XRCC3 deficiency results in a defect in recombination and increased endoreduplication in human cells

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XRCC3 was inactivated in human cells by gene targeting. Consistent with its role in homologous recombination, XRCC3^{-/-} cells showed a two-fold sensitivity to DNA cross-linking agents, a mild reduction in sister chromatid exchange, impaired Rad51 focus formation and elevated chromosome aberrations. Furthermore, endoreduplication was increased five- seven-fold in the mutants. The T241M variant of XRCC3 has been associated with an increased cancer risk. Expression of the wild-type cDNA restored this phenotype, while expression of the variant restored the defective recombinational repair, but not the increased endoreduplication. RPA, a protein essential for homologous recombination and DNA replication, is associated with XRCC3 and Rad52. Overexpression of RPA promoted endoreduplication, which was partially complemented by overexpression of the wild-type XRCC3 protein, but not by overexpression of the variant protein. Overexpression of Rad52 prevented endoreduplication in RPA-overexpressing cells, in XRCC3^{-/-} cells and in the variant-expressing cells, suggesting that deregulated RPA was responsible for the increased endoreduplication. These observations offer the first genetic evidence for the association between homologous recombination and replication initiation having a role in cancer susceptibility.

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Introduction

DNA replication frequently stalls when either the template DNA is damaged or the replication machinery malfunctions.

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The stalled replication forks generate DNA double-strand breaks (DSBs) that are repaired by homologous recombination between sister chromatids (Haber, 1999). Homologous recombination is a high-fidelity repair mechanism that serves to eliminate chromosome aberrations before cell division occurs. Thus, chromosome aberrations are increased in cells lacking homologous recombination proteins, including Rad51, Rad51 paralogs, Rad54, Rad50, Mre11, Nbs1, BRCA1 and BRCA2 (Morrison and Takeda, 2000; Thompson and Schild, 2001). In addition to the general link between replication and recombination, a relationship between recombination and the initiation of replication has been reported in *Schizosaccharomyces pombe* (Segurado *et al.*, 2002). The activation of DNA replication origins was associated with the generation of recombination intermediates. The frequency of homologous integration was extremely high at the DNA replication origins. These findings suggest that DNA replication is initiated in the close vicinity of homologous integration hot spots in *S. pombe*.

To ensure that the entire genome of a cell is replicated only once in each cell cycle, replication origins must be strictly regulated. As cells enter G1, replication origins are licensed by proteins such as Cdc6 and the Mcm2–7 complex. These proteins are recruited to the origin recognition complex (ORC) to form a prereplicative complex (Diffley and Labib, 2002). Cdc6 is replaced by Cdc45, which recruits DNA polymerase α -primase and replication protein A (RPA) to the replication origin (Walter and Newport, 2000). Cyclin-dependent kinases (CDKs) have been implicated in preventing the licensing of replicated DNA. In *Saccharomyces cerevisiae*, B-type CDKs prevent endoreduplication through multiple mechanisms, including phosphorylation of the ORC, downregulation of Cdc6 and nuclear exclusion of the Mcm2–7 complex (Nguyen *et al.*, 2001). In *S. pombe*, stable association of the mitotic B-type cyclin Cdc13/Cdc2 kinase with the replication origins prevents endoreduplication (Wuarin *et al.*, 2002). Despite these advances, there is limited information available regarding a link between DNA repair and endoreduplication.

The XRCC3 gene, sharing limited sequence homology with RAD51, is inactivated in the Chinese hamster ovary (CHO) cell line irs1SF (Tebbs *et al.*, 1995; Liu *et al.*, 1998). Constitutive expression of the gene confers resistance to DNA-damaging agents in irs1SF. A fluorescence-based assay that efficiently detects chromosomal gene-conversion events in response to DSBs revealed that XRCC3 promotes the repair of DSBs by homologous recombination in irs1SF (Pierce *et al.*, 1999). A reduction of sister chromatid exchange (SCE) levels was observed in DT40 cells lacking XRCC3 (Takata *et al.*, 2001). Focus formation of Rad51 in the nucleus, induced by treatment with DNA-damaging agents, was impaired in XRCC3-deficient cells (Bishop *et al.*, 1998; Takata *et al.*, 2001). An increase in chromosome mis-segregation was

observed in *irs1SF*, which was complemented by expression of XRCC3 (Griffin *et al*, 2000). This chromosome instability was associated with increased centrosome numbers and fragmentation of the centrosome. Thus, these findings in *irs1SF* suggest that XRCC3 plays a role in chromosome stability through its involvement in centrosome integrity (Griffin, 2002).

The Rad51-like proteins form two protein complexes that play roles in homologous recombination (Masson *et al*, 2001; Liu *et al*, 2002). One complex contains Rad51B/Rad51L1, Rad51C/Rad51L2, Rad51D/Rad51L3 and XRCC2 (BCDX2). This complex binds single-stranded DNA and single-stranded gaps in duplex DNA, suggesting that BCDX2 plays a presynaptic role in recombination. The other complex consists of XRCC3 and Rad51C, and may play a role in homologous pairing (Kurumizaka *et al*, 2001). Moreover, XRCC3 may function not only at the initiation of homologous recombination but also at late stages in the resolution of recombination intermediates (Brenneman *et al*, 2002).

A population-based breast cancer case-control study analyzing polymorphisms in several DNA double-strand break repair genes has revealed that the only amino-acid substitution significantly associated with increased risk was XRCC3 T241M (Kuschel *et al*, 2002). This association was also found for melanoma, bladder carcinoma and squamous cell carcinoma of the head and neck (Winsey *et al*, 2000; Matullo *et al*, 2001; Shen *et al*, 2002), while no association was found for lung carcinoma (Butkiewicz *et al*, 2001; David-Beabes *et al*, 2001). This variant protein complements defects in homologous recombination repair in XRCC3-deficient hamster cells, suggesting that the increased cancer risk associated with the variant is not attributable to repair defects (Araujo *et al*, 2002).

The functions of genes involved in homologous recombination are not always conserved throughout evolution (Lim and Hasty, 1996; Tsuzuki *et al*, 1996; Rijkers *et al*, 1998; Yamaguchi-Iwai *et al*, 1998). To determine the role of XRCC3 in human cells, the gene was deleted in the human colon cancer cell line HCT116 because it has normal mitotic and p53-dependent G1 tetraploidy checkpoints (Michel *et al*, 2001). Defects in homologous recombination and increased endoreduplication were observed in *XRCC3*^{-/-} cells. Furthermore, expression of the variant that is associated with increased cancer risk restored only the defective homologous recombinational repair, but not the increased endoreduplication. RPA was physically associated with XRCC3 and Rad52. RPA-overexpressing cells exhibited the same phenotype as the mutant expressing the variant XRCC3 protein, which was complemented by overexpression of the wild-type XRCC3 protein but not by overexpression of the variant protein. Rad52 prevented endoreduplication in cells overexpressing RPA, in *XRCC3*^{-/-} cells, and in the mutant expressing the variant XRCC3 protein. This observation suggests that endoreduplication resulting from altered XRCC3 function is associated with RPA dysfunction.

Results

Generation of human *XRCC3*^{-/-} cells

The human *XRCC3* locus was disrupted by the insertion of promoterless drug-resistance genes (Figure 1A). Five homozygous mutants were obtained from 193 hygromycin-resis-

tant clones (Figure 1B). Western blot analysis showed that no XRCC3 protein was expressed in the homozygous mutant (Figure 1C). In complementation experiments, the human *XRCC3* cDNA was expressed under the control of the MSV enhancer and the MMTV LTR in the homozygous mutant. A high level of XRCC3 expression was achieved in the presence of dexamethasone (Dex) (Figure 1C). However, the cells having high levels of XRCC3 (more than three-fold higher than endogenous levels) did not grow well. Moderate levels of expression comparable to that of endogenous expression were observed even in the absence of Dex. HCT116 cells were heterozygous for the T241M variation (data not shown).

Growth and sensitivity to DNA-damaging agents of *XRCC3*^{-/-} cells

The growth rate of *XRCC3*^{-/-} cells was almost the same as that of wild-type cells (Figure 2A). The doubling time of the cells was 16 h. *XRCC3*^{-/-} cells showed modest sensitivity to mitomycin C (MMC) (two-fold) (Figure 2B), while they showed no significant increase in sensitivity to ionizing radiation (data not shown). They also showed a similar modest sensitivity to cisplatin (two-fold) (data not shown). Expression of the wild-type (Wt) or variant (Var) *XRCC3* cDNA in the mutant restored this phenotype to levels of sensitivity comparable to that in wild-type cells. Thus, XRCC3 was shown to confer resistance to DNA cross-linking agents in human cells.

Homologous recombination in *XRCC3*^{-/-} cells

A reduction of SCE levels is the hallmark of a defect in sister chromatid-based recombination, and has been observed in DT40 cells lacking homologous recombination proteins, including Rad51 paralogs (Sonoda *et al*, 1999; Takata *et al*, 2001). *XRCC3*^{-/-} cells showed a mild reduction of the level of MMC-induced SCE ($P < 0.0001$, Mann-Whitney *U*-test), which was restored by the expression of the wild-type or variant cDNA (Table I). The frequency of targeted integration reflects that of homologous recombination without the use of sister chromatids. To examine the effect of XRCC3 on targeted integration, we measured the frequency at two independent loci, *RAD54B* and *RAD51C* (Miyagawa *et al*, 2002). No statistically significant difference in the targeted integration frequency between wild-type cells and *XRCC3*^{-/-} cells was observed (Table I), implying that XRCC3 plays a role in homologous recombination specifically by using sister chromatids.

Rad51 focus formation is impaired in *XRCC3*^{-/-} cells

DNA-damaging agents induce focus formation of the Rad51 protein in the nucleus, which is required for homologous recombination (Tashiro *et al*, 2000). A lack of Rad51 focus formation has been reported in cells with defects in homologous recombination (Bishop *et al*, 1998; Takata *et al*, 2001). Therefore, we examined the focus formation of Rad51 in *XRCC3*^{-/-} cells. Distinct foci were observed after irradiation in wild-type cells (7.03 ± 1.90 foci per cell, mean $\pm$ s.e.m.) and in the mutant expressing the wild-type (9.41 ± 1.75) or variant cDNA (9.00 ± 1.48), whereas the number of foci was significantly reduced in *XRCC3*^{-/-} cells (2.02 ± 0.79) (Figure 2C). These findings are consistent with the finding that sister chromatid-based recombination mediated by Rad51 is inhibited by the lack of a functional *XRCC3* gene.

Figure 1 Generation of $XRCC3^{-/-}$ HCT116 cells. (A) Schematic representation of the $XRCC3$ locus, the targeting vectors and the targeted alleles. Relevant restriction sites and the position of the probe used for Southern blot analysis are shown. (B) Southern blot analysis of DNA from HCT116 cells. (C) Western blot analysis of whole-cell extracts from HCT116 cells. For induction of $XRCC3$ expression by the MMTV promoter, the cells were cultured in the presence of 250 nM dexamethasone (Dex). The asterisk indicates a nonspecific background band that served as an internal control.

Chromosome aberrations in $XRCC3^{-/-}$ cells

To examine the effect of $XRCC3$ on chromosome stability, we performed chromosome analysis by preparing metaphase spreads in the presence of colcemid. To exclude clonal variation as the cause of chromosome aberrations, we examined five independent $XRCC3^{-/-}$ cell lines derived from a single $XRCC3^{+/+}$ cell line. In complementation experiments, $XRCC3$ cDNAs were introduced into the $XRCC3^{-/-}$ #83 cell line. Chromatid-type and chromosome-type aberrations were more frequently observed in $XRCC3^{-/-}$ cells than in wild-type cells and in the mutant expressing the transfected cDNAs (Table II), which is consistent with previous reports regarding *irs1SF* and the DT40 mutant (Tebbs *et al*, 1995; Liu *et al*, 1998; Takata *et al*, 2001). Furthermore, a significant increase in endoreduplication characterized by chromosome doubling was observed in $XRCC3^{-/-}$ cells (Figure 2D). Endoreduplicated cells were rare in wild-type cells (0.26%), while the number of these cells increased five- seven-fold in the mutants (Table II). The differences in the frequency of tetraploidy between wild-type cells and $XRCC3^{-/-}$ cells are statistically significant ($P < 0.01$, Fisher's exact test). There was no difference with respect to chromosome aberrations between wild-type cells and single-knockout cells, in which the variant allele was deleted. Expression of the wild-type $XRCC3$ cDNA in the mutant reduced the number of tetraploid cells to a level comparable to that in wild-type cells ($XRCC3^{-/-}$ #83 + Wt#18 and $XRCC3^{-/-}$ #83 + Wt#26), while expression

of the variant cDNA did not ($XRCC3^{-/-}$ #83 + Var#2 and $XRCC3^{-/-}$ #83 + Var#3) (Table II). The differences between the mutants expressing the wild-type versus the variant cDNA are statistically significant ($P < 0.001$). Despite this difference, expression of the variant restored chromosome aberrations to the wild-type level.

To further confirm endoreduplication as well as to examine aneuploidy, we performed fluorescence *in situ* hybridization (FISH) using chromosome-specific centromeric probes. Consistent with the findings of chromosome analysis, the numbers of cells with four chromosomes significantly increased in $XRCC3^{-/-}$ cells, whereas an increase in aneuploidy characterized by one or three chromosomes was not evident in $XRCC3^{-/-}$ cells (Figure 2E and Table III). The differences in the frequency of the four signals are statistically significant between wild-type cells and $XRCC3^{-/-}$ cells ($P < 0.05$). This phenotype was observed in all the five $XRCC3^{-/-}$ cell lines, excluding clonal variation as the cause of endoreduplication. A difference in the frequency of tetraploidy between the mutants expressing the wild-type cDNA and the mutants expressing the variant was also confirmed ($P < 0.01$).

Intact mitotic and G1 tetraploidy checkpoints in $XRCC3^{-/-}$ cells

Since cells with a defective mitotic checkpoint show tetraploidy in the presence of microtubule inhibitors (Cahill *et al*, 1998; Michel *et al*, 2001), we examined the mitotic index

Figure 2 A defect in homologous recombination and endoreduplication in *XRCC3*^{-/-} cells. (A) Growth curve. (B) Sensitivity to MMC. The error bars in (A) and (B) represent the standard error of the mean for three independent experiments. (C) Rad51 focus formation. Cells were nontreated or irradiated with 8 Gy and stained at 1.5 h after irradiation with anti-Rad51 antibody. Dex was added in the complementation experiment. (D) Endoreduplicated metaphase spread from *XRCC3*^{-/-} cells. (E) A microscopy image of interphase FISH on *XRCC3*^{-/-} cells using a probe for chromosome 17 (green) and a probe for chromosome 3 (orange). Magnification $\times 1000$.

Table 1 Frequencies of homologous recombination in HCT116 cell lines

Cell line	SCEs/cell ^a		Targeted integration ^b	
	Spontaneous ^c	MMC-induced (<i>P</i> -value ^d)	RAD54B-bsd	RAD51C-pur
<i>XRCC3</i> ^{+/+}	3.6 $\pm$ 0.3	8.9 $\pm$ 0.3 (<0.0001)	2.47% (7/283)	0.26% (5/1930)
<i>XRCC3</i> ^{-/-}	3.5 $\pm$ 0.2	6.5 $\pm$ 0.3	1.72% (6/349)	0.22% (3/1361)
<i>XRCC3</i> ^{-/-} + Wt ^e	4.0 $\pm$ 0.2	9.3 $\pm$ 0.3 (<0.0001)	ND	ND
<i>XRCC3</i> ^{-/-} + Var ^e	3.6 $\pm$ 0.2	8.2 $\pm$ 0.4 (0.022)	ND	ND

^aNumber of SCEs per cells represents the mean $\pm$ s.e.m. from at least 100 mitotic cells.

^bThe frequency of targeted integration is shown as a percentage of correctly targeted clones relative to the total number of drug-resistant clones analyzed; absolute numbers are given in parentheses.

^cThere is no statistically significant difference between *XRCC3*^{-/-} and *XRCC3*-expressing cell lines.

^dBased on Mann-Whitney *U*-tests, comparing either *XRCC3*^{+/+} with *XRCC3*^{-/-} or the *XRCC3*-transfected cell lines with *XRCC3*^{-/-}.

^eComplementation experiments were performed in the absence of Dex. ND, not determined.

of unsynchronized cells treated with nocodazole. Both wild-type and *XRCC3*^{-/-} cells showed accumulations of cells with condensed chromosomes characteristic of a sustained mitotic block, suggesting that the mitotic checkpoint is intact in *XRCC3*^{-/-} cells (data not shown).

Cells lacking p53, Rb and cyclin-dependent kinase inhibitors undergo microtubule inhibitor-induced endoreduplication (Cross *et al*, 1995; Di Leonardo *et al*, 1997; Hussain Khan and Wahl, 1998; Lanni and Jacks, 1998). Therefore, the G1 tetraploidy checkpoint pathway was investigated by

Table II Chromosome aberrations in HCT116 cell lines

Cell line	Chromatid type ^a	Chromosome type ^a	Abnormal cells ^a (excluding tetraploidy)	Tetraploidy ^b
XRCC3 ^{+/+}	9	1	8	0.26% (5/1936)
XRCC3 ^{+/-}	11	1	10	0.28% (2/708)
XRCC3 ^{-/-} #26	19	3	20	1.50% (15/1000)
XRCC3 ^{-/-} #83	20	4	23	1.80% (27/1496)
XRCC3 ^{-/-} #111	22	6	24	1.58% (16/1012)
XRCC3 ^{-/-} #117	18	8	23	1.21% (14/1161)
XRCC3 ^{-/-} #119	22	1	22	1.44% (13/902)
XRCC3 ^{-/-} #83 + Wt#18	10	1	10	0.29% (3/1034)
XRCC3 ^{-/-} #83 + Wt#26	7	0	6	0.19% (2/1063)
XRCC3 ^{-/-} #83 + Var#2	3	0	3	2.32% (23/993)
XRCC3 ^{-/-} #83 + Var#3	9	0	9	3.86% (46/1192)
XRCC3 ^{+/+} + RPA70	7	1	7	2.91% (43/1478)
XRCC3 ^{+/+} + RPA70 + Wt	6	0	4	0.93% (20/2141)
XRCC3 ^{+/+} + RPA70 + Var	2	1	2	3.06% (46/1501)
XRCC3 ^{+/+} + RPA32	9	0	9	3.30% (37/1122)
XRCC3 ^{+/+} + RPA32 + Wt	3	0	2	1.08% (15/1395)
XRCC3 ^{+/+} + RPA32 + Var	1	0	1	2.89% (36/1244)
XRCC3 ^{+/+} + RPA14	17	0	8	2.91% (31/1065)
XRCC3 ^{+/+} + RPA14 + Wt	2	0	2	0.71% (15/2109)
XRCC3 ^{+/+} + RPA14 + Var	2	0	2	2.01% (50/2492)
XRCC3 ^{+/+} + RPA14 + Rad52#1	4	0	4	0.67% (7/1051)
XRCC3 ^{+/+} + RPA14 + Rad52#2	7	1	8	0.56% (5/885)
XRCC3 ^{-/-} #83 + Rad52#1	6	0	5	0.79% (24/3041)
XRCC3 ^{-/-} #83 + Rad52#9	12	0	10	0.73% (10/1379)
XRCC3 ^{-/-} #83 + Var#3 + Rad52#14	4	1	5	0.93% (15/1621)
XRCC3 ^{-/-} #83 + Var#3 + Rad52#17	7	0	7	0.30% (2/669)
XRCC3 ^{-/-} #83 + Rad51C#2	7	4	10	3.45% (36/1043)

^aA total of 100 cells were scored for each line.

^bThe frequency of tetraploidy is shown as a percentage of tetraploid cells to the total number of metaphase analyzed; absolute numbers are given in parenthesis.

All experiments were performed in the absence of Dex.

Table III Distribution of chromosome numbers in HCT116 cell lines

Cell line	Number of FISH signals per cell (%)			
	1	2	3	4
Chromosome 9				
XRCC3 ^{+/+}	3.1	94.4	1.9	0.6
XRCC3 ^{-/-} ^a	5.8±1.4	89.0±1.4	2.3±0.4	2.9±0.3
Chromosome 17				
XRCC3 ^{+/+}	4.4	93.8	1.5	0.4
XRCC3 ^{-/-} ^a	3.9±0.4	91.0±0.4	2.7±0.2	2.5±0.4
XRCC3 ^{-/-} + Wt ^b	3.5	94.0	2.0	0.5
XRCC3 ^{-/-} + Var ^b	3.4	92.0	1.0	3.7

^aData are presented as the mean±s.e.m. of the pools of five individual clones.

^bData are presented as the mean of two individual clones. At least 500 cells were scored for each cell line.

examining the DNA content in unsynchronized cell populations treated with nocodazole. Although a small proportion of wild-type cells and XRCC3^{-/-} cells acquired 8N DNA content after up to 72 h in the presence of nocodazole, no clear difference between these cells was observed (data not shown). No increase in 8N DNA content in the mutants not having undergone nocodazole treatment was detected by FACS analysis, because only a small proportion of cells underwent endoreduplication. These observations exclude the possibility that a defect in the mitotic or G1 tetraploidy checkpoint causes endoreduplication in XRCC3^{-/-} cells.

RPA is physically associated with XRCC3 and Rad52

RPA is required for homologous recombination and DNA replication. To test whether RPA is involved in increased endoreduplication resulting from XRCC3 deficiency, we examined the physical association between these proteins. Since the potential complex was likely DNA bound, DNase I was used to release the possible interacting proteins from DNA (Zou and Stillman, 2000). Anti-RPA32 antibody pulled down XRCC3 in wild-type cells and in the mutants expressing the transfected cDNAs, but not in XRCC3^{-/-} cells, suggesting that these proteins physically associate *in vivo* (Figure 3A). To examine whether XRCC3 directly binds to RPA, we analyzed the association between XRCC3 and each subunit of RPA in transiently transfected COS7 cells. We observed a direct association between RPA32 and XRCC3 in transfected COS7 cells (Figure 3B), while no direct association between RPA14 and XRCC3 was observed (data not shown). This finding suggests that RPA32 mediates the direct association between RPA and XRCC3, although we do not exclude the possibility that RPA70 directly binds to XRCC3.

We next examined the association between RPA and Rad52 in unirradiated cells, because interaction between these proteins is required for homologous recombination (Park *et al*, 1996). Anti-RPA32 antibody pulled down the Rad52 protein in transfected COS7 cells (Figure 3C). Overexpression of XRCC3 did not affect the interaction between RPA32 and Rad52. We also examined the association between XRCC3 and Rad52, because the role of Rad52 overlaps with that of XRCC3 in homologous recombinational repair in DT40 cells (Fujimori *et al*, 2001). However, we

Figure 3 Association of RPA with XRCC3 and Rad52 in unirradiated cells. (A) Coimmunoprecipitation of XRCC3 with RPA32 from HCT116 cell lysates. Protein complexes were precipitated using anti-RPA32 antibody and visualized by Western blotting using anti-XRCC3 antibody. (B) Coimmunoprecipitation of XRCC3 with RPA32 from transiently transfected COS7 cell lysates. The RPA32 cDNA was transfected with or without the XRCC3 cDNA. Protein complexes were precipitated using anti-RPA32 antibody and visualized by Western blotting using anti-XRCC3 or anti-RPA32 antibody. (C) Coimmunoprecipitation of RPA32 with Rad52 from transfected COS7 cell lysates. The RPA32 cDNA was transfected with or without the RAD52 cDNA. Protein complexes were precipitated using anti-RPA32 antibody and visualized by Western blotting using anti-Rad52 or anti-RPA32 antibody.

found no direct association between these proteins in transiently transfected COS7 cells (data not shown).

Overexpression of RPA promotes endoreduplication

To provide evidence for the role of RPA in increased endoreduplication, each subunit of the RPA complex was stably overexpressed in wild-type cells. An approximately two-three-fold increase in the expression of each subunit was observed (Figure 4A). In RPA-overexpressing cells ($XRCC3^{+/+}$ + RPA70, $XRCC3^{+/+}$ + RPA32 and $XRCC3^{+/+}$ + RPA14), chromosome aberrations excluding tetraploidy were not increased, while the frequency of tetraploidy was increased 11–12-fold (Table II). This phenotype was observed in nine independent cell lines, thereby excluding clonal variation as the cause of endoreduplication. This observation suggests that increased RPA promotes endoreduplication.

RPA-induced endoreduplication is prevented by the wild-type XRCC3 protein, but not by the variant protein

To test whether the interaction between RPA and XRCC3 is important for endoreduplication, XRCC3 was overexpressed in RPA-overexpressing cells. Approximately a two-fold in-

crease in XRCC3 expression was observed (Figure 4B). Overexpression of the wild-type XRCC3 in RPA70-overexpressing cells reduced the frequency of tetraploidy ($XRCC3^{+/+}$ + RPA70 + Wt) ($P < 0.0001$). In contrast, the frequency by overexpression of the variant ($XRCC3^{+/+}$ + RPA70 + Var) was not significantly different from the frequency in the parental cells. Similarly, overexpression of the wild-type XRCC3 protein in RPA32-overexpressing cells reduced the frequency ($XRCC3^{+/+}$ + RPA32 + Wt) ($P < 0.0001$), whereas overexpression of the variant did not ($XRCC3^{+/+}$ + RPA32 + Var). Overexpression of the wild-type XRCC3 protein in RPA14-overexpressing cells reduced the frequency ($XRCC3^{+/+}$ + RPA14 + Wt) ($P < 0.001$), whereas overexpression of the variant did not ($XRCC3^{+/+}$ + RPA14 + Var). Thus, overexpression of the wild-type XRCC3 protein, but not the variant protein, is capable of preventing RPA-induced endoreduplication.

Rad52 prevents endoreduplication resulting from RPA and XRCC3 dysfunctions

Our finding that Rad52 associated with RPA in unirradiated cells prompted us to hypothesize that Rad52 may affect RPA

Figure 4 Overexpression of transfected cDNAs identified by Western blotting. (A) Overexpression of each subunit of RPA in HCT116 cells. (B) Overexpression of XRCC3 in RPA70-overexpressing cells. (C) Overexpression of Rad52 in *XRCC3*^{-/-} cells, in the variant protein mutant and in RPA14-overexpressing cells. (D) Overexpression of Rad51C in *XRCC3*^{-/-} cells.

functions other than homologous recombination. To address this, Rad52 was introduced in RPA14-overexpressing cells. Approximately a three- to five-fold increase in Rad52 expression was observed (Figure 4C). Overexpression of Rad52 reduced endoreduplication (*XRCC3*^{+/+} + RPA14 + Rad52) ($P < 0.0001$). This finding suggests that increased Rad52 prevents RPA-induced endoreduplication.

To provide evidence for the functional interaction between XRCC3 and Rad52 in increased endoreduplication, Rad52 was overexpressed in *XRCC3*^{-/-} cells. Overexpression of Rad52 reduced the frequency of tetraploidy (*XRCC3*^{-/-} #83 + Rad52). To examine whether the same pathway is responsible for increased endoreduplication associated with the variant XRCC3 protein, Rad52 was overexpressed in the variant protein mutants. Overexpression of Rad52 reduced the frequency of tetraploidy (*XRCC3*^{-/-} #83 + Var#3 + Rad52). The differences in the frequency of tetraploidy between the original cells and Rad52-overexpressing cells were statistically significant ($P < 0.01$). These findings suggest that endoreduplication associated with the variant XRCC3 protein may be caused by the deregulated RPA protein that can be regulated by overexpression of Rad52.

We also examined the effect of Rad51C on chromosome aberrations in *XRCC3*^{-/-} cells because XRCC3 stably associ-

ates with Rad51C (Masson *et al*, 2001; Liu *et al*, 2002). Approximately a two-fold increase in Rad51C expression was observed (Figure 4D). Overexpression of Rad51C complemented the chromosome aberrations excluding tetraploidy, whereas it did not affect the frequency of increased tetraploidy (*XRCC3*^{-/-} #83 + Rad51C#2). This finding supports the critical role of RPA and Rad52 in the link between homologous recombination and the initiation of DNA replication.

Discussion

Rad51 plays a central role in homologous recombination through its catalysis of homologous DNA pairing and strand exchange. Accumulating evidence suggests that Rad51 paralogs may function as cofactors assisting the action of Rad51. Rad51D binds to single-stranded DNA and exhibits DNA-stimulated ATPase activity (Braybrooke *et al*, 2000). The Rad51B–Rad51C complex facilitates the replacement of RPA from the nucleofilaments by Rad51 and promotes the DNA-strand exchange activity of Rad51 (Sigurdsson *et al*, 2001). Both Rad51B and Rad51C bind to single- and double-stranded DNA and exhibit DNA-stimulated ATPase activity. Moreover, Rad51C promotes double-stranded DNA separation (Lio *et al*,

2003). These results suggest that Rad51C may play a more direct role in the recombinational repair pathway in addition to its presynaptic mediator role.

Recent evidence from mutations in DT40 and CHO cell lines has suggested an important role for Rad51 paralogs in homologous recombination. Knockout mutants of five Rad51 paralogs in DT40 cells exhibited hypersensitivity to DNA-damaging agents, elevated chromosome aberrations, reduced frequencies of SCE and impaired damage-dependent Rad51 focus formation (Takata *et al*, 2000, 2001). XRCC2 and XRCC3 hamster mutants also exhibited similar phenotypes (Liu *et al*, 1998). Two Rad51C hamster mutants, *irs3* and *CL-V4B*, also showed defects in homologous recombinational repair (French *et al*, 2002; Godthelp *et al*, 2002). To help define a role for XRCC3 in homologous recombination, the gene was inactivated in human cells. We found much milder phenotypes with respect to recombinational repair in the HCT116 mutants, in which extreme sensitivity to MMC, as reported in *irs1SF* (60-fold), was not observed. This difference between hamster and human cells indicates a need for caution in extrapolating functional data for homologous recombination from one species to another. Despite this difference, the present results demonstrate that XRCC3 is involved in Rad51-dependent processes, in accord with the notion that it plays an early role in recombinational repair.

We did not observe an increased rate of chromosome mis-segregation in *XRCC3*^{-/-} cells. Aneuploidy in wild-type HCT116 cells is approximately 6% according to FISH analysis (Table III). A slight increase in aneuploidy might be undetectable due to the high incidence of aneuploidy in HCT116 cells. In *irs1SF*, the increased mis-segregation is associated with centrosome abnormalities. We examined *XRCC3*^{-/-} cells in interphase and mitosis for centrosome abnormalities by immunostaining with anti- γ -tubulin antibody (Griffin *et al*, 2000) and found no centrosome abnormalities (data not shown). It is well established that HCT116 has normal mitotic and p53-dependent checkpoints (Michel *et al*, 2001). The *irs1SF* cells were obtained from parent cell line CHO-AA8 after exposure to a DNA-damaging agent. This difference in genetic background may explain the phenotypic difference between these cells.

In addition to the role of XRCC3 in homologous recombination, this paper provides the first description of increased endoreduplication resulting from XRCC3 deficiency. Genetic studies of yeast proteins involved in cell cycle control are beginning to provide new insights into how endoreduplication is prevented. In fission yeast, the mitotic B-type cyclin Cdc13/Cdc2 kinase associates with replication origins, preventing endoreduplication. After the activation of Cdk and Cdc7, replication origins are unwound and RPA is recruited. Fractionation and reconstitution experiments using a cell-free system from human cells that initiates DNA replication have recently revealed that RPA is an initiation factor for human chromosomal DNA replication (Szűts *et al*, 2003). RPA is a heterotrimeric single-stranded DNA-binding protein. The nucleolar signal unique to RPA14, however, indicates the presence of RPA14 that is not associated with the RPA complex (Dimitrova and Gilbert, 2000). These reports are consistent with our finding that overexpression of each subunit of RPA promotes endoreduplication. Although we do not exclude the possibility that XRCC3 may interact with Cyclin/Cdk complexes, our observation that XRCC3 prevents

RPA-induced endoreduplication leads us to propose that the interaction of XRCC3 and RPA plays a role in the replication initiation.

XRCC3 and Rad51 have been shown to modulate the progression of replication forks on damaged vertebrate chromosomes. A similar result was observed with *XRCC2*^{-/-} cells, but not with *RAD54*^{-/-} cells (Henry-Mowatt *et al*, 2003). This finding suggests that fork slowing may require homologous recombination proteins that are involved in the early stages of recombination. It is therefore likely that Rad51 paralogs maintain replication fork integrity by allowing time for repair of damaged DNA. Given that fork progression is slowed on damaged chromosomes, a mechanism that prevents endoreduplication during fork slowing may be required. It is possible that XRCC3 may prevent endoreduplication that is related to fork slowing, although a direct model of this interaction remains to be demonstrated.

Our observation suggests that the association of RPA with XRCC3 plays a critical role in preventing endoreduplication. There is accumulating evidence for the interaction of RPA with proteins that are involved in homologous recombination. RPA also interacts with BRCA2, a protein that appears to function in homologous recombination. The breast cancer-predisposing mutation BRCA2 Y42C compromised the interaction between RPA and BRCA2 (Wong *et al*, 2003). Moreover, RPA interacts with WRN and BLM and stimulates their helicase activities (Brosh *et al*, 1999, 2000). These interactions have been shown to affect homologous recombination repair. Whether these interactions affect the replication initiation machinery remains to be determined.

Overexpression of Rad52 complemented chromosome aberrations, including gaps and breaks, and increased endoreduplication in HCT116 mutants; these findings suggest that the protein may play a role in maintaining chromosomal integrity and the initiation of DNA replication, despite the fact that Rad52-deficient DT40 and mouse ES cells exhibited no prominent phenotype (Rijkers *et al*, 1998; Yamaguchi-Iwai *et al*, 1998). This hypothesis is partly supported by the finding that the role of Rad52 overlaps with that of XRCC3 in homologous recombinational repair in DT40 (Fujimori *et al*, 2001). The prevention of RPA-induced endoreduplication by excess Rad52 suggests that the interaction between these proteins may play a role in regulating the initiation of DNA replication.

Overexpression of Rad51C restored the chromosome aberrations but not the increased endoreduplication in *XRCC3*^{-/-} cells. Among the protein complexes between Rad51 paralogs, Rad51C is a central player that interacts directly with XRCC3, Rad51B and Rad51D. This evidence supports the notion that Rad51C may play multiple roles in homologous recombination. Rad51C shows DNA-stimulated ATPase and DNA melting/separating activities, emphasizing a key role for Rad51C in recombinational repair. It is highly likely that increased Rad51C is capable of eliminating the chromosome aberrations in *XRCC3*^{-/-} cells through these significant functions.

The T241M variant is a nonconservative substitution, which might change protein structure and function. Present studies have demonstrated that the variation does not affect protein-protein interactions. However, our finding that increased Rad52 prevented endoreduplication in the variant XRCC3-expressing cells as well as in RPA-overexpressing cells and *XRCC3*^{-/-} cells strongly suggests that the variant protein

affects RPA function. T241M homozygote individuals have a significantly increased risk of developing tetraploid cells. HCT116 cells are heterozygous for this variation. Since we have not observed a statistically significant difference between wild-type cells and the mutant expressing the wild-type cDNA, heterozygote individuals are unlikely to have an increased risk of developing tetraploid cells.

Tetraploidy is a common feature of cancers. It has been proposed that chromosome doubling leads to an unstable state that is followed by a loss of chromosomes and rearrangements (Shackney *et al*, 1989; Atkin, 2000). Centrosome aberrations also arise from an intermediate tetraploid state (Borel *et al*, 2002). Therefore, tetraploidy resulting from endocycles may constitute an initial step of chromosome instability underlying tumor development. To date, very little is known about the genetic polymorphisms that are involved in endoreduplication. Our findings improve understanding of the germline genetic factors underlying cancer susceptibility.

Materials and methods

Inactivation of the XRCC3 locus in HCT116

Promoterless drug-resistance genes were inserted into Exon 3 in the frame. A 1.5 kb 5' targeting element was amplified from the isogenic DNA of HCT116 cells using I1-1 (5'-TGCGAGGTTTCATACTCC-3') and E3-2A (5'-GCATCGATGCAAATCCATTGTGTCGG-3'). A 3.0 kb 3' targeting element was amplified using E3-1 (5'-TACTGGACCTGAATCCAGAG-3') and I4-1 (5'-ATGGAACCTTGTCCTGCCA-3'). Both elements were cloned into pCR2.1 (Invitrogen) by the TA cloning method. The 3' element was cut out with *KpnI* digestion and subcloned into the *KpnI* site in the vector containing the 5' element. A neomycin resistance cassette was amplified from pMC1neo-polyA (Stratagene) using Cla4neo1 (5'-CTATCGATGTATGGGATCGGCCATT-3') and neo8cla2 (5'-TCATCGATGAAGCTTGGCTGCAGGT-3'). A hygromycin resistance cassette was amplified from pcDNA/Hygro (Invitrogen) using *Clal*-hyg (5'-CTATCGATGTATGAAAAAGCCT-3') and *hyg-Clal* (5'-TCATCGATGAGGCTTTACACTTT-3'). The drug resistance cassette was inserted into the *Clal* site of the vector containing both elements in the frame. A method for knockout experiments in HCT116 has been described (Miyagawa *et al*, 2002). Anti-XRCC3 antibody was obtained from Chemicon.

Expression of the XRCC3 gene

The human XRCC3 cDNAs were amplified from cDNA derived from normal blood using E3-5 (5'-CCTCCCACAGGCTTTGAATT-3') and 3UTR-1 (5'-GAAGAGCTGTGTCTGAACCA-3'). The cDNAs were inserted into pCR2.1, and the sequence was confirmed. The XRCC3 cDNAs were inserted downstream of the MSV enhancer and the MMTV promoter. XRCC3-deficient cells were transfected with the vectors and selected in the presence of 900 µg/ml Zeocin (Invitrogen). Overexpression of XRCC3 in RPA-overexpressing cells was performed using the same vectors and pKO SelectPuro (Lexicon Genetics), because the RPA-overexpressing cells had already been transfected with Zeocin resistance genes. XRCC3-overexpressing cells were isolated by PCR using primers specific to the expression vectors.

Growth rate and sensitivity to DNA-damaging agents

The cells were treated with MMC (Kyowa-Hakko) in suspension for 10 min, washed with phosphate-buffered saline (PBS) twice, and plated at a density of 2×10^3 cells per 60 mm dish. After 7 days of culture, colonies were counted. Sensitivity to ionizing radiation and growth rate were measured as described (Miyagawa *et al*, 2002).

Measurement of the homologous recombination frequencies and Rad51 focus formation

Methods for the measurement of SCE levels and the targeted integration frequency at the RAD54B locus have been described, and that for the RAD51C locus was modified (Miyagawa *et al*, 2002).

To improve the targeting frequency at the RAD51C locus, the 5' and 3' targeting elements were amplified from isogenic DNA. Rad51 focus formation was examined as previously described (Tashiro *et al*, 2000).

FISH analysis

Chromosome-specific centromeric probes were obtained from Vysis. DNA in cells was denatured in 70% formamide/2 × SSC at 72°C for 1.5 min. Hybridization was performed in CEP Hybridization Buffer (Vysis) at 37°C for 48 h. Slides were washed in 50% formamide/2 × SSC at 45°C for 5 min twice and in 2 × SSC at 45°C for 5 min twice. Cellular DNA was stained with DAPI.

Cell-cycle analysis

To measure the mitotic index, nocodazole was added to the culture medium to a final concentration of 200 ng/ml. Cells were harvested at 6 h time intervals, fixed in 70% ethanol and stained with Hoechst 33258. To measure the DNA content, cells cultured in the presence of 100 ng/ml nocodazole were taken at the indicated time points, washed three times with PBS and fixed in 70% ethanol. Fixed cells were treated with 500 µg/ml RNaseA for 30 min at 37°C, and propidium iodide was added to a final concentration of 50 µg/ml. Flow cytometry was performed using a FACS Caliber (Becton Dickinson).

Immunoprecipitation

Cell extracts were prepared in lysis buffer (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 5 mM EDTA, pH 8.0, 0.5% Nonidet P-40, 1 mM PMSF, 20 µg/ml aprotinin, 10 µg/ml leupeptin, 300 Kunitz U/ml DNase I), incubated for 30 min on ice and lysed by sonication. Insoluble material was removed by high-speed centrifugation. Whole cell extract was precleared with normal mouse IgG and 15 µl of recombinant protein-G agarose (Invitrogen). Samples were incubated with antibodies for 1 h at 4°C. For each sample, 15 µl of protein-G agarose was added, and the samples were incubated for 1 h at 4°C and washed six times with wash buffer (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 5 mM EDTA, pH 8.0, 0.5% Nonidet P-40, 1 mM PMSF). Complexes were visualized by Western blot analysis. Anti-RPA32 (12F3.3) and anti-RPA14 (11.1) antibodies were obtained from GeneTex. Anti-RPA70 (C-21) antibody was obtained from Santa Cruz Biotechnology. Anti-Rad51C antibody was obtained from Chemicon. Anti-XRCC3 (165-100) and anti-Rad52 (H-300) antibodies were obtained from Novus Biologicals and Santa Cruz Biotechnology, respectively.

Transient expression in COS7 cells

The XRCC3 cDNA and the RPA cDNAs were cloned into pcDNA3.1 (Invitrogen). The RAD52 cDNA was cloned into pcDNA3.1/Hygro. Plasmids were transfected using Superfect Transfection Reagent (Qiagen) according to the manufacturer's instructions. Cells cultured for 48 h after transfection were harvested in lysis buffer.

Expression of the RPA genes

The human RPA cDNAs were amplified from cDNA derived from normal blood. The following primers were used for the amplification of the RPA genes: RPA70 5' sense primer (5'-AAGTCTTGGCGGTGGAGCCA-3'); RPA70 3' antisense primer (5'-AAATCGATTCCATTCTGCC-3'); RPA32 5' sense primer (5'-TCGGCCTCTTTGCGGAGAAT-3'); RPA32 3' antisense primer (5'-GATTGTGAAACTAGGTCC-3'); RPA14 5' sense primer (5'-AGCCG CAGTCTTGGACCATA-3'); RPA14 3' antisense primer (5'-AAGCACA GAAATCTCTCC-3'). The cDNAs were inserted into pCR2.1, and the sequence was confirmed. The RPA cDNAs were inserted downstream of the MSV enhancer and the MMTV promoter. HCT116 cells were transfected with the vectors and selected in the presence of 900 µg/ml Zeocin.

Expression of the RAD52 and RAD51C genes

The human RAD52 cDNA was cloned from a human testis cDNA library (Clontech) and inserted downstream of the MSV enhancer and the MMTV promoter. RPA14-overexpressing cells and XRCC3^{-/-} cells were transfected with the vectors containing drug resistance genes and selected in the presence of 200 µg/ml hygromycin and 900 µg/ml Zeocin, respectively. The mutant expressing the variant XRCC3 protein was transfected with the RAD52 expression vector and pKO SelectPuro, and was selected in the presence of 1.25 µg/ml puromycin because the mutant cells had already been transfected

with hygromycin and Zeocin resistance genes. The mutant overexpressing Rad52 was isolated by PCR using primers specific to the expression vector. The human *RAD51C* cDNA was amplified from cDNA derived from normal blood. The cDNA was inserted into the same vector and introduced in *XRCC3*^{-/-} cells. Selection was performed in the presence of 900 µg/ml Zeocin.

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