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Physical interaction between SLX4 (FANCP) and XPF (FANCD1) proteins and biological consequences of interaction-defective missense mutations

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Abstract

SLX4 (FANCP) and XPF (FANCD1) proteins interact with each other and play a vital role in the Fanconi anemia (FA) DNA repair pathway. We have identified a SLX4 region and several amino acid residues that are responsible for this interaction. The study has revealed that the global minor allele, SLX4^{Y546C}, is defective in this interaction and cannot complement *Fancc* knockout mouse cells in mitomycin C-induced cytotoxicity or chromosomal aberrations. These results highly suggest this allele, as well as SLX4^{L530Q}, to be pathogenic. The interacting partner XPF is involved in various DNA repair pathways, and certain XPF mutations cause progeria, Cockayne syndrome (CS), and/or FA phenotypes. Because several atypical xeroderma pigmentosum (XP) phenotype-causing XPF missense mutations are located in the SLX4-interacting region, we suspected the disruption of the interaction with SLX4 in these XPF mutants, thereby causing severer phenotypes. The immunoprecipitation assay of cell extracts revealed that those XPF mutations, except XPF^{C236R}, located in the SLX4-interacting region cause instability of XPF protein, which could be the reason for the FA, progeria and/or CS phenotypes.

Graphical Abstract

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Conflict of interest statement

The authors declare that there are no conflicts of interest.

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Keywords

interstrand crosslink repair; protein-protein interaction; Fanconi anemia; FANCP; SLX4; XPF

1. Introduction

Fanconi anemia (FA) is a rare recessive hereditary disorder characterized by progressive bone marrow failure, developmental abnormalities and cancer predisposition [1]. The cells of FA patients show chromosomal instability and hypersensitivity to crosslinking agents such as mitomycin C and cisplatin, well-known cancer chemotherapeutics [2]. Crosslinking compounds physically connect two DNA strands and thereby block DNA replication and transcription. The biological and cytological characteristics of FA patients are believed to be primarily ascribed to their defect in a proper removal of interstrand crosslinks (ICLs) from DNA at a replication fork [3]. At present, 16 complementation groups have been identified: 8 FA proteins (FANCA, B, C, E, F, G, L and M) together with FA-associated proteins (FAAP20, FAAP24, FAAP100, MHF1, and MHF2) form a core complex that monoubiquitinates FANCD2-FANCI heterodimer [4, 5]. FANCL is a ubiquitin ligase. The monoubiquitinated FANCD2/FANCI heterodimer activates downstream ICL repair including ICL unhooking (strand incision on both sides of ICL), translesion DNA synthesis (TLS) and homologous recombination. The unhooking of an ICL from one strand at a replication fork generates a gap and a double strand break(s). The gap is sealed by TLS across an unhooked ICL-modified nucleotide, and the ICL-modified nucleotide is removed by nucleotide excision repair. This process generates one repaired molecule, which is then used to repair a double strand break(s) in a sister molecule by homologous recombination. A group of proteins such as FANCD1, FANCI, FANCN, and FANCO, function in this recombination step [4].

In the replication-associated ICL repair pathway, the monoubiquitinated FANCD2-FANCI heterodimer is thought to bind to a stalled fork [6] and recruits downstream FA proteins including SLX4 [7] directly or indirectly. SLX4, a protein platform, binds three structure-specific endonucleases, XPF-ERCC1, MUS81-EME1 and SLX1 and coordinates their activities during ICL unhooking [8, 9] and Holliday junction resolution [10–12] in recombination repair. XPF is an essential component of nucleotide excision repair and incises a DNA strand 5' to a DNA lesion. XPF also plays a critical role in ICL repair because *XPF*^{-/-} cells are much more sensitive to crosslinking agents than cells belonging to the other xeroderma pigmentosum (XP) complementation groups. The physical interaction

between SLX4 and XPF is critical to ICL unhooking [8, 9]. Although XPF missense mutations largely result in XP phenotypes, certain missense mutations cause complicated or different phenotypes such as progeria [13], FA [14] and XP/FA/CS [15]. XPF forms constitutively a heterodimer with ERCC1 and is recruited to nucleotide excision repair and ICL repair sites through ERCC1:XPA interaction [16, 17] and XPF:SLX4 interaction [18], respectively. This difference in the recruitment mechanism may contribute to the phenotypic variation of XPF patients. Although XPF and SLX4 are known to interact physically with each other, this interaction has not yet been characterized in detail. Here we have determined interacting regions of SLX4 and XPF and identified several critical amino acids. We have found that a SLX4 global minor allele destructs this interaction and sensitizes cells to crosslinking agents. We also studied whether XPF missense mutations, which cause atypical XP phenotypes and are located in the SLX4-interacting region, result in defective interaction with SLX4, thereby causing severer and complicated phenotypes.

2. Materials and methods

2.1. Cloning of XPF and SLX4 complementary DNAs and their fragments

Complementary DNAs (cDNAs) of SLX4 (1834 amino acids, aa) and XPF (916 aa) were obtained from human fibroblasts (GM637, Coriell Cell Repositories) by reverse transcription and polymerase chain reaction (PCR). The sequences of cDNAs were confirmed by DNA sequencing. Various fragments of these two cDNAs were cloned into vectors by PCR and restriction enzyme digestion. The sequences of cloned fragments were confirmed by sequencing.

2.2. Site-directed mutagenesis

Site-directed mutagenesis (base substitution and deletion) was conducted using Q5 Site-Directed Mutagenesis kit (New England BioLabs). Mutations intended were confirmed by DNA sequencing.

2.3. Yeast two-hybrid assay

Yeast two-hybrid assay was conducted using pGADT7 and pGBKT7 vectors (Clontech) and yeast AH109 cells (*MATa, trp1-901 leu2-3, leu2-112, ura3-52, his3-200, gal4, gal80, LYS2::GAL1_{UAS}-GAL1_{TATA}-HIS3, MEL1, GAL2_{UAS}-GAL2_{TATA}-ADE2, URA3::MEL1_{UAS}-MEL1_{TATA}-lacZ*). Because cloning of SLX4 cDNA into pGBKT7 results in autonomous transcriptional activation, SLX4 cDNA was cloned into pGADT7. XPF cDNA was cloned into pGBKT7.

2.4. Immunoblot assay

(FLAG)₃-tagged SLX4 cDNA fragments and (c-myc)₃-tagged XPF cDNA fragments were cloned into pIRESneo2 vector (Clontech) for a pull-down assay in human cells. HEK293T/17 (ATCC) cells were transfected, using Eugene6 (Roche), with constructs. Cloned genes were expressed for 72 hours, after which whole cell extracts were prepared from almost confluent flasks. Immunoprecipitation and immunoblot were conducted using anti-FLAG M2 and anti-c-myc 9E10 antibodies (Sigma). Target bands were identified according to a protein molecular weight marker following immunostaining.

2.5. Cytotoxicity assay

Immortalized *Fancp*^{-/-} mouse embryonic fibroblasts (MEFs) were kindly provided by K.J. Patel (MRC Lab, UK). The mouse *Fancp* gene was disrupted by deleting exon 3, which resulted in the introduction of a frameshift (out of frame) mutation at codon 80 [19]. (FLAG)₃-tagged wild-type and mutant FACNP cDNAs were cloned into the pQCXIH retroviral vector (Clontech) and transduced into cells using GP2-293 (Clontech) as packaging cells. Clonal lines were cultured in the presence of hygromycin B (400 µg/ml medium), established and analyzed for the expression of tagged SLX4 by reverse transcription-PCR and immunoblot (Supplementary Fig. S1). The cell growth assay was conducted using the CellTiter 96Aqueous One Solution Cell Proliferation Assay kit (Promega). Cells were plated at 1×10³ cells/well of a 12-well plate in triplicate and cultured for 2 days, after which mitomycin C (Nacalai tesque) was added to a well at 0, 12.5, 25, or 50 ng/ml. Cells were further cultured for 5 or 6 days, at which time cells in untreated control wells reached close to confluence. Surviving cells were measured by the Promega kit.

2.6. Cytogenetic assay

MEFs were treated in duplicate with 50 nM mitomycin C for 48 hours. Chromosomal preparations were made by the air drying method [20]. Fifty or 100 metaphases per group were analyzed for aberrations (break and exchange).

3. Results

3.1. Determination of an XPF-interacting region and critical amino acid residues of SLX4

The cDNAs of human SLX4 and XPF were cloned into the GAL4-based yeast two-hybrid assay vectors of pGADT7 and pGBKT7, respectively. Experiments employing various fragments of SLX4 revealed a critical interacting region to be located in SLX4₅₀₀₋₅₅₈ (#16 in Table 1). This region overlaps with SLX4₄₀₉₋₅₅₅ reported by Kim et al. [21]. The growth of the yeast host (AH109) transformed with SLX4₅₀₀₋₅₅₈ was slower on a selective medium plate (ade/his/trp/leu dropout medium, 4DO) compared with those transformed with larger fragments such as the constructs #15 and #17. The interaction with XPF may be stabilized by the flanking N and C terminal residues. This interacting core region is located downstream of MLR (Mei9-like region, [22]). Because SLX4₄₂₇₋₅₅₈ (#11), but not SLX4₄₂₇₋₅₂₇ (#12), interacts with XPF, critical amino acid residues are expected to be located in SLX4₅₂₇₋₅₅₈. The amino acid sequence alignment of human, mouse, chicken, zebrafish, *Xenopus*, and *Drosophila* SLX4s by Clustal Omega (<http://www.clustal.org/>) identified 3 conserved amino acid residues in this region (Fig. 1): L530, Y546 and L550. We found that L530A amino acid change (#7, Table 2) abolished the interaction, but Y546A (#6) or L550A (#3), as well as E532A (#2), did not affect appreciably the interaction. Interestingly, Y546C (rs150547487) was detected in the SLX4 mutation screening of non-BRCA1/2-mutated breast cancer families [23] and is recorded as a global minor allele with a frequency of 0.0038 (dbSNP, NCBI Resources). The Y546C mutation (#5) did not significantly impair the interaction. However, when the Y546C or Y546A mutation was combined with F545A mutation (#8, 9 vs. #4, 5, 6), the interaction was significantly impaired, revealing an additive effect. Thus, L530A was most deleterious to the interaction among the single amino acid substitutions examined. The L530A amino acid change is

generated by CTG to GCG tandem double base substitutions, but the double base substitutions are less likely to occur in cells. Therefore, we examined the effect of a single amino acid change that could be induced by a single base substitution at L530 (Table 3). There are five possible amino acid changes from leucine: two (methionine and valine) are hydrophobic, one (proline) is neutral, and two (glutamine and arginine) are hydrophilic. Among these five possible amino acid changes, SLX4^{L530M} does not interfere with the interaction; SLX4^{L530V} still weakly retains the interacting ability whereas SLX4^{L530P}, SLX4^{L530Q}, and SLX4^{L530R} failed to interact with XPF. These results suggest that the hydrophobicity and the length of a side chain are important to maintain the interaction with XPF.

3.2. Physical interaction of SLX4 mutants with XPF in human cells

To study the interaction in human cells, we cloned (FLAG)₃-SLX4₄₄₈₋₅₉₉ (20 kDa, #13 in Table 1) and (c-myc)₃-XPF₁₋₆₅₀ (79 kDa, #3 in Table 5) in pIRESneo2 and pIRESbla mammalian expression vectors, respectively, and these cloned genes were expressed transiently in HEK293T/17 cells. The immunoblot analysis revealed that c-myc-tagged XPF coimmunoprecipitated with FLAG-tagged SLX4 (Fig. 2A), indicating that these two fragments interact with each other in human cells. The effects of SLX4 missense mutations on the interaction were then examined (Fig. 2B). The results revealed that not only L530A but also F545A, Y546C and L550A impaired the interaction with XPF whereas E532A did not. These results indicate that L530, F545, Y546 and L550 residues are important to maintain stable interaction with XPF in human cells. These results appear to be inconsistent with the results obtained in the yeast two-hybrid assay (Table 2). However, stable interaction is necessary to yield coimmunoprecipitates in coimmunoprecipitation experiments whereas transient interaction, mediated by cloned gene fragments, between the GAL4 transcription activation domain and the GAL4 DNA binding domain may cause the temporary expression of marker genes (*HIS3*, *ADE2*), allowing host cells to grow on the selective medium (4DO).

3.3. The mutant SLX4s, L530Q and Y546C, fail to restore the resistance to the cytotoxic and genotoxic effects of mitomycin C

To study the biological effects of SLX4 missense mutations that impair the interaction with XPF, FLAG-tagged SLX4 wild type, L530Q and Y546C were expressed ectopically in *Fancp* gene knockout MEFs (Supplementary Fig. S1). These two mutations were selected because L530Q impairs the interaction most strongly (Table 3) and Y546C exists in the human gene pool. While the wild-type SLX4 restored the resistance to the cytotoxicity of mitomycin C, the two mutants failed to restore the resistance (Fig. 3), indicating that L530 and Y546 residues are critical for the SLX4 function in ICL repair. To confirm further the importance of these two amino acid residues in ICL repair, the induction of chromosomal aberrations was examined. The *Fancp*^{-/-} MEFs complemented with mutant SLX4s showed significantly higher percentage of cells with chromosomal aberrations compared with cells complemented with the wild-type SLX4 when they were treated with mitomycin C [column (A) in Table 4]. A clear difference was not observed in the absence of the mitomycin C treatment. Detailed results have been presented in Supplementary Table S1. Unexpectedly, cells complemented with SLX4^{L530Q} or SLX4^{Y546C} showed a much higher percent of cells with aberrations than cells complemented with the vector only. This enhanced effect was

also observed when the number of aberrations per 100 metaphases [column (B)] and the number of aberrations per metaphase with aberrations [column (C)] were measured. These results were confirmed in another independent experiment with *Fancp*^{-/-} MEFs complemented with SLX4^{Y546C}. However, there was no significant difference in the total number of chromosomal aberrations per 100 metaphases [column (B)] between cells complemented with the wild-type SLX4 and those complemented with the vector only. Therefore, the two mutants cause an enhancing effect on the induction of chromosomal aberrations. The reason for this enhancing effect is not clear at present, but these mutant SLX4s might trap and sequester structure-specific endonucleases, such as SLX1 and MUS81-EME1, from the resolution of recombination intermediates during recombination repair and double strand break repair. The cytotoxic and genotoxic data indicate that these two amino acid residues are critical in protecting cells from genomic instability inflicted by the crosslinking agent, mitomycin C. However, their biological importance in human cells remains to be confirmed using human FANCP mutant cells though L530 and Y546 are highly conserved among various organisms (Fig. 1). Based on these results, L550A and F545A are also expected to cause similar biological effects.

3.4. Identification of an SLX4-interacting region of XPF and the effects of pathogenic missense mutations in XPF on the interaction

The XPF region required for interaction with SLX4₃₇₉₋₈₇₁ (#5 in Table 1) in the yeast two-hybrid assay is quite large: XPF₁₂₋₆₅₀ is required, which corresponds to almost the entire superfamily II (SF2) helicase-like domain (Table 5). As mentioned in the Introduction, the phenotypes of XPF patients are not uniform: certain XPF patients show the phenotypes of progeria (XFE) [13], FA [14], and XP, FA and CS [15]. Interestingly, four XPF missense mutations (R153P, L230P, C236R and R589W) found in patients with the “atypical” XPF symptoms are located in the SF2 helicase-like region. This finding raises a possibility that these XPF mutations impair the interaction with SLX4₄₂₇₋₅₉₉ whereas those detected in patients with typical XP phenotypes do not. The yeast two-hybrid assay revealed that three (R153P, L230P and R589W) of the four mutations abolished the ability to interact with SLX4 whereas other XPF missense mutations, I225M, P379S, R454W and G513R, observed in typical XP patients did not (Table 6). XPF interacts constitutively with ERCC1 at its extreme C-terminal HhH (helix-hairpin-helix) region (Table 5) to form an XPF-ERCC1 heterodimer. Therefore, we also examined the effects of those XPF mutations on the interaction with ERCC1. The results were in parallel with those of the interaction with SLX4 (Table 6), suggesting that the R153P, L230P and R589W mutations catastrophically alter the whole structure of XPF and/or that these mutant XPFs are very unstable in yeast. Indeed, we failed to detect XPF^{L230P} in yeast extracts, suggesting that this mutant protein is unstable in yeast (Supplementary Fig. S2). However, target protein, in general, is not abundant in yeast extracts, and we did not pursue this question further. On the other hand, the C236R mutation causing the XP/CS/FA phenotype showed the same result as those observed in the typical XP patients, indicating no adverse effects on the structures of the SLX4- and ERCC1-interacting regions.

3.5. Physical interaction of mutant XPFs with SLX4 in human cells

The physical interaction was directly examined in human cells by immunoprecipitation. The wild-type (FLAG)₃-SLX4₄₄₈₋₅₉₉ and various (c-myc)₃-XPF₁₋₆₅₀ mutants were co-expressed transiently in HEK293T/17 cells. The FLAG-tagged SLX4₄₄₈₋₅₉₉ was immunoprecipitated with anti-FLAG antibody (Ab) agarose, and coimmunoprecipitated c-myc-tagged XPF₁₋₆₅₀ was detected with anti-c-myc Ab (79 kDa shown by arrows in Fig. 4A). The XPF mutants, I225M, C236R and P379S, and the wild-type XPF were coimmunoprecipitated with (FLAG)₃-SLX4₄₄₈₋₅₉₉ whereas L230P or R589W was not. Since there was a faint band, indicated by an asterisk, comigrating with (c-myc)₃-XPF in the immunoprecipitate of XPF^{R153P}₁₋₆₅₀, we repeated the experiment in parallel with XPF^{C236R}₁₋₆₅₀ and XPF^{P379S}₁₋₆₅₀ mutant extracts: an almost invisible, very faint band, just seen at its right and left edges, of XPF^{R153P}₁₋₆₅₀ was observed (Supplementary Fig. S3). In the immunoprecipitation experiments with these three mutants (R153P, L230P, R589W), a band corresponding to (c-myc)₃-XPF₁₋₆₅₀ was not observed not only in immunoprecipitate (IP) but also in supernatant (Su), which suggests that these mutant proteins are unstable in cells. To test this possibility, we collected cellular (c-myc)₃-XPF₁₋₆₅₀ with anti-c-myc Ab agarose from transfected HEK293T/17 extracts and tried to detect the collected protein with anti-c-myc Ab in the immunoblot assay (Fig. 4B). Immunoprecipitates of XPF^{Wt} and XPF^{I225M} showed a strong band corresponding to (c-myc)₃-XPF₁₋₆₅₀, but those of XPF^{R153P}, XPF^{L230P} and XPF^{R589W} revealed a very faint band at the position of (c-myc)₃-XPF₁₋₆₅₀ (shown by an arrow), indicating that these three mutant XPFs are very unstable in human cells. Coimmunoprecipitated (FLAG)₃-SLX4₄₄₈₋₅₉₉ was observed for XPF^{Wt} and XPF^{I225M}, but not for XPF^{R153P}, XPF^{L230P} or XPF^{R589W}. Thus, the negative results for these three mutants in the interaction with SLX4 are mainly due to the instability of those mutated XPF proteins. On the other hand, XP/FA/CS-causing C236R is stable and interacts with SLX4₄₄₈₋₅₉₉ normally.

4. Discussion

We studied the mutual physical interaction between SLX4 and XPF and showed that the small region of SLX4₅₀₀₋₅₅₈ is responsible for the interaction with XPF. Among well conserved amino acid residues in this region, SLX4 L530, F545, Y546 and L550, especially L530, are critical to the interaction as determined by the yeast two-hybrid assay (Table 2) and the immunoprecipitation assay (Fig. 2B). Furthermore, the interaction-defective mutants, L530Q and Y546C, cannot restore the resistance to the cytotoxicity (Fig. 3) and chromosome aberration induction by mitomycin C (Tables 4 and S1). Thus far, mutations found in SLX4-defective FA patients are all deletions [24, 25]. We predict, based on these results, that Y546C (global minor allele) and L530Q cause FA when combined with a nonfunctional *SLX4* allele. Because XPF is considered to play an essential role in ICL unhooking [8, 9] and to be recruited to ICL sites by SLX4 [7], not only L530Q and Y546C but also F545A and L550A mutants are also expected to be defective in ICL unhooking at a stalled replication fork, leading to FA. In addition, interaction of XPF with SLX4 is reported to increase the nuclease activity of XPF towards fork-like substrates [9]. From the cancer chemotherapeutic point of view, the development of a small molecule that binds to this SLX4 region may improve the efficacy of chemotherapeutic crosslinking agents.

Compared to SLX4, a much larger region of XPF is required for the interaction with SLX4: almost the entire SF2 helicase-like domain (XPF₁₂₋₆₅₀) is required (Table 5). The results of the interaction experiments with various XPF fragments and missense mutants (Tables 5 and 6 and Fig. 4) suggest that the integrity of the structure of XPF protein is very sensitive to subtle mutations in the helicase-like domain. The XPF mutations of R153P, L230P, C236R and R589W, which cause atypical XP phenotypes, fall onto the helicase-like domain. Three of these four mutations, except C236R, cause instability of XPF protein in cells and are negative in the interaction with SLX4 and ERCC1 in the two-hybrid assay (Table 6). In the immunoprecipitation assay, R153P was shown to retain the ability to interact with SLX4 (Fig. 4A). The cellular low levels of R153P [13] and L230P [14] have been reported. On the other hand, the mutations (I225M, P379S, R454W and G513R) observed in XPF patients with typical XP phenotypes are stable and do not affect the interaction with SLX4. These results, together with the requirement for the entire segment of the helicase-like domain, suggest that critical amino acids are spreading throughout the helicase-like domain to maintain the XPF structural integrity. The mutations of the critical amino acid residues may cause catastrophic misfolding of XPF protein, defective interaction with SLX4 and instability of XPF in cells. Compared to the other three mutations, the C236R mutation is unique: although this mutation does not induce protein instability or the impairment of the protein-protein interaction, C236R results in the combined phenotype of FA, CS, and XP [15]. This mutant is reported to have a reduced ability to interact with the XPB (p89) subunit of TFIIH, resulting in the CS phenotype, and also to retain reduced nuclease activity [15]. However, it is still unclear how this mutation in the helicase-like domain affects the nuclease activity and causes the FA phenotype. The mislocalization of mutant XPF protein to cytoplasm [26] might also contribute to the unique feature of C236R. Thus, the reason for the heterogeneity of phenotypes of XPF patients has not yet been fully elucidated, but the instability of XPF protein caused by a missense mutation, which results in the failure of nucleolytic incision of various forms of XPF substrates, could greatly contribute to those atypical XPF phenotypes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

Ab	antibody
aa	amino acids
cDNA	complementary DNA

CS	Cockayne syndrome
FA	Fanconi anemia
ICLs	interstrand crosslinks
MEFs	mouse embryonic fibroblasts
PCR	polymerase chain reaction
TLS	translesion DNA synthesis
XP	Xeroderma pigmentosum

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Highlights

- SLX4 L530, F545, Y546, and L550 are critical for the interaction with XPF.
- The global minor allele, SLX4 Y546C, may cause Fanconi anemia.
- SLX4 L530Q and Y546C enhance the degree of mitomycin C-induced chromosome aberrations
- We study the interaction of atypical XP phenotype-causing XPF missense mutations with SLX4.

	500	510	520	530	540	550	560
<i>H. sapiens</i>	STPPLPASRIK-EGWERAGQCP	PPPERKQSFL	WEGSALTGAW--	AMEDEYTARTVP	PLVPQRP	QAQGL-----	
<i>M. musculus</i>	CTPPLLASKISK-EELEPAGWRARL	PEGKRNF	WELSALTGAW--	AEESEYTVGLFPP	IVSQCP	SKE-----	
<i>G. gallus</i>	PTPRLPTSRIE-VEAGKAAWLLPL	PKNKDCF	WNFSALTGPC--	DPESEYAAGLTP	PIEPWKFVQ	NHKPEDV	
<i>D. rerio</i>	PTPTLSPS-----	SLPLQPHAPLWD	KSALHRGRQNSVSE	EYTS	ELSSFIQ	PWVAPVKENDKPL	
<i>X. tropicalis</i>	SNTALPQSWLSSIAEKEKHAWWRPL	QDKQICTL	WEGSNMMDNR--	DLLGYIYIQELN	PPITPWKPP	AQHK----	
<i>X. laevis</i>	GNTALPLSRLCNIEEREKYAWCRPL	RDDKICTL	WEGSKMMDNR--	DFLSYIYIQELN	PPITPWRDS	QLNHHNSQ	
<i>D. melanogaster</i>	-----ELRAE-----	GESRILRE	---	PSEGPIDDLES	YYVTDL	FEVSR-----	

Fig. 1.

Amino acid sequence alignment of XPF-interacting regions of SLX4 of various species.

Amino acid numbers correspond to those of human SLX4. Shaded area indicates the core of the interacting region. Conserved and similar amino acids are highlighted.

Fig. 2.

Physical interaction of SLX4 wild-type and missense mutants with XPF in HEK293T/17 cells. A. Cells were transfected with pIRESneo2[(FLAG)₃-SLX4₄₄₈₋₅₉₉] in the presence (lane 1) or absence (lane 2) of pIRESbla[(c-myc)₃-XPF₁₋₆₅₀]. Cell extracts were immunoprecipitated (**IP**) with anti-FLAG antibody (Ab) agarose and analyzed with anti-FLAG Ab (upper) or anti-c-myc Ab (lower) in an immunoblot (**IB**) assay. (c-myc)₃-XPF₁₋₆₅₀, 79 kDa; (FLAG)₃-SLX4₄₄₈₋₅₉₉, 20 kDa. B. Cells were transfected with wild-type or mutated pIRESneo2[(FLAG)₃-SLX4₄₄₈₋₅₉₉] and wild-type pIRESbla[(c-myc)₃-XPF₁₋₆₅₀]. Cell extracts were immunoprecipitated with anti-c-myc Ab agarose and analyzed with anti-FLAG Ab in an immunoblot assay. **IP**, immunoprecipitate; **Su**, supernatant of immunoprecipitation

Fig. 3. Complementation of the sensitivity of *Fancp*^{-/-} MEFs to mitomycin C by ectopically expressed SLX4^{Wt} and its missense mutants, SLX4^{L530Q} and SLX4^{Y546C}. Each point represents a mean±SD of three experiments.

Fig. 4.

Physical interaction of XPF missense mutants with SLX4 in HEK293T/17 cells. **A.** Cells were transfected simultaneously with wild-type or mutated pIRESbla[(c-myc)₃-XPF₁₋₆₅₀] and wild-type pIRESneo2[(FLAG)₃-SLX4₄₄₈₋₅₉₉]. Cell extracts were immunoprecipitated with anti-FLAG Ab agarose and analyzed with anti-c-myc Ab in an immunoblot assay. **IP** and **Su**: see the Figure 2 legend. Arrows indicate the position of (c-myc)₃-XPF₁₋₆₅₀. An asterisk indicates possible (c-myc)₃-XPF₁₋₆₅₀. **B.** Cell extracts were immunoprecipitated with anti-c-myc Ab agarose and analyzed with anti-c-myc Ab for XPF (upper) or with anti-FLAG Ab for SLX4 (lower). Arrows indicate the position of (c-myc)₃-XPF₁₋₆₅₀ or (FLAG)₃-SLX4₄₄₈₋₅₉₉.

Table 1

Interaction of various fragments of SLX4 with XPF in the yeast two-hybrid assay

Construct no.	SLX4 amino acid no.	SLX4					Interaction ^a
		U	M	B	S	C	
1	1-1834	—	—	—	—	—	+
2	867-1834			—	—	—	—
3	1-871	—	—	—			+
4	1-378	—					—
5	379-871		—	—			+
6	379-599		—	—			+
7	379-467		—				—
8	379-527		—				—
9	596-871			—			—
10	427-599		—	—			+
11	427-558		—	—			+
12	427-527		—	—			—
13	448-599		—	—			+
14	448-558		—	—			+
15	468-558		—	—			+
16	500-558		—	—			+
17	500-599		—	—			+
18	Δ(520-560)		—	—			—

^ainteraction with XPF1-916 cloned in pGBKT7; U, ubiquitin-binding domain; M, Mei9-like region; B, BTB/POZ domain; S, SAP domain; C, C terminal SLX1-interacting domain

Table 2Effects of SLX4 point mutations on the interaction with XPF₁₋₉₁₆ in the yeast two-hybrid assay

No.	pGAD (SLX4 ₄₂₇₋₅₉₉)	Growth on selective media		
		2DO	4DO	
1	Wild-type			+
2	E532A			+
3	L550A			+
4	F545A			+
5	Y546C			+
6	Y546A			+
7	L530A			–
8	F545A Y546C			±
9	F545A Y546A			±
10	L530A F545A Y546A			–
11	Δ(520-560)			–

2DO, lack of tryptophan and leucine;

4DO, lack of tryptophan, leucine, histidine and adenine

Table 3Effects of amino acid change of SLX4 L530 on the interaction with XPF₁₋₉₁₆

pGAD (SLX4 ₃₇₉₋₈₇₁)	Growth on selective media		
	2DO	4DO	
L530 (CTG)			+
L530A (<u>G</u> CG)			-
L530M (<u>A</u> TG)			+
L530V (<u>G</u> TG)			+
L530P (C <u>C</u> G)			-
L530Q (C <u>A</u> G)			-
L530R (C <u>G</u> G)			-

Letters underlined indicate mutations. See Table 2 for 2DO and 4DO.

Table 4

Induction of chromosomal aberrations by mitomycin C

<i>Fancp</i> ^{-/-} cells ^a (mitomycin C)	% of cells with aberrations (A)		Total no. of aberrations/100 metaphases, (B)		No. of aberrations/metaphase with aberrations, (C) ^c	
	0 nM	50 nM	0 nM	50 nM	0 nM	50 nM
Wild type	6	29	12	80	2.0	2.8
(Vector: pQCXIH)	4	52*	6	103	1.5	2.0
L530Q ^b	11	84**	11	330**	1.0	3.9
Y546C ^b	5	98**	5	582**	1.0	5.9**

^a *Fancp*^{-/-} MEFs complemented with the wild-type or mutant SLX4

* $p<0.01$;

** $p<0.001$ when compared with SLX4 wild type; χ^2 test for A and t -test for B and C

^b 50 metaphases were analyzed due to a high number of aberrations.

^c C=B/A

Table 5

Interaction of various fragments of XPF with SLX4 in the yeast two-hybrid assay

Construct no.	XPF (amino acid no.)	XPF			Interaction ^a
		SF2 helicase-like	Nuc	HhH	
1	1 - 916	—————	—————	—————	+
2	1 - 780	—————	———	—————	+
3	1 - 650	—————	—————	—————	+
4	1 - 580	—————	—————	—————	—
5	1 - 511	—————	—————	—————	—
6	1 - 407	—————	—————	—————	—
7	12 - 650	—————	—————	—————	+
8	100 - 650	———	—————	—————	—
9	266 - 916	—————	—————	—————	—
10	266 - 779	—————	———	—————	—
11	Δ(275-406)	—————	—————	—————	—

^a interaction with SLX4₃₇₉₋₈₇₁ cloned in pGADT7; Nuc, nuclease domain; HhH, helix-hairpin-helix

Table 6

Effects of XPF₁₋₉₁₆ missense mutations on the interaction with SLX₄₂₇₋₅₉₉ and ERCC1₁₋₂₉₇ in the yeast two-hybrid assay

Pheno- type	Cell line [reference]	Allele examined	(second allele)	SLX4		ERCC1	
				Growth on selective media			
				2DO	4DO	2DO	4DO
XFE	XP51RO [13]	R153P	(R153P)			—	—
FA	1333 [14]	L230P	(I800T/§*24)			—	—
XP/CS/ FA	XPCS1CD [15]	C236R				+	+
XP	XP32BR [15]	R589W	(R589W)			—	—
XP	XP24KY [15]	P379S	(N537R/§*8)			+	+
XP	XP24KY [15]	R454W				+	+
XP	XP101OS [15]	I225M				+	+
		G513R				+	+