MQP-BIO-DSA-BIO

THE ROLE OF SUB1 IN THE REPAIR OF UV INDUCED DNA DAMAGE IN *SACCHAROMYCES CEREVISIAE*

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ABSTRACT

Understanding the mechanisms involved in DNA repair is an important step in understanding and possibly controlling numerous human diseases. Previously the *S. cerevisiae* gene SUB1 and its human ortholog PC4 have been shown to play an important role in the repair of DNA lesions resulting from oxidative stress. In this project, I demonstrate that sub1 deletion mutants do not show an increase in UV sensitivity, however, in strains with combinations of other repair genes knocked out, the addition of the sub1 deletion does result in increased UV sensitivity.

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BACKGROUND

Importance of DNA Repair

DNA damage in all forms plays an important role in numerous human diseases including many cancers (Loft and Poulsen, 1996). Deficiencies in DNA repair mechanisms have been implicated as the cause of various disorders including Xeroderma pigmentosum (XP) and Cockayne syndrome (CS). XP results from a mutation in one of eight genes (XPA-G and XPV) and can result in extreme sensitivity to sunlight and increased incidence of skin cancer. CS results from mutations in either CSA or CSB, and can result in serious developmental disorders among other things (Lehmann, 2003). Below is a chart containing 15 known diseases caused by problems in DNA repair mechanisms. Obviously, the mechanisms of DNA repair are vitally important to maintaining normal cell function.

Disorder	Cancer	Genes	Repair process
XP	Yes	8	NER or translesion synthesis
CS	No	2–5	NER (TCR)
TTD	No	3	NER
HNPCC	Yes	3	Mismatch repair
Ataxia-telangiectasia (AT)	Yes	1	Radiation damage and checkpoints
AT-like disorder	Yes	1	Radiation damage and checkpoints
Nijmegen breakage	Yes	1	Radiation damage and checkpoints
Fanconi anaemia	Yes	7	DNA cross-links
Bloom's syndrome	Yes	1	Stalled forks
Werner's syndrome	Yes	1	Stalled forks
Rothmund–Thompson syndrome	Yes	1	Stalled forks
Familial breast cancer	Yes	2	Homologous recombination and checkpoints
Severe combined immunodeficiency	No	1	Non-homologous end-joining
LIG4 syndrome	No	1	Non-homologous end-joining
Seckel syndrome	No	1	Damage checkpoints
Li–Fraumeni syndrome	Yes	2	Damage checkpoints

Table 1. DNA Repair Related Diseases. This table illustrates the diseases known to result from DNA repair deficiencies (Lehmann, 2003).

Repair Pathways

One primary DNA repair pathway is Nucleotide Excision Repair (NER).

Nucleotide excision repair is when the DNA on either side of a lesion is cut, and the damaged section is removed and replaced with new DNA by the action of polymerase and ligase. NER is responsible for the repair of many different types of damage, including that resulting from UV exposure (Prakash and Prakash, 2000), and appears to be primarily responsible for the repair of lesions that significantly distort the DNA backbone (de Laat et al, 1999).

Transcription coupled repair (TCR) is a branch of nucleotide excision repair, the other major branch being global genomic repair (GGR) which, as its name implies, repairs DNA regardless of the strand. TCR is a process which causes the transcribed strand of a gene to be repaired quicker than the non-transcribed strand or inactive DNA regions (Svejstrup, 2002). For obvious reasons it is more important that the strand that is being transcribed be repaired quickly, since errors in the template would result in errors in the mRNA produced from it and could ultimately change the protein the gene encodes. Earlier data has shown that a UV-induced lesions will block the transcription mechanism. The stalled RNA polymerase then recruits DNA repair factors resulting in much faster repair of the transcribed strand than the non-transcribed strand (Lee et al, 2002; Tijsterman and Brouwer, 1999).

Oxidative Damage and PC4

Oxidative DNA damage has been shown to be a factor in the aging process (Schriner et al, 2005) and numerous cancers. Oxidative damage results from the interaction of reactive oxygen species (ROS), produced in cells during respiration, with the cell's DNA, although ROS can also come from external sources such as hydrogen

peroxide. There are likely over 100 different types of lesions produced by the ROS interactions with DNA, but the most abundant is 8-oxoguanine (8-oxo-G). The mutagenic properties of 8-oxo-G result primarily because it is often mispaired with adenine resulting in GC to TA transversions if it is not repaired. Consequently, every cell has mechanisms in place to prevent or repair the damage created by these ROS.

Role and Function of PC4

The human gene PC4 (positive cofactor 4) was isolated in our lab through a screen of a human cDNA library for genes that would suppress oxidative mutagenesis in an *E. coli* strain hypersensitive to this form of mutagenesis. SUB1 (the yeast homolog of PC4) was shown to be important for cellular resistance to oxidative damage since yeast cells lacking sub1 have higher spontaneous and peroxide induced mutation frequencies (Wang et al, 2004).

Previous experiments have shown that PC4 interacts with XPG, a fact which supports a role for PC4 in DNA repair. XPG (RAD2 in yeast) is an endonuclease responsible for cutting the lesion-containing strand of dsDNA on the 3' side of a lesion, and is critically important for both NER and TCR (Nouspikel et al, 1997). Knocking out rad2 on top of sub1 actually reduces sensitivity to hydrogen peroxide, suggesting that peroxide sensitivity of sub1 requires the action of Rad2 and suggests sub1 may function in a rad2 dependent pathway. Human PC4 has been shown to directly interact with XPG, and is capable of displacing XPG from single stranded DNA (Wang et al 2004). This suggests that PC4 may be involved in the removal of XPG from DNA after it has excised the lesion.

The PC4 protein contains two serine-enriched acidic domains on its N-terminus, and a single stranded DNA binding domain towards the C-terminal end (Figure 1). PC4 was initially recognized as a transcriptional coactivator, and has the ability to interact with both double stranded and single stranded DNA. However, only the double stranded binding activity is required for its function as a transcriptional coactivator

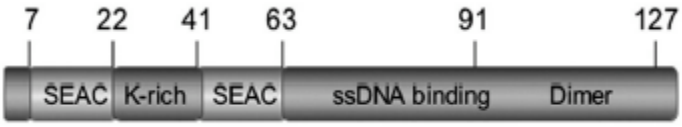

Figure 1. Structure of PC4. This figure shows the structure of the human PC4 gene**.**

(Kaiser et al, 1995). Interestingly, it has been shown that only the ssDNA binding domain is required for PC4s DNA repair function, and this same region was shown to have the ability to repress transcription (Werten et al., 1998; Malik et al., 1998). This is an odd function for something that acts as a transcriptional coactivator, but one that PC4s role in DNA repair might explain. Recently it has been shown that PC4 remains bound to the transcription mechanism throughout the process of transcription (Calvo and Manley, 2005), which is another fact that points to PC4 being involved in transcription coupled repair.

Repair Genes of Interest to This Project

There are countless numbers of other genes involved in the repair of DNA, below a few that play a role in this study are highlighted. Rad16 is a protein integral to the function of nucleotide excision repair. Data suggests that RAD16, in complex with RAD7, is important for the initial recognition of lesions in non-transcribed DNA regions

(Prakash and Prakash, 2000). In transcribed regions this function is replaced by RNAPII and Rad26 (Tijsterman and Brouwer, 1999). In this MQP rad16 is knocked out in order to disable the global genomic aspect of nucleotide excision repair. Another gene used in this study is RAD26. Previous studies have shown that deletion of rad26 affects the rate at which UV-induced damage is repaired on the transcribed strand of the DNA, but shows no affect on repair of non-transcribed strands (Tijsterman and Brouwer, 1999). This data indicates that RAD26 is involved in TCR, and when knocked out is believed to eliminate most TCR function (Lee et al, 2002). Interestingly, even after rad26 is knocked out there is still repair of the transcribed strand which was attributed to the actions of other repair mechanisms in global genomic repair covering up the lack of TCR (van Gool et al, 1994; Verhage, et al 1996), but may also be the result of other TCR pathways. RPB9 is a subunit of RNA polymerase II that is not required for cell survival that is believed to play a role in an alternate TCR pathway. Deletion of rpb9 results in no increase in UV sensitivity similar to rad26, and the rpb9, rad26 double mutant shows only a slight increase in sensitivity. The rad16 rpb9 double mutant shows a significant increase in UV sensitivity compared to rad16 alone, another similarity with rad26. The additional deletion of rad26 in this strain results in an even more sensitive strain. This indicates that rpb9 is involved in a TCR pathway independent of Rad26. Through tests of repair in yeast strains it was shown that in with rad16 rad26 rpb9 knocked out there is no repair of the transcribed region of the GAL1 gene, indicating that all TCR is knocked out (Li and Smerdon, 2002).

PROJECT PURPOSE

In this study, I used or created numerous strains containing different combinations of rad16, rad26, rad2, rpb9, and sub1, all in the same genetic background in an effort to narrow down how exactly sub1 functions in DNA repair. The data in this project suggests that sub1's role is not as straightforward as originally thought. I present data that suggests a more peripheral role for sub1, or the possibility that it is functioning in a pathway we are currently unaware of.

MATERIAL AND METHODS

Strains and Primers

Strain Number	Genotype
MVY101	Wild Type
MVY105	sub1Δ
MVY154	rad16Δ
MVY348	rad16Δ, sub1Δ
MVY352	rad16Δ, rad26Δ, sub1Δ
MVY357	rad16Δ, rad26Δ, rpb9Δ, sub1Δ
MVY360	rad16Δ,rad26Δ, rpb9Δ
MVY366	rad16Δ, rpb9Δ
MVY368	rad16Δ, rad26Δ, rpb9Δ, rad2Δ, sub1Δ
MVY376	rad16Δ, rad26Δ, rpb9Δ, rad2Δ
MVY379	rad16Δ, rpb9Δ, sub1Δ
MGSC107	rad16Δ, rad26Δ

Table 2 Strains used in this project

Primer Name	Sequence (5'-3')	Use
rpb9KO-L	AGGAGAAATTAGCGCTGGTG	rpb9 knock out primer
rpb9KO-R	ACGTTTCTGATCTGGGCAAC	rpb9 knock out primer
rpb9-L	CATCCTTGGCGACATTTTCT	rpb9 confirmation primer
rpb9-R	TCCATCATGACCCAACTG	rpb9 confirmation primer
rad2KO-L	AGCGCAGAAGGTACTCCTCA	rad2 knock out primer
rad2KO-R	CTGTTGCAGCCGTATTCTCA	rad2 knock out primer
rad2-L	TAAGCAGCGACGTATCGTGT	rad2 confirmation primer
rad2-R	ACCATGTTGGCAGGAATAGC	rad2 confirmation primer

Table 3 Primers used in this project

Construction of Yeast Knock Out Strains

Amplification of yeast knock out

First a culture of a strain already containing the knock out was started from a frozen glycerol stock and grown in YPD media at 30^{o}C. The genomic DNA was extracted using the Epicentre Masterpure Yeast DNA Purification kit. The knock out gene was amplified via PCR using a set of primers at least 200 base pairs outside of the gene. The primers were created based on sequences obtained from the yeast genome

database. The amplified DNA was then purified using the Qiagen PCR purification kit and transformed into the recipient cells.

Yeast Transformation

The cells were grown to an OD_{600} of approximately 1.6 overnight in 5ml of liquid YPD at 30°C. Next, the cells were centrifuged at 5,000rpm for 5min and then resuspended in 2.5mL of sterile water. The cells were centrifuged again for 5min at 5,000rpm and resuspended in 100uL of 100mM Lithium Acetate and transferred to a microfuge tube. The suspensions were spun in a microcentrifuge at top speed for 10 seconds to pellet the cells which were then resuspended in 50uL of 100mM Lithium acetate. Carrier DNA (Herring Sperm DNA 2ug/mL) was boiled for 5 minutes and then put on ice. The LiAc suspension was spun down to pellet the cells and then the following were added in order: 240uL 50% PEG, 36uL 1M LiAc, 25uL 2ug/mL Carrier DNA, 50ul DNA in TE with one control sample not receiving any DNA. The samples were incubated at 30°C for 30 minutes then heat shocked at 42°C for 15 minutes. After incubation, the samples were spun down to pellet the cells and then resuspended in 200uL of water. 100uL of sample were plated on a selection plate and allowed to grow for 3-5 days. The knock out is created by the yeast cells recombining the knock out segment transformed in with the wild type gene, consequently replacing the wild type with the cassette containing the selectable marker. In yeast the selectable markers that are generally used are URA3, TRP1, HIS3, LEU2 which confer a wild type phenotype on transformed cells, and KanMX4 which makes transformed cells resistant to Geneticin (G418). The first four would be selected on synthetic complete media with the

appropriate amino acid or nucleotide dropped out, which is written as SC-Ura, etc. For transformation with KanMX4 Geneticin is added to YPD media in varying amounts depending on the strain being transformed.

Confirmation of knock out

Several colonies were picked from the selection plates and grown overnight in 5mL of YPD. Genomic DNA was isolated and then amplified with confirmation primers. Confirmation primers are a set of primers designed to amplify a region slightly larger than the original knock out. This is done to confirm that the knock out recombined into the appropriate location in the yeast genome. Discrimination between wild type and replacement can be made either on the basis of size of the PCR product, restriction digest, or both.

UV Survival Curves

The strains were grown to mid-log phase (OD_{600} of approximately 2.5) in 5mL of YPD at 30^{o}C. The cultures were centrifuged at 5,000rpm for 5 minutes and then resuspended in 5mL of 1xPBS. The sample was poured into a Petri dish and exposed to UV for specific intervals. After each exposure a sample was taken. Appropriate dilutions of each sample were made, and 100uL was plated on YPD and incubated in the dark for 3 days, then the number of surviving colonies was counted.

RESULTS

Sub1Δ Alone Does Not Result in Increased UV Sensitivity

Previous work has suggested that a sub1 knock out strain is not any more sensitive to UV radiation than is a wild type (Wang et al, 2004). To test this, quantitatively a UV survival curve was completed of a wild type and a sub1Δ strain. This assay confirmed that a sub1Δ strain is no more sensitive than wild type to UV treatment, and may even be slightly less sensitive (Fig. 2).

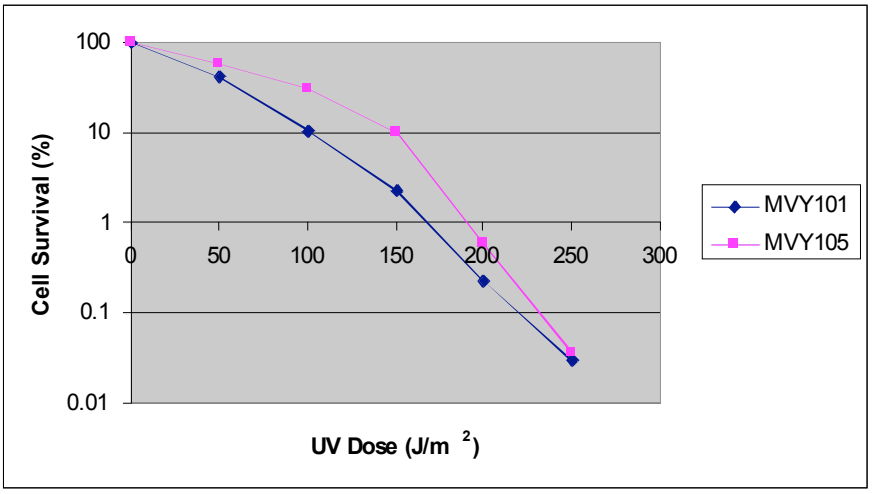

Figure 2. UV survival curve in wild type and sub1 Δ. MVY101(blue) is wt, MVY105 (pink) is sub1Δ.

Previous data has also suggested that sub1Δ could result in added sensitivity when added to a strain where rad16 is already knocked out. As mentioned before, RAD16 is a protein involved in nucleotide excision repair, without which global genomic repair will not function. The UV sensitivity of strains with rad16 knocked out, and the combination of rad16 and sub1 knocked out was compared (Fig. 3). From this data it is evident that the addition of sub1Δ to a rad16Δ strain does result in increased sensitivity to UV treatment, suggesting that when only sub1 is knocked out global genomic repair is able to

cover up for its absence. This confirms the previously stated idea (Wang at al, 2004) that sub1 is not involved in NER because if it was knocking out sub1 should have resulted in the same sensitivity seen when rad16 was knocked out.

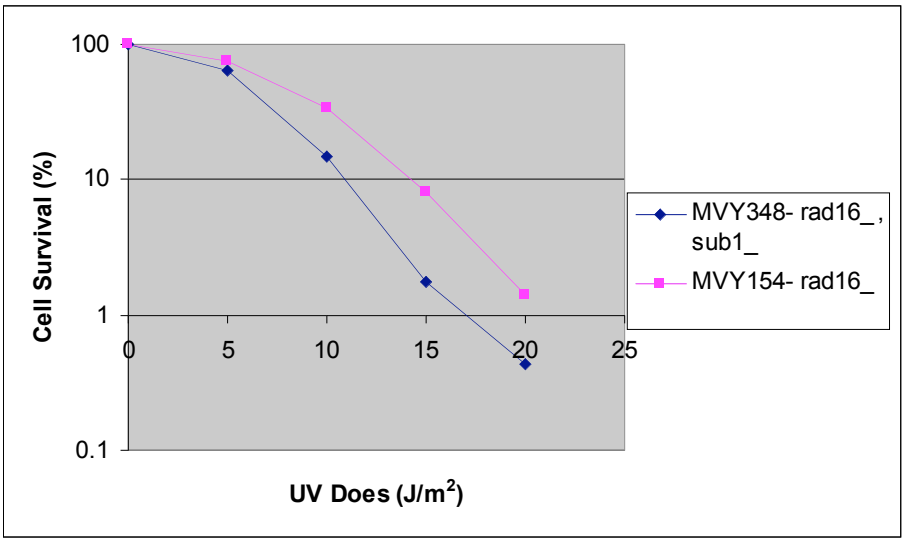

Figure 3. UV Survival Curves of rad16Δ (pink) and rad16Δ, sub1Δ (blue) strains.

Sub1Δ Increases UV Sensitivity When Transcription-Coupled Repair is Knocked Out

Since previous data suggested that SUB1 was involved in transcription coupled repair, the next step was to determine if knocking out sub1Δ affected UV sensitivity of a TCR deficient strain. Studies had shown that rad26 Δ strains were deficient in TCR, so the UV sensitivity of rad16Δ, rad26Δ and rad26Δ, rad16Δ, sub1Δ strains was compared (Fig 4). The data shows that the addition of sub1 Δ still results in an increase in UV sensitivity.

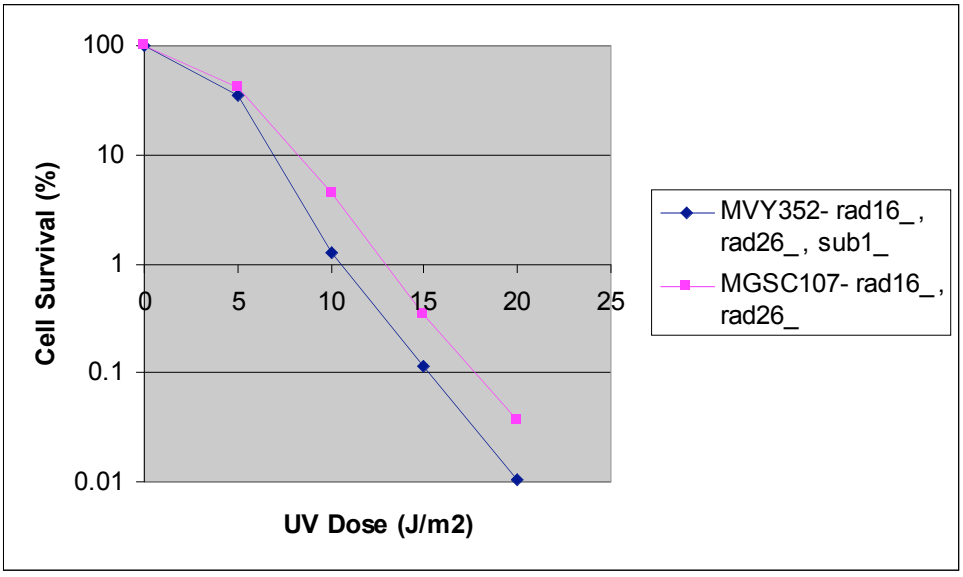

Figure 4. UV survival curves of rad16Δ, rad26Δ (pink) and rad16Δ, rad26Δ, sub1Δ (blue) strains.

Previously it was believed that knocking out rad26 would eliminate all TCR function, however, it was recently shown that RPB9 functions in an alternate TCR pathway. This raises the possibility that Sub1 may affect the rpb9 TCR pathway. To determine if this is the case, it was necessary to knock out rpb9 in the strains used in Figure 4. Figure 5 shows how a yeast knock out strain is created. From the data in Figure 6 it is seen that the addition of sub1 resulted in increased UV sensitivity even in the rad16, rad26, rpb9 mutant strain. Since knocking out RAD26 and RPB9 is believed to knock out all function of transcription coupled repair, knocking out anything else involved in TCR should not result in an increase of sensitivity. In order to better evaluate this data it has been placed in one graph as seen in Figure 7.

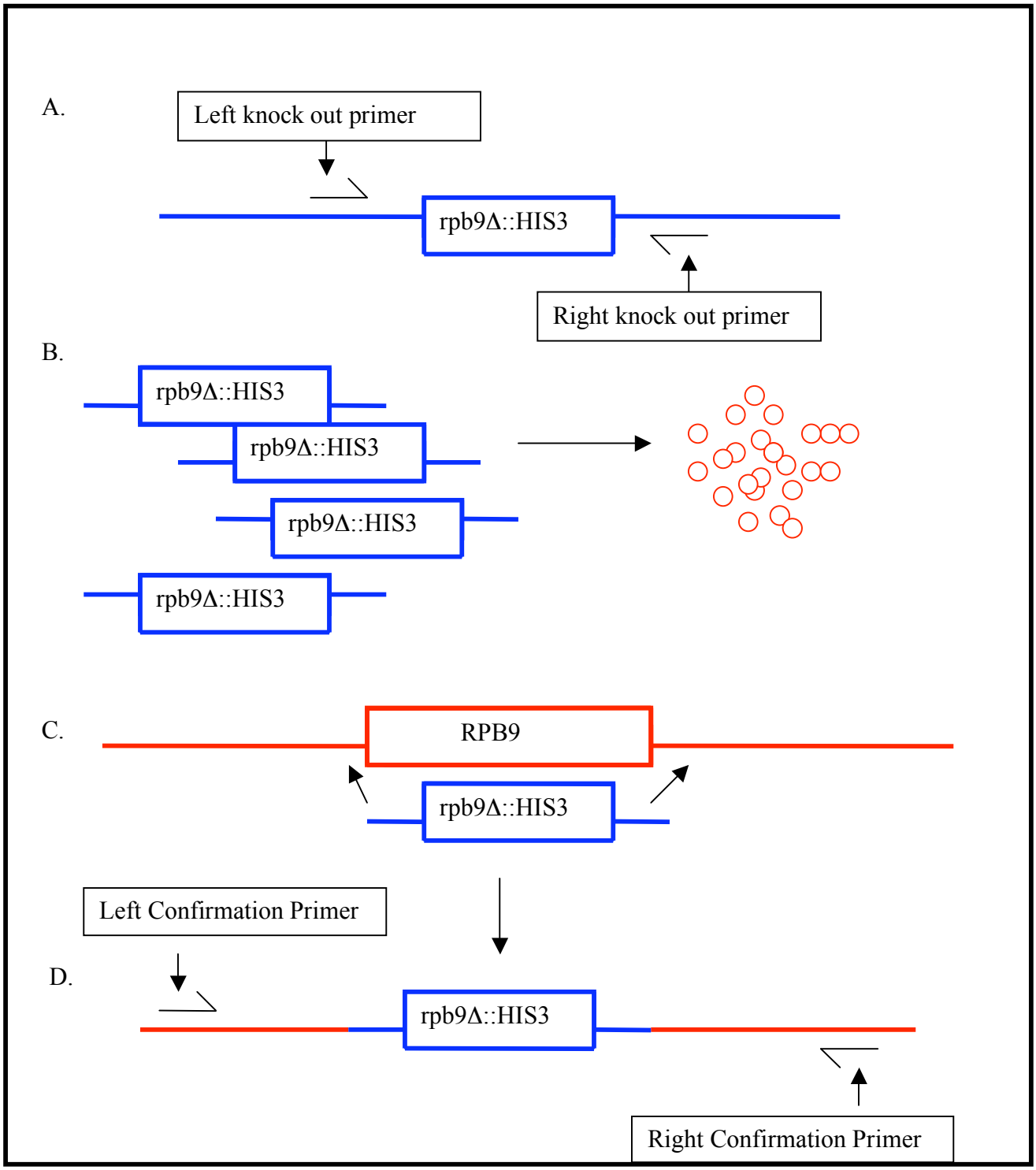

Figure 5. Creation of Yeast Knock Outs. A. The knock out is amplified using PCR from a strain already containing it. The knock out primers should be at least 200bp from the end of the gene. B. The amplified knock out segments are transformed into the receiving yeast cells. C. Through recombination the yeast incorporates the knockout into its genome replacing the wild type gene. D. Confirmation primers which are further out than the knock out primers are used to amplify the region to confirm that colonies picked from selection plates contain the knock out in the appropriate location.

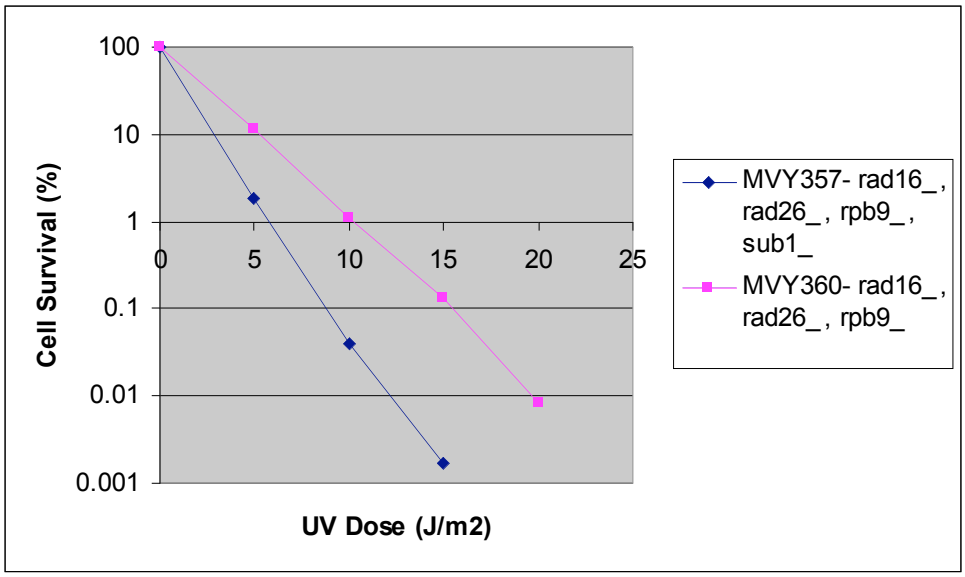

Figure 6. UV Survival Curves of rad16Δ, rad26Δ, rpb9Δ (pink) and rad16Δ, rad26Δ, rpb9Δ, sub1Δ (blue) strains.

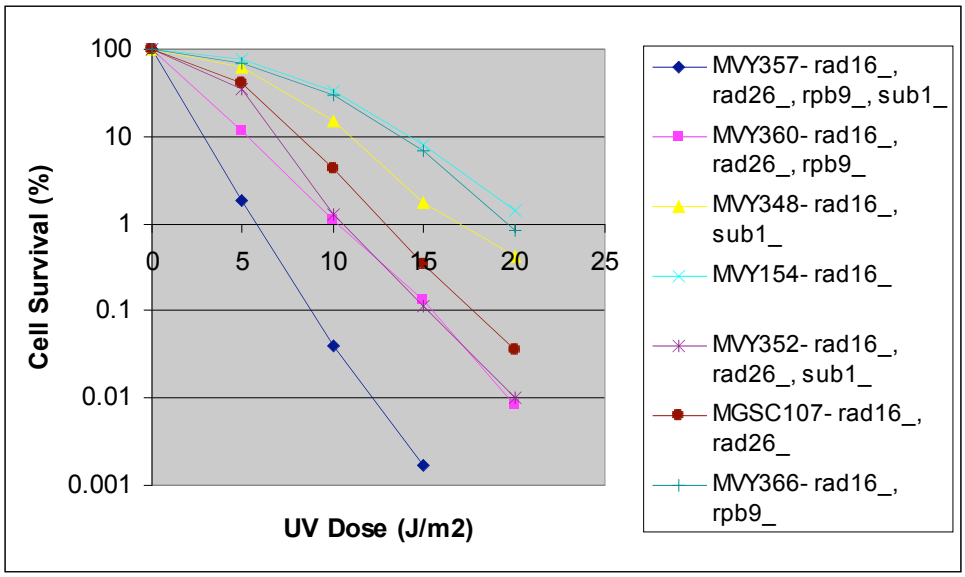

Figure 7. Combined UV Survival Curve Data. This figure shows all of the survival curve data together for comparison.

Figure 7 shows that in all cases, knocking out sub1 increases a strain's sensitivity to UV. This means that sub1 may be functioning in something other than TCR or possibly in a third TCR pathway that we are not yet aware of, or both.

Since earlier data had shown that sub1 functioned in a rad2 dependent pathway when involved in the repair of oxidative damage due to H_2O_2 stress, rad2 was then knocked out of the strains used in Figure 6 to determine what effect, if any, would be seen. Figure 8 shows the results of this experiment.

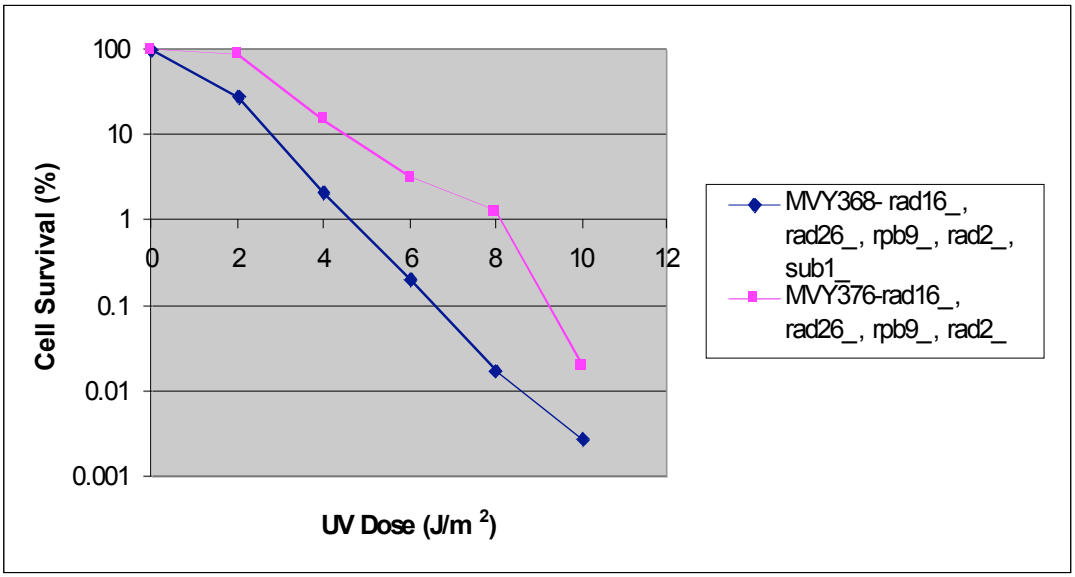

Figure 8. UV Survival Curve Data of rad2 Deletion Strains.

These rad2 deletion strains became even more UV sensitive, and the UV dose used had to be significantly decreased in order to obtain any useful data. From this data it is evident that the deletion of sub1 continues to increase UV sensitivity demonstrating that alternate pathways must be considered.

DISCUSSION

The goal of this project was to determine what role sub1 plays in DNA repair. Initial thoughts were that sub1 was directly involved in the transcription coupled repair pathway. Previous data pointed to a role in an XPG dependent pathway. The Sub1 deletion's phenotypic similarities to rad26 deletions also implied that sub1 was involved in TCR. The data from this project, however, indicates that sub1 is not simply a player in any one pathway and that its role may not be as straightforward as originally thought.

I was able to quantitatively show that a sub1 deletion by itself is no more sensitive to UV than wild type, and that, like rad26, rad16 must also be knocked out before any sensitivity will be seen. Knocking out rad26 in these strains increased sensitivity, and the triple mutant rad16 rad26 sub1 was more sensitive than either of the double mutants rad16 rad26 and rad16 sub1. This shows that sub1 is not involved solely in the rad26 dependent TCR pathway. This did not rule out a role in TCR, since another pathway involving RPB9 also exists. After knocking out rpb9 and still seeing sub1 result in an increase in UV sensitivity it became necessary to begin considering other roles for sub1. Finally, knocking out rad2, and creating strains that should then be deficient in all nucleotide excision repair, showed that sub1 deletion continues to increase sensitivity.

Since a simple role in TCR is now unlikely, it is necessary to consider other possibilities. One such possibility is a role in recombination, however, recent work in our lab using knockout strains deficient in recombination repair has shown that sub1 does not act in this pathway. The ability of PC4 to remove XPG from single-stranded DNA (Wang et al, 2004) could mean that PC4/SUB1 is necessary to get the excision machinery

off the DNA to allow polymerase to come in and replace the excised segment. It could then be possible that the deletion of sub1 forces the conversion of non-lethal DNA lesions to strand breaks which could explain the decreased survival of sub1 deletion strains. Another possibility is that sub1 is integral to restarting transcription or getting the transcription mechanism reassembled so that without it transcription cannot restart after DNA damage. One other possibility is that these strains simply contain too many mutations. With each added mutation the growth rate decreases with the quadruple and quintuple deletion strains being very slow growing, so it may be that these strains are just not healthy enough to recover from UV treatment. It is also possible that there is another TCR pathway that we are currently unaware of, although current data suggests that knocking out rad26 and rpb9 does knock out all of TCR (Li and Smerdon, 2002). Since sub1 deletion only results in an increase in sensitivity when rad16 is also knocked out, it seems that Sub1 is in some way involved in TCR, but at this point the data does not allow us to see how.

Work is currently being done in our lab to answer some of the questions resulting from this data. Experiments are being done to see if sub1 plays a role in recombination. Work is also being done to determine if sub1 is involved in the removal of UV damage from transcribed strands. I have also been doing work (detailed in my MQP submitted to the WPI Biochemistry department) with a TRP5 reversion system to determine if there is an increase in mutation rates in sub1 deletion strains, and if this increase is in a specific type of mutation.

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