Genetic and biochemical identification of a novel single-stranded DNA-binding complex in *Haloferax volcanii*

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**Introduction**

Genomic DNA must be unwound in order to be replicated or repaired, leaving it vulnerable to nuclease and chemical attack as well as open to the possibility of forming secondary structures. Binding of the single-stranded DNA (ssDNA)-binding proteins (SSB) RPA and SSB prevents any of these events from occurring (Lu et al., 2009). The SSB is denominated SSB in bacteria and replication protein A (RPA) and consists of three distinct subunits that function as a heterotrimer. In eukaryotes the ssDNA-binding protein (SSB) is known as replication protein A (RPA) and consists of three distinct subunits that function as a heterotrimer. The bacterial homolog is termed SSB and functions as a homotetramer. In the archaeon *Haloferax volcanii* there are three genes encoding homologs of RPA. Two of the *rpa* genes (*rpa1* and *rpa3*) exist in operons with a novel gene specific to Euryarchaeota; this gene encodes a protein that we have termed RPA-associated protein (*rpap*). The *rpap* genes encode proteins belonging to COG3390 group and feature OB-folds, suggesting that they might cooperate with RPA in binding to ssDNA. Our genetic analysis showed that *rpa1* and *rpa3* deletion mutants have differing phenotypes; only Δ*rpa3* strains are hypersensitive to DNA damaging agents. Deletion of the *rpa3*-associated gene *rpap3* led to similar levels of DNA damage sensitivity, as did deletion of the *rpa3* operon, suggesting that RPA3 and RPAP3 function in the same pathway. Protein pull-downs involving recombinant hexahistidine-tagged RPAs showed that RPA3 co-purifies with RPAP3, and RPA1 co-purifies with RPAP1. This indicates that the RPAs interact only with their respective associated proteins; this was corroborated by the inability to construct *rpa1 rpap3* and *rpa3 rpap1* double mutants. This is the first report investigating the individual function of the archaeal COG3390 RPA-associated proteins (RPAPs). We have shown genetically and biochemically that the RPAPs interact with their respective RPAs, and have uncovered a novel single-stranded DNA-binding complex that is unique to Euryarchaeota.

**Keywords:** archaea, *Haloferax volcanii*, RPA single-strand DNA-binding protein, COG3390 RPA-associated protein, DNA repair, protein overexpression, Cdc48d
Pyrococcus furiosus RPA consists of three subunits RPA1, 14, and 32, denominated RPA1, 2, and 3, respectively, which form a heterotrimer as seen in eukaryotes. Strand exchange and immunoprecipitation assays have shown that P. furiosus heterotrimeric RPA stimulates strand exchange, and interacts with the clamp loader RFC and both DNA polymerases B and D (Komori and Ishino, 2001). The heterotrimeric complex seen in P. furiosus is also found in P. abyssi and P. horikoshii. However, in other archaeal species the rpa genes have undergone lineage-specific duplications, resulting in differing numbers of SSBs with diverse structures. Unlike the RPA complex found in Pyrococcus spp., or eukaryotic RPA, these do not form trimeric complexes (Robbins et al., 2004).

Methanosarcina acetivorans possesses three RPA subunits, MacRPA1, 2, and 3, which are unlikely to form a heterotrimeric complex as seen in P. furiosus and in eukaryotes. MacRPA1 contains four DNA-binding domains (DBD) containing OB-folds, MacRPA2 and 3 both have two OB-fold containing DBDs. Each of the three MacRPs can function as SSBs, and are able to stimulate primer extension by M. acetivorans DNA polymerase BI (Robbins et al., 2004, 2005). This demonstrates an element of redundancy between the three MacRPs, and suggests that the heterotrimeric RPA structure observed in P. furiosus is the exception and not the rule. Lin et al. (2008) suggest that intramolecular recombination between RPA homologs may have led to the diversity of RPs found in euryarchaeota, which can function in different pathways or cellular processes.

A similar pattern of lineage-specific gene duplication is seen with the archaeal MCM helicase, where the number and type of MCM subunits that make up the hexameric helicase differ between archaeal species. The genes encoding the MCM subunits fall into distinct phylogenetic clades, but these do not correspond to specific subunits of eukaryotic MCM. Instead they have arisen through lineage-specific gene family expansion (Chia et al., 2010). Such gene duplication might allow different archaeal species to refine the structure and function of MCM (and potentially RPA) for differing conditions and specialized roles.

Sulfolobus solfataricus has a bacterial-like SSB consisting of a small 20 kDa peptide containing one OB-fold and an acidic C-terminus tail (Haseltine and Kowalczykowski, 2002; Rolfsmeier and Haseltine, 2010). The S. solfataricus SSB quaternary structure is similar to that of E. coli SSB, however the primary structure of the OB-fold shows greater homology to that of the eukaryotic RPA70 DNA-binding domain B (DBDB). This suggests that cre-archaeal SSBs may be structurally similar to bacterial SSB but at a protein sequence level show homology to the eukaryotic RPA (Haseltine and Kowalczykowski, 2002; Kerr et al., 2003). In S. solfataricus there is an absence of DNA damage recognition proteins such as homologs of XPA or XPC to initiate NER. The ability of S. solfataricus SSB to specifically bind and melt damaged duplex DNA in vitro suggests SSB may play a role in the identification and binding of damaged DNA, followed by the subsequent recruitment of NER repair proteins (Cubbeddu and White, 2005).

Haloferax volcanii encodes three RPA genes rpa1, rpa2, and rpa3 (Hartman et al., 2010). Recent studies have shown RPA2 to be essential while RPA1 and RPA3 are not (Skowyra and MacNeill, 2012). Note that these authors used the nomenclature rpaA1, A2, B1, B2, B3, and C to refer to rpa3, rpap3, rpa1, rpa1, rpe, and rpa2, respectively, while we have chosen to maintain the official nomenclature as described in Table 4 of the H. volcanii genome paper (Hartman et al., 2010). Both rpa1 and rpa3 are in operons with other genes; rpa1 is in an operon with genes encoding an OB-fold containing protein (hereby designated RPA-associated protein or RPAP) and a calcineurin-like phosphoesterase, while only one OB-fold rpa-associated protein (rpap) gene is present in the rpa3 operon (Figure 1). The presence of an rpap gene in the same operon as rpa can be found in other euryarchaeota, including Halobacterium marismortui, Halobacterium salinarum, and Natronomonas pharaonis, as well as in M. mazei and M. bark-eri. The rpap gene has been assigned to the cluster of orthologous groups (COG) 3390 (Herthom et al., 2008).

To examine if RPA1 and 3, as well as RPAP1 and RPAP3 play a role in DNA repair, as is true for both the bacterial SSB and eukaryotic RPA, DNA damage assays were performed using the single and operon deletion mutants. Cells with deletions of the rpa1 and rpa3 operons had previously been examined by Skowyra and MacNeill (2012). However, this is the first report investigating the individual function of the archaeal COG3390 RPA. We show genetically and biochemically that the RPAPs interact with their respective RPAs, and have thereby uncovered a novel SSB complex that is unique to Eurarchaeota.

MATERIALS AND METHODS
All chemicals were from Sigma and restriction enzymes from New England Biolabs, unless stated otherwise. Standard molecular techniques were used (Sambrook and Russell, 2001).

STRAINS AND PLASMIDS
Haloferax volcanii strains (Table 1) were grown at 45°C on complete (Hv-YPC), casamino acids (Hv-Ca), or minimal (Hv-Min) agar, or in Hv-YPC or Hv-Ca broth as described previously. Isolation of genomic and plasmid DNA, as well as transformation of H. volcanii were carried out as described previously (Allers et al., 2004).

![FIGURE 1 | Operon and domain structures of H. volcanii single-stranded DNA-binding proteins. Genes for RPA1 and RPAP3 are in operons with genes for RPA-associated proteins, RPAP1 and RPAP3, respectively. The gene for RPAP1 phosphoesterase (RPE) is present in the rpa1 operon. Domains (not to scale) comprising OB-folds, zinc fingers and a phosphoesterase motif are shown.](image-url)
Table 1 | *Haloferax volcanii* strains.

| Strain | Relevant genotype* | Source or reference |
|--------|--------------------|---------------------|
| DS2    | Wild-type          | Mullanahanbhai and Larsen (1975) |
| H195   | ΔpyrE2 bgHa-Bb leuB-Ag1 ΔtrpA ΔhdrB | Guy et al. (2006) |
| H1209  | ΔpyrE2 ΔhdrB Nph-pitA Δmrr | Allers et al. (2010) |
| H1216  | ΔpyrE2 bgHa-Bb leuB-Ag1 ΔtrpA ΔhdrB Δrapa1::trpA+ | H195 pTA1170 |
| H1217  | ΔpyrE2 bgHa-Bb leuB-Ag1 ΔtrpA ΔhdrB Δrapa1::trpA+ | H195 pTA1166 |
| H1244  | ΔpyrE2 bgHa-Bb leuB-Ag1 ΔtrpA ΔhdrB Δrapa3::trpA+ | H195 pTA1124 |
| H1246  | ΔpyrE2 bgHa-Bb leuB-Ag1 ΔtrpA ΔhdrB Δrapa1 operon | H195 pTA1189 |
| H1260  | ΔpyrE2 ΔhdrB bgHa-Bb Δrapa3 operon::trpA+ leuB-Ag1 ΔtrpA | H195 pTA1207 |
| H1280  | ΔpyrE2 bgHa-Bb leuB-Ag1 ΔtrpA ΔhdrB Δrapa1 | H1216 pTA1217 |
| H1281  | ΔpyrE2 bgHa-Bb leuB-Ag1 ΔtrpA ΔhdrB Δrapa1 | H1217 pTA1141 |
| H1282  | ΔpyrE2 bgHa-Bb leuB-Ag1 ΔtrpA ΔhdrB Δrapa1 operon rapa3 operon::[Δrapa3 operon::trpA+, pyrE2+] | H1246 pTA1207 pop-in |
| H1326  | ΔpyrE2 bgHa-Bb leuB-Ag1 ΔtrpA ΔhdrB Δrapa1 rapa3::[Δrapa3::trpA+ pyrE2+] | H1290 pTA1174 pop-in |
| H1333  | ΔpyrE2 ΔhdrB Nph-pitA Δmrr Hsa-cdc48d | H1209 pTA1240 |
| H1390  | ΔpyrE2 bgHa-Bb leuB-Ag1 ΔtrpA ΔhdrB Δrapa1 operon rapa3 operon::[Δrapa3 operon::trpA+, pyrE2+] <rapa1 operon::hdrB+pyrE2> | H1282 pTA1265 pop-in |
| H1410  | ΔpyrE2 bgHa-Bb leuB-Ag1 ΔtrpA ΔhdrB Δrapa3 | H195 pTA1284 |
| H1424  | ΔpyrE2 ΔhdrB Nph-pitA Δmrr cdc48d-Ct | H1333 pTA1294 |
| H1430  | ΔpyrE2 ΔhdrB Nph-pitA Δmrr cdc48d-Ct <p:tna::his tag::pyrE2+ | H1424 pTA963 |
| H1473  | ΔpyrE2 bgHa-Bb leuB-Ag1 ΔtrpA ΔhdrB Δrapa1 rapa3::[Δrapa3::trpA+ pyrE2+] | H1281 pTA1284 pop-in |
| H1480  | ΔpyrE2 ΔhdrB Nph-pitA Δmrr cdc48d-Ct <p:tna::his tag::rapa3 rapa3 pyrE2+ | H1424 pTA1280 |
| H1481  | ΔpyrE2 ΔhdrB Nph-pitA Δmrr cdc48d-Ct <p:tna::his tag::rapa3 rapa3 pyrE2+ | H1424 pTA1281 |
| H1482  | ΔpyrE2 ΔhdrB Nph-pitA Δmrr cdc48d-Ct <p:tna::his tag::rapa1 rapa1 pyrE2+ | H1424 pTA1326 |
| H1483  | ΔpyrE2 ΔhdrB Nph-pitA Δmrr cdc48d-Ct <p:tna::his tag::rapa1 rapa1 pyrE2+ | H1424 pTA1327 |

* Genes shown within <> are present on an episomal plasmid, genes shown within [] are present on an integrated plasmid (pop-in).

**CONSTRUCTION OF MUTANT STRAINS**

Deletion mutants were constructed as described previously (Allers et al., 2004). Plasmids for gene deletion are shown in Table 2, and were generated by PCR using oligonucleotides shown in Table 3. Template DNA for the PCRs was isolated from genomic DNA.

**CONSTRUCTION OF PROTEIN OVEREXPRESSION STRAINS**

Protein overexpression strains were constructed by transformation with episomal overexpression plasmids as described previously (Allers et al., 2010). Plasmids for protein expression are shown in Table 2, and were generated by PCR using oligonucleotides shown in Table 3. Template DNA for the PCRs was isolated from genomic DNA.

**UV IRRADIATION ASSAYS**

UV irradiation assays were carried out as described previously (Delmas et al., 2009).

**MITOMYCIN C ASSAYS**

Mitomycin C (MMC) assays were carried out as described previously (Lestini et al., 2010).

**PROTEIN OVEREXPRESSION AND PURIFICATION**

Protein overexpression was carried out as described previously (Allers et al., 2010) with the following amendments: cultures were incubated at 45°C overnight to an OD<sub>650</sub> of 0.5, when protein expression was induced by adding 3 mM Trp to the culture followed by incubation at 45°C, with shaking for a further 1 h until OD<sub>650</sub> ≈ 0.7.

**PROTEIN PRECIPITATION**

Deoxyscholate was added to 0.015%, vortexed, and incubated for 10 min at room temperature. Trichloroacetic acid was added to 7.2% and incubated at room temperature for 5 min. Samples were centrifuged at 14,000 × g at room temperature for 8 min. Supernatant was removed and precipitated protein resuspended in 15% trichloroacetic acid in 1 M NaOH, 5% glycerol, 0.25 mg/ml bromophenol blue). Samples were heated for 10 min at 94°C and cooled on ice before loading onto an SDS-PAGE gel.

**MASS SPECTROMETRY**

Mass spectrometry of excised protein bands was carried out as described previously (Allers et al., 2010). Details of protein identification are given in the Table 1 in Appendix.

**RESULTS**

**RPA3 BUT NOT RPA1 FUNCTIONS IN DNA REPAIR**

In eukaryotes, specifically *Saccharomyces cerevisiae*, all three RPA subunits have been shown to be essential for cell survival (Brill and Stillman, 1991). Work by Skowyra and MacNeill (2012) has shown that *H. volcanii* rpa2 is essential, which is in agreement with our fruitless attempts to delete rpa2 (data not shown). To examine if the other rpa genes of *H. volcanii* are also essential,
### Table 2 | Plasmids.

| Plasmid          | Relevant properties                                                                                                                                                                                                                                             | Source or reference          |
|------------------|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----------------------------|
| pBluescript II SK+ | Standard cloning vector                                                                                                                                                                                                                                          | Stratagene                  |
| pTA131           | Integrative vector based on pBluescript II, with pyrE2 marker                                                                                                                                                                                                   | Allers et al. (2004)         |
| pTA409           | Shuttle vector containing ampicillin, pyrE2 and hdr8 markers, and pHV1/4 replication origin                                                                                                                                                                        | Delmas et al. (2009)        |
| pTA884           | pBluescript II with H. volcanii 5,038-bp EcoRI/NotI genomic fragment containing rpa3 operon                                                                                                                                                                       | This study                  |
| pTA989           | pBluescript II with H. volcanii 7335-bp EcoRI/NotI genomic fragment containing rpa2                                                                                                                                                                           | This study                  |
| pTA937           | pBluescript II with H. volcanii 8,565-bp BspEI genomic fragment containing rpa1 operon                                                                                                                                                                          | This study                  |
| pTA963           | Overexpression vector with 6xHis-tag, pyrE2 and hdr8 markers, and pHV2 origin                                                                                                                                                                                      | This study                  |
| pTA1141          | pTA131 containing rpa1 deletion construct inserted at KpnI and XbaI sites, contains an internal Ndel site                                                                                                                                                       | This study                  |
| pTA1142          | pTA131 containing rpa3 deletion construct inserted at EcoRI and KpnI sites, contains an internal Ndel site                                                                                                                                                     | This study                  |
| pTA1166          | rpa1 deletion construct pTA1141 with trpA marker, amplified from pTA298 introducing Ndel restriction sites to insert at internal Ndel restriction site in pTA1141                                                                 | This study                  |
| pTA1170          | Deletion construct of rpa1 containing trpA marker from pTA298 inserted at EcoRI and KpnI sites in pTA131                                                                                                                                                        | This study                  |
| pTA1174          | rpa3 deletion construct containing trpA marker from pTA1166 inserted at Ndel restriction site                                                                                                                                                                     | This study                  |
| pTA1180          | pTA131 with cdc48d deletion construct                                                                                                                                                                                                                         | This study                  |
| pTA1189          | pTA131 with rpa1 operon deletion construct inserted at restriction sites XbaI and EcoRI with an internal Ndel site                                                                                                                                               | This study                  |
| pTA1196          | rpa3 operon deletion construct, using Ndel/EcoRI downstream fragment from pTA1282 (rpa3 deletion construct) inserted at Ndel/EcoRI sites in pTA1142 (rpa3 deletion construct), to replace the downstream fragment of the rpa3 deletion construct                                           | This study                  |
| pTA1207          | Deletion construct of rpa3 operon pTA1196 with insertion of the trpA marker from pTA1166 at internal Ndel site                                                                                                                                                | This study                  |
| pTA1217          | RPA1 deletion construct pTA1170 with upstream and trpA fragment replaced with the upstream fragment amplified from pTA937 by PCR, to introduce compatible Sphi sites, generating non-trpA-marked deletion construct                        | This study                  |
| pTA1218          | pTA983 with rpa3 inserted downstream of His-tag. Asel inserted after rpa3 stop codon to allow insertion of His-tagged rpa3 upstream of His-tagged rpa3 (Aasel is Ndel compatible)                                                                                       | This study                  |
| pTA1222          | pTA983 with rpa1 N-terminally His-tagged, has an Aasel site downstream of rpa1 to allow insertion of rpa1 (Ndel compatible)                                                                                                                                     | This study                  |
| pTA1223          | pTA983 overexpression vector with rpa1 N-terminally His-tagged inserted at PsiI and BamHI sites. rpa1 was amplified by PCR from pTA937 introducing BspHI and BamHI sites                                                                                      | This study                  |
| pTA1224          | pTA983 with rpa3 N-terminally His-tagged inserted at PsiI and EcoRI sites. RPA3 was amplified by PCR from pTA984 introducing BspHI and EcoRI sites                                                                 | This study                  |
| pTA1240          | Gene replacement construct with insertion of 896 bp Hsa-cdc48d gene (amplified from H. salinarum DNA) between upstream and downstream flanking regions of H. volcanii cdc48d deletion construct pTA1180                                                       | This study                  |
| pTA1265          | pTA409 with insertion of rpa1 operon from pTA937 at EcoRV site                                                                                                                                                                                                  | This study                  |
| pTA1280          | pTA1218 with rpa3 amplified from pTA884 by PCR and inserted at BstEII and EcoRI sites after the N-terminally His-tagged rpa3, maintaining reading frame                                                                                                               | This study                  |
| pTA1281          | pTA1224 with rpa3 amplified from pTA884 by PCR and inserted upstream of N-terminally His-tagged rpa3 at Ndel site                                                                                                                                                  | This study                  |
| pTA1282          | rpa3 deletion construct with upstream and downstream regions amplified from genomic clone pTA884, introducing external KpnI and EcoRI sites, used to ligate into pTA131, and internal Ndel site pTA1282 with trpA marker digested from pTA1166 using Ndel and inserted at Ndel site in pTA1282, generating trpA-marked rpa3 deletion construct | This study                  |
| pTA1284          | pBluescript II with H. volcanii 3,299-bp SalI/BspHI genomic fragment containing cdc48d gene                                                                                                                                                                        | This study                  |
| pTA1294          | pTA131 with 2,247 bp Hvo-cdc48d-CI gene replacement construct amplified from pTA1288: 1,797 bp EcoRI-Ahel fragment with C-terminally truncated cdc48d plus upstream region, ligated to 495 bp Nhel-KpnI fragment with downstream region of cdc48d, inserted at EcoRI and KpnI sites | This study                  |
| pTA1326          | pTA1222 with rpa1, amplified from pTA937 introducing BstEII and BamHI sites, and inserted downstream of His-tagged rpa1 at BstEII and BamHI sites                                                                                                                     | This study                  |
| pTA1327          | pTA1223 with rpa1 inserted upstream of His-tagged rpa1 at Ndel site. rpa1 was amplified from pTA937 introducing Ndel and Asel (Ndel compatible) sites                                                                                                                                 | This study                  |
### Table 3 | Oligonucleotides.

| Oligonucleotide | Sequence (5′–3′) | Relevant properties | Use (plasmid generated) |
|-----------------|-----------------|---------------------|-------------------------|
| Rpa1CF DS       | GTTCAGGAGTCAGTGTCGAGGC | Δrpa1 external downstream primer, KpnI site | pTA1141 |
| Rpa1CR DS       | AGGTGCAGCATGAGCGCCTGCC | Δrpa1 internal downstream primer, NdeI site | pTA1141 |
| Rpa1 CR US      | TACTAGCTCTAGAGCCAGGCTTGC | Δrpa1 external upstream primer, XbaI site | pTA1141 |
| Rpa1 CF US      | GTTGCAGGTTTCTATGTGCGAGTGGATCGGCC | Δrpa1 internal upstream primer, NdeI site | pTA1141 |
| Rpa3KpnI F      | GCCGGTGAGCACACACGCTTC | Δrpa3 internal upstream primer, KpnI site | pTA1142 |
| Rpa3NdeI R      | GACGTGAGTCAGGCGCGGGACGTTACGC | Δrpa3 internal downstream primer, EcoRI site | pTA1142 |
| Rpa3NdeI FC     | GCCGGGTGAGTCAGGCGCGGGACGTTACGC | Δrpa3 internal downstream primer, NdeI site | pTA1142 |
| Rpa3SeqF        | GGAAAAAGGGGCAGATGGTG | Forward primer to downstream flanking region of Hvo-cdc48d | |
| Hvo-cdc48d     | Cdc48dSeqF      | Revers primer to Cdc48dSeqR | |
| Hvo-cdc48d     | Hvo-cdc48d-CtrF | Internal forward primer to generate C-terminal truncated Hvo-cdc48d, Nhel site at cdc48d stop codon | pTA1294 |
| Hvo-cdc48d     | Hvo-cdc48d-CtrR | Internal forward primer to generate C-terminal truncated Hvo-cdc48d, Nhel site at cdc48d stop codon | pTA1294 |
| Hvo-cdc48d     | Hvo-cdc48d-SeqF | Forward primer to downstream flanking region of Hvo-cdc48d | PCR Figure 5C |
| Hvo-cdc48d     | Cdc48dSeqR      | Reverse primer to Hvo-cdc48d gene | PCR Figure 5C |
| Hvo-cdc48d     | Hvo-cdc48d-SeqR | Reverse primer to Hvo-cdc48d gene | PCR Figure 5C |
| Hvo-cdc48d     | Rpa1BstEI F     | rpai forward primer, native BstELI site | pTA1326 |
| Hvo-cdc48d     | Rpa1NdeI R      | rpai forward primer, NdeI site | pTA1327 |
Stroud et al. RPA mutants of Haloferax volcanii

Genomic deletions of rpa1 and rpa3 were generated using the counter selective pop-in/pop-out method (Allers et al., 2004). To generate the deletion constructs by PCR, rpa1 and rpa3 operons were first isolated from wild-type (WT) H. volcanii using native BspEI and EcoRI/NorI restriction sites, respectively, to generate genomic libraries. These were then screened for the presence of the rpa1 and rpa3 operons, individually, using colony hybridization. The isolated plasmids, pTA937 (rpa1 operon) and pTA884 (rpa3 operon) were confirmed by DNA sequencing. Deletion constructs for rpa1 and rpa3 were designed to avoid polar effects on the expression of the downstream rpap genes by maintaining the reading frame. Genomic deletions of both rpa1 and rpa3 (trpA-marked) were successful, generating strains H1217 and H1244, respectively (Figures 2 and 3, respectively). The ability to delete both rpa1 and rpa3 with relative ease, but not rpa2, indicates that the cellular requirement for each RPA is not equal, making it unlikely that they function collectively.

Both eukaryotic and bacterial SSB are involved in DNA repair. To examine if H. volcanii RPA1 and RPA3 function in DNA repair, the effects of DNA damage on cell survival of H1217 and H1244 were examined. UV irradiation results in the formation of cyclobutane pyrimidine dimers and 6-4 pyrimidine-pyrimidone dimer photoproducts, as well as ssDNA nicks that indirectly generate double-stranded DNA breaks (DSBs). The latter require repair by homologous recombination (HR) or single-strand DNA annealing (Fousteri and Mullenders, 2008; Rouillon and White, 2011). MMC is a chemotherapeutic agent that reacts with DNA generating covalent interstrand cross-links, requiring removal by nucleotide excision repair (NER) and HR (Tomasz et al., 1987). The Δrpa1 mutant H1217 was no more sensitive than the WT to UV and MMC-induced DNA damage, however the Δrpa3 mutant H1244 exhibited moderate sensitivity to both UV and MMC-induced DNA damage (Figure 4).

**FIGURE 2** | (A) Map of rpa1 operon indicating location of Δrpa1, Δrpap1, and Δrpa1 operon deletions, as well as the BspEI and NruI sites, and probes used to verify the deletions. (B) Southern blot of genomic DNA cut with BspEI and probed with flanking regions of rpa1 and rpap1, as shown in (A), to indicate deletion of rpa1 and rpap1, respectively. (C) Southern blot of genomic DNA cut with NruI and probed with flanking regions of rpa1 operon (rpa1 op.), as shown in (A), to indicate deletion of the rpa1 operon.
**FIGURE 3** (A) Map of rpa3 operon indicating location of Δrpa3, Δrpap3, and Δrpa3 operon deletions, as well as the AscI, MluI, Stul, and Xhol sites and probes used to verify the deletions. (B) Southern blot of genomic DNA cut with Stul and Xhol, and probed with flanking regions of rpa3, as shown in Figure 2A, to indicate deletion of rpa3. (C) Southern blot of genomic DNA cut with AscI and Stul, and probed with flanking regions of rpap3, as shown in Figure 2A, to indicate deletion of the rpap3 operon. (D) Southern blot of genomic DNA cut with MluI and probed with flanking regions of rpa3 operon (rpa3 op.), as shown in Figure 2A, to indicate deletion of the rpa3 operon.

**RPAP3 BUT NOT RPAP1 FUNCTIONS IN DNA REPAIR**

Analysis of predicted protein domains indicates that RPA1 and RPA3 both possess zinc finger domains, and that RPA1 has three OB-folds compared to the single OB-fold present in RPA3 (Figure 1). Both COG3390 RPAPs RPAP1 and RPAP3 possess a single OB-fold suggesting a possible role in DNA binding. The RPA1 phosphoesterase (RPE) has a calcineurin-like phosphoesterase domain, and was not investigated individually. However, our results and those of Skowyra and MacNeill (2012) show that rpe is a non-essential gene.

To study the roles of RPAP1 and RPAP3 in DNA repair, Δrpa1 (H1216) and Δrpap3 (H1410) mutants were generated, both using trpA-marked deletion constructs (Figures 2 and 3, respectively). As with Δrpa1 strain H1217, the Δrpa1 mutant H1216 showed no increased sensitivity to UV irradiation or to MMC-induced DNA damage. However, the Δrpap3 deletion mutant H1410 was hypersensitive to both types of DNA damage, and the level of sensitivity was similar to that exhibited by the single Δrpa3 and Δrpap3 mutants H1244 and H1410, respectively (Figure 4). This result suggests that RPA3 and RPAP3 function in the same pathway(s) of DNA repair.

**REDUNDANCY BETWEEN RPA1 AND RPAP3 OPERONS**

In order to test for redundancy between the two RPAs, an attempt was made to generate a double Δrpa1 operon Δrpap3 operon deletion. This involved constructing the strain H1282, which contained the pop-in of a trpA-marked Δrpa3 operon construct (pTA1207) in an unmarked Δrpa1 operon background (H1246). An episomal plasmid (pTA1265), marked with pyrE2 and providing in trans expression of the rpa1 operon was used for complementation during the pop-out step (note that this episomal plasmid is lost during counter-selection with 5-FOA). Neither of the two pop-outs generated from this strain (H1390) yielded the desired Δrpa1 operon deletion (Figures 2 and 3, respectively); deletions of the rpa1 and rpa3 operons have previously been reported by Skowyra and MacNeill (2012). The Δrpa1 operon mutant showed no increased sensitivity to UV irradiation or to MMC-induced DNA damage. However, the Δrpa3 operon deletion mutant was hypersensitive to both types of DNA damage, and the level of sensitivity was similar to that exhibited by the single Δrpa3 and Δrpap3 mutants H1244 and H1410, respectively (Figure 4). This result suggests that RPA3 and RPAP3 function in the same pathway(s) of DNA repair.
Δrpa3 operon mutant (see Figure A1 in Appendix). This indicates that the cell requires either RPA1 or RPA3 (and/or their respective RPAPs) for survival.

Next we attempted to generate Δrpa1 Δrpa3 and Δrpa3 Δrpa1 deletion mutants. This would test whether the RPAPs can complement each other, or whether they are instead specific for their respective RPAs. The trpA-marked Δrpa3 construct (pTA1284) was used in an unmarked Δrpa1 background (H1280), and the trpA-marked Δrpa3 construct (pTA1207) was used in an unmarked Δrpa1 background (H1281). In both cases two pop-outs were generated but none proved to be the desired deletions (see Figure 1 in Appendix). This suggests that the putative RPA:RPAP complex is dependent upon specific RPA:RPAP interactions for functionality.

CONSTRUCTION OF PROTEIN OVEREXPRESSION STRAIN WITH C-TERMINAL TRUNCATION OF CDC48D

In a previous publication (Allers et al., 2010), we constructed a strain of *H. volcanii* where the histidine-rich pitA gene is replaced by the ortholog from *N. pharaonis*. The latter protein lacks the histidine-rich linker region found in *H. volcanii* PitA and does not co-purify with His-tagged recombinant proteins. The absence of Hvo-PitA revealed an additional co-purifying protein, which we identified as Cdc48d (HVO_1907) and features a histidine-rich C-terminus (Figure 5A). We were unable to delete cdc48d, indicating that this gene is essential (Allers et al., 2010). The presence of this contaminating protein was problematic for purification of His-tagged RPA1 and RPAP1, due to similar molecular weights (Cdc48d, 53 kDa; RPA1, 46 kDa; RPAP1, 65 kDa).

All orthologs of Cdc48d from haloarchaea feature a histidine-rich C-terminus, however Cdc48d from *Halocarcula marismortui* and *H. salinarum* have only three and four histidines, respectively, compared to six in *H. volcanii* (Figure 5A). Therefore, we replaced the *H. volcanii* cdc48d gene in H1209 (Allers et al., 2010) with orthologous genes from *H. marismortui* and *H. salinarum*, generating *H. volcanii* strains H1405 and H1333, respectively. Unfortunately these strains grew poorly and were not suitable for recombinant protein overexpression. Instead, we generated a truncated allele of *H. volcanii* cdc48d, encoding a Cdc48d protein lacking the histidine-rich C-terminus (Cdc48d-Ct; Figure 5A). The cdc48d-Ct allele was used to replace the *H. salinarum* cdc48d gene in *H. volcanii* H1333, generating H1424 (Figures 5B,C). This strain exhibits normal cell growth and the Cdc48d-Ct protein no longer co-purifies with His-tagged recombinant proteins (Figure 5D). A number of minor histidine-rich contaminants are now apparent, which have been identified by mass spectrometry.

DIRECT RPAP INTERACTION WITH RESPECTIVE RPA

The genetic analysis of rpa1 and rpa3 and their respective rpa genes indicates not only that RPA3 and RPAP3 function in the same DNA repair pathway(s), but also that they function together as a specific RPA:RPAP complex. To establish whether this is achieved via a direct RPA:RPAP interaction, affinity pull-downs were employed (Allers et al., 2010). The rpa1 and rpa3 operons were cloned under control of the tryptophanase promoter in plasmid pTA963, where either the RPA or the RPAP was tagged with a hexahistidine tag.

Histidine-tagged RPA1 and RPA3 pulled down their respective RPAPs, and histidine-tagged RPAP1 and RPAP3 pulled down their respective RPAs (Figure 6). However, histidine-tagged RPA1 did not pull down RPAP3, and vice versa. This confirms that the RPAs interact specifically with their respective RPAPs, supporting our conclusions based on the failure to generate Δrpa1 Δrpa3 and Δrpa3 Δrpa1 deletion mutants. Neither RPA1 nor RPA3 pulled down RPA2, and histidine-tagged RPA2 did not pull down RPA1 or 3, or either of the RPAPs (data not shown). This supports the suggestion that the three RPAs of *H. volcanii* do not form a heterotrimer as observed in eukaryotes and *P. furiosus*, but instead form three separate ssDNA-binding factors.
FIGURE 5 | (A) Protein sequence alignment of C-terminus of Cdc48d from selected species of haloarchaea (Hvo, *H. volcanii*; Hsa, *Halobacterium salinarum*; Hma, *Haloarcula marismortui*; Hwa, *Haloquadratum walsbyi*; Hla, *Halorubrum lacusprofundi*; Nph, *N. pharaonis*; Hvo-Ct, *H. volcanii* C-terminal truncation Cdc48d-Ct). Histidine residues are indicated by a black background. (B) Colony hybridization of 5-FOA-resistant clones of *H. volcanii* H1333, after pop-in/pop-out gene replacement with pTA1294. *H. salinarum* cdc48d sequences (Hsa-cdc48d) were used as a probe, clones failing to hybridize therefore carry the truncated *H. volcanii* cdc48d-Ct allele present in pTA1294. (C) Verification of truncated cdc48d-Ct allele in H1424 by PCR (488 bp product), with primers specific to either *H. volcanii* or *H. salinarum* genes. H1209 genomic DNA was used as a control for wild-type *H. volcanii* cdc48d (563 bp product), and H1333 was used as a control for *H. salinarum* cdc48d (560 bp product). (D) *H. volcanii* strains H1209 and H1424 containing empty vector pTA963 (Allers et al., 2010) were used in mock protein overexpression. Histidine-rich cellular proteins were purified from the soluble fraction (lysate) by affinity chromatography on a Ni²⁺ chelating column, samples were taken from the flow-through (flow) and bound proteins were eluted using 50 and 500 mM imidazole. Precipitation using trichloroacetic acid and deoxycholate was used to enhance visualization and identification of the eluted proteins by mass spectrometry. Cdc48d (HVO_1907) eluted from cell extracts of H1209 but not from H1424 (cdc48d-Ct).
DISCUSSION

There is a unifying theme in archaea of a great variety in the number and type of proteins involved in DNA replication, and repair, whose counterparts in eukaryotes are much more uniform. This has been shown to be the case for RPA, where eukaryotes possess three subunits that all form unified clades in a phylogenetic analysis, but in archaea the number and structure of subunits varies widely. Some euryarchaea possess differing numbers of RPA subunits and some possess differing numbers of RPAs and RPAPs. Crenarchaea also possess varying numbers of SSB, however in both euryarchaea and crenarchaea none of the RPAs, RPAPs, or SSBs fall into unified clades. Again this is seen in the case of MCM, where eukaryotes possess six MCM subunits that each form unified clades, but in archaea there is a vast range in the number of MCM subunits, differing between individual species, and none of which fall into uniform clades (Chia et al., 2010). Characterizing the RPA-RPAP complexes of *H. volcanii* will shed light on how the RPAs and RPAPs function together in binding and stabilizing ssDNA. This in turn will provide insight for other RPAs and RPAPs in archaea, but also offer reasoning behind the driving force of such non-uniform evolution of archaea.

The genetic and biochemical analysis presented here indicates the three RPAs of *H. volcanii* do not form a heterotrimeric complex as in *P. furiosus* and eukaryotes. Instead, RPA1 and RPA3 form complexes with their respective RPAPs. Unlike *rpa2*, both *rpa1* and *rpa3* genomic deletions were generated with relative ease, showing that the latter are not essential for cell survival and supporting the hypothesis that the three RPAs do not form a heterotrimeric complex.

The ease at which the *rpa1*, *rpap1*, and *rpa1* operon deletion mutants were made, coupled with a lack DNA damage sensitivity, signifies the *rpa1* operon does not play a major role in DNA replication or repair. The moderate DNA damage sensitivity shown by the individual *rpa3*, *rpap3*, and the *rpa3* operon mutants indicates that the efficient repair of UV and MMC-induced DNA damage requires the products of the *rpa3* operon but not the
Both single Δrpa3 and Δrpa3 Δrpa1 mutants showed a similar DNA damage sensitivity to each other, and to the rpa3 operon mutant, providing genetic evidence that RPA3 and RPAP3 act in the same DNA repair pathway. Furthermore, we were unable to generate Δrpa1 Δrpa3 and Δrpa3 Δrpa1 Δrpa31 deletion mutants, indicating that RPA1 could not substitute for RPAP3 (and vice versa), and suggesting that the RPA3 interacts specifically with RPAP3 (and likewise for RPA1 and RPAP1). However it is unclear what role the associated proteins play, since the presence of an OB-fold does not necessarily indicate direct ssDNA binding. Instead, the RPAPs may provide a platform for protein:protein interactions. This is seen in eukaryotes, where the RPA 14 kDa subunit possesses a single OB-fold, this subunit is essential for formation of the RPA heterotrimer by facilitating protein:protein interactions (Fanning et al., 2006).

The co-purification of histidine-tagged RPA1 and RPA3 with their respective untagged RPAPs (and vice versa) supports our hypothesis that H. volcanii RPA1 and RPA3 form complexes with their respective RPAPs. This observation, and the differing outcomes of rpa1, rpa2, and rpa3 deletions, indicates that the three RPAs of H. volcanii do not function as a heterotrimer. Similar results have been obtained in M. acetivorans, where the three RPAs are able to bind ssDNA individually, in addition to stimulating primer extension by M. acetivorans DNA polymerase BI in vitro. (Robbins et al., 2004).

This study has shown genetically and biochemically that RPAPs interact with RPA, and that this interaction is RPA-specific. This is the first report investigating the function of the archaeal COG3390 RPA-associated proteins (RPAPs), thus providing an important insight of the structure and function of H. volcanii single-strand DNA-binding proteins.

AUTHOR CONTRIBUTIONS
Amy Stroud and Thorsten Allers wrote the paper; Amy Stroud and Thorsten Allers designed the experiments; Amy Stroud and Thorsten Allers performed the microbiological and biochemical experiments; Susan Liddell carried out the mass spectrometry; Amy Stroud, Susan Liddell, and Thorsten Allers analyzed the data.

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cle distributed under the terms of the Creative Commons Attribution Non Commercial License, which permits non-commercial use, distribution, and reproduction in other forums, provided
the original authors and source are credited.
### Table A1 | Identification of proteins present in cellular soluble fraction after purification by affinity chromatography on a Ni²⁺ chelating column.

| Prot accession | Protein name          | HVO_# | Predicted MW | Observed MW | MASCOT score | Number of peptides | % coverage | Peptide sequences |
|----------------|-----------------------|-------|--------------|-------------|--------------|-------------------|------------|------------------|
| gi[292655491]  | RPAP1                 | 1337  | 64,829       | 57,781      | 671          | 12                | 18         | 8                |
| gi[292655492]  | RPA1                  | 1338  | 45,954       | 36,960      | 940          | 15                | 40         | 11               |
| gi[292655491]  | RPAP1                 | 1337  | 64,829       | 62,780      | 578          | 10                | 21         | 9                |
| gi[292655492]  | RPA1                  | 1338  | 45,954       | 45,686      | 808          | 15                | 40         | 12               |
| gi[292654471]  | RPAP3                 | 0291  | 21,979       | 15,074      | 435          | 10                | 53         | 7                |
| gi[292654471]  | RPAP3                 | 0291  | 21,979       | 16,217      | 484          | 10                | 59         | 8                |
| gi[292654472]  | RPA3                  | 0292  | 34,562       | 28,262      | 796          | 13                | 43         | 10               |
| gi[292654472]  | RPA3                  | 0292  | 34,562       | 31,741      | 735          | 13                | 48         | 11               |
| gi[292656508]  | Hypothetical protein  | 2381  | 52,319       | 55,300/50,200 | 240      | 5                | 11         | 5                |
| gi[292493992]  | Hypothetical protein  | 80053 | 13,897       | 4,73,600    | 259          | 4                | 33         | 3                |
| gi[292656425]  | Deoxyhypusine synthase | 2297  | 38,616       | 39,500      | 451          | 7                | 25         | 4                |
| gi[292655899]  | Thioredoxin reductase | 1758  | 36,505       | 38,000      | 312          | 6                | 21         | 6                |
| gi[292493992]  | Hypothetical protein  | 80053 | 13,897       | 36,300      | 208          | 6                | 40         | 4                |
| gi[292656448]  | Htr-like protein      | 2629  | 30,266       | 31,700      | 93           | 4                | 17         | 4                |
| gi[292654704]  | Ferritin              | 0536  | 19,892       | 24,900      | 66           | 1                | 6          | 1                |
| gi[292653907]  | Transcriptional regulator | A0389  | 20,201       | 22,300      | 586          | 15               | 54         | 8                |
| gi[292493992]  | Hypothetical protein  | 80053 | 13,897       | 13,500      | 170          | 3                | 33         | 3                |

**Prot Accession**, the database entry, e.g., gi[292655491]; predicted MW, predicted molecular weight (Da) of the protein sequence identified by MASCOT; observed MW, molecular weight estimated from migration on SDS-PAGE; MASCOT score, MASCOT score associated with protein identification; number of peptides, no. of peptides associated with protein identification by MASCOT; % coverage, % of the database sequence entry that is covered by the peptides matched to it in the MASCOT data. Peptide sequences, the number of distinct peptide sequences associated with the protein identified by MASCOT.
Map of rpa3 operon indicating location of Δrpa3, Δrpa3, and Δrpa3 operon deletions, as well as the Ascl and StuI sites and the probe used to verify the deletions. The size of this fragment in the wild-type (H195) is 8 kb. (B) Southern blot of genomic DNA cut with Ascl and StuI, and probed with flanking regions of rpa3 operon (rpa3 op.), as shown in (A), indicates failure to generate Δrpa1 Δrpa3 mutant as bands of the expected size for deletion are not seen (3.4 and 4.4 kb). (C) Southern blot of genomic DNA cut with Ascl and StuI, and probed with flanking regions of rpa3 op., as shown in (A), indicates failure to generate Δrpa1 Δrpa3 mutant as bands of the expected size for deletion are not seen (2.8 and 3.4 kb). (D) Southern blot of genomic DNA cut with Ascl and StuI, and probed with flanking regions of rpa3 op., as shown in (A), indicates failure to generate Δrpa1 operon Δrpa3 operon mutant as bands of the expected size for deletion are not seen (2.8 and 3.4 kb).