Oncogene (2002) 21, 4176–4180

www.nature.com/onc

Variant XRCC3 implicated in cancer is functional in homology-directed repair of double-strand breaks

Felipe D Araujo[1], Andrew J Pierce[1], Jeremy M Stark[1] and Maria Jasin*[,1,2]

[1]*Cell Biology Program, Memorial Sloan-Kettering Cancer Center, 1275 York Avenue, New York, NY 10021, USA;* [2]*Cornell University Weill Graduate School of Medical Sciences, 1275 York Avenue, New York, NY 10021, USA*

Polymorphisms in DNA repair genes, including double-strand break (DSB) repair genes, are postulated to confer increased cancer risk. A variant of the *XRCC3* gene, which is involved in DSB repair, has been associated with increased risk of malignant skin melanoma and bladder cancer. We tested the hypothesis that this variant, Thr241Met, may affect cancer risk by disrupting a critical function of XRCC3, i.e., promoting homology-directed repair (HDR) of chromosomal DSBs. Using a quantitative fluorescence assay, we find that the variant XRCC3 protein is functionally active for HDR, complementing the HDR defects of an *XRCC3* mutant cell line as well as the wild-type protein. We also examined cells expressing this variant for sensitivity to the interstrand cross-linking agent, mitomycin C (MMC), as HDR mutant cell lines, including the *XRCC3* mutant, have been found to be hypersensitive to this DNA damaging agent. Cells expressing the variant protein were found to be no more sensitive than cells expressing the wild-type protein. These results suggest that the increased cancer risk associated with this variant may not be due to an intrinsic HDR defect.
Oncogene (2002) **21,** 4176–4180. doi:10.1038/sj.onc.1205539

Keywords: homologous recombination; DNA double-strand break repair; XRCC3; RAD51; polymorphism

XRCC3, a paralog of the strand-exchange protein RAD51 (Liu *et al*., 1998), functions in homology-directed repair (HDR) of double-strand breaks (DSBs) in mammalian cells (Pierce *et al*., 1999). XRCC3-deficient irs1SF cells are severly impaired in their ability to undergo HDR (Pierce *et al*., 1999; Brenneman *et al*., 2000) and also to form DNA damage-induced RAD51 nuclear foci (Bishop *et al*., 1998). Moreover, irs1SF cells are extremely sensitive to DNA cross-linking agents and have a high incidence of spontanteous and mutagen-induced chromosomal aberrations (Cui *et al*., 1999; Liu *et al*., 1998; Tebbs *et al*., 1995). Defects in chromosome segregation have also been observed in these cells (Griffin *et al*., 2000). Several lines of evidence indicate that XRCC3 interacts with both RAD51C, another RAD51 paralog, and possibly RAD51 itself (Kurumizaka *et al*., 2001; Liu *et al*., 1998; Masson *et al*., 2001; Schild *et al*., 2000), and recent experiments demonstrate that the XRCC3-RAD51C complex binds DNA, with a higher affinity for single-stranded DNA than for double-stranded DNA (Kurumizaka *et al*., 2001; Masson *et al*., 2001).

Emerging evidence suggests that DSB repair genes, like other genes involved in DNA repair, act as genomic caretakers, by preventing cells from accumulating the genetic alterations that promote tumorigenesis (Pierce *et al*., 2001b). Therefore, inheritance of repair genes with reduced DNA repair activity is predicted to lead to an increased cancer risk. Population studies have been undertaken to identify XRCC3 variants (Shen *et al*., 1998) and to determine their association with the development of cancer (Butkiewicz *et al*., 2001; David-Beabes *et al*., 2001; Matullo *et al*., 2001; Winsey *et al*., 2000). The *XRCC3* variant allele Thr241Met has been identified in healthy individuals with a frequency ranging from 0.231 to 0.382 (David-Beabes *et al*., 2001; Matullo *et al*., 2001; Shen *et al*., 1998; Winsey *et al*., 2000). Two epidemiological studies reported no association of the Thr241Met variant with the development of lung cancer (Butkiewicz *et al*., 2001; David-Beabes *et al*., 2001); however, a statistically signficant increase in variant allele frequency was reported in melanoma skin cancer and bladder cancer groups (allele frequencies 0.43 and 0.48, respectively) (Matullo *et al*., 2001; Winsey *et al*., 2000).

To test whether the variant XRCC3 protein could restore the ability of XRCC3-deficient irs1SF cells to repair DSBs by homologous recombination, wild-type and variant cDNAs (Figure 1a) were cloned into a pCAGGS expression vector. The cDNAs differ by a C to T transition, which results in the distinct *Nla*III restriction patterns for the expression vectors (Figure 1b). To assess the ability of each of these proteins to promote HDR, we used a quantiative *in vivo* DSB repair assay previously developed for the irs1SF cell line (Pierce *et al*., 1999). The assay utilizes the DR-GFP reporter substrate, which quantifies HDR of an I-*Sce*I endonuclease-generated chromosomal DSB by cellular green fluorescence (Figure 2a). This substrate

*Correspondence: M Jasin; E-mail: m-jasin@ski.mskcc.org
Received 14 December 2001; revised 27 March 2002; accepted 2 April 2002

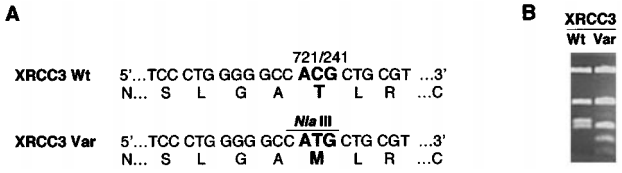

Figure 1 (**a**) Wild-type (Wt) and variant (Var) XRCC3 amino-acid and nucleotide sequences illustrating the region encompassing the polymorphism at nucleotide position 721 and amino-acid 241. (**b**) The polymorphism results in distinct *Nla*III restriction enzyme patterns in which a 171 bp fragment is cleaved to 108 and 63-bp fragments. Wild-type and variant Thr241Met *XRCC3* cDNAs were cloned into a pCAGGS expression vector (Miyazaki *et al*., 1989) by amplifying the *XRCC3* cDNA from pXR3-10 (a gift from L Thompson, Livermore), utilizing the high fidelity Vent DNA polymerase (New England Biolabs). Wild-type *XRCC3*, amplified utilizing the sense primer-A (5′-CCGGAATTCGCCAC-CATGGATTTGGA-TCTA-3′) and anti-sense primer-D (5′-CCGGAATTCTCAGTGGGACTGGGT-3′), was cloned into pCAGGS as an *Eco*RI fragment. Variant *XRCC3* was created by amplifying two independent fragments. Fragment 1 was amplified utilizing the sense primer-A and anti-sense primer-B (5′-GGCACTGCTCAGCTCACGCAGCATGGCCCCCAGGG-ACTG-3′). Fragment 2 was amplified utilizing the sense primer-C (5′-CTGCGTGAGCTGAGCAGTGCC-3′) and anti-sense primer-D. Amplified fragments were digested with *Blp*I, ligated together, and then cloned into pCAGGS as an *Eco*RI fragment. PCR fidelity was verfied by DNA sequencing. Both proteins are expressed from a hCMV enhancer/chicken β-actin promoter in the pCAGGS vector, as is I-*Sce*I in the expression vector pCBASce (see Pierce *et al*., 2001a)

is composed of two different mutated green fluorescence protein (*GFP*) genes: *SceGFP*, mutated to contain the I-*Sce*I site so as undergo a DSB when I-*Sce*I is expressed *in vivo*, and *iGFP*, a 5′ and 3′ truncated (internal) fragment that can correct the mutation in the *SceGFP* gene.

Wild-type AA8 hamster cells and the derivative XRCC3-deficient irs1SF cell line were previously constructed to contain the DR-GFP substrate at single copy in their genome, creating the DRaa-40 and DRirs-10 cell lines, respectively (Pierce *et al*., 1999). Using the cell lines we have analysed the requirement for expression of both I-*Sce*I and XRCC3 for efficient HDR. In the absence of I-*Sce*I expression, GFP positive cells are rare in both cell lines (Figure 2b). With I-*Sce*I expression, homologous recombination is induced in the wild-type DRaa-40 cells, such that 0.70% of the cell population is GFP positive in this experiment. This strong induction of HDR is not seen in the *XRCC3*-deficient cells unless an XRCC3 expression vector is co-transfected with the I-*Sce*I expression vector, in which case similar levels of HDR are found in the wild-type and complemented mutant cells. Thus, HDR is dependent upon expression of both I-*Sce*I and XRCC3, consistent with previous results (Pierce *et al*., 1999).

We next compared the activities of the wild-type and variant XRCC3 proteins to complement the HDR defect of the irs1SF cells. Using a wide range of concentrations of each XRCC3 expression plasmid, we found no difference in the ability of the wild-type and variant XRCC3 expression vectors to complement the HDR defect of the XRCC3-deficient cells (Figure 2b–d). Even at the lowest vector concentrations, similar levels of complementation are observed for transfection of either the wild-type or variant XRCC3 expression vector (e.g., for 50 ng, $0.15 \pm 0.02\%$ for wild-type versus $0.13 \pm 0.04\%$ for variant, Figure 2d). We also coexpressed I-*Sce*I with wild-type or variant XRCC3 in the wild-type cell line (DRaa-40) to test whether any dominant-negative effect on HDR would occur, since individuals carrying a single variant allele appear to have a higher cancer risk (Matullo *et al*., 2001; Winsey *et al*., 2000). Our results indicate no dominant negative effect when the variant allele is expressed (Figure 2e). Furthermore, we compared the effect of the wild-type and variant XRCC3 proteins on DSB repair pathways in addition to HDR, utilizing a PCR assay that quantifies total homologous and nonhomologous repair events leading to loss of the genomic I-*Sce*I site in the DRirs-10 cells (Pierce *et al*., 2001a). In these experiments, we found similar levels of I-*Sce*I site loss whether the wild-type or variant XRCC3 protein is expressed (data not shown). Thus, by examining repair of an induced DSB we find that there is no detectable difference between the wild-type and variant XRCC3 proteins in the ability to complement the HDR defect of XRCC3-deficient cells.

Apart from repair of a single DSB, XRCC3 also plays a role in the repair of more global DNA damage arising from carcinogen treatment, and it is conceivable that repair of these other types of damage are not being repaired as efficiently by the variant protein. Previous studies have shown that XRCC3 promotes the repair of DNA damage arising from the interstrand cross-linking agent mitomycin C (MMC) (Tebbs *et al*., 1995). We compared the ability of the wild-type and variant XRCC3 to correct the hypersensitivity of the irs1SF cells to this DNA damaging agent. Our results indicate that wild-type AA8 cells, as expected, are not affected by 0.2 μM MMC (surviving fraction of 1.2 ± 0.1), whereas the irs1SF cell line showed a >100-fold hypersensitivity (surviving fraction of 0.008 ± 0.004) (Figure 3). Transient transfection of the expression vector for either the wild-type or variant XRCC3 protein just prior to MMC exposure partially corrected the irs1SF MMC hypersensitivity to a similar extent (surviving fraction of 0.18 ± 0.005 for wild-type protein versus 0.17 ± 0.001 for the variant protein). Therefore, we are not able to detect a significant difference between the wild-type and variant proteins in their ability to correct the hypersensitivity of the irs1SF cells to this DNA damaging agent.

Thus, our results indicate that the variant XRCC3 protein is able to complement the HDR defect in XRCC3-deficient irs1SF cells to a similar extent as the wild-type protein, whether this is measured directly using a homologous recombination reporter substrate or indirectly by the ability of cells to repair DNA damage arising from a cross-linking agent. The finding that this variant protein has HDR activity is perhaps not surprising considering that individuals homozygous for the variant allele are viable. Although an *Xrcc3*

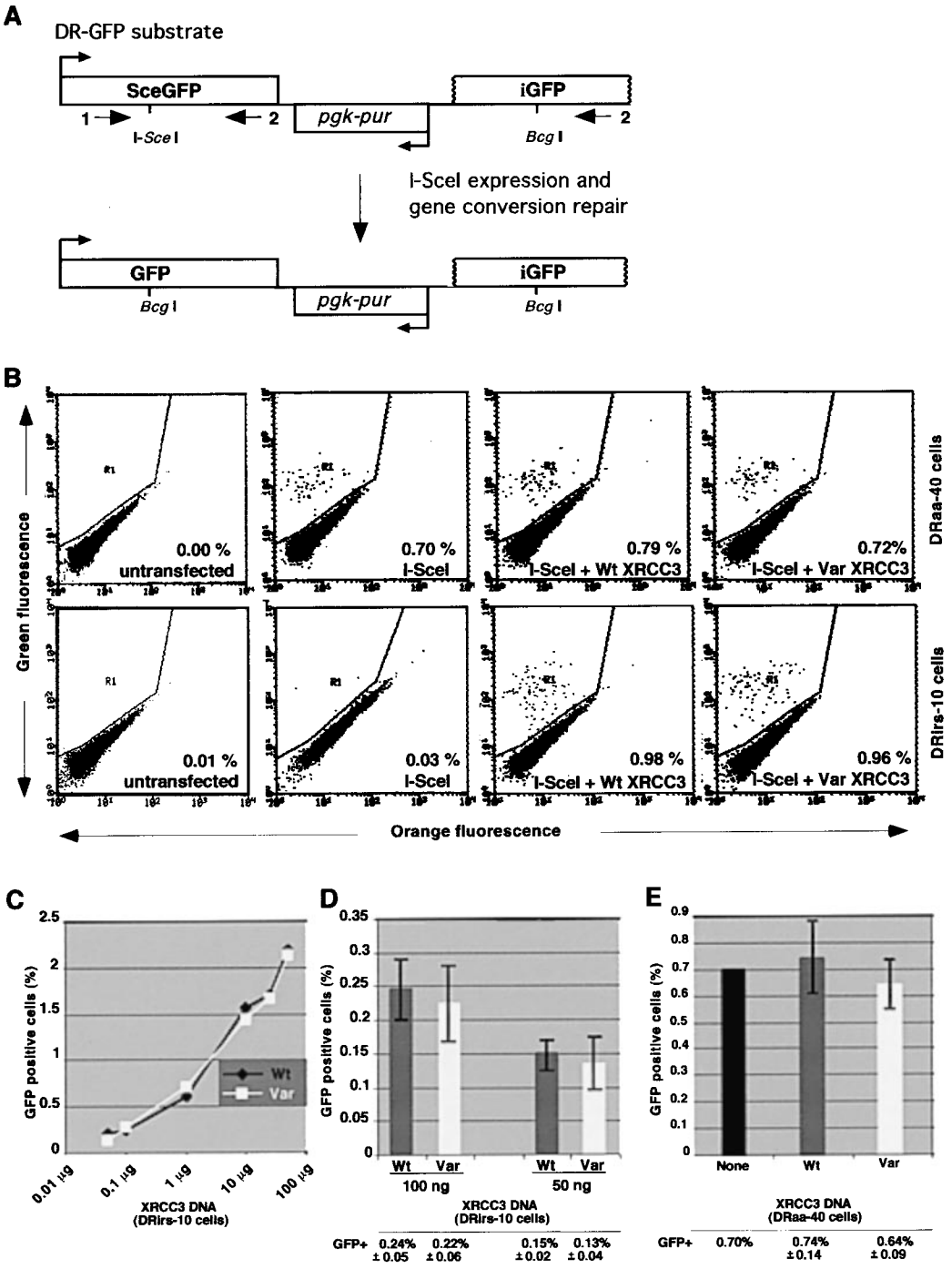

Figure 2 Wild-type and variant XRCC3 proteins complement a DSB-induced homologous recombination defect in XRCC3-deficient cells. (**a**) The DR-GFP reporter contains an I-*Sce*I endonuclease cleavage site within the coding region of a *GFP* gene (*SceGFP*). Downstream of *SceGFP* and in the same orientation is a 0.8 kb truncated *GFP* gene (*iGFP*), which can template the repair of the DSB in the *SceGFP* gene to restore functionality to the *GFP* gene. Arrows 1 and 2 indicate the position of PCR primers. (**b**) Flow cytometric analysis of HDR in wild-type (DRaa-40) and XRCC3-deficient (DRirs-10) cell lines containing the DR-GFP reporter after electroporation with the indicated expression vectors. The GFP positive population is shifted 'greenward' (R1 gate) from the GFP negative population, and the percentage of green fluorescent cells falling above the diagonal is indicated. Differences in HDR are not observed after expression of either wild-type or variant XRCC3. Titration of the XRCC3 expression vectors in the DRirs-10 cell line (**c**) or transfection of a limiting amount of the vectors (**d**) also shows no differences between the wild-type or variant XRCC3. (**e**) Expression of the variant XRCC3 protein in wild-type DRaa-40 cells does not reduce HDR. DRaa-40 and DRirs-10 cell lines have been previously described (Pierce *et al*., 1999). Electroporations were performed with log-phase cells in 650 μl phosphate buffered saline using a Bio-RAD Gene Pulser II set to 250 V, 1000 μF in a 0.4 cm cuvette. In each case, 50 μg of the I-*Sce*I vector were electroporated in each case, except in the untransfected panels in (**b**); the amount of the XRCC3 expression vectors was variable (**b**, 10 μg; **c** and **d**, as indicated; **e**, 20 μg)

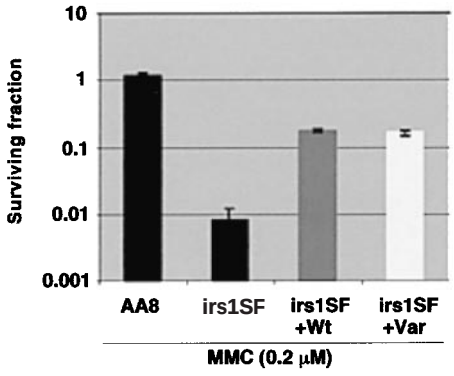

Figure 3 Correction of MMC sensitivity by wild-type and variant XRCC3 proteins in XRCC3-deficient cells. Wild-type and variant XRCC3 expression vectors (20 μg) were electroporated into irs1SF cells (1×10^7 cells per dish), except in the untransfected controls, followed by a 24-h incubation period to allow transient expression. Electroporations were performed as described above in the legend to Figure 2. Survival was measured for exponentially growing cells exposed to MMC (0.2 μM) for 1 h at 37°C. Single-cell suspensions were prepared by trypsin treatment, and cells were plated at various concentrations into 10 cm dishes. After a 7-day period, the cells were fixed in methanol and stained in 5% Giemsa. Surviving fractions were determined as the number of colonies on treated plates divided by the number of colonies on untreated plates

mouse disruption has not as yet been reported, disruption of three of the five *Rad51* paralogs leads to embryonic lethality (see Pierce *et al*., 2001b) suggesting that a null mutation in *Xrcc3* would also lead to developmental defects. Nevertheless, partial loss of function *XRCC3* alleles may be compatible with viability; yet in our assay, in which the only variable is the single amino acid change in the *XRCC3* allele, no detectable difference in HDR activity was observed between the variant and wild-type alleles.

The Thr241Met variant XRCC3 has been, on one hand, associated with the development of melanoma skin cancer (Winsey *et al*., 2000), and bladder cancer (Matullo *et al*., 2001), while on the other hand, no association has been observed with the development of lung cancer including adenocarcinoma, squamous plus small cell carcinoma, and other cell types (Butkiewicz *et al*., 2001; David-Beabes *et al*., 2001). Several explanations are possible for the seeming discrepancies between these epidemiological studies and by the lack of an observed DNA repair defect. First, diverse tissue types are known to be differentially impacted for tumor progression as a result of DNA repair defects, as for example colon and skin, from mismatch repair and nucleotide excision repair defects, respectively (Vogelstein and Kinzler, 1998). Thus, the observed cancer association may be due to skin and bladder specific factors not present in lung. However, a cell-type restriction in tumor formation is not usually associated with a similar restriction in the manifestation of the DNA repair defect. For example, for DSB repair defects, i.e., as a result of *BRCA1* and *BRCA2* mutation, tumors occur primarily in breast and ovary, but the HDR repair defect is present in other cell types as well (see Moynahan *et al*., 2001). Nevertheless, we cannot formally rule out a cell-type specific defect in DSB repair as a result of the XRCC3 polymorphism, which is not recapitulated in our hamster cell assay. Nor can we rule out genetic interactions between the variant XRCC3 and polymorphic alleles of other DNA repair genes that may lead to an HDR defect, or even an extremely mild HDR defect, that would not be detectable in our assays.

Other possible explanations relate to the case-control studies. XRCC3 could be in linkage disequilibrium with another gene responsible for the cancer association. Additionally, it is notable that allele frequencies between different control groups in the case–control studies showed a large range for the variant allele (range 0.30 to 0.38) (Butkiewicz *et al*., 2001; David-Beabes *et al*., 2001; Matullo *et al*., 2001; Shen *et al*., 1998). The difference in allele frequency within heathly subjects is even more pronounced when comparing different ethnic populations, such as African–Americans (0.231) with Caucasians (0.382) (David-Beabes *et al*., 2001). This variation points to the need for well-matched control groups in order to rule out population variation as the underlying reason for an apparent cancer risk.

Finally, it is possible that XRCC3 participates in other cellular pathways not assayed here. Thus far, separation-of-function alleles have not been identified for XRCC3 in which HDR is intact yet some other cellular phenotype is defective. It has, however, been presumed that chromosome instability and other repair phenotypes of the XRCC3-deficient cells are due to the HDR defect, as the same phenotypes are found in other HDR mutants (Pierce *et al*., 2001b).

In summary, we found that, within the limits of detection of our assays, the common and variant *XRCC3* alleles are functionally equivalent for HDR, suggesting that the reported cancer risk associated with this variant XRCC3 is not caused by an overt HDR defect.

Acknowledgments
We thank Marianne Berwick and Adam Olshen (MSKCC) for their assistance. This work was funded by NIH grant GM54688 to M Jasin.

References

Bishop DK, Ear U, Bhattacharyya A, Calderone C, Beckett M, Weichselbaum RR and Shinohara A. (1998). *J. Biol. Chem.,* **273,** 21482–21488.

Brenneman MA, Weiss AE, Nickloff JA and Chen DJ. (2000). *Mutat. Res.,* **459,** 89–97.

Butkiewicz D, Rusin M, Enewold L, Shields PG, Chorazy M and Harris CC. (2001). *Carcinogenesis,* **22,** 593 – 597.

Cui X, Brenneman M, Meyne J, Oshimura M, Goodwin EH and Chen DJ. (1999). *Mutat. Res.,* **434,** 75 – 88.

David-Beabes GL, Lunn RM and London SJ. (2001). *Cancer Epidemiol. Biomarkers Prev.,* **10,** 911 – 912.

Griffin CS, Simpson PJ, Wilson CR and Thacker J. (2000). *Nat. Cell Biol.,* **2,** 757 – 761.

Kurumizaka H, Ikawa S, Nakada M, Eda K, Kagawa W, Takata M, Takeda S, Yokoyama S and Shibata T. (2001). *Proc. Natl. Acad. Sci. USA,* **98,** 5538 – 5543.

Liu N, Lamerdin JE, Tebbs RS, Schild D, Tucker JD, Shen MR, Brookman KW, Siciliano MJ, Walter CA, Fan W, Narayana LS, Zhou ZQ, Adamson AW, Sorensen KJ, Chen DJ, Jones NJ and Thompson LH. (1998). *Mol. Cell,* **1,** 783 – 793.

Masson JY, Stasiak AZ, Stasiak A, Benson FE and West SC. (2001). *Proc. Natl. Acad. Sci. USA,* **98,** 8440 – 8446.

Matullo G, Guarrera S, Carturan S, Peluso M, Malaveille C, Davico L, Piazza A and Vineis P. (2001). *Int. J. Cancer,* **92,** 562 – 567.

Miyazaki J, Takaki S, Araki K, Tashiro F, Tominaga A, Takatsu K and Yamamura K. (1989). *Gene,* **79,** 269 – 277.

Moynahan ME, Cui TY and Jasin M. (2001). *Cancer Res.,* **61,** 4842 – 4850.

Pierce AJ, Hu P, Han M, Ellis N and Jasin M. (2001a). *Genes Dev.,* **15,** 3237 – 3242.

Pierce AJ, Johnson RD, Thompson LH and Jasin M. (1999). *Genes Dev.,* **13,** 2633 – 2638.

Pierce AJ, Stark JM, Araujo FD, Moynahan ME, Berwick M and Jasin M. (2001b). *Trends Cell Biol.,* **11,** S52 – S59.

Schild D, Lio Y, Collins DW, Tsomondo T and Chen DJ. (2000). *J. Biol. Chem.,* **275,** 16443 – 16449.

Shen MR, Jones IM and Mohrenweiser H. (1998). *Cancer Res.,* **58,** 604 – 608.

Tebbs RS, Zhao Y, Tucker JD, Scheerer JB, Siciliano MJ, Hwang M, Liu N, Legerski RJ and Thompson LH (1995). *Proc. Natl. Acad. Sci. USA,* **92,** 6354 – 6358.

Vogelstein B and Kinzler KW. (1998). *The Genetic Basis of Human Cancer*. New York: McGraw-Hill.

Winsey SL, Haldar NA, Marsh HP, Bunce M, Marshall SE, Harris AL, Wojnarowska F and Welsh KI. (2000). *Cancer Res.,* **60,** 5612 – 5616.