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The *Saccharomyces cerevisiae* DNA damage checkpoint is required for efficient repair of double strand breaks by non-homologous end joining

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Abstract In this work we report that the *Saccharomyces cerevisiae RAD9, RAD24, RAD17, MEC1, MEC3* and *RAD53* checkpoint genes are required for efficient non-homologous end joining (NHEJ). *RAD9* and *RAD24* function additionally in this process. Defective NHEJ in *rad9Δ–rad24Δ*, but not *yku80Δ* cells, is only partially rescued by imposing G1 or G2/M delays. Thus, checkpoint functions other than transient cell cycle delays may be required for normal levels of NHEJ. Epistasis analysis also indicated that *YKU80* and *RAD9*/*RAD24* function in the same pathway for repair of lesions caused by MMS and γ-irradiation. Unlike NHEJ, the checkpoint pathway is not required for efficient site-specific integration of plasmid DNA into the yeast genome, which is *RAD52*-dependent, but *RAD51*-independent.

Key words: DNA damage; Checkpoint; Repair; Non-homologous end joining; Ku

1. Introduction

DNA damage and the efficiency of its repair are key determinants in the development of cancer and may also be relevant to ageing. Highly conserved DNA repair pathways have evolved to repair specific lesions [1]. These lesions also activate the DNA damage checkpoint pathway, resulting in transient delays in the G1, S and G2/M phases of the cell cycle that have been well characterised in yeast [2,3]. Checkpoint controlled delays are believed to increase the efficiency of DNA repair by allowing more time for DNA repair, prior to important cell cycle transitions. The DNA damage checkpoint pathway of budding yeast also controls the transcriptional induction, after DNA damage, of a large regulon of DNA repair genes termed the DNA damage regulon or DDR [4,5]. The relevance of DDR activation for the efficiency of any DNA repair pathway has not yet been determined. It is also possible that the DNA damage checkpoint pathway might control the efficiency of individual repair pathways by post-transcriptional means. For example, re-localisation of Ku and Sir proteins from telomeres to double strand breaks (DSBs) is under checkpoint control [6–8]. Ku and Sir proteins are involved in illegitimate recombinational repair of DSBs by error free non-homologous end joining (NHEJ) [9,10]. Indeed, interactions between DNA repair and checkpoint pathways are becoming more evident (recently reviewed in [11]). Not only can the DNA damage checkpoint target DNA repair pathways, but repair proteins may also be required for the checkpoint to sense specific lesions.

In this study we use a plasmid based re-ligation assay [12] to investigate the effects of the checkpoint pathway on illegitimate recombination. We observed that the efficiency of NHEJ, the major illegitimate recombination pathway in yeast [12], is markedly diminished by mutations in the DNA damage checkpoint pathway. Moreover, mutations that result in residual checkpoint activity have a modest effect on repair whereas those that effectively abolish checkpoint activity have a marked effect. Conversely, mutation of the checkpoint pathway had no measurable effect on a related plasmid based assay that measures site-specific integration by homologous recombination, a *RAD51*-independent but *RAD51*-dependent process.

2. Materials and methods

2.1. Strains, plasmids and culture conditions

The yeast strains and plasmids used have been previously described [5]. Cells were grown in YNB (2% glucose, 1× yeast nitrogen base plus required amino acids) prior to transformation and then plated onto YNB agar plates. Overproduction of Rad9 or Rad24 (confirmed by Western blotting) was achieved by activation of the *GAL1* promoter as previously described [5].

2.2. End-joining ligation assay

Cells exponentially growing in minimum medium (YNB plus 2% glucose plus the required amino acids) were transformed in parallel with either 250 ng of the centromeric plasmid pRS315 digested to completion with *Bam*HI or with 250 ng of undigested pRS315. The values plotted as % repair efficiency correspond to the number of transformants obtained with the digested plasmid normalised to the number of transformants recovered with the undigested plasmid. This value was then normalised to the value obtained with the wild type, which was assigned a value of 100%. In most case nine, but at least six, independent transformations were assayed. Standard deviations are indicated but in some cases are too small to be detectable in the histogram.

2.3. Cell synchronization and survival curves

Synchronisation in G1 or G2 was achieved by incubation of exponentially growing cells for 2–3 h with either 20 μg/ml α-factor or 5 μg/ml nocodozole (Sigma), respectively. Cell survival after DNA damage was assessed as follows: exponentially (YPD) growing cells were treated with 0.02% MMS (methylmethanesulphonate, Sigma) for the times indicated and dilutions of cells were plated onto YPD agar plates. Alternatively, cells were plated directly onto YPD agar plates and immediately γ-irradiated with a ^{60}Co source. In both cases % survival relative to untreated cells (normalised to 100%) were plotted relative to exposure time to MMS or to dose of γ-irradiation.

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3. Results and discussion

3.1. DNA damage checkpoint genes play a role in error-free NHEJ

We used an in vivo plasmid-rejoining assay [12,13] to assess the role of the DNA damage checkpoint in illegitimate recombinational repair of a DSB. All the single checkpoint mutants tested displayed a 4–5-fold decrease in illegitimate recombinational repair relative to the isogenic wild type, WT (Fig. 1A). Significantly, the efficiency of illegitimate recombinational repair in the *rad9Δ–rad24Δ* double mutant was reduced still further, to 6.0% of that observed in WT cells. *RAD9* and *RAD24* have previously been shown to function additionally after DNA damage with respect to survival, cell cycle delays and the transcriptional induction of the DDR [5,14]. Deletion of *YKU80*, which encodes a protein required for NHEJ (the major error free end joining pathway of yeast cells [9,13]), resulted in 2.3% residual illegitimate recombination (Fig. 1A). These data suggest a role for the DNA damage checkpoint pathway in illegitimate recombinational repair almost equivalent to deletion of *YKU80*. In *yku80Δ* cells, all plasmids recovered from the rare transformants obtained were not accurately repaired (data not shown, but see [13]). These rare transformants are produced by a less efficient error prone pathway that result in deletion of sequences on one or both sides of the DSB ([12,13] and data not shown). However, in all cases examined, the plasmids recovered from *rad9Δ–rad24Δ* cells could be re-linearised with *Bam*HI (data not shown) indicating that error free NHEJ can occur in the absence of both *RAD9* and *RAD24*. Thus, it is unlikely that the checkpoint proteins are playing an essential enzymatic role in end joining, rather our results suggest that the deficiency observed in checkpoint mutant cells is due to inefficient, but accurate, Ku80-dependent NHEJ. This hypothesis is sup-

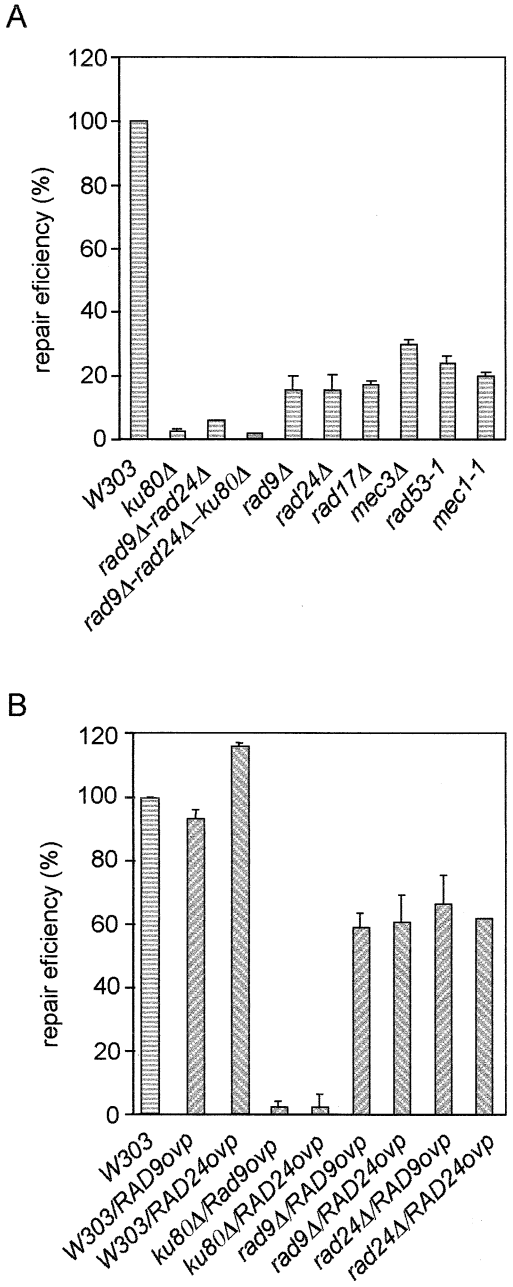

Fig. 1. Decreased efficiency of non-homologous end joining (NHEJ) in DNA damage-dependent checkpoint mutant cells. A: The efficiency of re-ligation of linearised relative to covalently closed circular plasmids in the indicated strains. B: The effects of overexpressing *RAD9* or *RAD24* on the efficiency of plasmid re-ligation in the indicated strains.

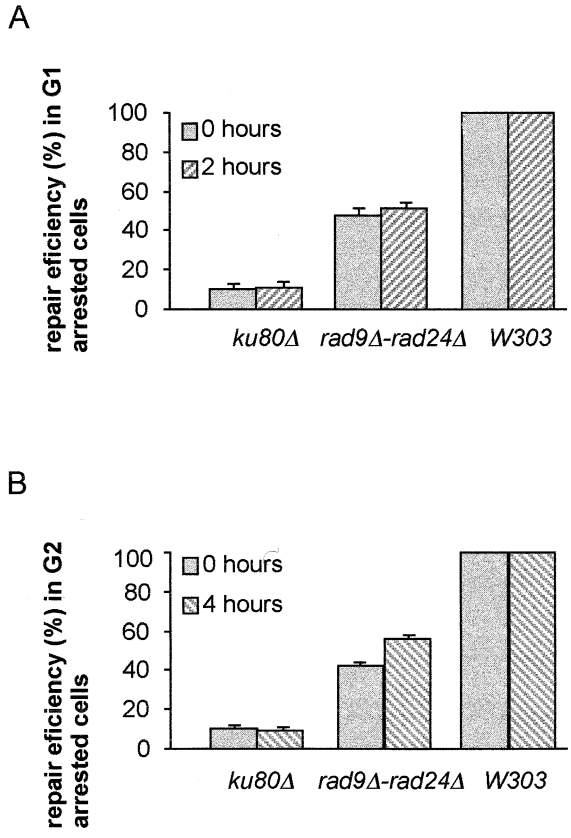

Fig. 2. Partial rescue of NHEJ by G1 and G2/M arrest. Exponentially growing cells were blocked with either (A) α-factor (10 μg/ml) or (B) nocodazole (10 μg/ml) for 2 h at 30°C. Arrested cells were either transformed with *Bam*HI digested or undigested pRS315. Transformed cells were either plated immediately after the transformation (0 h) or incubated in minimum media plus α-factor for a further 2 h, or nocodazole for a further 4 h, prior to plating as indicated.

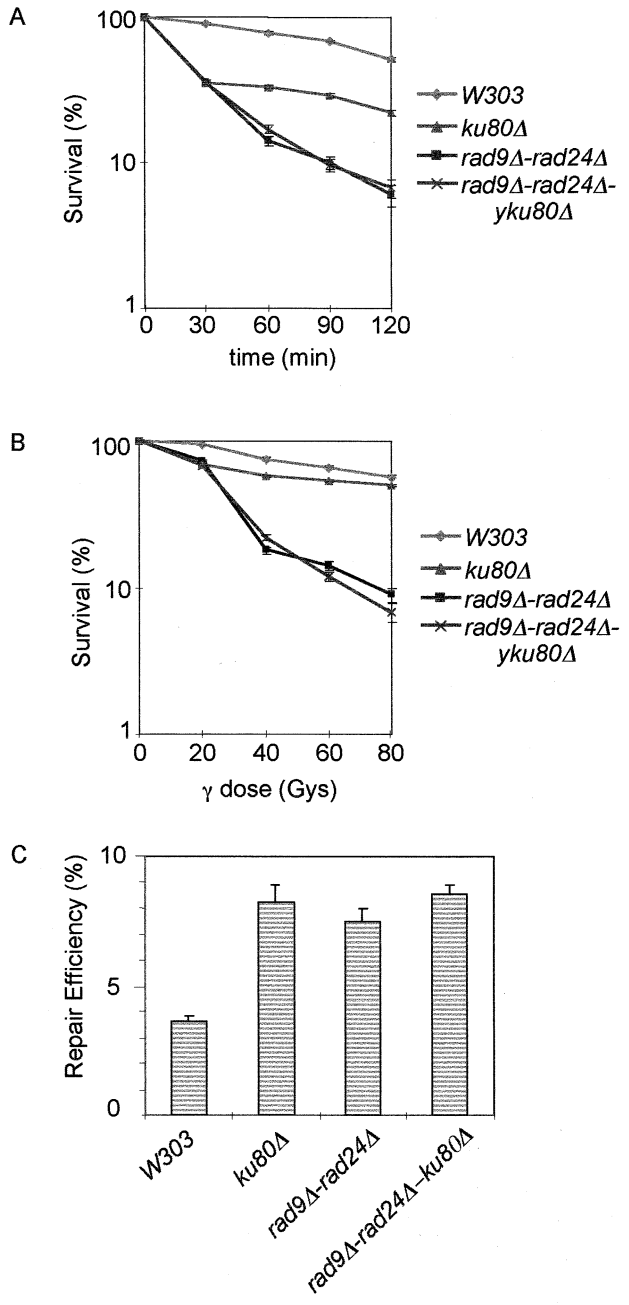

Fig. 3. RAD9 and *RAD24* are epistatic to *KU80* for survival after MMS treatment or γ-irradiation. A: Exponentially growing cells in YPD were exposed to 0.02% MMS for the times indicated and then plated onto YPD. Percentage cell survival was plotted against time in MMS. B: Exponentially growing cells in YPD were directly plated onto YPD plates and immediately γ-irradiated with the doses indicated. Percentage cell survival was plotted against dose. C: Repair of blunt ended linearised plasmids in *ku80*Δ cells and mutants of the DNA damage-dependent checkpoint pathway. The percentage of DNA repair was calculated as described in Fig. 1, the only variation was that the plasmid pRS315 was digested to completion with *Sma*I to produce blunt ended linear plasmid.

ported by overexpression of *RAD24* or *RAD9* in checkpoint mutant and *YKU80* deficient cells (Fig. 1B). Both *RAD9* and *RAD24* overexpression efficiently rescue the end-joining defect seen in *rad9*Δ and *rad24*Δ mutants (similar results were obtained with *rad17*Δ and *mec3*Δ cells, data not shown), but not the *yku80*Δ mutant. Furthermore, all transformants were accurately re-ligated (data not shown). *RAD9* or *RAD24* overexpression rescues defects observed in *rad9*Δ, *rad24*Δ, *rad17*Δ and *mec3*Δ cells after UV-irradiation [5]. These defects include survival, G1 and G2/M checkpoints, DDR induction and Rad53 phosphorylation. Rescue of the defects in NHEJ observed in checkpoint mutant backgrounds by *RAD9* or *RAD24* overexpression can now be added to this list of phenotypes.

Using single mutations of the *RAD9* or *RAD17* genes other reports have suggested that NHEJ, as measured by the plasmid re-ligation assay used here, is not appreciably dependent on the checkpoint pathway [15,16]. However, Lee et al. have also reported that repair of a single DSB introduced into a version of chromosome III lacking *HML* and *HMR* by continuous expression of the HO endonuclease was reduced three-fold in *rad9*Δ compared to WT cells [16]. Differences in growth conditions are likely to explain the discrepancies between the results presented here and those of Lee et al. with the plasmid re-ligation assay [15,16]. Growth of *rad9*Δ and *rad24*Δ cells in minimal medium prior to transformation resulted in a five-fold decrease in end re-joining (Fig. 1A), whereas growth of these cells in rich medium resulted in a reproducible but only two-fold reduction (data not shown). Nevertheless, the *rad9*Δ–*rad24*Δ double mutant resulted in a 10-fold reduction and *yku80*Δ in a 30-fold reduction, even when cells were grown in rich medium (data not shown). Thus, the relative difference between transformants obtained with *rad9*Δ-*rad24*Δ and *yku80*Δ cells is maintained, irrespective of growth conditions. We have previously noted growth condition-dependent effects on the checkpoint pathway [17]. In cells growing optimally, Rad9 hyperphosphorylation after DNA damage requires both *MEC1* and *TEL1*, whereas, in sub-optimally growing cells *MEC1* is sufficient for this modification.

3.2. The efficiency of NHEJ increases moderately in G1 or G2 blocked rad9Δ–rad24Δ cells

Failure to activate sufficient cell cycle delays after introduction of the linearised plasmid into cells could be one reason why checkpoint mutants have a deficiency in plasmid re-ligation. To test this possibility, cells arrested in G1 or G2/M were transformed with linearised or covalently closed plasmids. Arrests were maintained for up to 4 h (Fig. 2). Transformation of linearised plasmid DNA into cells arrested in either G1 or G2 did result in rescue of the number of recovered transformants in *rad9*Δ–*rad24*Δ cells to approximately 50% of that observed in WT cells, whereas *ku80*Δ cells were only rescued to 12% of the WT level. Furthermore, there was no additional rescue in the repair efficiency if transformed cells were maintained in G1 for 2 h or G2/M for 4 h after transformation. These results indicate that the efficiency of repair of linearised plasmids transformed into *rad9*Δ–*rad24*Δ cells increases in G1 and G2 blocked cells relative to cycling cells. It is possible that Ku-independent pathways might operate in G1 and G2 blocked cells to repair DSBs. However, such pathways are not very efficient as *ku80*Δ cells arrested in either G1 or G2 have only 12% of the transformation efficiency of WT cells. Furthermore, all plasmids recovered from G1 and G2/M arrested WT and *rad9*Δ–*rad24*Δ cells were accurately re-joined, suggesting that repair under these

conditions is also Ku-dependent (data not shown). Plasmids recovered from arrested *ku80*Δ cells were repaired as expected by error prone processes. Recent evidence indicates that the importance of the DNA damage checkpoint for cell survival after DNA damage is not limited to cell cycle delays [18]. It is possible that the efficiency of many types of DNA repair may be under DNA damage checkpoint control. Our data indicate that this checkpoint is required for efficient and accurate end joining and suggest that this effect cannot be totally explained by G1 or G2 cell cycle delays. It is likely that checkpoint-dependent cell cycle delays and the induction of more efficient repair both contribute to the efficiency of NHEJ in budding yeast. Mechanistic insight into how the DNA damage checkpoint might control the efficiency of end joining has been provided by studies of Sir and Ku proteins. These proteins are re-localised from telomeres to the sites of double strand breaks and this re-localisation requires an intact DNA damage checkpoint pathway [6–8]. However, even in *rad9*Δ–*rad24*Δ cells, where re-localisation of Ku and Sir proteins should not occur, some error free re-ligation of introduced linearised plasmids is detectable (Fig. 1 and data not shown). Thus re-localisation of Ku and Sir proteins must facilitate, but may not be absolutely required for, NHEJ. Other checkpoint dependent mechanisms, in addition to Ku and Sir re-localisation, might also operate to facilitate NHEJ. Genes encoding enzymes required for NHEJ might be part of the checkpoint regulated transcriptional response to DNA damage. Similarly, components necessary for efficient NHEJ may require checkpoint pathway mediated post-transcriptional activation, perhaps by phosphorylation.

3.3. *RAD9 and RAD24 operate in the same NHEJ pathway as KU80*

A requirement for both the DNA damage checkpoint pathway and the Ku proteins for efficient NHEJ would predict that mutations that prevent checkpoint pathway activation and mutation of *YKU80* are epistatic. Deletion of both *RAD9* and *RAD24* abolishes G1/S checkpoint activity and results in only residual G2/M checkpoint activity [5]. Therefore, we compared the survival of WT, *yku80*Δ, *rad9*Δ–*rad24*Δ and *rad9*Δ–*rad24*Δ–*ku80*Δ cells after MMS and γ-irradiation (Fig. 3A,B). After either treatment there is no further increase in sensitivity of the *rad9*Δ–*rad24*Δ–*yku80*Δ triple mutant compared to the *rad9*Δ–*rad24*Δ mutants. This suggests that the DNA damage checkpoint pathway and Ku function use the same pathway to repair damage caused by γ-irradiation or MMS treatment. However, as the effect of the *yku80*Δ single mutation after γ-irradiation is so small, a further increase in sensitivity caused by combining the *yku80*Δ single mutation with the *rad9*Δ–*rad24*Δ double mutation might not be easily detectable. Nevertheless, this increase should have been easily detected after MMS treatment. Therefore, *YKU80* and *RAD9–RAD24* must function in the major pathway for repair of damage caused by MMS. Our data can not rule out a role for *RAD9* and *RAD24* in other repair pathways that can repair lesions induced by γ-irradiation.

Further support for the DNA damage checkpoint pathway functioning in Ku-dependent NHEJ comes from transforming blunt-ended plasmids into WT, *yku80*Δ, *rad9*Δ–*rad24*Δ and *rad9*Δ–*rad24*Δ–*yku80*Δ cells. Surprisingly, linearised plasmids with blunt ends are inefficiently repaired by an error prone process in WT cells [12,13]. In *yku80*Δ cells this low level of repair actually increases, suggesting that normally Ku suppresses this mutagenic type of end joining. In *rad9*Δ–*rad24*Δ and *rad9*Δ–*rad24*Δ–*yku80*Δ cells transformed with blunt-ended plasmids, we also observed an increase in recovered transformants similar to that observed with *yku80*Δ cells (Fig. 3C). Thus our results suggest that *RAD9* and *RAD24* also function with Ku in suppression of this error prone repair of blunt-ended DSBs. The *rad9*Δ–*rad24*Δ–*yku80*Δ triple mutant and the *yku80*Δ single mutant have very similar levels of re-joined plasmid (1.2 ± 0.05 and $2.3 \pm 1.06\%$, respectively, see Fig. 1). It is believed that one of the roles that the Ku proteins are playing in NHEJ is protecting DNA ends from nucleases [19]. In agreement with this possibility, all residual repair observed in the single *yku80*Δ mutant is error-prone. Interestingly, however, in *rad9*Δ–*rad24*Δ–*yku80*Δ cells 66% of the rescued plasmids were efficiently repaired by an error-free mechanism. An explanation for this observation might be that in the absence of Ku-dependent protection of DNA ends, nuclease activity involved in strand degradation is checkpoint dependent to some degree. In the absence of activation of this nuclease some residual accurate repair occurs.

3.4. *RAD9 and RAD24 are not involved in RAD52-dependent homologous recombination repair*

A role for the DNA damage checkpoint pathway in the efficient operation of NHEJ raises the possibility that other DNA repair pathways may also have a degree of checkpoint pathway dependence. We have tested this possibility using a related plasmid based assay to measure site-specific integration by homologous recombination, a *RAD52*-dependent but *RAD51*-independent process [20,21]. We could not observe any dependence on the DNA damage-dependent checkpoint pathway for integration of plasmid DNA by homologous recombination (data not shown). This result does not rule out the possibility that other homologous recombination pathways, perhaps *RAD51*-dependent, are dependent on the checkpoint pathway to some degree. This hypothesis is supported by the observation that *RAD51* and *RAD54* are transcriptionally induced after DNA damage [4,5]. Furthermore, the role of *YKU80* in cell survival following ionising radiation is negligible, whereas *RAD9* and *RAD24* have significant roles (Fig. 3B). Previously we have shown that artificial holding of γ-irradiated *rad9*Δ cells in G2/M for 4 h rescued cell survival to WT levels [4]. Similar observations have also been made after irradiation with X-rays [22]. These data support a role for the DNA damage checkpoint pathway in surviving γ- and X-irradiation by simply providing enough time for repair to be completed. However, given that these cells were held for so long in G2/M (4 h for both treatments), repair by homologous recombination could proceed without the need for an inducible component. Under these conditions, any contribution from the checkpoint pathway to increasing the efficiency of homologous recombination may not have been detected. Therefore, the possibility that DNA damage checkpoints contribute to the efficiency of homologous recombination by mechanisms additional to providing more time for repair needs to be examined further.

In yeast DSBs are mainly repaired by homologous recombination, whereas in mammalian cells such lesions are primarily repaired by NHEJ. Thus, our observations suggest that an intact DNA damage checkpoint in human cells may be critically important for repairing DSBs via NHEJ. The hypoth-

esis that DNA damage checkpoints might control the efficiency of other DNA repair pathways has already found some support in human cells. Cells deficient for p53 function are also deficient in global genome, but not transcription-coupled nucleotide excision repair [23]. Furthermore, in mouse embryonic stem cells, transcription-coupled repair of oxidative damage, but not UV lesions, requires Brca1 [24], which functions in the G2/M checkpoint [25]. The involvement of the DNA damage checkpoint in multiple DNA repair pathways might be widespread.

In conclusion, our results indicate a role for the DNA damage-dependent checkpoint pathway in promoting efficient NHEJ. They also suggest that the checkpoint proteins are not absolutely required for the enzymology of the end joining process, rather they are required for its efficient operation. Furthermore, our data suggest that transient G1 or G2/M cell cycle arrests also contribute to the efficiency of NHEJ. However, WT levels of NHEJ were not restored in checkpoint defective cells even after prolonged arrests in either G1 or G2/M. Thus, the checkpoint pathway must have additional roles in regulating the efficiency of NHEJ. Consistent with this possibility, re-localisation of Ku and Sir proteins is dependent upon the DNA damage checkpoint protein kinase, Mec1 [6–8]. It is also possible that other transcriptional or post-transcriptional processes regulated by the checkpoint may be important for efficient NHEJ. An interesting possibility raised by our results is that the DNA damage dependent checkpoint pathway might regulate nuclease activity involved in processing DSBs prior to the ligation process. Our observation that the DNA damage checkpoint promotes efficient DSB repair via NHEJ may be generally applicable to other DNA repair pathways.

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References

[1] Friedberg, E.C., Walker, G.C. and Siede, W. (1995) ASM Press, Washington, DC.
[2] Weinert, T. (1998) Curr. Opin. Genet. Dev. 8, 185–193.
[3] Rhind, N. and Russell, P. (1998) Curr. Opin. Cell Biol. 10, 749–758.
[4] Aboussekhra, A., Vialard, J.E., Morrison, D.E., de la Torre-Ruiz, M.A., Cernakova, L., Fabre, F. and Lowndes, N.F. (1996) EMBO J. 15, 3912–3922.
[5] de la Torre-Ruiz, M.A., Green, C.M. and Lowndes, N.F. (1998) EMBO J. 17, 2687–2698.
[6] Martin, S.G., Laroche, T., Suka, N., Grunstein, M. and Gasser, S.M. (1999) Cell 97, 621–633.
[7] Mills, K.D., Sinclair, D.A. and Guarente, L. (1999) Cell 97, 609–620.
[8] McAinsh, A.D., Scott-Drew, S., Murray, J.A. and Jackson, S.P. (1999) Curr. Biol. 9, 963–966.
[9] Featherstone, C. and Jackson, S.P. (1999) Mutat. Res. 434, 3–15.
[10] Featherstone, C. and Jackson, S.P. (1999) Curr. Biol. 9, R759–761.
[11] Lowndes, N.F. and Murguia, J.R. (2000) Curr. Opin. Genet. Dev., in press.
[12] Boulton, S.J. and Jackson, S.P. (1996) EMBO J. 15, 5093–5103.
[13] Boulton, S.J. and Jackson, S.P. (1996) Nucleic Acids Res. 24, 4639–4648.
[14] Lydall, D. and Weinert, T. (1995) Science 270, 1488–1491.
[15] Lee, S.E., Moore, J.K., Holmes, A., Umezu, K., Kolodner, R.D. and Haber, J.E. (1998) Cell 94, 399–409.
[16] Lee, S.E., Paques, F., Sylvan, J. and Haber, J.E. (1999) Curr. Biol. 9, 767–770.
[17] Vialard, J.E., Gilbert, C.S., Green, C.M. and Lowndes, N.F. (1998) EMBO J. 17, 5679–5688.
[18] Sanchez, Y., Bachant, J., Wang, H., Hu, F., Liu, D., Tetzlaff, M. and Elledge, S.J. (1999) Science 286, 1166–1171.
[19] Teo, S.H. and Jackson, S.P. (1997) EMBO J. 16, 4788–4795.
[20] Schiestl, R.H., Zhu, J. and Petes, T.D. (1994) Mol. Cell. Biol. 14, 4493–4500.
[21] Sugawara, N., Ivanov, E.L., Fishman-Lobell, J., Ray, B.L., Wu, X. and Haber, J.E. (1995) Nature 373, 84–86.
[22] Weinert, T.A. and Hartwell, L.H. (1988) Science 241, 317–322.
[23] Hwang, B.J., Ford, J.M., Hanawalt, P.C. and Chu, G. (1999) Proc. Natl. Acad. Sci. USA 96, 424–428.
[24] Gowen, L.C., Avrutskaya, A.V., Latour, A.M., Koller, B.H. and Leadon, S.A. (1998) Science 281, 1009–1012.
[25] Xu, X. et al. (1999) Mol. Cell 3, 389–395.