The role of homologous recombination in mitotic and meiotic double-strand break repair

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Chapter 4

The role of SUMO modification in repair of DNA double-strand breaks in *D. melanogaster* and *S. pombe*.

4 The role of SUMO modification in repair of DNA double-strand breaks in *D. melanogaster* and *S. pombe*

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Abstract

Homologous recombination is one of the two principle pathways for the repair of double-strand breaks in DNA. To identify novel proteins involved in this pathway, *Drosophila* DmRad51 and both Rad52 homologs from *S. pombe*, Rad22A and Rad22B, were used as bait proteins in yeast two-hybrid studies. In these screens components of the SUMO conjugation pathway were identified as associating factors. DmRad51 shows complex formation with Uba2, Ubc9 and Su(Var)2-10, the SUMO E1 activating, E2 conjugating and E3 ligating enzymes in *Drosophila*, respectively. Su(Var)2-10, which was originally identified as a dominant suppressor of position-effect-variegation, is essential for viability and is involved in the regulation of chromosome structure. In heterozygous *Su(Var)2-10* flies an increased X-ray sensitivity was observed, implicating sumoylation in DNA damage responses to X-rays. Both Rad22A and Rad22B display interaction with Hus5, the SUMO E2 conjugating enzyme in *S. pombe*. However, mutations in the single SUMO consensus motif of Rad22A did not influence the complementation of *rad22A* mutant strains. Screening of the *S. pombe* database resulted in the identification of the SP-RING domain protein Pli1, which belongs to the PIAS family of SUMO ligases. Targeted disruption of $pli1^+$ did not result in hypersensitivity to X-rays and hydroxyurea. Although, the protein-protein interaction studies suggest a connection between SUMO modification and the repair of double-strand breaks by homologous recombination, the role for SUMO in DNA repair may be rather indirect as defects in chromosome structure in *Su(Var)2-10* mutants imply an essential role for SUMO in maintaining proper nuclear organization. Subtle alterations in nuclear architecture could have serious effects on repair and other DNA metabolizing processes.

Introduction

Posttranslational modification of proteins by the ubiquitin related protein SUMO (small ubiquitin-like modifier) affects various cellular processes. SUMO modification has been shown to play an important role in regulating transcription, redistribution and transport of proteins through nuclear pores, maintaining proper chromosome organization, formation of PML nuclear bodies, activation of DNA damage responses and antagonizing ubiquitination (Verger et al., 2003; Johnson, 2004; Watts, 2004). In mammals three variants of SUMO have been identified: SUMO-1 and the closely related SUMO-2 and SUMO-3 proteins

(reviewed in Su and Li, 2002; Johnson, 2004; Watts, 2004). In *S. cerevisiae* only one SUMO gene, *SMT3*, has been identified which is essential for viability. In *S*. *pombe* the SUMO protein is encoded by the *pmt3*$^+$ gene. Strains having a mutated allel of *pmt3*$^+$, are barely viable and display serious defects in genome maintenance.

SUMO is synthesized as a precursor which is processed by hydrolytic cleavage at the C-terminal end. It is subsequently conjugated to proteins by means of E1 activating, E2 conjugating and E3 ligating enzymes. E2 catalyses the formation of an isopeptide bond between the C-terminal glycine of SUMO and a lysine residue in the target protein. This residue is usually found within the SUMO ψKxE consensus motif (where ψ is a large hydrophobic residue and x is any residue). The E3-like proteins might serve to increase the affinity between E2 and substrate proteins by bringing them into close proximity in catalytically favourable orientations, allowing sumoylation to occur more efficiently. The resulting isopeptide bond is stable and its disruption requires the action of a desumoylating enzyme (Su and Li, 2002; Schmidt and Muller, 2003; Johnson, 2004; Watts, 2004). The SUMO activating E1 enzyme is a heterodimer and consists of AOS1 (SAE1) and UBA2 (SAE2). UBC9 is the SUMO E2 conjugating enzyme. In most organisms *AOS1*, *UBA2* and *UBC9* genes are essential. *S. pombe* strains mutated in homologs of *AOS1* (*fub2*$^+$), *UBA2* (*rad31*$^+$) and *UBC9* (*hus5*$^+$) are not or barely viable, displaying various phenotypes including slow growth, aberrant mitosis, high-frequency loss of minichromosomes and increased sensitivity to various genotoxic agents as ionizing radiation, UV and the DNA synthesis inhibitor hydroxyurea (al Khodairy et al., 1995; Shayeghi et al. 1997; Tanaka et al., 1999; Ho and Watts, 2003).

The E3 SUMO ligases are members of three distinct protein families. The first includes E3 ligases of the PIAS family. PIAS family members were originally discovered as protein inhibitors of activated STAT transcription factors (Chung et al., 1997; Liu et al.,1998; Schmidt and Muller, 2003). PIAS proteins interact with and modulate activities of various transcription factors and the PIAS/SUMO pathway is important for transcriptional regulation of various important cellular pathways. The different PIAS proteins may direct SUMO to different substrates or support SUMO modification by different SUMO isoforms in mammals (Schmidt and Muller, 2003). In *S. cerevisiae* three PIAS family proteins have been identified, Siz1/Ull1, Siz2/Nfi1 and recently it was shown that Mms21 also has SUMO E3 activity (Johnson and Gupta, 2001; Takahashi et al., 2001; Hoege et al.,2002; Takahashi et al., 2003; Zhao and Blobel, 2005). Siz1/Ull1 was found to be an E3 ligase specific for septin components and PCNA. Although Siz2/Nfi1 promotes sumoylation of septin components *in vitro*, the *in vivo* substrates of Siz2/Nfi1 remain to be elucidated (Takahashi et al., 2003). Siz2/Nfi1 and other members of the PIAS family contain a highly conserved SP-RING domain, a nuclear localization signal (NLS) and ψKxE consensus motifs, suggesting autosumoylation. In *Drosophila melanogaster*, the *Su(Var)2-10* gene was identified as a member of the PIAS family of proteins. Su(Var)2-10 is required for viability and regulates chromosome organisation in the nucleus. *Su(Var)2-10* mutant larvae have defects in chromosome inheritance and show abnormal

chromosome structures (Hari et al., 2001). A second type of SUMO E3 ligases was discovered within the nuclearporin factor RanBP2 /Nup358 (Wu et al., 1995; Yokoyama et al., 1995; Pichler et al., 2002). A domain within this nuclear pore protein was found to stimulate *in vitro* sumolyation of the RanGAP1, Sp100 and HDAC4 subunits of the nuclear pore complex. Enhanced sumoylation of these substrates by PIAS proteins was not observed. Most likely, the RanBP2 E3 ligase coordinates the positioning of the SUMO E2 intermediate for optimal substrate conjugation (Reverter and Lima, 2005). A third SUMO E3 ligase is the polycomb group protein Pc2. Pc2 can stimulate SUMO modification of the transcriptional repressor CtBP, that can also be sumolyated by PIAS proteins (Lin et al., 2003; Kagey et al., 2003; Kagey et al., 2005).

DNA damage can occur as a result of exposure to genotoxic agents such as ionizing radiation or certain chemical compounds. A particular harmfull type of damage is the breakage of both DNA strands leading to the formation of double-strand breaks (DSBs). Repair of DSBs in eukaryotes can occur through homologous recombination (HR) or non-homologous endjoining. Rad51, one of the key proteins in HR, forms nucleoprotein filaments at the site of the break. Stimulated by among others Rad52, Rad51 mediates pairing of the nucleofilament with homologous DNA, invasion and strand exchange (Pâques and Haber, 1999). Sumoylation of proteins involved in HR has been shown for Rad22A, one of the two Rad52 homologs in *Schizosaccharomyces pombe* (Ho et al., 2001; van den Bosch et al., 2001). Interaction with the SUMO E2 enzyme Ubc9 in a yeast two-hybrid system has been shown for mammalian Rad51 and Rad52 (Shen et al., 1996). Sumolyation of Rad51 could not be detected, but the existence of a Rad51-Rad52-SUMO complex, in which neither Rad51 nor Rad52 is sumolyated, has been demonstrated (Li et al., 2000). Perturbation of Ubc9 in mammalian cells prevents the redistribution of Rad51 and the formation of foci after exposure to radiation (Saitoh et al., 2002). It is conceivable that the SUMO pathway modulates the intracellular trafficking of Rad51 and other proteins involved in HR. Upon DNA damage, SUMO modification may direct these repair factors to the DNA damage and/or sequester them into foci (Müller et al., 2004). A role for sumoylation in the control of intracellular localization of repair factors has also been shown for XRCC4 and BLM's protein (Eladad et al., 2005, Yurchenko et al., 2006).

In this work, we describe the interactions between proteins involved in HR and components of the SUMO pathway in *Drosophila melanogaster* and *Schizosaccharomyces pombe*. Heterozygosity for *Su(Var)2-10*, encoding one of the E3 ligating enzymes in *Drosophila*, results in increased X-ray sensitivity. Alteration of the unique SUMO consensus sequence of Rad22A in *S. pombe* did not result in enhanced radiation sensitivity nor did disruption of the *pli1*$^{+}$ gene, coding for one of the PIAS E3 ligases in *S. pombe*.

Materials and Methods

Two-hybrid analysis
The single-copy two-hybrid vectors pPC97, carrying the GAL4 DNA binding domain, and pPC86, containing the GAL4 activation domain, have been described (Chevray and Nathans, 1992). The *Drosophila melanogaster DmRad51* gene and the *S. pombe rad22A*$^+$*, rad22B*$^+$*, rad51*$^+$*, rad54*$^+$*, rad55*$^+$*, rad57*$^+$*, ssb1*$^+$*, ssb2*$^+$*, hus5*$^+$ and *pli1*$^+$ open reading frames (ORFs) were amplified using Platinum Pfx DNA polymerase (Invitrogen) according to the manufacturers instructions and PCR primers that create 5'-*Sal*I and 3'-*BamH*I sites flanking the start and stop codons, respectively. PCR fragments were cloned in PCRscript using the PCRscript Amp Cloning Kit (Stratagene) and subcloned in pPC97 and pPC86, as *Sal*I-*BamH*I fragments. A *Drosophila melanogaster* matchmaker library (BD Biosciences) was screened using DmRad51 as a bait. *S. pombe* matchmaker (BD Biosciences) and pBI771 two-hybrid libraries (Kohalmi et al., 1998) were screened using Rad22A and Rad22B as baits. For the two-hybrid screens the *S. cerevisiae* Y190 strain was used (Harper et al., 1993). Transformants were selected on YNB media containing 30 mg/l adenine and 50 mM 3-aminotriazole (Fields and Sternglanz, 1994). Protein-protein interactions were verified by a filter assay for β-galactosidase activity in permeabilized yeast cells. DNA from positive colonies was isolated using glass-beats and used for PCR, sequencing and BLAST analysis to determine the identity of the prey plasmid inserts.

GST pull down experiments
Two-hybrid interactions were confirmed by GST pull-down assays. DNA inserts from positive two-hybrid clones were isolated as *Sal*I-*Not*I fragments and subcloned in pGEX-6P-2 and pMV2-HA. GST pull-down experiments were performed essentially as described elsewhere (van den Bosch et al., 2002).

Fly strains
Flies were grown on standard sugar-agar medium at 25°C and adults were handled under CO_2 anesthesia. The *Su(Var)2-10[2]/CyO* strain was obtained from the Bloomington Drosophila Stock Centre. Heterozogous *Su(Var)2-10[2]/ CyO* flies were maintained using the *Pm*/*CyO* strain, containing the second chromosome balancers *In(2LR)bw*^{V1} and SM5 (Lindsley and Zimm, 1992).

Survival studies of Su(Var)2-10 *heterozygous flies*
In *Drosophila*, the sensitivity to ionizing radiation is dependent on the developmental stage of the fly and therefore embryos and larvae of different stages were used for treatment. *Su(Var)2-10[2]/CyO* females and *Pm*/*CyO* males were crossed and 0- to 24-hr embryos, 24- to 48-hr larvae and 48- to 72-hr larvae were exposed to a single dose of 10 Gy using a 225 SMART X-ray source (200 kV, 4mA, 1.0 mm Al filter and a dose rate of ~1 Gy/min; Andrex SA, Copenhagen, Denmark). After irradiation, fly cultures were kept at 25°C, and the offspring was scored after 12 to 18 days. Untreated embryos/larvae of

the same cross were used as controls. To determine the relative sensitivity, the ratio between heterozygous *Su(Var)2-10[2]/CyO* and *Su(Var)2-10[2]/Pm* mutants and *Pm/CyO* flies was calculated. In the untreated control the ratio of the three genotypes is 1:1:1 according to Mendelian laws. If the sensitivity to DNA-damaging agents is increased, the relative number of *Su(Var)2-10[2]* heterozygotes will decrease after exposure to 10 Gy of X-rays.

Fission yeast strains
Growth media and general handling of fission yeast were essentially as described (Gutz et al., 1974). The *S. pombe rad22A, rad22B* and *rad22Arad22B* double mutant strains used in this study have been described (van den Bosch et al., 2001; de Vries et al., 2005). The Y4 wildtype strain was used for control studies.

Mutational analysis of the SUMO modification motif in Rad22A
An expression construct of the *rad22A*$^+$ gene was made by cloning the *rad22A*$^+$ ORF in pREP1 (Maundrell, 1993). The *rad22A*$^+$ ORF was amplified as described before using primers containing *Nde*I and *BamH*I restriction sites, respectively. After cloning in PCRscript (Stratagene) and confirmation of the sequence, a *Nde*I-*BamH*I DNA fragment was subcloned in the *LEU2* containing autonomously replicating expression vector pREP1. Analysis of the amino acid sequence revealed a single sumoylation consensus sequence CKKE between residues 134 and 138. To study the role of this sequence motif, several amino acid changes were introduced: 135 K>R, 135 K>A, 136 K>R, 135,136 KK>RR. Site-directed mutagenesis was performed using the QuikChange Multi kit (Stratagene). To introduce sequence alterations, primers containing the mutations were synthesized for one strand of the pREP1/*rad22A*$^+$ template. The mutagenic primers were extended by PfuTurbo enzyme, generating a strand bearing the mutation and also a unique restriction site to facilitate selection of mutant plasmids. pREP1plasmids expressing wildtype or mutant Rad22A were introduced into *rad22Arad22B* double mutant *S. pombe* strains.

Isolation of the pli1$^+$ *gene*
BLAST searches exploiting *S. cerevisiae* and human PIAS family members led to the identification of the *S. pombe pli1*$^+$ gene, which is homologous to *NFI1* from *S. cerevisiae*. The *pli1*$^+$ gene was obtained by amplifying a 4142 bp region encompassing the *pli1*$^+$ ORF using Pfx DNA Polymerase (Invitrogen) and primers PliFW2 (5'– CCA GGC GTT TGC AGA GAT TC – 3') and PliREV2 (5' – TCT ATG CTC GCC AGA GAC AA – 3'). To amplify the *pli1*$^+$ ORF a second PCR was performed using Pfx Polymerase and primers PliSkip (5' – CGC GTC GAC CAT GAA CCA GGC GAA CTT TTT ACA GGA GCT TCC AAA TGT ACT AAA GCG ACT GGA AAC TGG TCT T – 3'), which creates a *Sal*I restriction site flanking the start codon and skips the 67 bp intron and PliStop (5' – CCG GGA TCC GTT AAT CTA TAC TCT GAA A – 3') which creates a *BamH*I site downstream of the stop codon. The *pli1*$^+$ cDNA was cloned in PCRscript and verified by sequencing. The *pli1*$^+$ cDNA was

subsequently subcloned in pPC97 and pPC86 and used in two-hybrid studies (see elsewhere).

Disruption of the pli1$^+$ *gene*
A *pli1* deletion mutant was constructed by replacing the open reading frame with a kan^r marker by PCR-based gene disruption (Wach et al., 1994). PliS1 primer (5′ – ATG AAC CAG GCG AAC TTT TTA CAG GAG CTT CCA GTA AGC CCA ATA ATA AGA TAT TTA CAA GTT AAC TAA CAA AAT GAA TGC GTA CGC TGC AGG TCG AC – 3′) and PliS2 primer (5′ – TTA ATC TAT ACT CTG AAA AGT GTT TCC CGT TCC TTC AAA TCC GGA ATT ATG ATA GGA GTT ATT TGA CTG TAA CTC AGA ATT CCA TCG ATG AAT TCG AGC TCG – 3′) were used in combination with plasmid pFA6a-*kanMX4* to create a kan^r fragment with arms flanking the *pli1*$^+$ gene (the nucleotide sequences underlined overlap with the *pli1*$^+$ DNA). The *S. pombe* diploid sp.101 strain (Murray et al., 1991) was transformed with 5 µg of PCR product according to the LiAc method (Keeney and Boeke, 1994). G418-resistant colonies were screened by PCR using primers flanking the *pli1*$^+$ gene in combination with primers specific for the kan^r cassette and targeted disruption was confirmed by Southern blot analysis. An h^{90}/h+ derivative was selected and used to isolate haploid G418-resistant cells. Both Southern blot and PCR analysis were performed to assure correct integration of the kan^R cassette.

S. pombe *survival studies*
Survival studies to measure the sensitivity of *S. pombe* cells to ionizing radiation, hydroxyurea (HU) and methyl methanesulphonate (MMS) were performed as described before (de Vries et al., 2005). To test for sensitivity to MMS, 5 µl of 10-fold dilutions of log-phase cells were spotted onto YES plates containing 0.0001-0.0005% MMS. Cell survival was determined after 3 days of incubation at 30°C. All experiments were repeated at least twice.

Results

Two-hybrid interactions of recombinational repair proteins and components of the SUMO pathway in flies and fission yeast
To identify new partners of proteins involved in homologous recombination (HR), *Drosophila* and *S. pombe* two-hybrid libraries were screened using *Drosophila* Rad51 and the two Rad52 homologs from *S. pombe*, Rad22A and Rad22B, as bait proteins. Yeast transformants were selected for histidine prototrophy and subsequently screened for ß-galactosidase activity. Screening of 10^6 transformants with DmRad51 resulted in 41 positive clones. Among the proteins that display interaction with DmRad51 were Uba2, homologous to the mammalian SUMO E1 activating enzyme, Ubc9, homologous to the mammalian SUMO E2 conjugating enzyme and Su(Var)2-10, homologous to mammalian SUMO E3 ligases belonging to the PIAS family. Analysis of Gal4 binding domain and activation domain fusions of Uba2, Ubc9 and Su(Var)2-10 also showed interactions among the E1, E2 and E3 enzymes of the SUMO conjugation pathway (see Figure 2A). Among the other clones identified in the screen with

DmRad51, were Tosca and PCNA. Tosca belongs to the RAD2 family of nucleases involved in DNA repair. Within this group of proteins, Tosca is closely related to Exo1, a 5'-3' double-stranded DNA exonuclease from *S. pombe*. In *Drosophila Tosca* is specifically expressed in female germ cells (Digilio et al., 1996). The expression pattern may suggest a role for Tosca in the specific resection of 5'-ends of DSBs during meiotic recombination. Screening of two different *S. pombe* libraries (10^6 transformants in total) with Rad22A resulted in the isolation of 28 clones and with Rad22B in the recovery of 17 clones. Sequence analysis identified Hus5, homologous to the mammalian SUMO E2 conjugating enzyme, as one of the proteins interacting with both Rad22A and Rad22B.

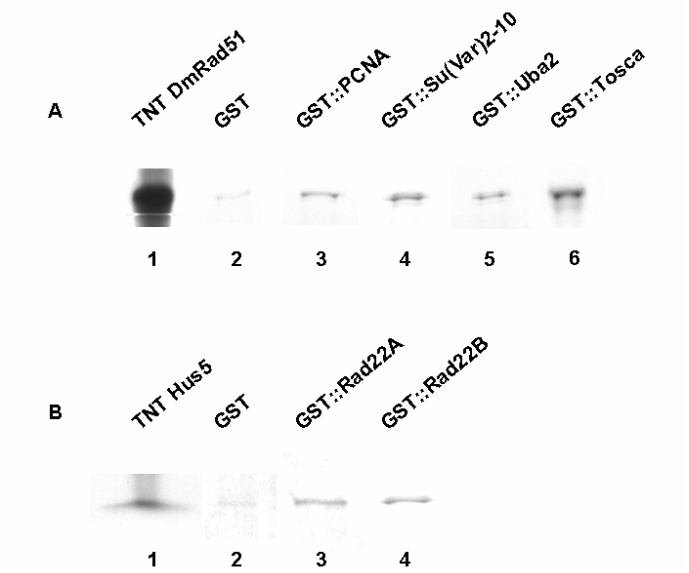


Figure 1:Protein-protein interactions investigated by GST pull down assays.
(A) 3µl of [^{35}S]methionine labelled DmRad51 (lane 1); 15µl of [^{35}S]methionine labelled DmRad51 was mixed with glutathione-Sepharose beads loaded with 2.5µg of GST (lane 2), 2.5µg of GST::PCNA (lane 3), 2.5µg of GST::Su(Var)2-10 (lane 4), 2.5µg of GST::Uba2 (lane 5), 2.5µg of GST::Tosca (lane 6). **(B)** 2µl of [^{35}S]methionine labelled Hus5 (lane 1); 15µl of [^{35}S]methionine labelled Hus5 was mixed with glutathione-Sepharose beads loaded with 2.5µg of GST (lane 2), 2.5µg of GST::Rad22A (lane 3), 2.5µg of GST::Rad22B (lane 4).

The associations detected by two-hybrid screens were validated by reciprocal combinations of Gal4 binding domain and activator domain fusions and confirmed by GST pull-down assays. Full length PCNA, Su(Var)2-10 and Tosca proteins were expressed in *E. coli* as glutathione S-transferase (GST) fusions. GST-fusion proteins were bound to glutathione-Sepharose beads and incubated with [^{35}S]methionine labelled DmRad51. After washing, proteins bound to the beads were detected by electrophoresis on polyacrylamidegels. The results shown in Figure 1 indicate *in vitro* interaction of DmRad51 with PCNA, Su(Var)2-10, Uba2 and Tosca (Figure 1A, lanes 3-6). Specific binding of [^{35}S]methionine

labelled Hus5 to GST-Rad22A and GST-Rad22B bound to glutathione-Sepharose beads is shown in Figure 1B, lanes 3 and 4. Interactions of DmRad51 with Ubc9 and among Uba2, Ubc9 and Su(Var)2-10 were also confirmed by GSTpull-down assays (results not shown).
To examine putative interactions of Hus5 with other members of the RAD52 group which are involved in HR in *S. pombe*, additional two-hybrid studies were performed. In addition to the interactions of Hus5 with Rad22A and Rad22B, we observed interaction of Hus5 with Ssb1, one of the three subunits of RPA. No interaction was detected with Rhp51, Rhp54, Rhp55, Rhp57 and Ssb2 (data not shown). To localize the regions that mediate association of Rad22A and Rad22B with Hus5, various deletion constructs were tested (gene fragments are indicated in Figure 2B).

A

		DNA binding fusions			
		DmRad51	Uba2	Ubc9	(no insert)
Activator fusions	Uba2	+	++	+	-
	Ubc9	+	+	+	-
	Su(Var)2-10	+	++	+	-
	(no insert)	-	-	-	-

B

Hus5								
	Amino acid			Amino acid			Amino acid	
Rad22A-a	1 – 469	+/–	Rad22A-e	168 – 258	+/–	Rad22B-a	1 – 158	–
Rad22A-b	1 – 167	+	Rad22A-f	72 – 167	+	Rad22B-b	1 – 232	+
Rad22A-c	1 – 258	–	Rad22A-g	72 – 224	+	Rad22B-c	159 – 371	+
Rad22A-d	168 – 469	–	Rad22A-h	168 – 224	+	Rad22B-d	233 – 371	+

Figure 2: Protein-protein interactions detected by two-hybrid studies.
(A) Analysis of DNA binding domain fusions of *D. melanogaster* DmRad51, Uba2 and Ubc9 and activation domain fusions of Uba2, Ubc9 and Su(Var)2-10. Positive interactions were confirmed by β-galactosidase filter assays. ++, strong positive interaction; +, positive interaction; +/–, weak interaction; –, no interaction. **(B)** Two-hybrid analysis of Hus5 and fragments of *S. pombe* Rad22A and Rad22B. Amino acid regions of Rad22A and Rad22B used for two-hybrid analysis are indicated. In all experiments genes and gene fragments were tested both as DNA binding and activation domain fusions.

Two-hybrid experiments revealed that the region required for interaction of Rad22A with Hus5 is within gene fragment Rad22A-b coding for amino acids 1 to 258. A weak interaction with Hus5 was observed in case of Rad22A-a and Rad22A-e fragments. To localize the region of association more precisely, gene fragments Rad22A-f, Rad22A-g and Rad22A-h were tested. Two-hybrid experiments indicated that the region(s) required for interaction between Rad22A and Hus5 is located between amino acid 72 and 224. (Figure 2B). This clarifies that the region interacting with Hus5 is probably located in the

proximity of the (putative) SUMO modification site, the lysine residue at amino acid 135.
The small Rad22B-a N-terminal deletion fragment did not interact with Hus5, while all other fragments showed an interaction using the two-hybrid system with Hus5, indicating that the site(s) of interaction of Rad22B and Hus5 is located C-terminal of amino acid 158 (Figure 2B).

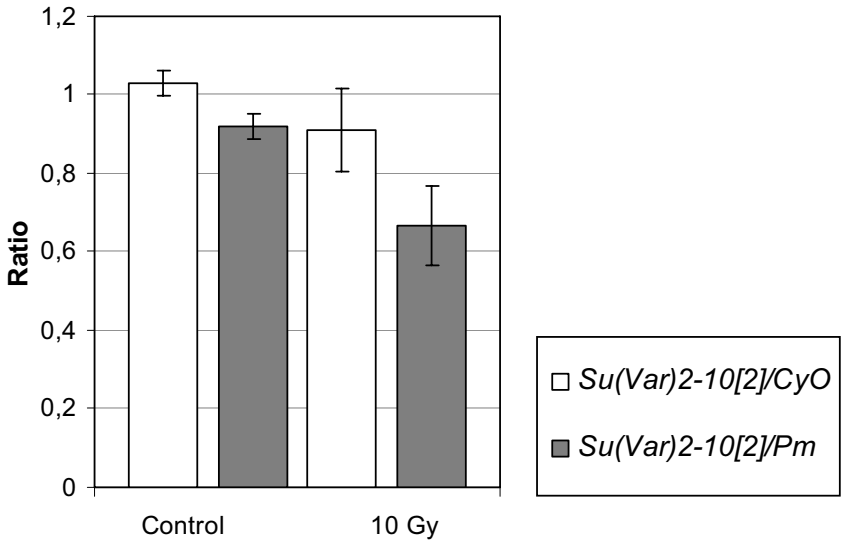


Figure 3: Survival of 0- to 24-hr *Drosophila* embryo's heterozygous for *Su(Var)2-10* after exposure to radiation. On the y-axis the ratio of *Su(Var)2-10 [2]/CyO* to *Pm/CyO* and of *Su(Var)2-10 [2]/Pm* to *Pm/CyO* flies from the same cross is indicated. If the defect in *Su(Var)2-10* leads to increased radiation sensitivity, these ratios will drop after exposure to X-rays. In agreement with Mendelian laws, the ratios in the untreated control are approximately 1.0. Standard errors of the mean are indicated for every experiment.

Su(Var)2-10 *heterozygous flies are sensitive to X-rays.*
To investigate the role of the Su(Var)2-10 E3 ligase in the cellular responses to damage inflicted by ionizing radiation, we tested a *Drosophila melanogaster* strain mutated in *Su(Var)2-10* for increased X-ray sensitivity. Since homozygous *Su(Var)2-10* mutant flies are embryonic lethal, heterozygous flies were tested. *Su(Var)2-10[2]/CyO* females were crossed with *Pm/CyO* males and 0- to 24-hr embryos, 24- to 48-hr larvae and 48- to 72-hr larvae were exposed to a dose of 10 Gy of X-rays. In untreated offspring, the ratio of *Su(Var)2-10[2]/CyO*, *Su(Var)2-10[2]/Pm* and *Pm/CyO* flies and mutants was 1.0 : 0.9 : 1.0. The theoretically expected ratio for the three genotypes is 1:1:1 according to Mendelian laws (*CyO/CyO* flies are embryonic lethal). Increased radiation sensitivity will lead to a decrease in the *Su(Var)2-10[2]/Pm* to *Pm/CyO* and *Su(Var)2-10[2]/CyO* to *Pm/CyO* ratios. After irradiation of 0- to 24-hr embryos a slight increase in sensitivity was observed for the *Su(Var)2-10[2]/CyO* flies. For the *Su(Var)2-10[2]/Pm* flies the increase in radiation sensitivity was more

pronounced (Figure 3). Similar results were obtained for the reciprocal cross of *Su(Var)2-10[2]/CyO* males with *Pm/CyO* females (results not shown). Irradiation of 24- to 48-hr and 48- to 72-hr larvae did not result in enhanced radiation sensitivity (data not shown). These data indicate that Su(Var)2-10 mediated SUMO modification is directly or indirectly involved in proper cellular responses to radiation-induced damage in *Drosophila*.

Mutational analysis of a putative SUMO modification site in Rad22A

Screening of the Rad22A amino acid sequence for putative SUMO modification sites (ψKxE) revealed only one lysine residue at position 135 that may be a target for sumoylation. The putative acceptor lysine is flanked by a cysteine, a lysine and a glutamic acid ($C_{134}K_{135}K_{136}E_{137}$). To determine if sumoylation of K_{135} is required for proper repair through HR, the *rad22A*$^{+}$ ORF was inserted in the pREP1 expression vector and mutations were introduced at position 135 and 136 or both (see materials and methods). Wildtype and mutated plasmid DNAs were introduced into *rad22Arad22B* mutant *S. pombe* cells and tested for X-ray sensitivity.

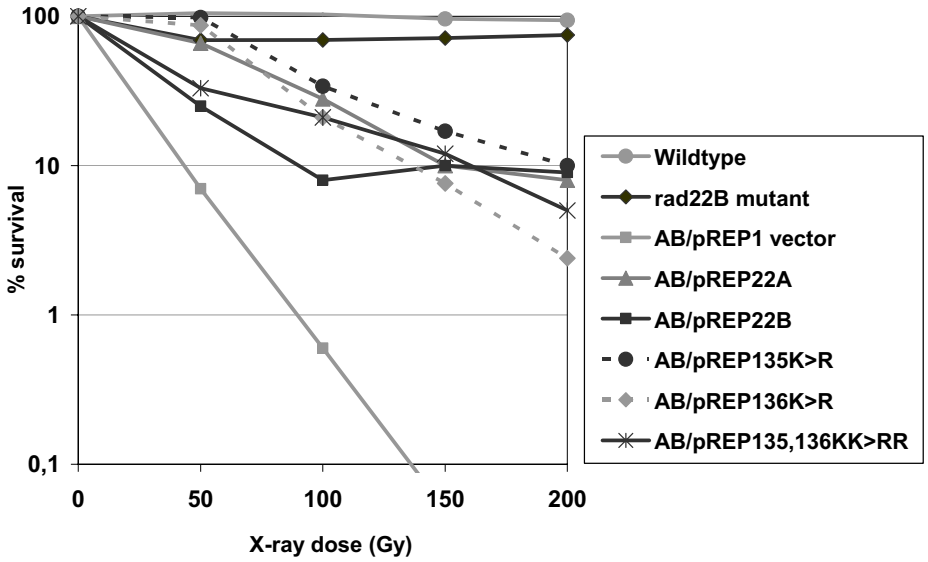


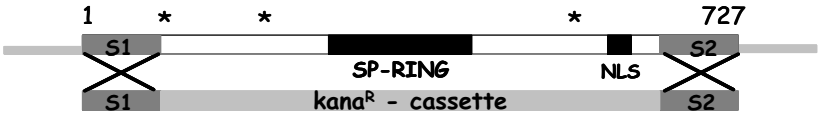
Figure 4: Survival of *S. pombe* strains after irradiation with X-rays. After irradiation of exponentially growing cells, appropriate dilutions were plated and colonies were counted after incubation of the plates for 3 days at 30ºC. Each survival experiment was repeated at least twice. Strains used in this experiment: wildtype (Y4), rad22B$^{-/-}$ (rad22B mutant), and double mutant rad22A$^{-/-}$rad22B$^{-/-}$ strains containing expression vectors without insert (AB/pREP-), with Rad22A insert (AB/pREP22A), with Rad22B insert (AB/pREP22B) and with Rad22A inserts in which the putative SUMO acceptor site has been mutated (AB/pREP135 K>R, AB/pREP136 K>R, AB/pREP135,136 KK>RR). For details see Materials and methods. **See appendix for a colour version of this figure.**

As previously observed, *rad22Arad22B* mutant cells containing only vector DNA display a severely increased X-ray sensitivity (Figure 4) (van den Bosch et al., 2001). Introduction of pREP1 containing wildtype *rad22A*$^+$ resulted in a considerable correction of the hypersentivity to X-rays of the double mutant (see Figure 4). Overexpression of *rad22B*$^+$ also resulted in significant complementation as was previously observed (van den Bosch et al., 2001). Constructs expressing mutated Rad22A in which the putative acceptor lysine residue was changed into an arginine (135 K>R) also corrected the radiation sensitivity of the *rad22Arad22B* mutant. Similar results were obtained using a 135 K>A allele (data not shown). Two hybrid experiments indicated that mutating Rad22A protein does not influence the interaction with Hus5. Conversion of the lysine flanking the putative lysine acceptor residue (136 K>R) or changing both lysines in the SUMO consensus motif into arginine (135, 136 KK>RR) did not influence the complementation of the X-ray hypersensitivity of the mutant drastically (see Figure 4).

A

Pli1					
Rad22A	+/−	Rhp55	−	Ssb2	+
Rad22B	+/-	Rhp57	−	Hus5	+
Rhp51	−	Ssb1	−	no insert	−
Rhp54	−				

B



C

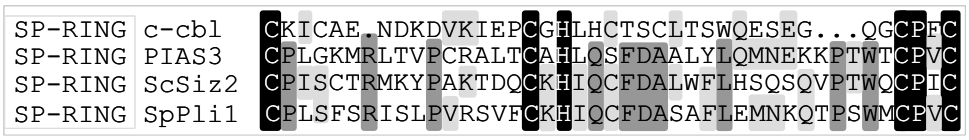


Figure 5

Analysis of *S. pombe* Pli1. **(A)** Interactions of Pli1 as determined by two-hybrid studies. All gene fragments were tested both as DNA binding and activator fusions. Positive interactions were confirmed by β-galactosidase filter assays. +, positive interaction; +/−, weak interaction; −, no interaction. **(B)** Schematic representation of the *pli1*$^+$ gene (the 52 base pair intron is not shown). SP-RING domain and NLS coding sequences are indicated. Stars (*) represent the three putative SUMO modification motifs at position 41, 274 and 442 of the protein. The location of the arms (S1 and S2) used for disruption of the *pli1*$^+$ gene by replacement with a kanamycine resistance cassette, is indicated. **(C)** Sequence alignment of SP-RING domains of the human ubiquitin ligase c-Cbl (GenPept accession number P22681), human PIAS3 (Q9Y6X2), *S. cerevisiae* Siz2 (NP_014799) and *S. pombe* Pli1 (AL031181). Identical amino acids are highlighted.

Isolation and disruption of the pli1$^+$ *gene*
BLAST searches of the *S. pombe* database for sequences homologous to *S. cerevisiae* and human PIAS family members, led to the identification of a hypothetical open reading frame (ORF) on chromosome 1 with the reserved name of *pli1*$^+$ (*S. pombe* geneDB). The predicted protein of 727 amino acid residues is homologous to Siz1 (Ull1) and Siz2 (Nfi1) from *S. cerevisiae* (21% and 25% identity, respectively). Like Siz1/2 and other members of the PIAS family, the Pli1 protein in *S. pombe* contains a SP-RING domain (Figure 5B), which is highly conserved (Figure 5C). In addition, a nuclear localization signal (NLS) and three putative sumoylation sites could be identified in Pli1 (Figure 5B). Two-hybrid studies were performed after cloning the *pli1*$^+$ ORF in bait and prey vectors. The results showed weak interaction of Pli1 with Rad22A and Rad22B and a strong interaction with Hus5 and Ssb2. No interaction was observed with Rhp51, Rhp54, Rhp55 and Rhp55 (Figure 5A).

To examine the function of *pli1*$^+$ and its role in DNA damage responses, a deletion mutant was generated (see materials ands methods). When treated with hydroxyurea, *Pli1* mutant cells displayed the same sensitivity as wildtype cells (data not shown). The survival after X-ray irradiation is also very similar to the survival of the wildtype Y4 strain and the *rad22B* mutant. The data presented in Figure 6B, however, may indicate a slight but reproducable increase in radiation sensitivity of *pli1* (and *rad22B*) mutant cells.

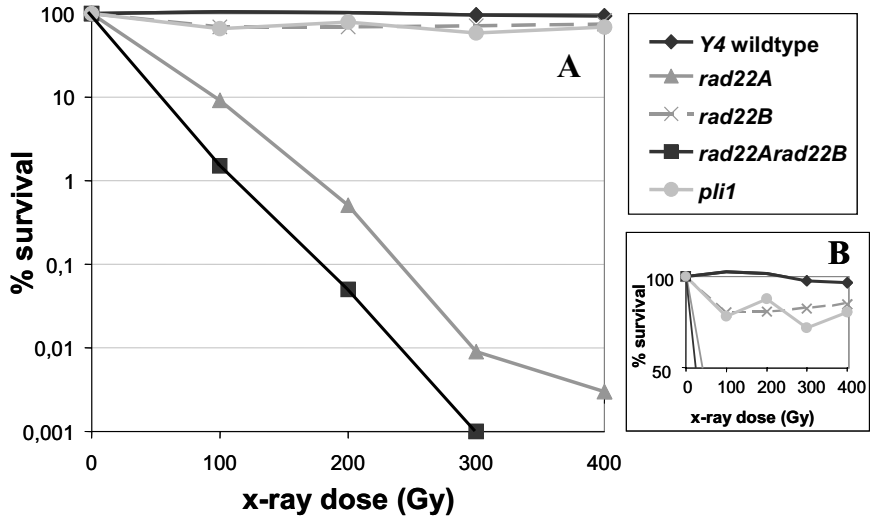


Figure 6
Survival following irradiation with X-rays of wildtype and mutant *S. pombe* strains after irradiation with X-rays **(A)** and a magnification of the survival curve of wildtype, *rad22B* mutant and *pli1* mutant cells **(B)**.

Discussion

To identify novel factors involved in the repair of DSBs in DNA through homologous recombination in *D. melanogaster* and *S. pombe*, yeast two-hybrid screens were performed. The use of *Drosophila* DmRad51 and Rad22A and Rad22B from *S. pombe* as baits, resulted in the identification of components of the sumoylation pathway. Interaction was detected between DmRad51 and SUMO activating, conjugating and ligating enzymes. Two-hybrid experiments and GST pull-down assays also showed interaction among the three components of the SUMO conjugation pathway itself (see Figure 2A). These data may suggest the formation of a sumoylation complex, in which SUMO is activated, conjugated and ligated to the substrate. The interactions between the various constituents may be highly dynamic and not all proteins have to be present in the complex at the same time during sumoylation.
The putative Su(Var)2-10 SUMO E3 ligase identified in our screen, was originally isolated as a suppressor of position-effect-variegation (PEV). Abnormal juxtaposition of genes near heterochromatic regions in the genome results in silencing of expression. Mutations in genes like *Su(Var)2-10* result in a mosaic or variegated expression of genes near centromeric or telomeric heterochromatin (for a review see Wallrath, 1998). At the chromosomal level abnormally condensed chromosomes and segregation defects were observed in *Su(var)2-10* homozygous mutants (Hari et al., 2001). In addition telomere-telomere and telomere-lamina associations are affected in this mutant. To investigate a role in the cellular responses to DNA damage, *Su(Var)2-10* mutant flies were assessed for increased sensitivity to ionizing radiation. As homozygous mutant flies die as late larvae and early pupae, heterozygous flies were tested. After a dose of 10 Gy, a decreased survival of 0- to 24-hr embryos was seen (see Figure 3). In contrast to most genes involved in DNA damage responses, increased radiation sensitivity was already manifest in heterozygous flies. The difference in hypersensitivity between *Su(Var)2-10[2]/Pm* and *Su(Var)2-10[2]/CyO* heterozygotes is most likely due to differences in the genetic background of *Pm* and *CyO* flies. At larval stages, no increased sensitivity was seen. Fly strains in which Rad54 or Ligase IV activity is abolished, also exhibit less severe radiation sensitivity at later stages of development (Gorski et al., 2003). The radiation-hypersensitivity seen in *Su(Var)2-10* heterozygous mutant flies indicates an important role for SUMO modification in signalling and/or repair of DNA damage. However, the role for SUMO in the repair of DSBs may be rather indirect. Alterations in chromosome architecture and organization in heterozygous *Su(Var)2-10* flies, as manifested by the PEV phenotype, may have serious effects on signalling and repair of damage and lead to reduced survival after irradiation.
Screening of two-hybrid libraries with Rad22A and Rad22B resulted in the identification of Hus5 as an interacting partner of both Rad52 homologs in *S. pombe*. Sumolyation of Rad22A *in vivo* has already been shown (Ho et al., 2001). Analysis of the Rad22A amino acid sequence revealed only one putative ψKxE sumoylation consensus motif ($C_{134}K_{135}K_{136}E_{137}$). To test for sumoylation of

Rad22A at this position, various amino acid changes were introduced by PCR. However, overexpression of 135 K>R, 135 K>A, 136 K>R or 135,136 KK>RR alleles did not result in altered levels of complementation of *rad22rad22B* mutant cells as compared with the wildtype allele (see Figure 4). Apparently, SUMO conjugation of the lysine residue at position 135 is not strictly required for the role of Rad22A in HR. The possibility that Rad22A is sumoylated at other positions cannot be ruled out, neither can we exclude the possibility for a minor role of SUMO modification of Rad22A in HR, or a role in only certain types of recombination. The specific sumoylation of certain proteins in HR may be achieved by E3 ligases of the PIAS family. Two-hybrid studies demonstrated the interaction between proteins belonging to the RAD52 group and PIAS family homologs from various eukaryotes (Su(Var)2-10 with Rad51 in *Drosophila* (this study), PIASy with Mre11 in mice (C. Heyting and F. de Vries, unpublished results)). BLAST searches for PIAS family homologs in *S. pombe* revealed the Pli1 protein which is homologous to *S. cerevisiae* Siz1 (Ull1) and Siz2 (Nfi1). Recently, the *pli1*$^+$ gene was also identified in a two-hybrid screen using the chromatin-associated switch-activating protein as a bait (Xhemalce et al., 2004). Disruption of the *pli1*$^+$ gene did not result in increased ionizing radiation sensitivity (Figure 6). Possibly, Pli1 is not required for sumoylation of proteins involved in cellular responses to X-rays or its role is compensated by other SUMO ligases. Xhemalce and coworkers reported increased sensitivity of *pli1* mutant cells to the microtubule-destabilizing drug TBZ and enhanced loss of a minichromosome (Xhemalce et al., 2004).

Recently a second SUMO E3 ligase has been identified in *S*. *pombe*. Nse2, one of the components of the Smc5-6 complex, which is probably involved in recombinational repair mechanisms, contains an SP-RING motif and exhibits SUMO ligase activity (Andrews et al., 2005). Mutations in the SP-RING domain resulted in increased sensitivity to DNA damaging agents. Inactivation of the E3 ligase activity of Mms21, the *S*. *cerevisiae* homologue of Nse2, also leads to increased sensitivity to genotoxic agents as well as defects in nucleolar integrity and clustering of telomeres (Zhao and Blobel, 2005). Reduced survival after treatment with methyl methanesulfonate was observed in human cells after RNAi-mediated depletion of Mms21 (Potts and Yu, 2005). The phenotypical analysis of *Su(Var)2-10* and *mms21* mutants imply a crucial role for SUMO modification in maintaining proper chromosome architecture and nuclear organisation. Reduced levels of sumoylation lead to defects in the regulation of chromosome structure and functioning. Chromatin immunoprecipitation showed an enrichment of Hus5 in heterochromatin in *S*. *pombe* (Shin et al., 2005). In this study also the specific sumoylation of heterochromatin proteins was shown.

Although the data presented here and those of others imply that SUMO modification of proteins involved in homologous recombination is probably important for the repair of DSBs (*e.g*. in the control of intracellular localization), sumoylation presumably also affects DSB-repair indirectly as a consequence of its essential role in maintaining proper chromosome structure and organization. Minor deviations in the spatial organization of chromosomes probably have

severe consequences for the efficacy of DNA repair mechanisms in preservation of the integrity of the genome.

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