The single-strand DNA binding activity of human PC4 functions to prevent mutagenesis and killing by oxidative DNA damage.

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Human positive cofactor 4 (PC4) is a transcriptional coactivator with a highly conserved single-strand DNA (ssDNA) binding domain of unknown function. We identified PC4 as a suppressor of the oxidative mutator phenotype of the *Escherichia coli fpg mutY* mutant and demonstrate that this suppression requires its ssDNA binding activity. Yeast mutants lacking their PC4 ortholog Sub1 are sensitive to hydrogen peroxide and exhibit spontaneous and peroxide induced hypermutability. PC4 expression suppresses the peroxide sensitivity of the yeast *sub1Δ* mutant, suggesting that the human protein has a similar function. A role for yeast and human proteins in DNA repair is suggested by the demonstration that Sub1 acts in a peroxide-resistance pathway involving Rad2 and by the physical interaction of PC4 with the human Rad2 homolog XPG. We show XPG recruits PC4 to a bubble-containing DNA substrate with resulting displacement of XPG and formation of a PC4-DNA complex. We discuss the possible requirement for PC4 in either global or transcription-coupled repair of oxidative DNA damage to mediate the release of XPG bound to its substrate.

INTRODUCTION

Oxidative DNA damage and the mutations it causes have been implicated in a number of human diseases, including cancer and neurodegenerative diseases, and is a contributing factor to aging (6, 12, 35, 36). Thus, a thorough understanding of genes involved in the prevention and repair of oxidative DNA damage and its mutagenic consequences is important to our understanding of the mechanisms mitigating these diseases and exacerbating normal degenerative processes associated with aging.

Oxidative DNA damage results from the interaction of reactive oxygen species (ROS) with DNA. ROS are produced as by-products of normal aerobic metabolism and by exogenous factors, such as ionizing radiation and chemical oxidants (6, 12, 35, 36). The deleterious consequences of ROS to an organisms genetic material are held in check by proteins that prevent or repair oxidative DNA damage (7, 12, 13, 15, 21). Unrepaired oxidative lesions result in increased mutagenesis, lethality, and apoptosis (23, 27). A balance between DNA repair and damage prevention mechanisms and ROS production is required to maintain a low spontaneous mutation rate. Factors that increase ROS production, reduce ROS detoxification, or factors that affect repair of oxidative DNA lesions result in increased mutagenesis. This is best demonstrated in *E. coli*; mutations that inactivate the *fpg* and *mutY* genes, whose products repair the predominant oxidative lesion 8-oxoguanine (8-oxoG) and its mispaired intermediate 8-oxoG:A respectively, result in a mutator phenotype that specifically increases GC→TA transversion mutagenesis (37).

In this study, we screened a human cDNA library and describe the isolation and characterization of the human transcription *p*ositive *c*ofactor 4 (PC4) gene as a suppressor of oxidative mutagenesis in the *E. coli fpg mutY* strain. We demonstrate that PC4 and its yeast ortholog *SUB1* are required for resistance to hydrogen peroxide and function to prevent spontaneous and induced oxidative mutagenesis. The oxidation protection function of PC4 requires its single-strand DNA (ssDNA) binding activity, which is not required for the transcription co-activator function of PC4 (56, 57). A function of PC4 in repair of oxidative DNA damage is suggested by its physical and biochemical interactions with the multi-functional human DNA repair protein XPG, the structure-specific endonuclease activity of which is essential for nucleotide excision repair (NER) but which also plays important non-enzymatic roles in base excision repair (BER) and transcription-coupled repair (TCR) of oxidative damage (Tsutakawa and Cooper, 2000).

MATERIALS AND METHODS

Bacterial strains. The *lacZ* mutant *E. coli* strain cc104 and its isogenic derivatives MV4705 (cc104 *Δfpg::Tn10*), MV4707 (cc104 *ΔmutY::Cat*^{r}) and MV4709 (cc104 *Δfpg::Tn10 ΔmutY::Cat*^{r}) were constructed by P1 transduction selecting for the appropriate antibiotic resistance marker.

Bacterial mutagenesis assays.. The papillation assay medium (39) contains 0.2% D-glucose, 1X A salts, 1 mM $MgSO_4$, 5 μg/ml thiamine hydrochloride, 0.5 mM IPTG, 40 μg/ml X-Gal, 0.5 mg/ml P-Gal, 50 μg/ml carbenicillin and 2% agar. For pBAD24 induction, 0.2% L-arabinose (20) was added and the glucose concentration was reduced to 0.05%. Papillation was scored 5-6 days after plating. Spontaneous mutation frequencies (revertants/total cells) were quantitated by plating dilutions of the overnight cultures of individual transformants on lactose and glucose minimal medium, incubating 3 days at 37°C. LacZ revertants are detected on lactose plates, total colonies on glucose plates.

Construction of Fpg, hOGG1 and hMutY expression vectors. To overexpress the *E. coli* Fpg protein, a 1 kbp *Eco*RI/*Hind*III fragment from V243 (39) was subcloned into pTrc 99A (Pharmacia, Inc.) (pTrc-Fpg). To construct pTrc-hOGG1, a 5.7 kbp *Xba*I/*Hind*III fragment from pET8c-OGG1-1a (42) (Dr. Y. Nakabeppu, Kyushu Univ.) was subcloned into pTrc 99A. To construct pTrc-hMYH, a 4.5 kbp *Stu*I/*Bam*HI fragment from pT7blue-hMYHα3-2 (43) (Dr. Y. Nakabeppu, Kyushu Univ.) was subcloned into *Sma*I/*Bam*HI linearized pTrc 99A.

Construction of wild type and mutant PC4 expression vectors. Plasmids carrying the full-length coding sequences of the wild-type (PC4-wt) or ssDNA-binding defective mutants (PC4-W89A and PC4-β2β3) (57) were obtained from M. Meisterernst (Munich, Germany). The full-

length PC4 wild type and mutant protein coding sequences were isolated as *Nde*I and *Eco*RI fragments and subcloned into the corresponding sites in pET-28b(+) to attach the 6X histidine tag to their amino termini. The insert containing *Nco*I and *Sal*I fragments from the pET-28(+) derivatives were subcloned into the corresponding sites of pBAD24 for *araBAD* promoter-dependent expression. Expression of the 6X his-tagged PC4 proteins was verified by western blotting using the anti-Penta· His antibody (Qiagen).

Construction of yeast mutant strains. The *Saccharomyces cerevisiae* wild-type strain FY833 (*MATa his3Δ200 leu2Δ1 lys2Δ202 trp1Δ63 ura3-52*) and the *sub1Δ* mutant strain YMH476 (FY833 *sub1Δ::hisG*) (59) were obtained from M. Hampsey (Rutgers University). Additional *sub1Δ* mutants were constructed by PCR-mediated one-step gene replacement methods (8). Cells were cultured either in YPD (1% yeast extract, 2% peptone, 2% dextrose) or, for plasmid bearing strains, in synthetic complete medium lacking leucine or uracil (SC-leu or SC-ura). To express PC4 in yeast strains, the coding sequence for amino acids 40-127 was amplified by PCR using the PC4 cDNA clone initially isolated from the genetic screen as the template with primers PC4-N (acgc*gtcgac*ATGcaaaagacaggtgagacttcgagagctctg) and PC4-C (ccg*ctcgag*tcatcttacaaattcctctgc). The ATG initiation codon shown in upper case letters along with the italicized sequences for *Sal*I and *Xho*I restriction sites were added for protein expression and cloning purposes. The *Sal*I and *Xho*I treated PCR product was inserted into pMV611, which carries the LEU2 gene from pRS315 (49) and the GPD promoter from p426-GPD (41). To express the Sub1 protein, the *SUB1* ORF was amplified from the FY833 genomic DNA by PCR using primers X001-SUB1f (gc*tctaga*tgtcatattacaacaggtatagg) and E292-SUB1r (gc*gaattc*ttattcttcttcacttatgtcg). The italicized sequences for *Xba*I and *Eco*RI restriction sites were introduced to the ends of the PCR product for cloning into p416-GPD, which carries the URA3 gene for selection (41). MVY219

(*rad2Δ□□□RPι*) and MVY221 (*sub1Δ rad2Δ□□□□□ι*) RAD2 deletion strains were constructed by transforming FY833 (*wt*) and YMH476 (*sub1Δ*) with *Sal*I-digested pWS521 (W. Siede, Univ. North Texas Heath Science Center).

H_2O_2 sensitivity and induced mutagenesis. Yeast strains were grown to mid-log phase at 30°C, washed and resuspended in PBS. Cells were then treated with H_2O_2 by shaking at 30°C for 1 hr as indicated. Cells were harvested by centrifugation, washed and resuspended in sterile deionized H_2O, diluted and plated on YPD for survival analysis of non plasmid bearing strains, or SC-leu or SC-ura for plasmid bearing strains. Survival experiments were repeated from 3 to 10 times and representative data indicating the reproducible differences between strains are shown. To measure the *can1*^{r} mutagenesis, cells were plated on synthetic medium lacking arginine but containing 60 μg/ml canavanine and incubated at 30 °C for 3-4 days for survival and 4-5 for mutagenesis.

UV and MMS sensitivity tests. Yeast strains were grown to mid-log phase at 30°C, washed with water, suspended in PBS and incubated with the indicated concentrations of Methyl methanesulfonate (MMS; Sigma Chemical) for 1 hr at 30°C, diluted and plated on YPD plates. Surviving colonies were counted after 3 to 4 days incubation at 30°C. For UV dose response tests, approximately 1000 log phase cells were placed in each spot on YPD plates. Spots were irradiated with UV (λ=254 nm), in 30 J/m^2 increments using a Stratalinker UV crosslinker (Stratagene). Plates were then incubated in the dark at 30°C for 3-4 days.

Expression and purification of PC4 and XPG. Full length wild type PC4 was expressed in the *E. coli* BL21(DE3) strain and purified according to the methods of Ge et al. (18). The cDNA for human XPG (a generous gift from Stuart Clarkson) was inserted into a pFASTBAC vector,

expressed in High5 insect cells, and purified to 95% homogeneity essentially as described (16). For use only in the Far Western assays, a heart muscle kinase (HMK) recognition sequence tag, RRASV, was added at the C-terminus.

Protein-protein interaction assays. For Far Western analysis, human PC4 protein (1.5 μg), human Nth1 protein (2.1 μg) and *E. coli* EndoIII (3 μg) were separated on SDS-PAGE (pre-cast 4-20% gel, BioRad), transferred to a PVDF membrane, and stained with Ponceau S to visualize the proteins on the membrane. The membrane was then blocked with a blocking buffer (25 mM Hepes-KOH (pH 7.7), 25 mM NaCl, 5 mM $MgCl_2$, 1 mM DTT, 1% nonfat milk and 0.1% NP-40) for 2 hr at 4 ^{0}C and probed overnight at 4 ^{0}C on a rocker with blocking buffer containing 150 mM KCl and ^{32}P-labeled XPG with a heart muscle kinase (HMK) tag . The membrane was washed with washing buffer (25 mM Hepes-KOH (pH 7.7), 25 mM NaCl, 150 mM KCl, 5 mM MgCl2, 1 mM DTT, 1% nonfat milk and 0.5% NP-40), and interactions were visualized by both phosphorimager and autoradiography. For slot-blot analysis 1 μg each of PC4, hNth1 and EndoIII were applied to a nitrocellulose membrane, and the membrane was processed as above.

Electrophoretic mobility shift assay. The sequence of the oligonucleotides used to form the bubble DNA substrate for the electrophoretic mobility shift assay were as follows, with the central unpaired region of the substrates highlighted in bold: 10T-strand,

5’-GGGCAGACAACGTGGCGCTG**TTTTTTTTTT**GTGTCCTAGCACAGCGTATG-3’ and 10C-strand,

5’-CATACGCTGTGCTAGGACAC**CCCCCCCCCC**CAGCGCCACGTTGTCTGCCC-3’.

The 10T-strand was 5'-end labeled with T4 polynucleotide kinase and (γ-^{32}P) ATP and annealed with the complementary 10C-strand by heating for 3 min. at 90 ^{0}C and cooling to room temperature. The resulting DNA bubble substrate was gel purified. Fifty fmol of ^{32}P-labeled bubble-DNA was incubated at room temperature with XPG, PC4, or both for 20 min in a 20 µl reaction mixture containing 10 mM Hepes-KOH (pH 7.5), 110 mM KCl, 1 mM EDTA, 1 mM DTT, 4% glycerol, and 0.2 µg poly[d (I.C)-d (I.C)]. After the incubation, the samples were loaded on a 4.5% non-denaturing polyacrylamide gel (19:1, acrylamide:bis-acrylamide). Electrophoresis was performed under refrigeration at 150 V for 2 h in 0.5 X TBE buffers . The gels were analyzed by phosphorimager and ImageQuant software (Molecular Dynamics).

RESULTS

Suppression of the *E. coli* oxidative mutator phenotype The *fpg mutY* strain of *E. coli* was used to screen for human cDNAs that express proteins that either prevent spontaneous DNA oxidation or repair the DNA lesions produced. This *E. coli* strain is a spontaneous mutator because Fpg repairs 8-oxoG, the predominant oxidative DNA lesion, and MutY removes A mispaired with 8-oxoG; a high frequency mispairing event during replication of template 8-oxoG lesions resulting in GC→TA transversion mutations (37, 38, 40). Production of ROS by normal metabolic processes leads to accumulation of 8-oxoG lesions in DNA and results in elevated spontaneous mutagenesis (37). The GC→TA transversions are detected using the CC104 allele of *lacZ*, which reverts only by GC→TA transversion (14). On indicator medium, *E. coli* cells carrying *lacZ*(cc104) produce distinctive white LacZ^{-} colonies containing dark blue LacZ^{+} revertant microcolonies, or papillae (Fig. 1) (37). Fig. 1 compares the mutator phenotype of the *fpg mutY* double mutant strain (Fig.

1B) with that of wild type *E. coli* (Fig. 1A) and shows the suppression of mutagenesis resulting from the expression of *E. coli* and human DNA repair genes. Complete suppression of the *fpg mutY* strains mutator phenotype is seen upon high level expression of the bacterial *fpg* gene (Fig. 1 C), reducing the frequency of transversions to levels below that seen in wild type (Fig. 1 A). Since most of the transversion mutations seen in the wild type strain can also be prevented by *mutM* overexpression (60), GC→TA transversions can be considered a signature of oxidative DNA damage. Suppression of the *fpg mutY* mutator phenotype is also seen upon expression of the human 8-oxoG glycosylase, OGG1 (Fig. 1D), or the human mutY ortholog, MYH (Fig. 1E) (1, 3, 43, 46, 48, 50).

Isolation of the human PC4 gene. The PC4 gene was isolated by transforming the *E. coli fpg mutY* strain with a human cDNA library constructed in a bacterial expression vector (45, 54), then screening individual transformed colonies for suppression of the spontaneous mutator phenotype. PC4 is one of the strong antimutators identified (Fig. 1F). Expression of PC4 provides complete mutation suppression under these conditions, as confirmed by a quantitative mutagenesis assay (Table 1).

PC4 is a transcription cofactor mediating activator-dependent transcription by RNA polymerase II (19, 32) through interactions with sequence-specific activators and TFIIA of the basal transcription machinery (19, 32). PC4 encodes a polypeptide of 127 amino acids (a.a.) (Fig. 2). Functional deletion analyses revealed a bipartite structure of PC4 comprising an amino-terminal regulatory domain (a.a. 1-62) and a carboxyl-terminal, ssDNA binding/dimerization domain (CTD; a.a. 63-127) (10, 28, 56, 57). The X-ray crystal structure of the PC4-CTD shows that it forms a dimer with two ssDNA binding channels running in opposite directions (10). Previously *in vitro* transcription studies showed that a peptide comprised of a.a. 22-87 of PC4 is necessary and

sufficient for co-activation (28, 32, 56, 57), and that the lysine rich motif between amino acids 22 and 41 is required for transcription activation (26, 28). It has also been demonstrated that inactivation of the ssDNA binding activity does not affect the ability of PC4 to function in transcription activation (57).

The PC4 cDNA clone isolated in this study is truncated at its 5' end, lacking the codons for the first 39 amino acid residues, but containing a short heterologous DNA sequence of unknown origin encoding MPSNSAPAHGTSS fused to glutamine 40 of PC4. The N-terminal truncation of PC4 removes the lysine rich motif required for coactivation (28), but leaves intact the ssDNA binding and dimerization domains (10, 56). This suggests that the ssDNA binding and dimerization motifs alone are sufficient for PC4 to function as an oxidative antimutator protein in *E. coli*. To examine this further and to rule out effects of the heterologous upstream sequence, we obtained the cloned full length wild type PC4 gene, transferred it into the L-arabinose inducible pBAD24 bacterial expression vector (20), and tested its ability to function as an antimutator. Fig. 3A shows that wild type PC4 is able to fully suppress the mutator phenotype of the *E. coli fpg mutY* strain, indicating that the full length and truncated fusion forms of PC4 isolated in this study are functionally similar.

ssDNA binding activity of PC4 is required for suppression of oxidative mutagenesis. Since the PC4 clone we originally isolated contained only the ssDNA binding and dimerization domains of PC4, we tested if ssDNA binding activity is required for the antimutator activity in the *fpg mutY* strain of *E. coli* by comparing the β2β3 and W89A ssDNA binding deficient mutants of PC4 constructed by Werten et al. (56) with the full length, wild type PC4. In the absence of L-arabinose, no protein expression was detectable by western blot (Fig. 3B, left panel) and no

mutation suppression was detected (Fig. 3A, upper panels). In the presence of inducer, all forms of PC4 were expressed equally well (Fig. 3B). However, compared to wild type PC4, the ssDNA binding deficient mutants were severely impaired in their ability to suppress the oxidative mutator phenotype when expressed in the *fpg mutY* strain (Fig. 3A), thus demonstrating that the ssDNA binding activity is required for the antimutator function of PC4 in the bacterial assay and that the wild type protein functions as well as the truncated form of PC4 in this assay.

Yeast *sub1Δ* mutants are hypersensitive to hydrogen peroxide. To determine if PC4 functions to prevent oxidative mutagenesis in eukaryotes, we turned to yeast genetics. Sequence analysis reveals that PC4 orthologs exist in all sequenced eukaryotic genomes and that the most conserved region is the C-terminal ssDNA binding and dimerization domains. The *S. cerevisiae* PC4 ortholog, termed Sub1 (31) or Tsp1p (22), shows 48% identity and 58% similarity when compared with the C-terminal region (a.a residues 63-127) of PC4 (2). Like its human ortholog, Sub1 is involved in various aspects of transcription, but is not essential for viability (11, 22, 31, 59).

To determine if Sub1 plays a role in oxidation protection, we tested yeast *sub1Δ* mutants for phenotypes associated with oxidative stress, DNA damage, or repair. Fig. 4A shows that the yeast *sub1Δ* mutant (59) obtained from M. Hampsey (Rutgers University) is extremely hyper-sensitive to hydrogen peroxide compared to its wild-type parent. To confirm this, we disrupted the *SUB1* gene in two other laboratory strains, W303-1B (lab strain) and RDKY3023 (R. Kolodner, UC San Diego), and found similar degrees of sensitization (data not shown). Reintroduction of the wild type *SUB1* gene on the p416-GPD yeast expression vector (41) fully restores peroxide resistance to the *sub1Δ* mutant, demonstrating that the observed peroxide sensitivity is solely due to the *sub1Δ*

mutation (Fig. 4A). In contrast, the *sub1Δ* mutant strain does not cause increased sensitivity to methylation or UV treatments (Fig. 5), suggesting that Sub1 does not play a role in either NER or BER of alkylation damage but rather is specific for protection from oxidative DNA damage.

hPC4 can suppress the peroxide sensitivity of the yeast *sub1Δ* mutant. To determine if the human PC4 gene can function to protect against oxidative DNA damage, we tested if it suppresses the peroxide sensitivity of the yeast *sub1Δ* mutant strain. We constructed a plasmid that expresses the truncated form of PC4 by adding an ATG codon to the 5' end of PC4 beginning with the glutamine 40 codon. This construct corresponds to the PC4 coding sequence of the truncated form of PC4 originally isolated (see Fig. 2). Expression of this clone in yeast results in a complete restoration of peroxide resistance (Fig. 4B), indicating that the truncated form of PC4 can fully substitute for the yeast *SUB1* gene and strongly suggests that the human PC4 gene, like its yeast counterpart, functions in oxidation protection.

The yeast *sub1Δ* mutation increases spontaneous and induced mutagenesis. Since many mutations affecting peroxide sensitivity also affect spontaneous and peroxide induced mutagenesis, we tested if the *sub1Δ* mutation has such effects by measuring the forward mutation frequency to canavanine resistance. Canavanine resistance can result from a wide variety of genetic changes including GC→TA transversions and OGG1 mutants of yeast show a 7-fold increase in spontaneous canavanine resistance mutagenesis (52). Fig. 6 shows that the *subΔ* mutant exhibits a two-fold increase in both spontaneous and peroxide-induced mutagenesis, indicating that Sub1 protects against mutations arising from the low spontaneous production of endogenous ROS and exposure to high levels of exogenous hydrogen peroxide.

***rad2Δ* partially suppresses peroxide sensitivity of *sub1Δ*.** A non-enzymatic function of XPG is essential for transcription-coupled repair of oxidative damage (Le Page et al., 2000) and has also been implicated in transcription *per se* (9, 34). XPG also stimulates initiation of BER by the NTH1 glycosylase that removes oxidized pyrimidines, again in a non-enzymatic capacity (4, 30, 33). Furthermore, XPG also interacts with and stimulates APE1, the major human AP endonuclease activity in BER, and appears to coordinate NTH1 and APE1 function in vitro (B. Haltiwanger and P.K. Cooper, unpublished results). Because PC4 and Sub1 also have roles in both transcription and resistance to oxidative damage, we wondered whether they might interact with XPG and its yeast homolog Rad2, respectively. We therefore constructed a *sub1Δ rad2Δ* double mutant strain in order to see if these mutations are epistatic or result in increased peroxide sensitivity. Interestingly, the *rad2Δ* mutation partially rescues the *sub1Δ* mutant strain, reducing its peroxide sensitivity (Fig. 7). This finding suggests an interplay between Rad2 and Sub1 in minimizing oxidative damage. In particular, it raises the possibility that in the absence of Sub1, an activity of Rad2 responding to oxidative damage is deleterious.

Direct interaction of PC4 with XPG. Since Sub1 evidently functions in a pathway involving Rad2, we tested the possibility of direct interaction between the human PC4 and XPG proteins by Far Western analysis. To test PC4-XPG interaction, ^{32}P-labeled XPG was used as a probe for PC4 transferred onto a membrane from an SDS-PAGE gel. A strong signal appears at the position corresponding to PC4, indicating that XPG and PC4 directly interact in the absence of DNA (Fig. 8A). Under these conditions, XPG bound the positive control human NTH1 but did not bind its *E. coli* homologue EndoIII, the negative control. To eliminate the possibility that the

interaction might be due to denaturation of PC4 during the electrophoresis step, we also tested native PC4 bound directly to a membrane in a slot blot assay (Fig. 8B). Notably, the interaction of PC4 and XPG is preserved, providing additional evidence for its specificity.

PC4 binding to bubble substrates is enhanced by XPG. The preferred DNA substrates for binding by PC4 are DNA bubble structures (56). Perhaps not coincidentally, XPG also binds stably, specifically and with high affinity to DNA bubbles resembling in size the open regions associated with transcription (A.H. Sarker, S.E. Tsutakawa, and P.K. Cooper, unpublished results). In order to determine if these two proteins compete for this substrate or interact synergistically, we tested if the presence of XPG affects binding by full length PC4 protein to a DNA bubble substrate having a central 10 bp unpaired region. XPG alone bound to this substrate to form a slowly-migrating complex (Fig. 9A, lane 2). In the absence of XPG, PC4 also bound specifically to the 10 bp DNA bubble (Fig. 9A, lanes 9-13), with complete binding at 352 nM (lane 12). We also observed an additional shifted band that appears only at the highest concentration assayed, 704 nM (Fig 9A, lane 13). Since it is known that PC4 binds as a dimer (Werten et al. 1998b), we postulate that the slower-migrating shifted species represents a double complex of PC4 dimers bound to DNA as has been previously observed by others (Werten et al., 1998b). To determine if XPG affects PC4 interaction with DNA bubbles, we pre-incubated the 10 bp bubble DNA with XPG and then added PC4 at varying concentrations (Fig. 9A, lanes 3-7). XPG strongly stimulated PC4 binding, with complete binding achieved at the lowest PC4 protein concentration analyzed (44 nM, lane 3) as compared to 352 nM in the absence of XPG. Quantitation from the phosphorimage in Fig. 9A of the amount of substrate shifted as a function of PC4 concentration reveals dramatically increased binding of PC4 in the presence of XPG (Fig. 9B). Correspondingly, the PC4 tetramer-bound complex appears at lower concentrations (352 nM, lane

6) in the presence of XPG. Interestingly, in no case did we observe a trimeric complex of XPG, PC4, and DNA. At approximately equimolar ratios of PC4 and XPG (44 nM, lane 3), there appears to be a complete replacement of XPG by PC4 in the protein-bound DNA complexes. This observation is significant, since XPG itself remains stably bound to bubble DNA substrate even in the presence of high concentrations of competitor or other DNA binding proteins with which XPG interacts (Sarker, Tsutakawa, and Cooper, unpublished results). To test if PC4-mediated displacement of XPG is dependent upon the order of addition, the proteins were added in either order, or simultaneously. In all cases, only the complexes migrating to the positions detected when PC4 alone is added are seen (Fig. 9C, lanes 3-5). These results strongly suggest that XPG recruits PC4 to bubble-containing DNA substrates and that binding of PC4 displaces XPG from the bubble substrate.

DISCUSSION

In this study we identified PC4 as a human protein capable of suppressing the oxidative mutator phenotype of the *fpg mutY* strain of *E. coli.* We characterize the PC4 gene and its yeast ortholog, demonstrating that a yeast mutant devoid of its PC4 ortholog, *SUB1,* is sensitive to hydrogen peroxide, exhibits a spontaneous mutator phenotype, and is hypermutable upon treatment with hydrogen peroxide. These results indicate that Sub1 is a eukaryotic peroxide resistance protein. The result demonstrating that expression of the human gene can restore peroxide resistance to the *sub1Δ* mutant of yeast suggests a similar function for the human PC4 protein. These observations, combined with our previous results (54), demonstrate the utility of the bacterial screen for the identification of unique human oxidation resistance proteins.

The previously identified transcriptional coactivator function of PC4 does not require the ssDNA binding activity (56, 57) contained in the most highly conserved region of this family of proteins. A novel function in DNA repair for the ssDNA binding activity of PC4 is indicated by the result that the truncated form of human PC4, lacking sequences required for transcription coactivation, can function to suppress oxidative mutagenesis in bacteria and can complement the peroxide sensitivity of a yeast *sub1Δ* mutant. This conclusion is further supported by the observation that the DNA binding-defective mutant forms of human PC4 are incapable of functioning as antimutators in the bacterial oxidative mutagenesis assays. Thus, we propose that PC4 functions both in transcription and in repair of oxidative DNA damage. These two functions are genetically separable; mutations in PC4's amino terminal domain affect primarily transcription coactivation and mutations in its ssDNA binding domain affect primarily DNA repair.

Taken together, the observations that Sub1 functions in a repair pathway involving Rad2 and that PC4 directly and functionally interacts with the DNA repair protein XPG suggest a role for PC4 in repair of oxidative damage. XPG functions in multiple DNA repair pathways in human cells. Both its enzymatic activity as a structure-specific endonuclease and a non-enzymatic function evidently involve interactions with other proteins required in NER (16, 55). In addition, a non-enzymatic function of XPG is required for TCR of oxidative DNA damage, evidently at an early step presumably involving recognition of RNA polymerase stalled at a lesion (Le Page et al., 2000). XPG also both stimulates BER enzymes in vitro through direct interactions (Klungland, et al., 1999a; Bessho, 1999; B. M. Haltiwanger and P.K. Cooper, unpublished results) and stimulates removal of oxidative lesions in the cell (Le Page et al., 2000). A role for PC4

in NER is unlikely, because we have shown that deletion of its yeast homolog does not affect sensitivity to UV. Thus the interaction of PC4 with XPG in repair of oxidative damage could conceivably affect either global BER or TCR. The stable, specific binding of XPG to dsDNA containing unpaired regions is functionally separate from its structure-specific endonuclease activity ((25); A.H. Sarker, S.E. Tsutakawa, and P.K. Cooper, unpublished results), and an attractive possibility is that its preferential binding to transcription-sized bubble is relevant to the TCR function of XPG. In TCR, rapid preferential repair is initiated on transcribed strands after an RNA polymerase is stalled at a DNA lesion, and it is thought that the RNA polymerase must be removed or remodeled in order for the repair enzymes to gain access to the lesion (for review see: (51). XPG is apparently required along with CSB and TFIIH for this early step in TCR (Le Page et al., 2000; Tsutakawa and Cooper, 2000). Our finding that XPG bound to a DNA bubble substrate recruits PC4 to the complex with resulting displacement of XPG suggests the possibility that PC4 may be involved in TCR at a step immediately following XPG.

The observed reduction of peroxide sensitivity seen when the *sub1Δ rad2Δ* double mutant is compared with the *sub1Δ* single mutant strain is consistent with the idea that Rad2 produces a potentially lethal intermediate in repair of oxidative damage that requires Sub1 for efficient further processing. Inability to release Rad2 from the partially repaired lesion (or lesion plus stalled RNA polymerase) may block access to proteins required to complete subsequent steps of repair. According to this model the accumulation of unrepaired or inefficiently repaired intermediates leads to increased lethality in the *sub1Δ* mutant. Blocking production of the intermediates by eliminating Rad2 improves survival because the initial damage is not as lethal as the DNA repair intermediate. It should be

noted that the effect of the *rad2Δ* mutation is only partial, suggesting that Sub1 may either have additional functions in DNA repair, or may perform similar functions for other DNA repair enzymes.

In the context of this model, our finding that PC4 displaces XPG that is stably bound to a DNA bubble structure suggests the possibility that PC4 is required in TCR for release of XPG to allow subsequent processing either of the lesion itself or of the stalled RNA polymerase. Significantly, a requirement for an XPG release factor in NER has recently been suggested by the results of Riedl et al., who found that XPG was not released from the DNA substrate after excision of the lesion *in vitro* without the addition of an unknown factor present in nuclear extracts (47). While this NER release factor is presumably not PC4 since deletion of SUB1 does not result in sensitivity to UV, it is possible that XPG similarly is not released on its own after its function in TCR but requires PC4 in order to turn over. The observation that PC4 stimulates XPG release is particularly intriguing in light of results demonstrating that PC4 can stimulate DNA synthesis via an interaction with RPA protein (44). A possible explanation combining all these results is that PC4 may function in TCR as an intermediary between RNA polymerase removal or remodeling by XPG together with other TCR proteins, possibly clearing the initial TCR machinery from the damaged region, and subsequent steps including recruitment of repair enzymes and synthesis of the repair patch. The observation that PC4 blocks RNA polymerase elongation in vitro and the ability of TFIIH to alleviate this block (17, 57) raises the possibility that PC4 may also have additional functions in TCR that affect the resumption of transcription. Clearly the close association of PC4 with other transcriptional processes and components of the transcription machinery makes a possible role for PC4 in transcription-coupled DNA

repair processes particularly interesting. However, it is presently unclear if PC4 functions in XPG-stimulated BER, XPG-dependent TCR of oxidative DNA damage, or both, and further experimentation will be required to elucidate its possible roles in these processes. In this connection it should be noted that the lack of sensitivity of the *sub1* mutant to UV does not rule out a requirement for PC4 in TC-NER (TCR of UV damage), since loss of TCR by deletion of RAD26 (the yeast homolog of CSB) does not render yeast sensitive to UV (53). Thus the postulated role for PC4 in TCR as a release factor for XPG could conceivably apply to transcription-coupled repair of either UV or oxidative lesions.

The function of PC4 in XPG-related DNA repair processes does not readily explain the ability of this protein to function in the *E. coli* oxidative antimutator assay. However, since the highly sensitive *E. coli* antimutator assay requires only a small number of repair events per cell per generation to reveal suppression of spontaneous mutagenesis, the repair enhancing activity of PC4 need not be very efficient. The activity we observe could be due to a general affect of PC4 binding to damaged DNA, or to unpaired DNA regions produced as intermediates of repair, creating a more accessible environment for additional *E. coli* DNA repair factors. It is unlikely that protein-protein interactions between human PC4 protein and bacterial DNA repair proteins would be functional, and moreover *E. coli* does not encode any proteins homologous to PC4. While the interaction of PC4 with XPG implicates it in an XPG-dependent DNA repair pathway in human cells, it may also function in a more general fashion to stimulate, stabilize, or assist DNA repair by other factors via a direct interaction with DNA, as it evidently can do in *E. coli*. Expression of hPC4 in either wild type of the *fpg mutY* strains of *E. coli* does not result in enhanced resistance to the lethal effects of hydrogen peroxide exposure (data not shown).

However, since *fpg* and *mutY* mutations have little or no effect on peroxide lethality (unpublished observations), it is unclear if this observation indicates a specificity of PC4 for repair of non-lethal lesions, such as 8 oxoG, or that the DNA repair activity required for mutation suppression when PC4 is expressed in bacteria is weak.

Sub1 mutations in yeast result in increased spontaneous and induced oxidative mutagenesis and lethality. Since expression of the PC4 protein in yeast can restore the wild type phenotype, it suggests that the human PC4 protein may also function to prevent mutations resulting from oxidative DNA damage in human cells. Mutations that cause increased spontaneous and damage induced mutagenesis cause an increased risk of cancer (24). PC4 maps to chromosome 15p13, a region that frequently suffers from loss of heterozygosity in bladder and lung tumors (5, 58). This has been interpreted to suggest that the regulatory properties of PC4 may be important in tumor suppression, and support for this hypothesis has been presented (29). However, our findings suggests an alternative, or additional, mechanism for PC4 in tumor suppression. Loss of PC4 function may also increase the rate of mutagenesis resulting from spontaneous or induced oxidative DNA damage in humans. This can increase the level of mutations leading to transformation, or secondary mutations within tumors leading to tumor promotion.

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Table 1. Mutation frequency of *mutM mutY* strains expressing bacterial and human DNA repair genes.

Strain	Genotype	Plasmid	Mutation Frequency (mutants/10^8 Cells)[a]
MV4724	Wild Type	pTrc99A vector	3
MV4755	*mutM mutY*	pTrc99A vector	2750
MV4763	*mutM mutY*	pTrc99A-*mutM*	0
MV4761	*mutM mutY*	pTrc99A-hOGG1	11
MV4762	*mutM mutY*	pTrc99A-hMYH	58
MV4722	*mutM mutY*	pS380[b]-PC4	0

[a]Representative data are shown.

[b]pSE380 is identical to pTrc99A except for the presence of additional cloning sites in the vector.

FIG. LEGENDS

Fig. 1. Mutator phenotype of *E. coli fpg mutY* and its suppression by bacterial and human DNA repair genes. Upper panels show the phenotype of (A) wild type E. coli cc104 carrying the GC→TA transversion specific allele of *lacZ*, and (B) its isogenic *fpg mutY* double mutant derivative. The antimutator activity resulting from (C) expression of bacterial *fpg* protein (*E. coli fpg mutY/pfpg*); (D) expression of the human 8-oxoG DNA glycosylase OGG1 (*E. coli fpg mutY/phOGG1*); (E) expression of the human MutY ortholog hMYH (*E. coli fpg mutY/phMYH*); and (F) expression of the truncated form of PC4 isolated in this study (*E. coli fpg mutY/*pSE380-PC4).

Fig. 2. Structure of PC4 and its derivatives. The upper panel shows the domains of wild type PC4. The protein region designated amino acids 22-87 is the minimal coactivator clone described by Kaiser et al., (28) and is shown for comparison. The initial PC4 clone is the form of PC4 initially isolated in our screen. The white amino-terminal box indicates the in frame vector sequence fused to the 40-127 amino acid region of PC4. The PC4-CTD expressed in yeast was constructed by adding an ATG codon 5' to sequences encoding PC4 a.a. residues 40 through 127. The *S. cerevisiae* SUB1 gene is also shown, the boxes containing the dotted lines (not to scale) depict the heterologous 39 amino acid amino-terminal and 187 amino acid carboxyl-terminal domains of unknown function.

Fig. 3. Single-strand DNA binding activity of PC4 is required for mutation suppression in *E. coli fpg mutY*. Histidine tagged forms of PC4 and its ssDNA binding defective mutants W89A and β2β3 are expressed from the L-arabinose inducible *araBAD* promoter present on the pBAD24 vector. (A) upper panels show the mutator activity in the absence of L-arabinose, lower panels

show the mutator activity of wild-type and mutant forms of PC4 after induction by L-arabinose. (B) left section shows the western blot using anti-histidine antibody to determine levels of wild type and mutant protein expression; the right section shows the same gel stained with Coomassie brilliant blue.

Fig. 4. Peroxide sensitivity of the yeast *sub1Δ* mutant strain, its suppression by yeast *SUB1* and truncated PC4 gene expression. (A) (◆) *S. cerevisiae* Wild-type yeast carrying the vector p416-GPD (❑) *S. cerevisiae sub1Δ* mutant carrying the vector p416-GPD; (▲) Wild-type carrying the full length *SUB1* gene; (o) *S. cerevisiae sub1Δ* mutant carrying the full length *SUB1* gene expression plasmid. (B) (◆) *S. cerevisiae* Wild-type yeast carrying the vector pMV611, (❑) *S. cerevisiae sub1Δ* mutant carrying the vector pMV611; (▲) Wild-type carrying the truncated PC4 gene expression plasmid; (o) *S. cerevisiae sub1Δ* mutant carrying the truncated PC4 gene expression plasmid.

Fig. 5. MMS and UV survival in the wild type and *sub1Δ* mutant of *S. cerevisiae.* (A) MMS survival (◆) wild type yeast; (■), *sub1Δ* mutant. Data shown represent the average of 3 experiments. Error bars indicate standard error of the mean and are shown when they extend beyond the symbol. (B) UV spot test, undiluted spots of overnight cultures diluted to inoculate each spot with approximately 1000 cells of wild type (upper row) and *sub1Δ* mutant (lower row) were placed on YPD agar plates and exposed to increasing doses of UV.

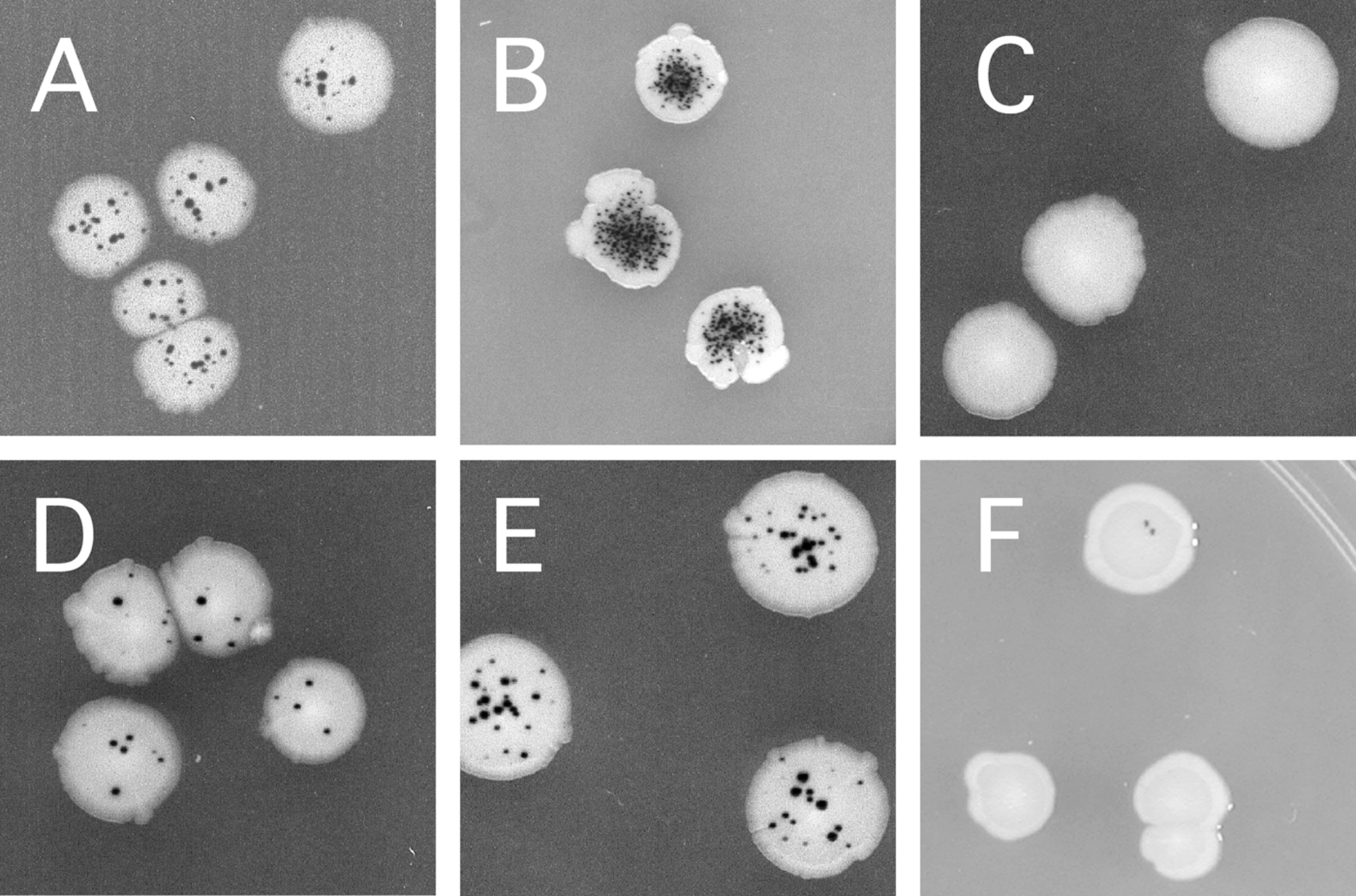
Fig. 6. The yeast *sub1Δ* mutation results in elevated mutagenesis. Spontaneous and induced mutagenesis in the wild type (□) and the *sub1Δ* (■) mutant of *S. cerevisiae.* The insert expands the view of the mutagenesis in the absence of exogenous peroxide addition.

Fig. 7. Partial suppression of *sub1* hydrogen peroxide sensitivity by *rad2*. (❍) Wild Type, (▲) *sub1Δ* mutant strain, (▼) *rad2Δ* mutant strain, (◆) *sub1Δ rad2Δ* double mutant strain. A and B, strains were plated on SC minimal medium plates, C. cells were plated on YPD medium plates. Representative data are shown.

Fig. 8. Interaction of PC4 and XPG. Slot-blot and far-western tests were performed as described in Materials and Methods. Full-length hNth1 and *E. col*i EndoIII were used as the positive and negative controls, respectively. Left panels of (A) and (B) show Ponceau S-stained images of the membrane, right panels show the results of incubation of membranes with ^{32}P-labelled XPG-HMK. The Arrow indicates the position of protein in the membrane.

Fig. 9. (A) XPG protein enhances PC4 binding to DNA bubble substrates. 2.5 nM of the 10-nt bubble DNA substrate was incubated with 41 nM of purified XPG (lanes 2-7). or without XPG (lanes 9-13) as described in Materials and Methods. Reaction mixtures were supplemented with 44nM (lanes 3 and 9), 88 nM (lanes 4 and 10), 176 nM (lanes 5 and 11), 352 nM (lanes 6 and 12) and 704 nM (lanes 7 and 13) of human PC4 protein, respectively. Samples were loaded onto a 4.5% native gel and electrophoresis was conducted at 150 V for 2 hr at 4^{O} C. The gel was dried and exposed on a phosphorimager screen. No protein was added in lane 1 and 8. (B) Quantitative representation of the effect of XPG on PC4 binding. (C) Displacement of XPG by PC4 does not depend on the order of addition of the proteins. In all experiments 44 nM of XPG and 352 nM PC4 protein and the same DNA bubble substrates shown in Fig. 8 were used. XPG was first incubated with the DNA bubble substrate (lane 2 and 3); PC4 protein was then added (lane 3), or PC4 protein was first incubated with the DNA bubble substrate followed by XPG addition (lane 4); or XPG and PC4 mixed first, then added to the DNA bubble substrate (lane 5); or PC4 alone was incubated with the substrate (lane 6). Samples were run in a 4.5% native gel

and electrophoresis was conducted at 150 V for 2 h in the cold. The gel was dried and exposed on a phosphorimager screen.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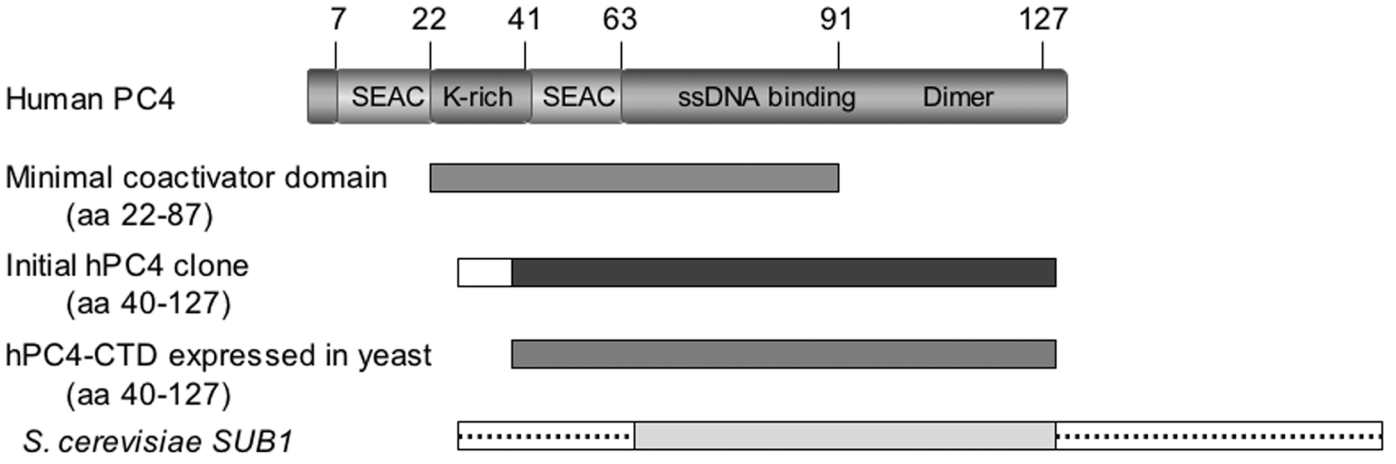
7
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41
63
91
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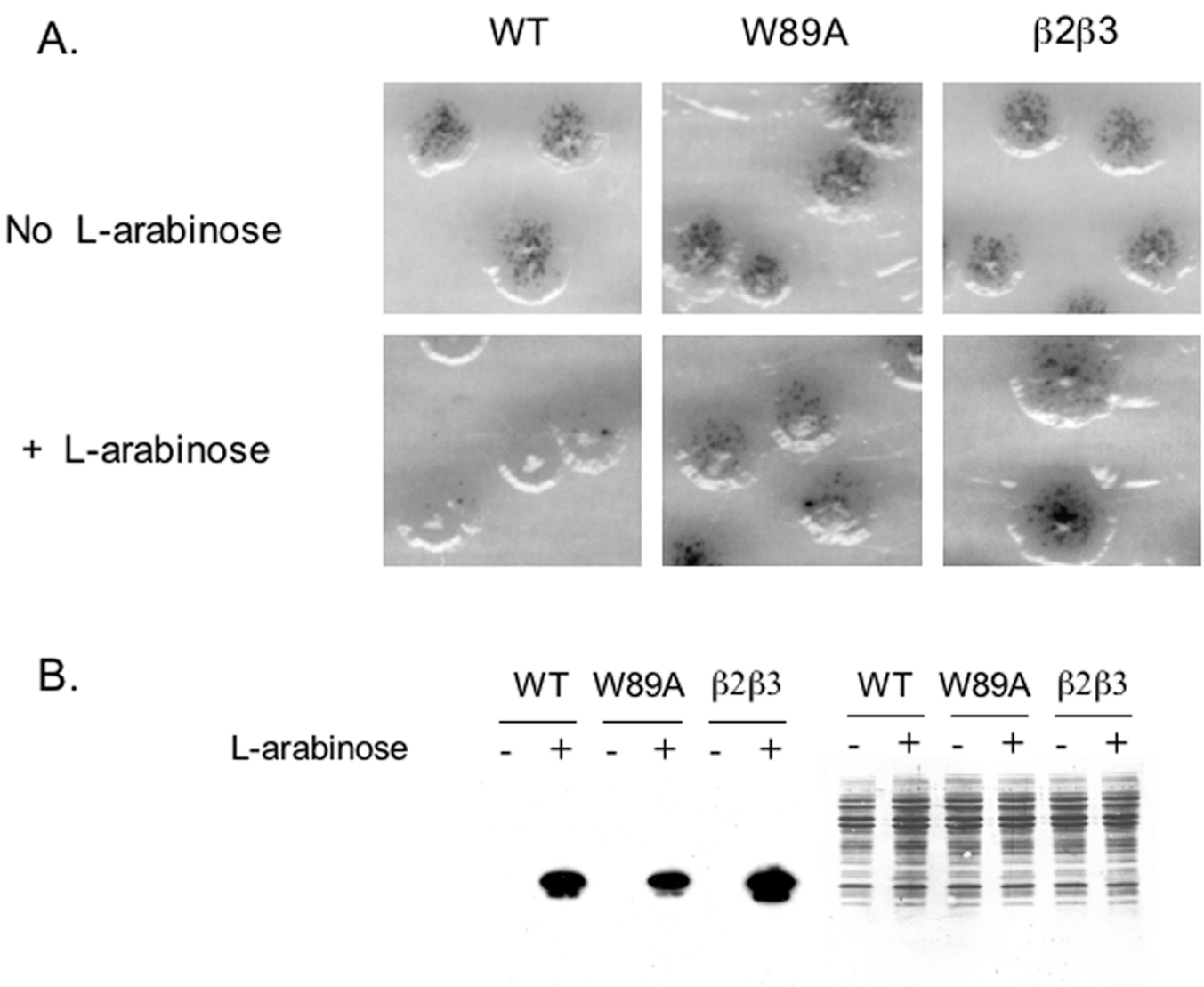
Human PC4
SEAC
K-rich
SEAC
ssDNA binding
Dimer
Minimal coactivator domain
(aa 22-87)
Initial hPC4 clone
(aa 40-127)
hPC4-CTD expressed in yeast
(aa 40-127)
S. cerevisiae SUB1


A.
WT
W89A
β2β3
No L-arabinose
+ L-arabinose
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W89A
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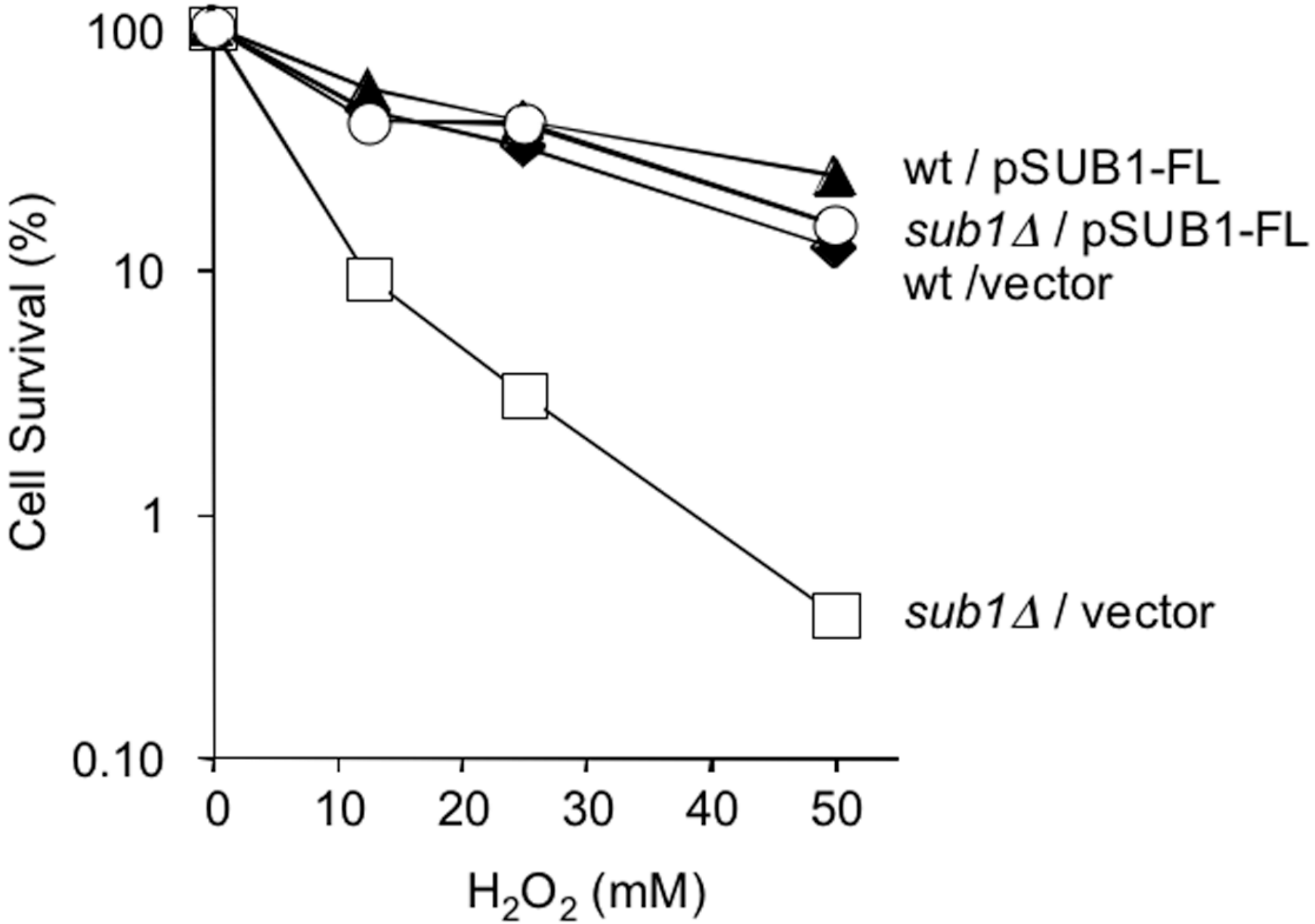
A.


100
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1
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Cell Survival (%)
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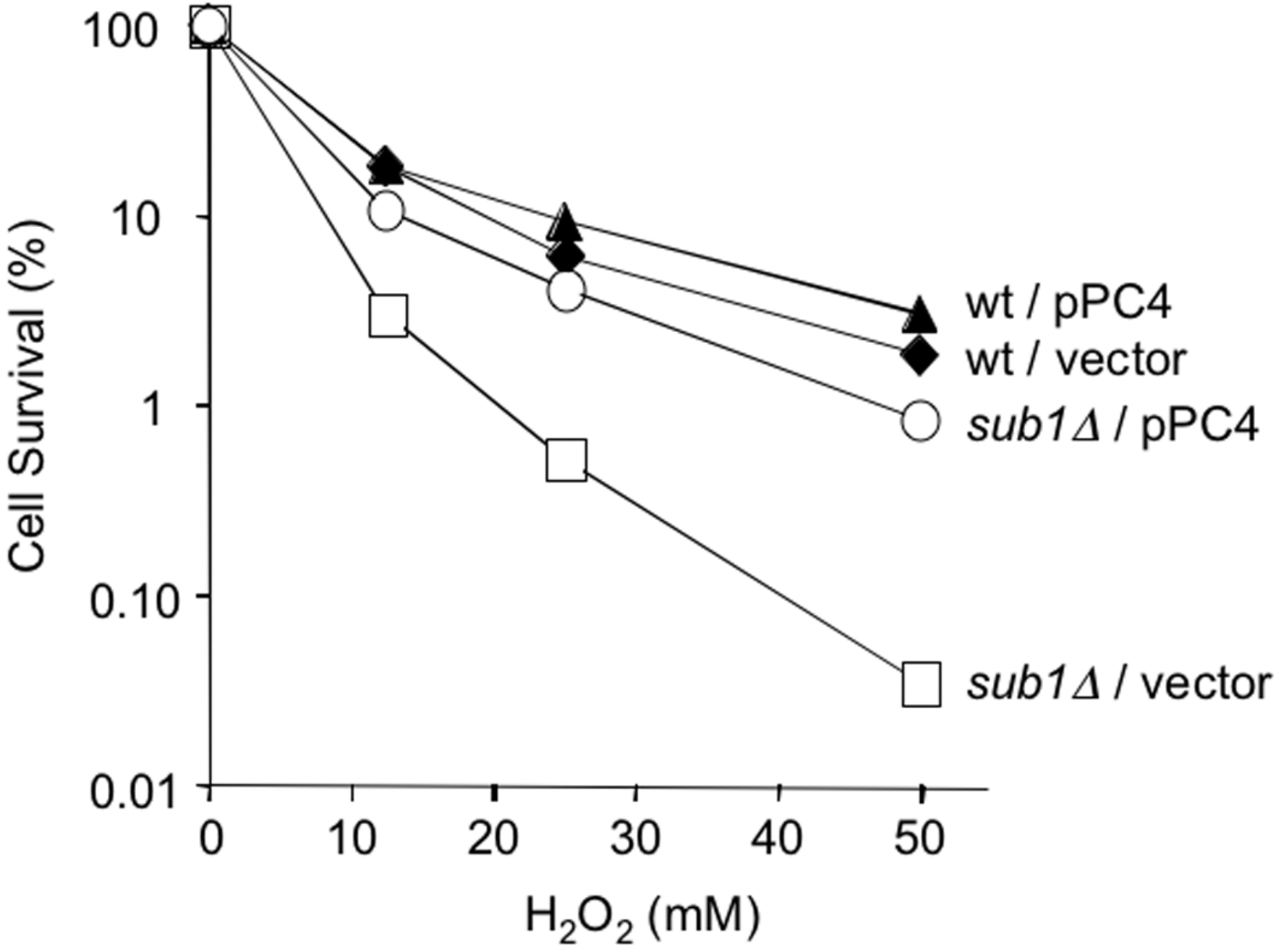
20
30
40
50
H2O2 (mM)
wt / pSUB1-FL
sub1Δ / pSUB1-FL
wt /vector
sub1Δ / vector

B.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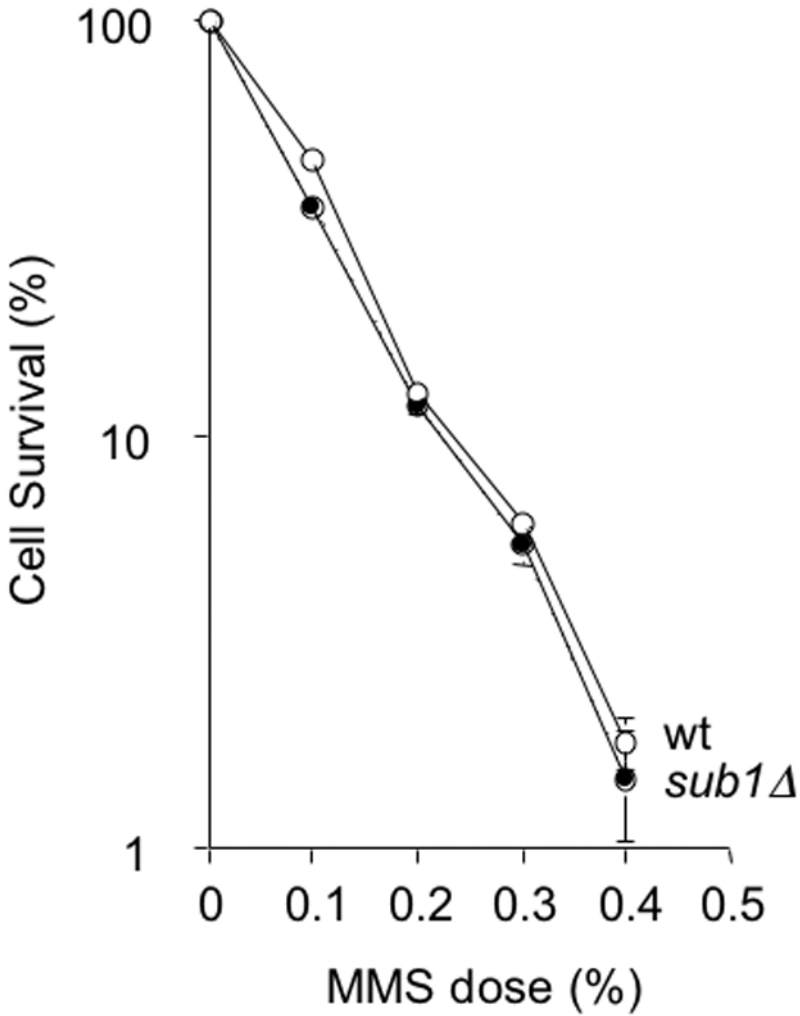
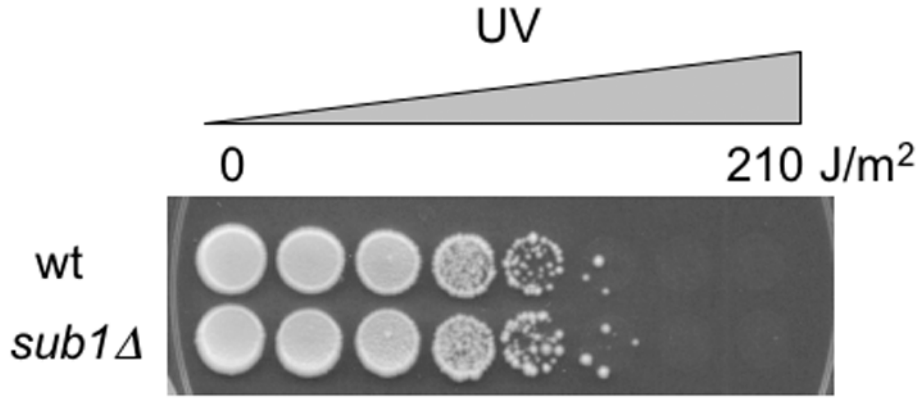
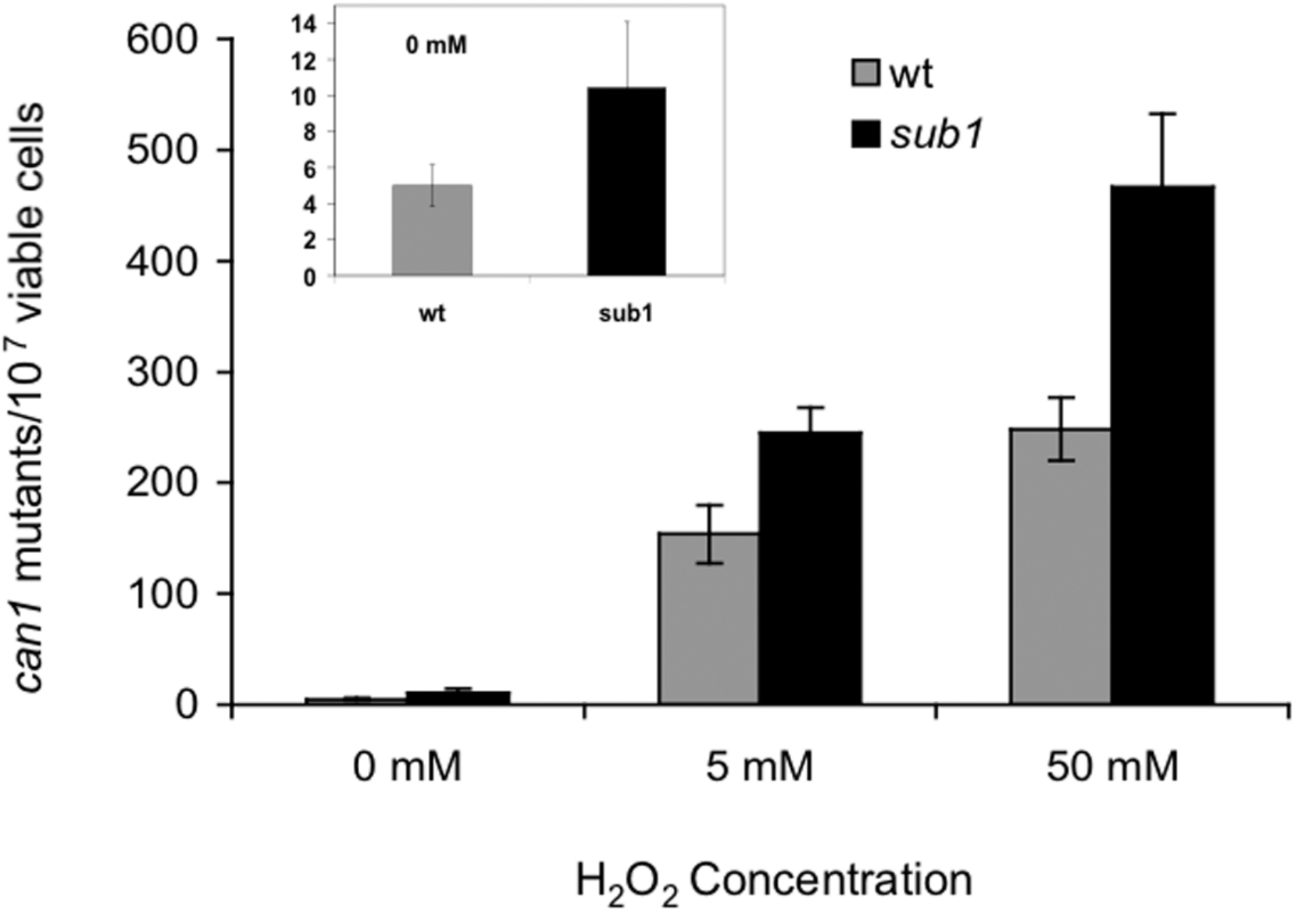
0.10
0.01
Cell Survival (%)
0
10
20
30
40
50
H2O2 (mM)
wt / pPC4
wt / vector
sub1Δ / pPC4
sub1Δ / vector

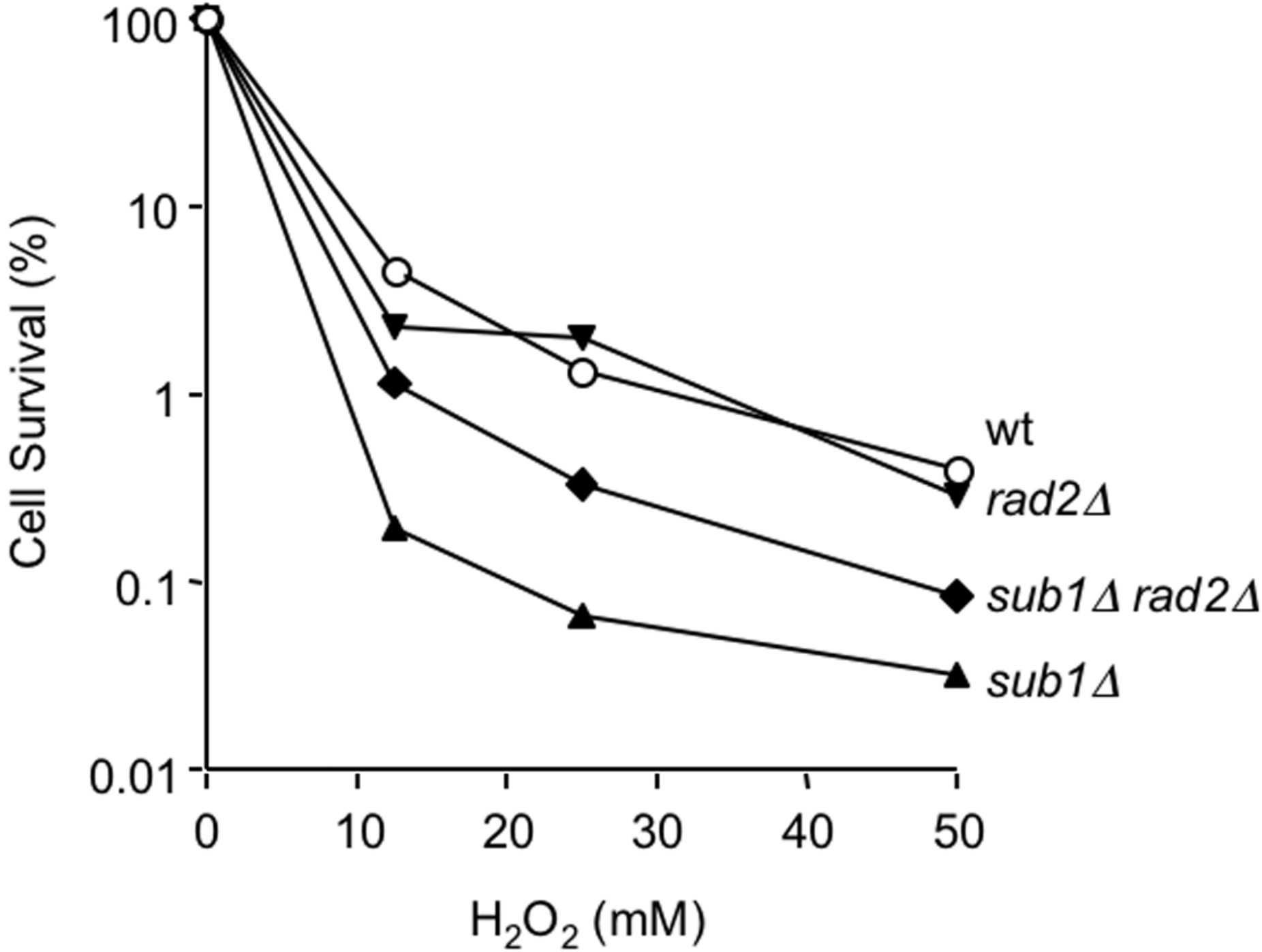
A.


100
10
1
Cell Survival (%)
0
0.1
0.2
0.3
0.4
0.5
MMS dose (%)
wt
sub1Δ

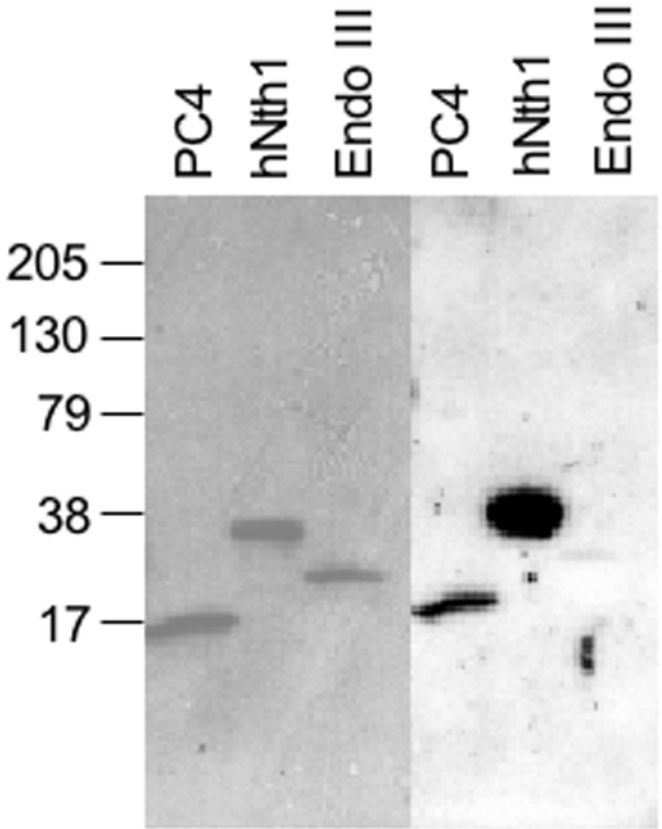
B.


UV
0
210 J/m2
wt
sub1Δ


600
500
400
300
200
100
0
can1 mutants/10 7 viable cells
0 mM
5 mM
50 mM
H2O2 Concentration
wt
sub1
14
12
10
8
6
4
2
0
0 mM
wt
sub1

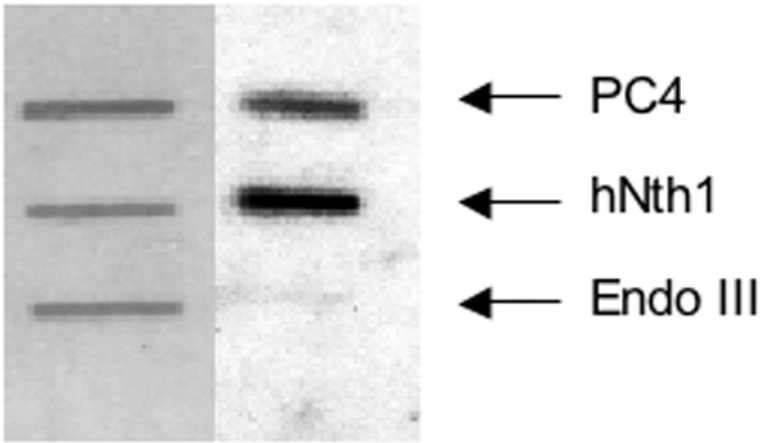

100
10
1
0.1
0.01
Cell Survival (%)
0
10
20
30
40
50
H2O2 (mM)
wt
rad2Δ
sub1Δ rad2Δ
sub1Δ

A.

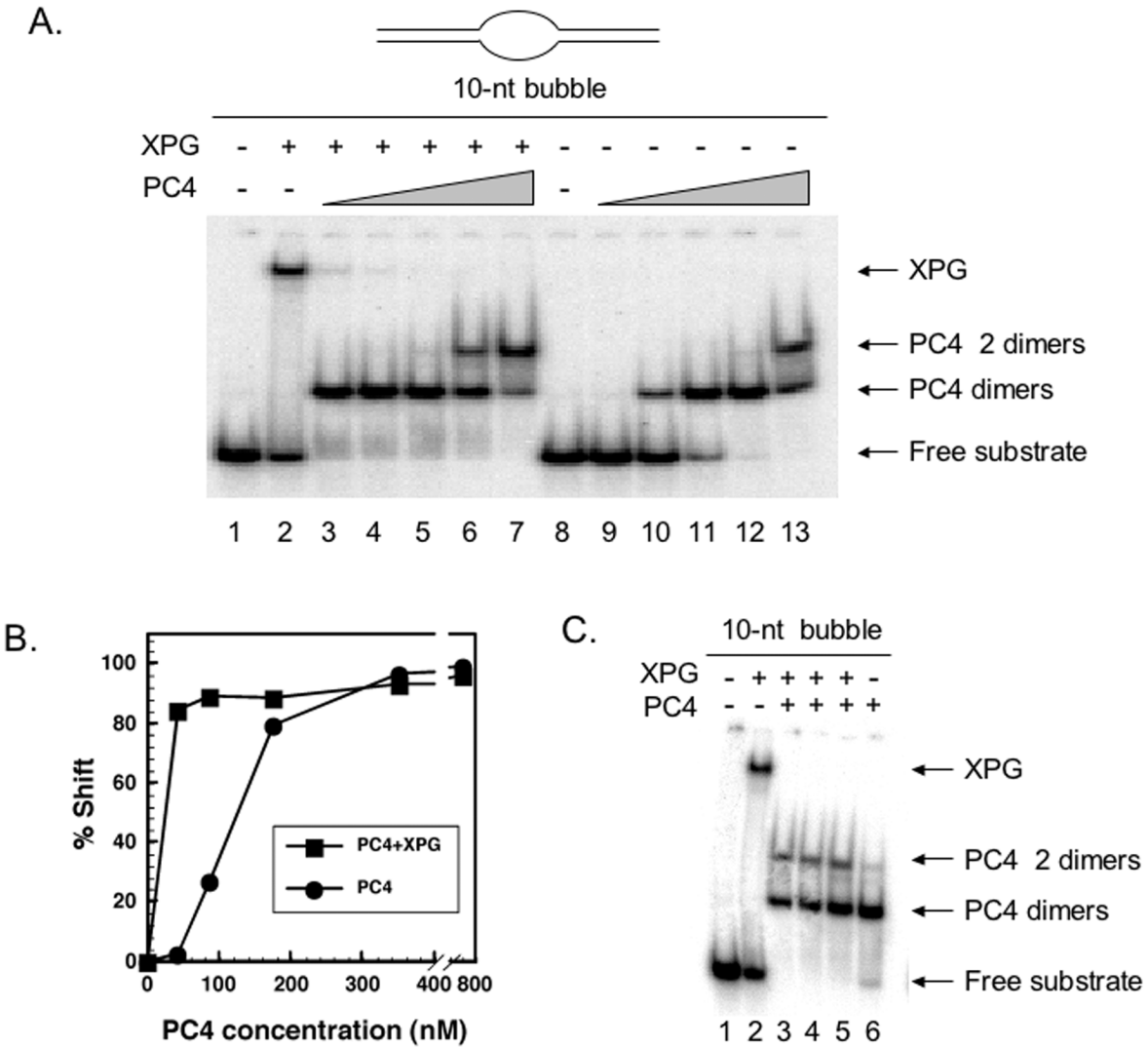


Ponceau Far Western

B.



Ponceau Slot blot


A.
10-nt bubble
XPG - + + + + + + - - - - - -
PC4 - - - -
XPG
PC4 2 dimers
PC4 dimers
Free substrate
1 2 3 4 5 6 7 8 9 10 11 12 13
B.
% Shift
100
80
60
40
20
0
0 100 200 300 400 800
PC4+XPG
PC4
PC4 concentration (nM)
C.
10-nt bubble
XPG - + + + + -
PC4 - - + + + +
XPG
PC4 2 dimers
PC4 dimers
Free substrate
1 2 3 4 5 6