfa2 (or hybrid) protein (Figure 5C; 30 μg total protein/lane). This increase in expression was not observed for any of the other damage treatments. Second, PHL (and perhaps CPT) treatment results in an apparent increase in the abundance of the higher mobility species of post-translationally modified Rfa2-h2NT and Rfa2-h4NT relative to the unmodified species. Although qualitative, this suggests not only that different DNA damaging agents (and by inference, different types of damage) are eliciting different damage responses in the cells, but also that Rfa2-h2NT is being modified differently in response to these agents. This indicates that the Rfa2-h2NT hybrid could be a useful tool for assessing these differences in responses, especially after the kinases have been identified that target the Rfa2-h2NT domain.

Does phosphorylation of the hybrid Rfa2-h2NT in yeast cells occur at the same sites and same (cell cycle and damage) conditions as human Rpa2 in human cells? To address this, western blotting was performed with phospho-specific antibodies to human Rpa2 serines 4 and 8 (anti-pS4/pS8), serine 12 (anti-pS12), threonine 21 (anti-pT21), serine 29 (anti-pS29), and serine 33 (anti-pS33). Figure 6A shows two independent experiments examining WT (y2NT) or hybrid Rfa2 (h2NT) protein extracted from unstressed and stressed cells (treated with MMS to induce DNA damage) for human Rpa2 N-terminal phosphorylation at serines 4 and 8. Although the anti-yeast Rfa2 antibody recognizes both the WT and hybrid forms in either condition (left blots), the only form that is detected by the anti-pS4/S8 antibody is the Rfa2-h2NT form. Furthermore, this hybrid form is only detected when the cells have been

treated with MMS. We conclude that phosphorylation is occurring in the N-terminal region of the hybrid Rfa2-h2NT, and that serines 4 and 8 are two of the targets.

In human cells, phosphorylation of the Rpa2 N-terminus at serines 4 and 8 is downstream of almost all other phosphorylation events in this region (11, 12, 17). To address if this might be true for hybrid Rfa2-h2NT in yeast cells, phosphorylation at other sites using phospho-specific antibodies was examined. Figure 6A shows that the phospho-specific antibodies (right half) only recognize hybrid Rfa2. Furthermore, phosphorylation of Rfa2-h2NT is detected in both unstressed and stressed conditions for T21, S29, and S33 (Figure 6A), and damage-specific phosphorylation was observed for S12 and S4/S8 (as described above). Thus, phosphorylation of Rfa2-h2NT in yeast cells occurs at the same sites where phosphorylation is observed in human cells.

DISCUSSION

Replication Protein A (RPA) functions in the cell not only through binding single-stranded DNA (ssDNA), but also through its role as a sensor and recruiter of factors necessary to process ssDNA intermediates correctly. Since RPA functions in multiple processes (DNA replication, repair/recombination, cell cycle regulation), it is presumed that its function is different for each of these processes. It is also presumed that RPA must somehow be regulated to function in different capacities. Post-translational modification is a common method by which a protein's function can be quickly regulated, and human RPA is post-translationally modified, especially in response to DNA damage. In human tissue culture cells, this predominantly occurs as hyper-phosphorylation of the Rpa2 N-terminus. It is clear that phosphorylation of the human Rpa2 N-terminus is important to prevent hyper-recombination, promote checkpoint arrest, and delay replication restart; however, this is studied over the course of a few generations in human cells and the long-term consequences of such defects have not been directly examined due to technical limitations of knockdown and expression studies. In budding yeast, consequences of RFA subunit mutation can be examined over the course of 50 or more generations, allowing for the assessment of physiological consequences of defects in DNA processes; however, hyper-phosphorylation of the Rfa2 N-terminus does not occur in response to DNA damage in budding yeast. It is unclear how the yeast Rfa2 (or RFA) is regulated in response to DNA damage, but it is clear that lack phosphorylation of the N-terminus in response to DNA damage is not obviously detrimental over the long-term (at least 50 cell divisions).

In an attempt to study human RPA function in a simpler system (yeast), we explored the possibility of replacing a complex for a complex, rather than a subunit for a subunit. Unfortunately, the human RPA complex (in either the canonical or alternative form) does not support viability in yeast cells and precludes further study. Human *Rpa1* and *Rpa2* mRNA expression levels are about 50% of the levels of their homologous *RFA* genes, and we can clearly detect substantial full-length protein for each expressed in yeast cells. We can conclude that human Rpa3 and Rpa4 are also expressed in yeast cells through indirect means. If Rpa4 or Rpa3 were not expressed from the plasmid, addition of the plasmid expressing Rpa4 only would not show auto-activation for BD-Rpa1, and addition of the plasmid expressing Rpa4 and Rpa3 would not quench some of the auto-activation observed

in Figure 2C. We cannot rule out that human *Rpa3* mRNA (and by inference protein) expression levels (~10% of yeast *RFA3*) might be too low to support viability. It is also conceivable that the human Rpa1 and Rpa2 expression levels might be below the threshold to support viability. However, given that the human *RPA* subunit genes are driven from their homologous yeast gene promoters, the only way to address whether lack of complementation is due to expression levels in the future would be through the use of an inducible promoter (*e.g.*, galactose-inducible) or a high-copy plasmid to produce high-level expression of human *RPA* genes (most likely much higher than physiological, endogenous *RFA* gene expression).

Through these studies, we have provided evidence that heterotrimeric complex formation is mediated through Rpa2 (or Rpa4), and that Rpa1 interaction with Rpa2 (or Rpa4) does not require the presence of Rpa3. This does not preclude the possibility of human Rpa2 interacting with yeast Rfa3 to form a subcomplex that can interact with human Rpa1 (we cannot currently measure this due to auto-activation observed for all bait constructs containing human or yeast Rpa2 or Rpa3). However, human Rpa1 does not interact with yeast Rfa3, suggesting again that human Rpa2 is the main driving force for complex formation. This is consistent with the idea of a stable subcomplex of Rpa2 and Rpa3 in human cells that can then interact with Rpa1 (34). Our data indicate that subcomplex interaction with human Rpa1 is predominantly through Rpa2, or alternatively that Rpa2 (or Rpa4) expression is what stabilizes Rpa3 to allow interaction with Rpa1.

Loop 3–4 function appears to be human cell-specific

Although whole complexes could not be swapped (at least in this system), individual domains could be. This allowed for examination of the importance of the loop 3–4 (L34) region of human Rpa2 and Rpa4. It had been demonstrated that the replacement of the human Rpa2 L34 region with the Rpa4 L34 region resulted in a human Rpa2 hybrid protein that can no longer support DNA replication in human tissue culture and *in vitro* (28, 32). We demonstrated that the replacement of the yeast Rfa2 predicted L34 region with either the human Rpa2 L34 or Rpa4 L34 had very little effect on yeast Rfa2 function in yeast cells. This highlights two important points. First, the human Rpa4 L34 region does not inhibit Rfa2 function in DNA replication or repair in yeast cells, indicating that the negative effect of the Rpa4 L34 region on cellular replication is specific to human cells. We propose that the negative effect observed in human cells is mediated through human-specific protein interactions (or lack thereof). Second, the L34 region of yeast Rfa2 has been previously reported to be phosphorylated (39–41). While we cannot rule out the possibility that this region is still phosphorylated when either the human Rpa2 or Rpa4 L34 region is present, we can conclude that the amino acid sequence in this region appears to be relatively unimportant. Both of the points above suggest that this loop is there for structural purposes in yeast and may be important for additional activities in human cells (and potentially other higher eukaryotes).

The human Rpa2 N-terminus is a target for phosphorylation by yeast kinases

The domain swap of particular interest was the replacement of the Rfa2 N-terminus with the human Rpa4 or Rpa4 N-terminus. Although this domain in yeast Rfa2 is not hyper-

phosphorylated like human Rpa2, it is clearly required for yeast Rfa2 function in the DNA damage response. Swapping the yeast Rfa2 N-terminus for either the human Rpa2 or Rpa4 N-terminus revealed a number of features. Both the human Rpa2 and Rpa4 N-termini support DNA replication and repair, as both displayed viability and DNA damage resistance in yeast cells. Also, the human Rpa2 and Rpa4 N-termini are phosphorylated during the cell cycle and in response to DNA damage in yeast cells; however, the phosphorylation of human Rpa2 is much more pronounced. Also, phosphorylation occurs on the same serines/threonines on the hybrid Rfa2-h2NT in yeast as it does for human Rpa2 in stressed human cells. This suggests that the difference in phosphorylation of yeast Rfa2 *vs*. human Rpa2 (or human Rpa4) is simply sequence – the serines/threonines in the yeast Rfa2 N-terminus are not surrounded by appropriate residues that allow for recognition by yeast kinases. However, the observation that yeast kinases recognize and act on the human Rpa2 N-terminus suggests that if phosphorylation of other eukaryotic Rpa2 N-termini is occurring, the mechanism of phosphorylation might be conserved.

An interesting difference in human Rpa2 N-terminal phosphorylation in yeast *vs*. human cells lies in residues that display damage-specific phosphorylation. In human cells, phosphorylation of S4/S8, S12, T21, and S33 is DNA damage-dependent. In yeast cells, only phosphorylation of S4/S8 and S12 appear to depend on DNA damage. Recently, it was shown that human cells in G2 display chromatin-bound Rpa2 that is phosphorylated on S33 in the absence of DNA damaging agent and is increased upon damage induction (51). Yeast cells that are grown exponentially are predominantly in G2/M phase, and this could explain the apparent phosphorylation of S33 on Rfa2-h2NT in undamaged cells. However, there is not an obvious increase in S33 phosphorylation upon DNA damage in yeast cells. The other major difference lies in modification of T21, which in yeast cells appears to be phosphorylated in both unstressed and stressed cells. Phosphorylation of T21 is one of the first residues to be phosphorylated in a damage-specific manner in human cells (51) and appears to be important for priming phosphorylation of other sites (17). It is possible that low levels of damage that occur naturally in exponentially growing yeast cells are enough to trigger phosphorylation of T21 in the absence of damage induction. However, like phosphorylation of S33, this phosphorylation is not obviously increased in damaged yeast cells (at least those treated with MMS). Consistent with human Rpa2 phosphorylation in human cells, the Rfa2-h2NT hybrid protein is also phosphorylated in a damage-specific manner in yeast.

An alternative explanation for condition-specific phosphorylation differences could simply lie in the sequence and the kinase(s) that recognize it. Figure 6B shows a summary of the phosphorylation of the human Rpa2 N-terminus in human and yeast cells. In human cells, generally ATR/ATM/DNA-PK recognizes S/TQ motifs (T21 and S33), CDK recognizes the SP motifs (S23 and S29), and DNA-PK recognizes the other non-consensus sites (S4/S8 and S12) (17, 52, 53). It should be noted that in yeast, S29 is not condition-specific, indicating that SP motifs are potentially recognized by yeast cyclin-dependent kinase during the cell cycle. The residues T21 and S33 are within SQ and TQ motifs that would presumably be recognized by Mec1/Tel1 (ATR/ATM homologs); however, these kinases are normally activated by DNA damage. It is possible that some fraction of Mec1/Tel1 is active in

exponentially-growing cells and phosphorylates the human Rpa2 SQ and TQ sites, similar to ATR/ATM regulating replication initiation during the cell cycle in unperturbed human cells (54). It is also possible that another kinase(s) is active during the cell cycle in yeast recognizes and phosphorylates these motifs. Perhaps most interesting is the observation that serines (S4/S8 and S12) without an SQ motif displayed damage-specific phosphorylation in both human and yeast cells. The lack of an obvious DNA-PK homolog in yeast makes identification of the kinase involved in phosphorylating S4/S8 and S12 in yeast cells potentially interesting.

Why is hyper-phosphorylation not observed in the N-terminus of yeast Rfa2?

Yeast Rfa2 requires an intact N-terminus to support its function in response to DNA damage. This is not only based on the observation that mutation of this domain to an unphosphorylated form supports DNA damage resistance, but also on the observation that substituting the human Rpa2-A_x (h2A_x; cannot be phosphorylated) mutant form into yeast Rfa2 also results in damage-resistant cells. Taken together with the fact that deletion of the Rfa2 N-terminus results in damage-sensitive cells, this indicates that this domain needs to be present, but does not need to be phosphorylated for yeast cells to respond to DNA damage. This also suggests that a substantially (and/or constitutively) negatively-charged N-terminal region, while potentially beneficial in human cells, might be detrimental in yeast cells. This is based on the observation that yeast Rfa2-y2D_x and hybrid Rfa2-h2D_x mutant cells display DNA damage-sensitive phenotypes. In both mutants, the N-terminus is constitutively negatively-charged to mimic hyper-phosphorylation of this region. We propose that hyper-phosphorylation of human Rpa2 has potentially co-evolved with machinery necessary for DNA repair and cell cycle regulation specifically found in higher eukaryotes.

One final observation is the apparent molecular weight differences between the WT Rfa2 protein and the hybrid Rfa2-h2NT or Rfa2-h4NT proteins. Both of the hybrid proteins have an apparent molecular weight that is smaller by approximately 2–4 kilodaltons, despite all three proteins being the exact same length in amino acids. This suggests that the N-terminus of yeast Rfa2 is more negatively-charged than the human Rpa2 or Rpa4 N-termini, which does appear to be reflected by a cursory examination of positively- and negatively-charged residues found in these domains. Perhaps this feature substitutes for an apparent lack of phosphorylation of this domain in yeast – it is already in the negatively-charged state necessary to function in yeast cells, especially in response to DNA damage. Consistent with this is the apparent qualitative ranking of damage-sensitivities for mutants (Figure 4C). The yeast *rfa2-A_x* (*y2A_x*) mutant is nearly as resistant as WT (yeast-derived with same charged residue makeup as the Rfa2 N-terminus), followed by *rfa2-h4L34* and *rfa2-h2L34* (contain the exact same N-terminus as yeast Rfa2), followed by *rfa2-h4NT* and *rfa2-h2NT* (human-derived N-terminus that is less negative than Rfa2 but post-translationally modified to give these proteins an apparent molecular weight nearly indistinguishable from Rfa2), followed by *rfa2-h2A_x* (human-derived and less negatively-charged with no opportunity for N-terminal phosphorylation), followed by the remainder of mutants that either have no domain or contain a constitutively negatively-charged domain.

Implications of hybrid Rfa2 phosphorylation studies

The first implication of these studies is that *Saccharomyces cerevisiae* provides the potential for studying the human Rpa2 N-terminus. This is not to imply that studies in yeast cells will yield insights into human Rpa2 N-terminus cellular function (although it is possible), as it is clear that phosphorylation of the yeast Rfa2 N-terminus is not necessary for cellular resistance to DNA damage in standard assays commonly used in yeast. Rather, this is to imply that we now have the opportunity to compare and contrast features of phosphorylation that occur in human cells. For example, one can address the following: *1)* whether phosphorylation is sequential or primed by phosphorylation of other sites, *2)* what damaging conditions elicit phosphorylation (initially investigated in this study), *3)* what yeast kinases are involved, and *4)* if phosphorylation of the human Rpa2 N-terminus is phase-specific. Although some the above questions have been addressed in human tissue culture cells (12, 17), examination in yeast cells might provide insight (and perhaps consensus) into the mechanism by which phosphorylation occurs in higher eukaryotes. It might also provide insight into how yeast RFA does similar functions in the absence of obvious hyper-phosphorylation of the Rfa2 N-terminus and other proteins that are higher eukaryote-specific (*i.e.*, DNA-PK).

The second implication is the use of *S. cerevisiae* as a tool for understanding the relevance of (hyper-) phosphorylation of Rpa2 subunits from other organisms. It is difficult to study the function of Rpa2 in the context of the whole organism. This is why yeast or human tissue culture is so powerful. However, there are many organisms in which it is not clear if RPA functions in a similar manner or whether or not it is post-translationally modified (it is often just assumed to be similar). As an example, in plants, there are multiple Rpa2 subunits, which have been proposed to have potentially different roles (55–57) – post-translational modification of these Rpa2 forms has not been addressed. In fact, in most organisms besides humans, *Xenopus*, *Candida*, and now *Saccharyomyces* (22–24, 58–60), Rpa2 NT phosphorylation has not been examined in detail. Given that the human Rpa2 N-terminus is phosphorylated in yeast, we propose that this system would provide a relatively simple tool for addressing whether other Rpa2 N-termini from other organisms might also be phosphorylated. Extending this to organisms with multiple Rpa2 subunits might provide insight or additional evidence for which Rpa2 subunits might be actively playing a role in the DNA damage response.

MATERIALS AND METHODS

Yeast strains

Yeast strains used in this study are listed in Table S1. For plasmid shuffle (determining viability and recovering *rfa2* mutants), the W303 derivative RMY122-A (*rfa1*Δ*::TRP1 rfa2*Δ*::TRP1*) containing the plasmid pJM132 (*RFA1 RFA2 URA3*) (61) was used. *In vivo* cloning was performed using the strains EGY40 or EGY48. The strain EGY48 ($6xO_{lexA}$-*LEU2*) was used for all two-hybrid analyses.

Plasmid construction

All primers used and plasmids generated are listed in Table S2 and S3, respectively. To generate a yeast vector containing the human *Rpa1*, *Rpa2*, and *Rpa3* genes driven by native *RFA1*, *RFA2*, and *RFA3* promoters, PCR and *in vivo* cloning (62) were utilized. Briefly, primers containing 40 nucleotides (nt) immediately upstream of the coding region for each RFA subunit gene and the initial 20 nt of coding region of the homologous human RPA subunit gene were designed. Primers were also designed containing 40 nt immediately downstream of the coding region for each RFA subunit gene and the last 20 nt of coding region (including the stop codon) of the homologous RPA subunit gene. Utilizing plasmid templates containing *Rpa1*, *Rpa2*, *Rpa4*, or *Rpa3* full-length cDNA, Phusion DNA polymerase (New England Biolabs) was used to generate *rfa1*Δ*::Rpa1*, *rfa2*Δ*::Rpa2* (or *rfa2*Δ*::Rpa4*), and *rfa3*Δ*::Rpa3* PCR fragments.

RFA2 was first replaced with *Rpa2* (or *Rpa4*) by digesting pJM132 with *Hpa*I. This linearized vector was co-transformed with the *rfa2*Δ*::Rpa2* (or *rfa2*Δ*::Rpa4*) PCR fragment into EGY40 cells. Transformants were selected on synthetic complete (0.5% ammonium sulfate, 0.34% yeast nitrogen base without amino acids) media containing 2% dextrose and lacking uracil (SD-Ura). Genomic and plasmid DNA were isolated from a scrape of yeast transformants and electroporated into DH10B bacterial cells. Bacterial transformants were selected on LB media containing 100 μg/mL ampicillin (LB+Amp). Plasmid DNA was isolated from independent bacterial colonies, and correct substitution candidates were verified by restriction digestion and DNA sequencing (Eton Bioscience). The resulting verified vectors were called pPLG35 and pPLG36.

Once verified, the *RFA3* gene was replaced with *Rpa3* by digesting pPLG35 (or pPLG36) vectors with *Msc*I and co-transforming them into EGY40 cells along with the *rfa3*Δ*::Rpa3* PCR fragment. The same procedure as above was followed, and candidates were verified by DNA sequencing. The resulting verified vectors were designated pPLG37 and pPLG38. Next, the *RFA1* gene in these plasmids was replaced with *Rpa1* by digesting pPLG37 (or pPLG38) vectors with *Mfe*I and co-transforming with the *rfa1*Δ*::Rpa1* PCR fragment into EGY40 cells. Following the same procedure as previously, candidates were isolated and verified by sequencing. These resulting plasmids are pPLG39 and pPLG40, respectively. Following a similar procedure, the *URA3* gene of pPLG39 and pPLG40 was deleted and replaced with a *kanMX* cassette (*ura3*Δ*::kanMX*) to generate pPLG41 and pPLG42, respectively. *kanMX* insertion was verified by restriction digest.

To generate chimeric *RFA2* genes encoding for yeast Rfa2 with the human Rpa2 N-terminus (NT), human Rpa4 NT, or corresponding human Rpa2 NT phospho-mutants, *in vivo* homologous recombination cloning was utilized. Double-stranded DNA fragments encoding for the human Rpa2 NT (h2NT), human Rpa4 NT (h4NT), or the Rpa2 NT phospho-mutant forms, Rpa2-D_X (h2D_X), Rpa2-A_X (h2A_X), or Rpa2-ΔN_X (h2ΔN_X), were generated as double-stranded gBlock fragments (Integrated DNA Technologies). The gBlock fragments also contained sequence upstream and downstream that encoded for the yeast *RFA2* endogenous promoter and the remainder of the *RFA2* coding region starting at amino acid 39, respectively (Table S4). These fragments were combined with *Nco*I-digested pAW10 and

transformed into the yeast strain EGY40. Leucine prototrophs were selected, and genomic/plasmid DNA was isolated. This DNA was electroporated into DH10B bacterial cells, and plasmid DNA was isolated from ampicillin-resistant transformants. To verify incorporation of the human Rpa2 NT forms into the N-terminus of the yeast *RFA2* gene, the resulting plasmid DNA was digested and verified by DNA sequencing (Eton Bioscience).

Yeast/human chimeric genes encoding *RFA2*, where the Rfa2 loop 3–4 region was substituted with the acidic loop 3–4 region of human Rpa2 (Rpa2-L34) or the basic loop 3–4 region of Rpa4 (Rpa4-L34), were also generated via *in vivo* homologous recombination cloning (*rfa2-h2L34* and *rfa2-h4L34*, respectively). gBlock fragments were generated in a similar fashion as above, except that the loop 3–4 region encoding amino acid (aa) residues 102–132 of Rfa2 was substituted with the region encoding aa 108–124 of human Rpa2 or aa 108–123 of human Rpa4. These fragments were combined with *Sna*BI-digested pAW07 and transformed into EGY40 yeast cells. After isolation of DNA as described above, the resulting plasmid DNA were identified by double-digestion with *Nco*I-*Sna*BI and verified by DNA sequencing.

Human RPA and yeast RFA subunit-expressing bait (pENM5) and prey (pENM10, pENM11, pENM12, pENM13, pENM14, pENM15, and pENM16) constructs for two-hybrid analysis were generated as follows. PCR using the appropriate subunit template DNA and Phusion DNA polymerase was performed with sets of primers listed in Table S2. Briefly, *RFA1*, *RFA2*, *RFA3*, *Rpa1*, *Rpa2*, *Rpa3*, and *Rpa4* were amplified by PCR using primers with 40 nt (on the 5′ end) of homologous sequence to the appropriate cloning vector and 20 nt (on the 3′ end) of complementary sequence to the desired yeast RFA or human RPA subunit gene. pEG202K or pJG4-5 were digested with *Nco*I or *Eco*RI, respectively, to linearize each fragment. The linearized vectors were co-transformed with the corresponding PCR-amplified RFA subunit gene into EGY48, and transformed cells were plated onto media lacking histidine (SD-His) or lacking tryptophan (SD-Trp) for cells transformed with pEG202 or pJG4-5, respectively. The resulting colonies, some containing recombined vectors, were then scraped from the plates, DNA (both genomic and plasmid) was isolated and electroporated into DH10B bacterial cells. The bacterial cells containing plasmids were selected for on LB plate media containing 100 μg/mL ampicillin (LB+Amp). Plasmid DNA was isolated from individual bacterial colonies and analyzed by diagnostic restriction digests. Plasmids with inserts were sequenced (Eton Bioscience).

Yeast two-hybrid assay

Protein interactions between yeast RFA and human RPA subunits were examined using the DupLEX-A Yeast Two-Hybrid System (Origene). Bait (lexA BD) constructs (described in (33)) were each co-transformed into EGY48 (*leu2::6xO*$_{lexA}$*-LEU2*) with prey (B42 AD) constructs containing each of the individual RFA or RPA subunits and the reporter construct pSH18-34 (*8xO*$_{lexA}$*-lacZ*). Transformants were selected on synthetic complete media containing dextrose (2%) and lacking histidine, tryptophan, and uracil (SD-HTU). Independent colonies were patched to SD-HTU and replicated to the following media: synthetic dextrose (2%) media lacking histidine, tryptophan, uracil, and leucine (SD-HTUL), synthetic galactose (2%) media lacking histidine, tryptophan, uracil, and leucine

(SG-HTUL), and synthetic galactose media lacking histidine, tryptophan, and uracil containing 40 μg/mL 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (SG-HTU+X-gal). Plates were grown for 2–4 days at 30°C.

Assessing viability and isolation of yeast cells expressing Rfa2:Rpa2/4 hybrid proteins via plasmid shuffle

To examine the viability of yeast cells containing human RPA (all three subunits), the strain RMY122-A containing a deletion of both the *rfa1* and *rfa2* endogenous genes and a plasmid (pJM132) with the wild-type (WT) forms of *RFA1* and *RFA2* driven by their native promoters was transformed with pPLG41 (*rfa1*Δ*::Rpa1 rfa2*Δ*::Rpa2 rfa3*Δ*::Rpa3*) or pPLG42 (*rfa1*Δ*::Rpa1 rfa2*Δ*::Rpa4 rfa3*Δ*::Rpa3*). Transformants were selected on YPD (1% yeast extract, 2% peptone, and 2% dextrose) containing 200 μg/mL G418 sulfate (YPD +G418). Control cells were transformed with pRS313-RFA1 and pAW07 containing WT *RFA1* and RFA2, respectively, and transformants were selected for using synthetic dextrose (2%) media lacking histidine, leucine, and uracil (SD-HLU). Transformed (control and experimental) cells were grown in liquid YPD or YPD+G418, respectively, at 30°C overnight. The next day, the cells were sonicated (Branson Sonifier 450) using a microtip and pulsed at 20% for 1 sec (0.2 sec on/0.5 sec off). Cells were then quantitated on a cell counter (Nexcelom), and diluted in 1x PBS to 1×10^7 cells/mL. Ten-fold serial dilutions were made in 1x PBS and 5 μL cells of each dilution were spotted onto YPD+G418, SD-HLU, synthetic dextrose media containing 0.8 μg/mL 5-fluororotic acid (5-FOA), and YPG (1% yeast extract, 2% peptone, 2% glycerol) plates and incubated at 30°C for 3–5 days. Viability was assessed qualitatively based on the ability of cells to lose pJM132 and grow on 5-FOA-containing media.

Plasmid shuffle was also utilized to generate yeast cells expressing a chimeric *RFA2-Rpa2/4* gene as the only form in the cell. The strain RMY122-A was transformed with pRS313-RFA1 and pAW07 (or a derivative plasmid expressing a chimeric *RFA2* gene). Yeast cells containing all three plasmids were selected for and grown overnight at 30°C in SD-HLU. Cells were treated and spotted as described above. To recover individual shuffle-out cells, 5×10^4 cells were spread onto 5-FOA plates.

DNA damage assays of Rfa2:Rpa2/4 hybrid protein-expressing yeast cells

Once isolated, *RFA2-Rpa2/4* hybrid protein-expressing yeast cells were grown overnight in YPD at 30°C with shaking. Again, cells were sonicated and counted as described above. The cells were then diluted to 2.5×10^5 cells/mL, and three-fold dilutions were made in 1x PBS. The dilutions were then spotted to the following plates: YPD containing 0.0019–0.015% methyl methanesulfonate (MMS), YPD containing 0.2–25 μg/mL camptothecin (CPT), YPD containing 40–320 mM hydroxyurea (HU), YPD containing 0.2–25 μg/mL phleomycin (PHL), YPD, SD-HLU, and YPG plates. All of the plates were incubated at 30°C. The duration of incubation was between 2–5 days, depending on the media used.

Quantitative real-time PCR to examine RPA or RFA subunit gene expression

RMY122-A cells containing no additional plasmid, pPLG41 (canonical RPA-expressing), or pPLG42 (alternative RPA-expressing) were grown overnight in YPD. RNA was isolated

from these cells using the YeaStar RNA Kit (Zymo Research). Genomic DNA was removed from the samples using the DNA-Free RNA Kit (Zymo Research), and the RNA was quantitated using a Nano-Drop (Thermo). cDNA was then generated using the AMV First Strand cDNA Synthesis Kit (New England BioLabs). Primer sets were designed for the human RPA or yeast RFA subunit genes using Primer Express (Applied Biosystems) or PrimerQuest (Integrated DNA Technologies), respectively. Primers were also designed for the normalizing control yeast gene *UBC6*. All primers are listed in Table S2. cDNA was then amplified using PerfeCTa SYBR Green FastMix Low ROX (Quanta Biosciences) and performed on an AB7500 Real-Time PCR System (Applied Biosystems). Results were analyzed using Sequence Detection System (SDS) Software v1.2 (Applied Biosystems).

Protein extraction and western blotting of Rfa2:Rpa2/4 hybrid proteins

To demonstrate that the *RFA2-Rpa2/4* hybrid proteins are expressed, stable, and can be post-translationally modified following DNA damage treatment, RMY122-A cells containing the hybrid forms as the exclusive forms of the 32 kDa subunit of yeast RFA were grown in YPD at 30°C overnight to exponential phase. Cells were then diluted to 2.5×10^6 cells/mL and grown for an additional 4 hr. Finally, cells were treated with listed concentration of damaging agent for 3–5 hr. After this incubation, both treated and untreated cells were isolated and protein was extracted using a sample buffer lysis method (63). Protein from ~5×10^6 cells was separated on a 12%, 8%, or 6% SDS-PAGE gel (37.5:1 acrylamide:bis-acrylamide). Where noted, protein was quantitated using the RC-DC Assay Kit (Bio-Rad), and 30 μg/lane was loaded. After transfer to nitrocellulose, Rfa2 or Rfa2:Rpa2/4 hybrid proteins were detected using a 1:20,000 dilution of rabbit polyclonal anti-Rfa2 antibody (kindly provided by Steve Brill), followed by a 1:40,000 dilution of goat anti-rabbit HRP antibody (ab97051; Abcam). To detect human specific phosphorylation, the following phospho-specific antibodies and dilutions were used: rabbit anti-Rpa2 pS4/S8 antibody (A300-245A; Bethyl Laboratories) at a 1:2,000 dilution, rabbit anti-Rpa2 pS12 (Oakley lab) at a 1:3,000 dilution, rabbit anti-Rpa2 pT21 (ab61065; Abcam) at a 1:1,000 dilution, rabbit anti-Rpa2 pS33 (A300-246A; Bethyl Laboratories) at a 1:2,500 dilution, and rabbit anti-Rpa2 pS29 (Borowiec lab) at a 1:3,000 dilution. Activation (phosphorylation) of Rad53 was detected using rabbit anti-Rad53 (ab104232; Abcam) at a 1:8,000 dilution. Yeast Rfa1 and Rfa2 expression was detected using a 1:40,000 dilution of rabbit polyclonal antibody to yeast Rfa1 or a 1:20,000 dilution of rabbit polyclonal antibody to yeast Rfa2 (both kindly provided by Steve Brill). Human Rpa1 and Rpa2 proteins were detected using a 1:1,000 dilution of mouse monoclonal antibody to human Rpa1 (ab176467; Abcam) or a 1:5,000 dilution of rabbit polyclonal antibody to human Rpa2 (kindly provided by Marc Wold).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. Wold MS. Replication protein A: a heterotrimeric, single-stranded DNA-binding protein required for eukaryotic DNA metabolism. Annu Rev Biochem. 1997; 66
2. Iftode C, Daniely Y, Borowiec JA. Replication protein A (RPA): the eukaryotic SSB. Crit Rev Biochem Mol Biol. 1999; 34:141–180. [PubMed: 10473346]
3. Fanning E, Klimovich V, Nager AR. A dynamic model for replication protein A (RPA) function in DNA processing pathways. Nucleic Acids Res. 2006; 34:4126–4137. [PubMed: 16935876]
4. Walther AP, Gomes XV, Lao Y, Lee CG, Wold MS. Replication protein A interactions with DNA. 1 Functions of the DNA-binding and zinc-finger domains of the 70-kDa subunit. Biochemistry (Mosc). 1999; 38:3963–3973.
5. Wyka IM, Dhar K, Binz SK, Wold MS. Replication protein A interactions with DNA: differential binding of the core domains and analysis of the DNA interaction surface. Biochemistry (Mosc). 2003; 42:12909–12918.
6. Bochkareva E, Korolev S, Lees-Miller SP, Bochkarev A. Structure of the RPA trimerization core and its role in the multistep DNA-binding mechanism of RPA. EMBO J. 2002; 21:1855–1863. [PubMed: 11927569]
7. Dickson AM, Krasikova Y, Pestryakov P, Lavrik O, Wold MS. Essential functions of the 32 kDa subunit of yeast replication protein A. Nucleic Acids Res. 2009; 37:2313–2326. [PubMed: 19244309]
8. Bastin-Shanower SA, Brill SJ. Functional analysis of the four DNA binding domains of replication protein A. The role of RPA2 in ssDNA binding. J Biol Chem. 2001; 276:36446–36453. [PubMed: 11479296]
9. Vassin VM, Wold MS, Borowiec JA. Replication protein A (RPA) phosphorylation prevents RPA association with replication centers. Mol Cell Biol. 2004; 24:1930–1943. [PubMed: 14966274]
10. Oakley GG, Tillison K, Opiyo SA, Glanzer JG, Horn JM, Patrick SM. Physical interaction between replication protein A (RPA) and MRN: involvement of RPA2 phosphorylation and the N-terminus of RPA1. Biochemistry (Mosc). 2009; 48:7473–7481.
11. Liaw H, Lee D, Myung K. DNA-PK-dependent RPA2 hyperphosphorylation facilitates DNA repair and suppresses sister chromatid exchange. PloS One. 2011; 6
12. Anantha RW, Vassin VM, Borowiec JA. Sequential and synergistic modification of human RPA stimulates chromosomal DNA repair. J Biol Chem. 2007; 282:35910–35923. [PubMed: 17928296]
13. Olson E, Nievera CJ, Klimovich V, Fanning E, Wu X. RPA2 is a direct downstream target for ATR to regulate the S-phase checkpoint. J Biol Chem. 2006; 281:39517–39533. [PubMed: 17035231]
14. Ashley AK, Shrivastav M, Nie J, Amerin C, Troksa K, Glanzer JG, Liu S, Opiyo SO, Dimitrova DD, Le P, Sishc B, Bailey SM, Oakley GG, Nickoloff JA. 2014DNA-PK phosphorylation of RPA32 Ser4/Ser8 regulates replication stress checkpoint activation, fork restart, homologous recombination and mitotic catastrophe. DNA Repair.
15. Oakley GG, Patrick SM. Replication protein A: directing traffic at the intersection of replication and repair. Front Biosci Landmark Ed. 2010; 15
16. Binz SK, Sheehan AM, Wold MS. Replication protein A phosphorylation and the cellular response to DNA damage. DNA Repair. 2004; 3:1015–1024. [PubMed: 15279788]
17. Liu S, Opiyo SO, Manthey K, Glanzer JG, Ashley AK, Amerin C, Troksa K, Shrivastav M, Nickoloff JA, Oakley GG. Distinct roles for DNA-PK, ATM and ATR in RPA phosphorylation and checkpoint activation in response to replication stress. Nucleic Acids Res. 2012; 40:10780–10794. [PubMed: 22977173]
18. Binz SK, Lao Y, Lowry DF, Wold MS. The phosphorylation domain of the 32-kDa subunit of replication protein A (RPA) modulates RPA-DNA interactions. Evidence for an intersubunit interaction. J Biol Chem. 2003; 278:35584–35591. [PubMed: 12819197]

19. Vassin VM, Anantha RW, Sokolova E, Kanner S, Borowiec JA. Human RPA phosphorylation by ATR stimulates DNA synthesis and prevents ssDNA accumulation during DNA-replication stress. J Cell Sci. 2009; 122:4070–4080. [PubMed: 19843584]
20. Schramke V, Neecke H, Brevet V, Corda Y, Lucchini G, Longhese MP, Gilson E, Geli V. The set1Delta mutation unveils a novel signaling pathway relayed by the Rad53-dependent hyperphosphorylation of replication protein A that leads to transcriptional activation of repair genes. Genes Dev. 2001; 15:1845–1858. [PubMed: 11459833]
21. Clifford DM, Marinco SM, Brush GS. The meiosis-specific protein kinase Ime2 directs phosphorylation of replication protein A. J Biol Chem. 2004; 279:6163–6170. [PubMed: 14634024]
22. Ghospurkar PL, Wilson TM, Severson AL, Klein SJ, Khaku SK, Walther AP, Haring SJ. Distinct roles for the Rfa2 N-terminus in the DNA damage response and adaptation in Saccharomyces cerevisiae. Genetics. submitted.
23. Wang H, Gao J, Wong AH-H, Hu K, Li W, Wang Y, Sang J. Rfa2 is specifically dephosphorylated by Pph3 in Candida albicans. Biochem J. 2013; 449:673–681. [PubMed: 23140133]
24. Gao J-X, Wang H-T, Wong AH-H, Zeng G-S, Huang Z-X, Wang Y-M, Sang J-L, Wang Y. Regulation of Rfa2 phosphorylation in response to genotoxic stress in Candida albicans. Mol Microbiol. 2014
25. Lee D-H, Pan Y, Kanner S, Sung P, Borowiec JA, Chowdhury D. A PP4 phosphatase complex dephosphorylates RPA2 to facilitate DNA repair via homologous recombination. Nat Struct Mol Biol. 2010; 17:365–372. [PubMed: 20154705]
26. Majka J, Binz SK, Wold MS, Burgers PMJ. Replication protein A directs loading of the DNA damage checkpoint clamp to 5′-DNA junctions. J Biol Chem. 2006; 281:27855–27861. [PubMed: 16864589]
27. Brill SJ, Stillman B. Replication factor-A from Saccharomyces cerevisiae is encoded by three essential genes coordinately expressed at S phase. Genes Dev. 1991; 5:1589–1600. [PubMed: 1885001]
28. Haring SJ, Humphreys TD, Wold MS. A naturally occurring human RPA subunit homolog does not support DNA replication or cell-cycle progression. Nucleic Acids Res. 2010; 38:846–858. [PubMed: 19942684]
29. Melendy T, Stillman B. An interaction between replication protein A and SV40 T antigen appears essential for primosome assembly during SV40 DNA replication. J Biol Chem. 1993; 268:3389–3395. [PubMed: 8381428]
30. Philipova D, Mullen JR, Maniar HS, Lu J, Gu C, Brill SJ. A hierarchy of SSB protomers in replication protein A. Genes Dev. 1996; 10:2222–2233. [PubMed: 8804316]
31. Keshav KF, Chen C, Dutta A. Rpa4, a homolog of the 34-kilodalton subunit of the replication protein A complex. Mol Cell Biol. 1995; 15:3119–3128. [PubMed: 7760808]
32. Mason AC, Haring SJ, Pryor JM, Staloch CA, Gan TF, Wold MS. An alternative form of replication protein a prevents viral replication in vitro. J Biol Chem. 2009; 284:5324–5331. [PubMed: 19116208]
33. Piya G, Mueller EN, Haas HK, Ghospurkar PL, Wilson TM, Jensen JL, Colbert CL, Haring SJ. Characterization of the interaction between Rfa1 and Rad24 in Saccharomyces cerevisiae. PLoS One. submitted.
34. Henricksen LA, Umbricht CB, Wold MS. Recombinant replication protein A: expression, complex formation, and functional characterization. J Biol Chem. 1994; 269:11121–11132. [PubMed: 8157639]
35. Haring SJ, Mason AC, Binz SK, Wold MS. Cellular functions of human RPA1. Multiple roles of domains in replication, repair, and checkpoints. J Biol Chem. 2008; 283:19095–19111. [PubMed: 18469000]
36. Araya R, Hirai I, Meyerkord CL, Wang H-G. Loss of RPA1 induces Chk2 phosphorylation through a caffeine-sensitive pathway. FEBS Lett. 2005; 579:157–161. [PubMed: 15620706]
37. Kneissl M, Pütter V, Szalay AA, Grummt F. Interaction and assembly of murine pre-replicative complex proteins in yeast and mouse cells. J Mol Biol. 2003; 327:111–128. [PubMed: 12614612]

38. Deng X, Habel JE, Kabaleeswaran V, Snell EH, Wold MS, Borgstahl GEO. Structure of the full-length human RPA14/32 complex gives insights into the mechanism of DNA binding and complex formation. J Mol Biol. 2007; 374:865–876. [PubMed: 17976647]

39. Gnad F, de Godoy LMF, Cox J, Neuhauser N, Ren S, Olsen JV, Mann M. High-accuracy identification and bioinformatic analysis of in vivo protein phosphorylation sites in yeast. Proteomics. 2009; 9:4642–4652. [PubMed: 19795423]

40. Soulard A, Cremonesi A, Moes S, Schutz F, Jeno P, Hall MN. The rapamycin-sensitive phosphoproteome reveals that TOR controls protein kinase A toward some but not all substrates. Mol Biol Cell. 2010; 21:3475–3486. [PubMed: 20702584]

41. Holt LJ, Tuch BB, Villen J, Johnson AD, Gygi SP, Morgan DO. Global analysis of Cdk1 substrate phosphorylation sites provides insights into evolution. Science. 2009; 325:1682–1686. [PubMed: 19779198]

42. Brush GS, Morrow DM, Hieter P, Kelly TJ. The ATM homologue MEC1 is required for phosphorylation of replication protein A in yeast. Proc Natl Acad Sci U S A. 1996; 93:15075–15080. [PubMed: 8986766]

43. Bartrand AJ, Iyasu D, Marinco SM, Brush GS. Evidence of meiotic crossover control in Saccharomyces cerevisiae through Mec1-mediated phosphorylation of replication protein A. Genetics. 2006; 172

44. Umezu K, Sugawara N, Chen C, Haber JE, Kolodner RD. Genetic analysis of yeast RPA1 reveals its multiple functions in DNA metabolism. Genetics. 1998; 148

45. Pellicioli A, Lee SE, Lucca C, Foiani M, Haber JE. Regulation of Saccharomyces Rad53 checkpoint kinase during adaptation from DNA damage-induced G2/M arrest. Mol Cell. 2001; 7

46. Kantake N, Sugiyama T, Kolodner RD, Kowalczykowski SC. The recombination-deficient mutant RPA (rfa1-t11) is displaced slowly from single-stranded DNA by Rad51 protein. J Biol Chem. 2003; 278:23410–23417. [PubMed: 12697761]

47. Wu X, Yang Z, Liu Y, Zou Y. Preferential localization of hyperphosphorylated replication protein A to double-strand break repair and checkpoint complexes upon DNA damage. Biochem J. 2005; 391:473–480. [PubMed: 15929725]

48. Notredame C, Higgins DG, Heringa J. T-Coffee: A novel method for fast and accurate multiple sequence alignment. J Mol Biol. 2000; 302:205–217. [PubMed: 10964570]

49. Sanchez Y, Desany BA, Jones WJ, Liu Q, Wang B, Elledge SJ. Regulation of RAD53 by the ATM-like kinases MEC1 and TEL1 in yeast cell cycle checkpoint pathways. Science. 1996; 271:357–360. [PubMed: 8553072]

50. Shi W, Feng Z, Zhang J, Gonzalez-Suarez I, Vanderwaal RP, Wu X, Powell SN, Roti Roti JL, Gonzalo S, Zhang J. The role of RPA2 phosphorylation in homologous recombination in response to replication arrest. Carcinogenesis. 2010; 31

51. Borgstahl GEO, Brader K, Mosel A, Liu S, Kremmer E, Goettsch KA, Kolar C, Nasheuer H-P, Oakley GG. Interplay of DNA damage and cell cycle signaling at the level of human replication protein A. DNA Repair. 2014; 21:12–23. [PubMed: 25091156]

52. Block WD, Yu Y, Lees-Miller SP. Phosphatidyl inositol 3-kinase-like serine/threonine protein kinases (PIKKs) are required for DNA damage-induced phosphorylation of the 32 kDa subunit of replication protein A at threonine 21. Nucleic Acids Res. 2004; 32

53. Block WD, Merkle D, Meek K, Lees-Miller SP. Selective inhibition of the DNA-dependent protein kinase (DNA-PK) by the radiosensitizing agent caffeine. Nucleic Acids Res. 2004; 32:1967–1972. [PubMed: 15060176]

54. Shechter D, Costanzo V, Gautier J. ATR and ATM regulate the timing of DNA replication origin firing. Nat Cell Biol. 2004; 6:648–655. [PubMed: 15220931]

55. Aklilu BB, Soderquist RS, Culligan KM. Genetic analysis of the Replication Protein A large subunit family in Arabidopsis reveals unique and overlapping roles in DNA repair, meiosis and DNA replication. Nucleic Acids Res. 2013

56. Ishibashi T, Kimura S, Furukawa T, Hatanaka M, Hashimoto J, Sakaguchi K. Two types of replication protein A 70 kDa subunit in rice, Oryza sativa: molecular cloning, characterization, and cellular & tissue distribution. Gene. 2001; 272:335–343. [PubMed: 11470540]

57. Marwedel T, Ishibashi T, Lorbiecke R, Jacob S, Sakaguchi K, Sauter M. Plant-specific regulation of replication protein A2 (OsRPA2) from rice during the cell cycle and in response to ultraviolet light exposure. Planta. 2003; 217:457–465. [PubMed: 14520573]
58. Recolin B, Van der Laan S, Maiorano D. Role of replication protein A as sensor in activation of the S-phase checkpoint in Xenopus egg extracts. Nucleic Acids Res. 2012; 40:3431–3442. [PubMed: 22187152]
59. Francon P, Lemaitre J-M, Dreyer C, Maiorano D, Cuvier O, Mechali M. A hypophosphorylated form of RPA34 is a specific component of pre-replication centers. J Cell Sci. 2004; 117:4909–4920. [PubMed: 15456845]
60. Recolin B, Maiorano D. Implication of RPA32 phosphorylation in S-phase checkpoint signalling at replication forks stalled with aphidicolin in Xenopus egg extracts. Biochem Biophys Res Commun. 2012; 427:785–789. [PubMed: 23047005]
61. Maniar HS, Wilson R, Brill SJ. Roles of replication protein-A subunits 2 and 3 in DNA replication fork movement in Saccharomyces cerevisiae. Genetics. 1997; 145
62. Ma H, Kunes S, Schatz PJ, Botstein D. Plasmid construction by homologous recombination in yeast. Gene. 1987; 58:201–216. [PubMed: 2828185]
63. Kushnirov VV. Rapid and reliable protein extraction from yeast. Yeast Chichester Engl. 2000; 16:857–860.
64. Golemis EA, Brent R. Fused protein domains inhibit DNA binding by LexA. Mol Cell Biol. 1992; 12:3006–3014. [PubMed: 1620111]
65. Gyuris J, Golemis E, Chertkov H, Brent R. Cdi1, a human G1 and S phase protein phosphatase that associates with Cdk2. Cell. 1993; 75:791–803. [PubMed: 8242750]
66. Hegnauer AM, Hustedt N, Shimada K, Pike BL, Vogel M, Amsler P, Rubin SM, van Leeuwen F, Guenole A, van Attikum H, Thoma NH, Gasser SM. An N-terminal acidic region of Sgs1 interacts with Rpa70 and recruits Rad53 kinase to stalled forks. EMBO J. 2012; 31:3768–3783. [PubMed: 22820947]

HIGHLIGHTS

- The human RPA complex does not function in yeast cells.
- Human RPA and yeast RFA subunits do not interact (explains lack of function).
- Regions affecting human RPA function characterized in the context of yeast Rfa2.
- Unlike yeast Rfa2, the human Rpa2 N-terminus is hyper-phosphorylated in yeast.
- Human Rpa2 phosphorylated on same residues in yeast and human cells.
- Yeast is a potential tool for studying other eukaryotic Rpa2 N-termini.

IMPORTANCE

The accumulation of mutations in the cellular genome can lead to cellular disease. To prevent mutation, prokaryotic and eukaryotic cells have systems to recognize and repair DNA lesions before they become mutations. Replication Protein A (RPA) is the eukaryotic form of single-stranded binding (SSB) protein essential for proper DNA duplication and maintenance. In human cells, the 32-kilodalton (kDa) subunit of RPA, called Rpa2, is hyper-phosphorylated on its N-terminus in response to DNA damage. It is important to examine Rpa2 phosphorylation in other eukaryotic organisms to fully understand how phosphorylation contributes to Rpa2 function. When expressed in a single-celled eukaryotic organism (budding yeast), the human Rpa2 N-terminus is recognized and phosphorylated similarly to that observed in human cells. We propose that budding yeast could be a powerful tool to study phosphorylation of Rpa2 N-termini from other eukaryotes where technical limitations to studying phosphorylation currently exist.

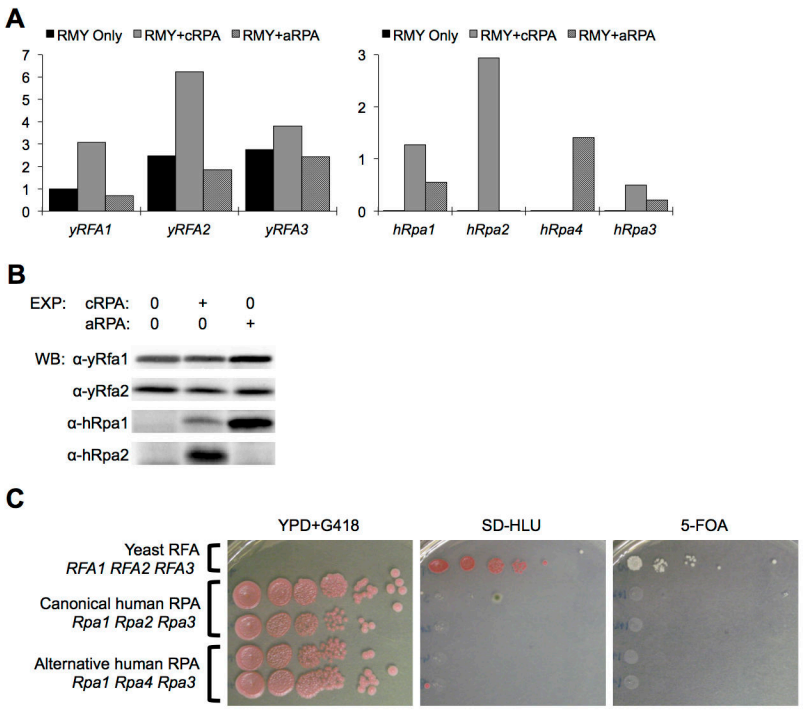

Figure 1. Human RPA does not support viability in yeast cells

[A] *Quantitative PCR measurement of yeast and human RPA subunit gene expression.* mRNA expression was measured for all yeast and human RPA subunit genes for RMY122-A (RMY Only; no additional plasmid), RMY122-A expressing human canonical RPA (*Rpa1*, *Rpa2*, and *Rpa3*) genes (RMY122+cRPA), and RMY122-A expressing human alternative RPA (*Rpa1*, *Rpa4*, and *Rpa3*) genes (RMY122+aRPA). All values were normalized to expression of the yeast *UBC6* gene.

[B] *Detection of human Rpa1 and Rpa2 expression in yeast cells.* Using the same three strains in [A], protein expression was examined for human Rpa1 (hRpa1), Rpa2 (hRpa2), yeast Rfa1 (yRfa1), and Rfa2 (yRfa2) via western blotting (WB). EXP denotes which expression plasmid the yeast cells contained.

[C] *Plasmid shuffle to examine human canonical and alternative RPA function in yeast cells.* The ability of cells to lose the pJM132 vector, which can only occur if the human RPA complex (cRPA or aRPA) can substitute for yeast RFA function, was assessed by spot assay to SD media containing 0.8 μg/mL 5-FOA. Two independent transformants expressing canonical human RPA and alternative human RPA are shown, as well as a positive control (Yeast RFA) in which the WT yeast genes *RFA1* and *RFA2* were co-transformed into RMY122-A cells. YPD+G418 is a control to show that cells originally contained the "humanized" vector, and -HLU is a control to show that cells originally contained yeast *RFA1* and *RFA2* vectors.

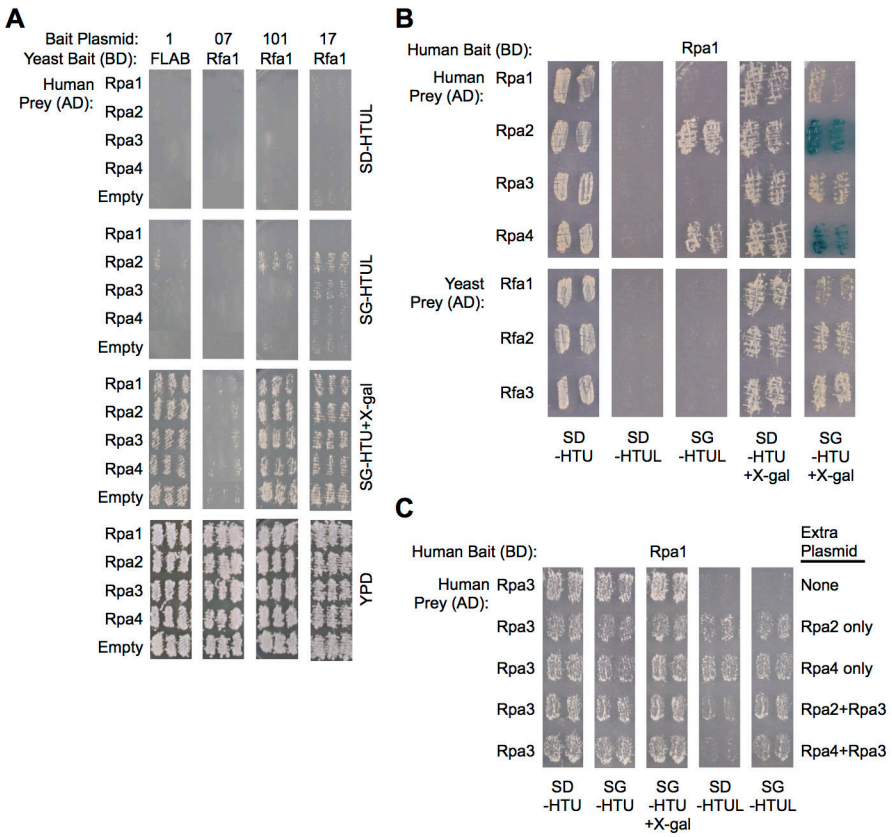

Figure 2. Examination of inter-species RPA subunit interactions

[A] *Yeast Rfa1 does not interact with human RPA subunits*. Two-hybrid analysis was performed by co-transforming EGY48 cells with bait (BD) plasmids (P_{ADH1}-*lexA*) expressing yeast *RFA1* and prey (AD) plasmids (P_{GAL}-*B42-HA*) expressing each possible human RPA subunit. Included in each co-transformation was the reporter plasmid pSH18-34 ($8xO_{lexA}$-*lacZ*). Interaction was measured for three independent transformants by the ability to grow on synthetic media containing 2% galactose and lacking histidine, tryptophan, uracil, and leucine (SG-HTUL). Qualitative interaction strength was measured by replica plating to SG-HTU+X-gal. FLAB = BD-Rfa1-FLAB (missing DBD-C; does not interact with yeast Rfa2 or Rfa3); 07 = BD-Rfa1 expressed from galactose-inducible promoter; 101 = BD-Rfa1 expressed from constitutive promoter; 17 = BD-Rfa1 constitutively expressed with leucine (instead of histidine) immediately preceding Rfa1 start codon. YPD plate is a control for growth after replica plating.

[B] *Human Rpa1 does not interact with yeast RFA subunits.* Experiment performed as in [A], except bait plasmid was human Rpa1 and prey plasmids contain either human RPA subunits or yeast RFA subunits. SD-HTU plate shown to verify that each patch contained a bait, prey, and reporter plasmid. Again, growth on SG-HTUL indicates interaction, and blue color of SG-HTU+X-gal represents strong interaction.

[C] *Rpa2 or Rpa4 is required for Rpa1 and Rpa3 to interact.* No interaction between Rpa1 and Rpa3 was observed in [B] (SG-HTUL and SG-HTU+X-gal plates); however, when a pJM132 derivative expressing either Rpa2 or Rpa4 (extra plasmid) was co-transformed, Rpa1 and Rpa3 now display weak interaction, as indicated by growth on SG-HLTU. SG-

HTU is a growth control; SD-HTUL is to detect auto-activation. Although auto-activation complicates analysis in cells expressing Rpa2 or Rpa4 only, cells expressing Rpa2+Rpa3 or Rpa4+Rpa3 show reduced auto-activation, yet display increased growth on SG-HTUL (*i.e.*, when AD-Rpa3 expression is induced).

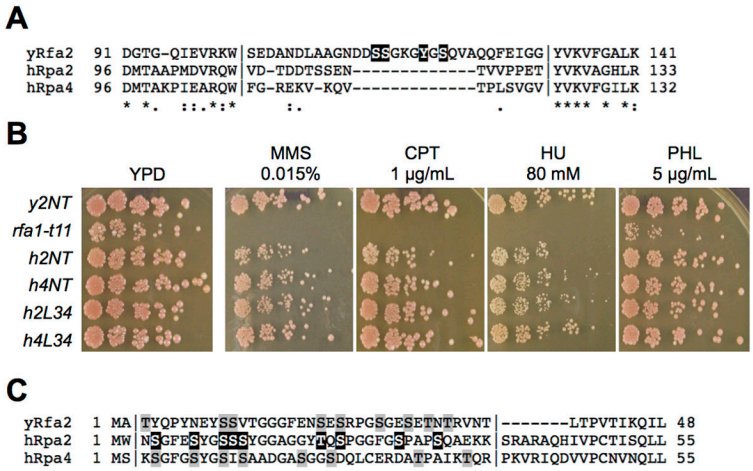

Figure 3. Domain swapping of the yeast Rfa2 loop 3–4 or N-terminal region with the analogous human Rpa2 or Rpa4 region

[A] *Sequence comparison of the yeast Rfa2, human Rpa2, and human Rpa4 loop 3–4 regions*. Alignment of conserved amino acid sequence on either side of the loop region is displayed. Also denoted (black highlight with white font) are sites of phosphorylation of Rfa2 that have been reported through mass spectrometry analysis (39–41). * = identical residue; : = strongly similar properties; . = weakly similar properties.

[B] *DNA damage phenotypes of Rfa2 loop 3–4 or N-terminal swaps.* Sensitivity to DNA damage was measured by growing WT *RFA2*-expressing (*y2NT*), loop hybrid (*h2L34* or *h4L34*)-expressing, N-terminal (NT) hybrid (*h2NT* or *h4NT*)-expressing, and *rfa1-t11*-expressing (damage-sensitive control) cells and serial diluting (three-fold dilutions) equivalent numbers of cells. The diluted cells were then spotted onto rich media (YPD) or rich media containing the DNA damaging agents methyl methanesulfonate (0.015% MMS), camptothecin (1 µg/mL CPT), hydroxyurea (80 mM HU), or phleomycin (5 µg/mL PHL). Results from additional concentrations of damaging agents are shown in Figure S1.

[C] *Sequence alignment of the yeast Rfa2, human Rpa2, and human Rpa4 N-termini (with gaps removed).* Gaps were removed from the T-COFFEE sequence alignment shown in Figure S1B for the first 38 aa residues to display that known sites (black highlights with white font) or putative sites (grey highlights) of phosphorylation lie in similar positions.

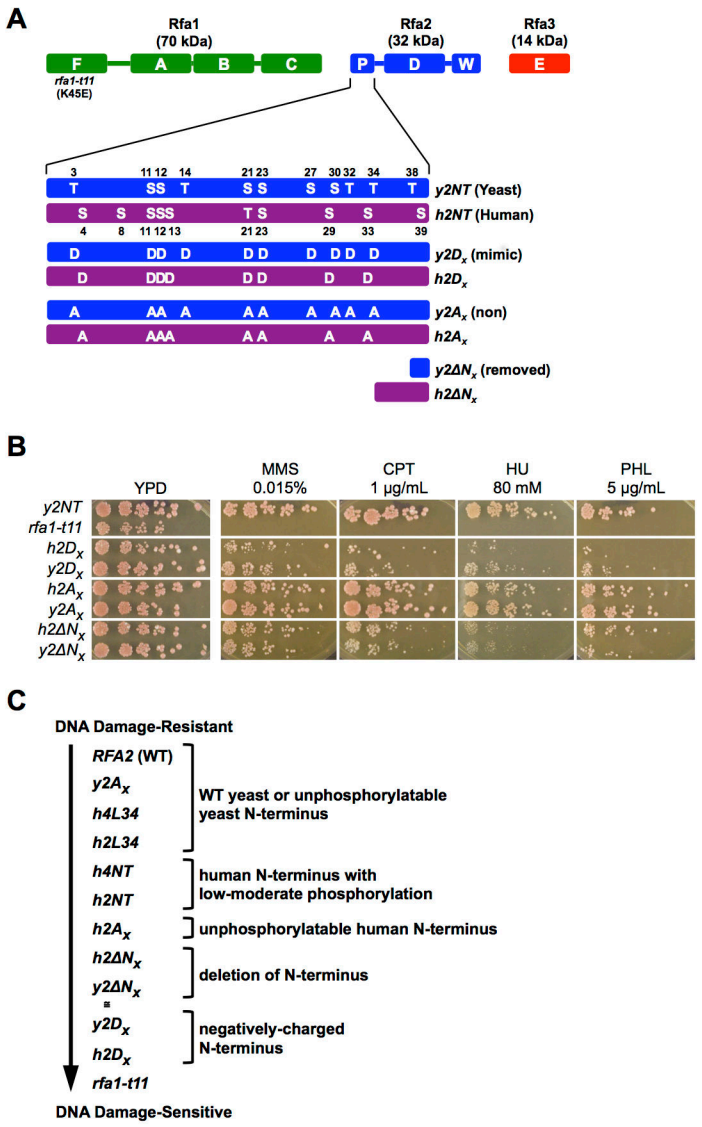

Figure 4. Domain swapping of the yeast Rfa2 N-terminus with human Rpa4, human Rpa2, or extensive phospho-mutant forms of human Rpa2

[A] *Schematic representing the human N-terminal hybrid proteins examined.* Sites (known or putative) of phosphorylation were either mutated to aspartic acids (D_x) to mimic phosphorylation, alanines (A_x) to be non-phosphorylatable, or the N-terminus was removed (ΔN_x). Yeast forms are designated with the prefix *y* and are denoted by the color blue; human forms are designated with the prefix *h* and are denoted by the color purple. The *rfa1-t11* mutation (K45E) is in DBD-F of yeast Rfa1. Amino acid positions of serines/threonines for yeast Rfa2 or human Rpa2 are denoted above or below their respective N-termini.

[B] *DNA damage phenotypes of domain swaps compared to their analogous yeast N-terminal mutants.* Yeast *RFA2* chimeric mutants were generated that expressed the yeast Rfa2 N-terminus swapped for the equivalent human Rpa2 or Rpa4 N-terminus, denoted as *rfa2-h2NT* or *rfa2-h4NT*, respectively (examined in Figure 3B). *RFA2* chimeric genes were also generated to express extensive phospho-mutant forms of the Rpa2 N-terminus in which

every serine/threonine (S/T) within the first 33 aa of human Rpa2 are mutated to aspartic acids (*rfa2-h2D$_x$*), alanines (*rfa2-h2A$_x$*), or the first 33 aa are deleted (*rfa2-h2ΔN$_x$*), as represented in [A]. The equivalent *rfa2* mutations are designated as *rfa2-yD$_x$*, *rfa2-y2A$_x$*, and *rfa2-y2ΔN$_x$*, respectively. Sensitivity to DNA damage of Rfa2 hybrid cells was measured as described in Figure 3B.

[C] *Qualitative ranking of DNA damage resistance of rfa2 N-terminal hybrid forms.* Mutant (hybrid or aa substitution) forms were ranked from most DNA damage-resistant to most DNA damage-sensitive based on the results of spot assays on media containing varying concentrations of DNA damaging agents (Figure 3B, 4B, S1A, and S2). Designations to the right describe the N-terminus of each cluster of mutants. ≈ designates that the mutant above and below the symbol are approximately equal with respect to damage sensitivity phenotype.

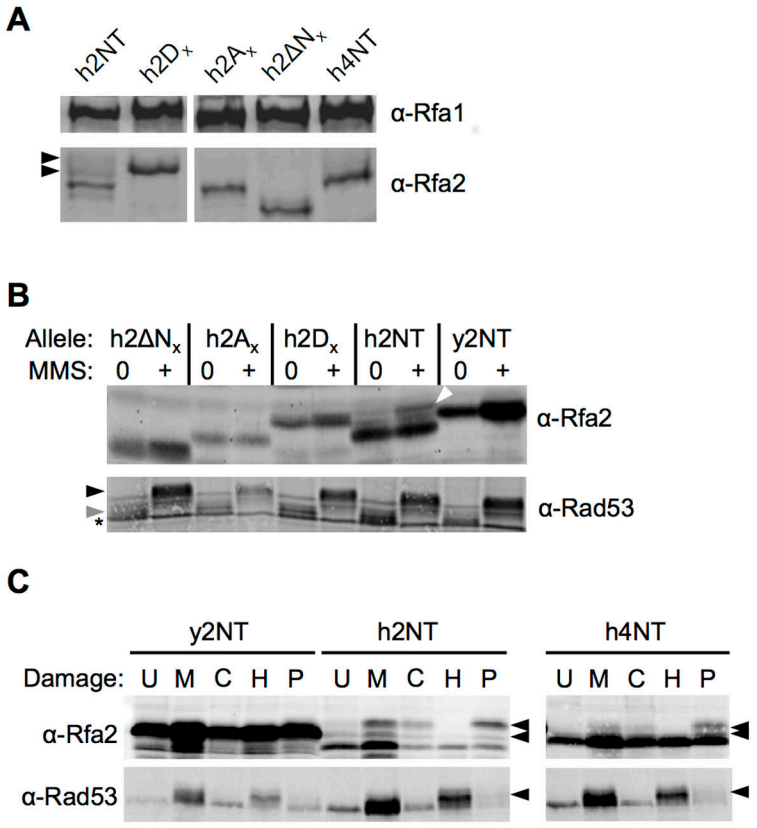

Figure 5. Hybrid Rfa2 is phosphorylated on its N-terminus

[A] *Phosphorylation occurs in undamaged cells and is specific to the human Rpa2 N-terminus*. Cells containing hybrid forms of Rfa2 were grown to exponential phase and protein was collected using the method described by Kushnirov (63). Western blotting with anti-yeast Rfa2 antibody reveals slower-migrating species (denoted by black arrows) that are not detected in Rfa2 phospho-mutant hybrid forms. Rfa1 blotting was performed to demonstrate that yeast Rfa1 expression was unaffected, indicating proper complex formation.

[B] *DNA-damaged cells display additional N-terminal phosphorylation on the human N-terminus*. Cells were grown as in [A]; however, half of each cell culture was treated with 0.03% methyl methanesulfonate (MMS; denoted above each lane; 0 = none, + = added). Protein separation and detection were performed as in [A], and a more intense species (denoted by white arrow) is detected by anti-yeast Rfa2 antibody. Again, no additional species are observed for any of the human Rpa2 N-terminal S/T mutants in either unstressed or stressed conditions. Blot below shows Rad53 (lower grey arrow) and activated Rad53 (upper black arrow) as a marker indicating that cells were damaged and initiated a G2/M checkpoint. A background species is observed in all lanes and denoted with an asterisk.

[C] *Phosphorylation of the Rfa2-h2NT or Rfa2-h4NT hybrid proteins in response to different DNA damaging agents.* Cells were grown to exponential phase and either left untreated (U) or treated with 0.03% methyl methanesulfonate (M), 20 μg/mL camptothecin (C), 400 mM hydroxyurea (H), or 20 μg/mL phleomycin (P) for 4 hr. For hybrid Rfa2 detection, 30 μg of

total protein were loaded per lane. For Rad53 detection, 120 μg of total protein were loaded per lane. Black arrows denote phosphorylated species of the hybrid Rfa2 or Rad53 proteins.

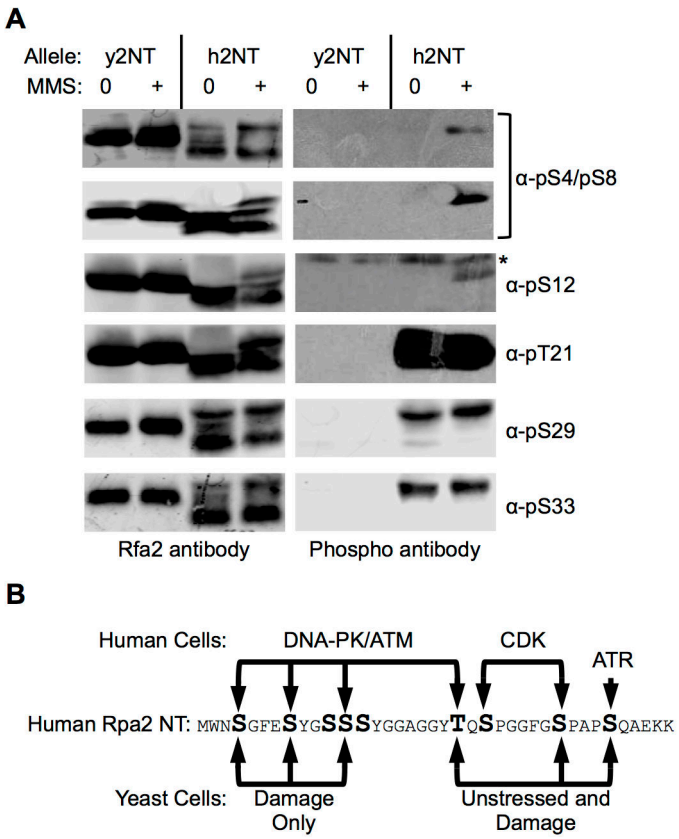

Figure 6. Hybrid Rfa2 containing the human Rpa2 N-terminus is phosphorylated at the same residues as in human cells

[A] *The hybrid Rfa2-h2NT is phosphorylated on multiple serines/threonines.* To determine if phosphorylation is occurring on specific S/T residues, western blotting with human Rpa2 phospho-specific antibodies (right-side blots with specific antibody used denoted to the right of each blot) was performed. Blots on left half represent resolution of approximately equal amounts of total Rfa2 or Rfa2-h2NT (not total protein). MMS treatment and designations are as in Figure 5B. The anti-yeast Rfa2 antibody recognizes all species, and the phospho-specific antibodies recognize only the Rfa2-h2NT species that have been phosphorylated at that particular residue. The asterisk for the blot detected with pS12-specific antibody indicates a non-specific species.

[B] *Comparison of phosphorylation of human Rpa2 in human cells vs. the human Rfa2 hybrid in yeast cells.* Shown are the first 38 amino acid residues of the human Rpa2 N-terminus. Sites of phosphorylation are shown in larger font and bold. Human kinases that recognize and phosphorylate each residue in human cells are designated above the sequence. Only CDK sites are not strictly damage-specific in human cells. Denoted below the sequence are residues identified to be phosphorylated on Rfa2-h2NT in yeast cells. The condition in which phosphorylation is observed is denoted below the arrows.

Table 1

Qualitative summary of phosphorylation observed under different damage conditions.

		Damage Treatment			
Protein	**Mitotic growth**	**0.03% Methyl methanesulfonate (MMS)**	**20 µg/mL Camptothecin (CPT)**	**400 mM Hydroxyurea (HU)**	**20 µg/mL Phleomycin (PHL)**
Rad53	0	++++	0	+++++	++
Rfa2	0	0	0	0	0
Rfa2-h2NT	+	+++	++++	+	+++++
Rfa2-h4NT	0	++	++	0	+++

0 = no detectable post-translational modification; number of + represents the intensity of the post-translationally modified species compared the unmodified species.