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Mutations of the *SLX4* gene in Fanconi anemia

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Fanconi anemia is a rare recessive disorder characterized by genome instability, congenital malformations, progressive bone marrow failure and predisposition to hematologic malignancies and solid tumors[1]. At the cellular level, hypersensitivity to DNA interstrand crosslinks is the defining feature in Fanconi anemia[2]. Mutations in thirteen distinct Fanconi anemia genes[3] have been shown to interfere with the DNA-replication–dependent repair of lesions involving crosslinked DNA at stalled replication forks[4]. Depletion of SLX4, which interacts with multiple nucleases and has been recently identified as a Holliday junction resolvase[5–7], results in increased sensitivity of the cells to DNA crosslinking agents. Here we report the identification of biallelic *SLX4* mutations in two individuals with typical clinical features of Fanconi anemia and show that the cellular defects in these individuals' cells are complemented by wildtype SLX4, demonstrating that biallelic mutations in *SLX4* (renamed here as *FANCP*) cause a new subtype of Fanconi anemia, Fanconi anemia-P.

SLX4 is a multidomain scaffold protein that interacts with three distinct nucleases: SLX1, ERCC4/XPF-ERCC1 and MUS81-EME1[5–7]. Although the SLX4-SLX1 interaction is largely responsible for the Holliday junction resolvase activity seen in the complex, SLX4 can also stimulate the activity of ERCC4/XPF and MUS81 nucleases, both of which have been previously implicated in the processing of interstrand crosslinks (ICLs)[8]. The finding that depletion of SLX4 leads to increased sensitivity to crosslinking agents and to camptothecin[5–7] prompted us to investigate SLX4 as a candidate gene for Fanconi anemia[1,2].

So far, mutations in thirteen genes have been shown to be responsible for Fanconi anemia[3]. Eight of the Fanconi anemia proteins (FANCA, FANCB, FANCC, FANCE, FANCF, FANCG, FANCL and FANCM) form a core complex, a nuclear E3 ubiquitin ligase which ubiquitinates FANCI and FANCD2 (refs. 9,10). These two activated proteins subsequently localize as an FANCI-FANCD2 complex to chromatin and direct repair[4] partly through interaction with the newly identified nuclease FAN1 (refs. 11–14). Cells with mutations in the Fanconi anemia core complex (except for FANCM) lack monoubiquitination of FANCD2. The other Fanconi anemia proteins are FANCJ (also known as BRIP1), a helicase, and the homologous recombination effectors FANCN (also known as PALB2) and FANCD1 (also known as BRCA2). Recently, RAD51C, also involved in homologous recombination repair, has been found to be mutated in three individuals with a Fanconi anemia–like disorder[15]. Cells mutated in *FANCJ* (*BRIP1*), *FANCN* (*PALB2*), *FANCD1* (*BRCA2*) and *RAD51C* have normal FANCD2 monoubiquitination, and their products are thought to work downstream of the FANCI-FANCD2 complex.

As depletion of SLX4 in a U2OS cell line does not affect FANCD2 ubiquitination (**Fig. 1a**,**b**), we sequenced *SLX4* in the families from the International Fanconi Anemia Registry[16] with unassigned Fanconi anemia complementation groups and normal FANCD2 modification (**Fig. 1c**) and identified two families carrying germline mutations, IFAR1084 and IFAR414 (**Fig. 1d**). Phenotypes of the two affected individuals are summarized in **Table 1**. The lymphoblastoid cell line (LCL) (RA3042) and fibroblasts (RA3083) from individual 1084/1 showed increased genomic instability (**Fig. 1e** and **Table 2**) and increased sensitivity to mitomycin C (MMC) (**Supplementary Fig. 1a**). The 414/1 individual's LCL (RA 1376) was not sensitive to MMC, suggestive of reversion (**Supplementary Fig. 1b**); however, his skin fibroblasts (RA 3331) displayed a high degree of diepoxybutane (DEB)-induced chromosomal instability (**Fig. 1e** and **Table 2**) and sensitivity to MMC. We observed no ultraviolet sensitivity in fibroblasts from either of the affected individuals (**Supplementary Fig. 1c**,**d**). Fibroblasts from individual 414/1 (RA3331) but, interestingly, not individual 1084/1 (RA3083) were sensitive to camptothecin, a topoisomerase I inhibitor (**Supplementary Fig. 1e**,**f**).

Sequencing of the complementary DNA (cDNA) from the 1084/1 individual's cells revealed skipping of exon 5 (**Supplementary Fig. 2a**) due to a homozygous point mutation in the canonical splice donor dinucleotide GT in intron 5 (c.1163+2T>A) in the genomic DNA (**Supplementary Fig. 2b**). We found both of this individual's parents to be heterozygous and found an unaffected sibling to be negative for this mutation (**Supplementary Fig. 2b**). The predicted effect of this mutation is a 70-amino-acid deletion of amino acids 317–387 of SLX4 (p.Arg317_Phe387del) leading to an in-frame deletion of the conserved cysteine and leucine of the first UBZ domain and the whole second UBZ domain (**Fig. 2a** and **Supplementary Fig. 2c**).

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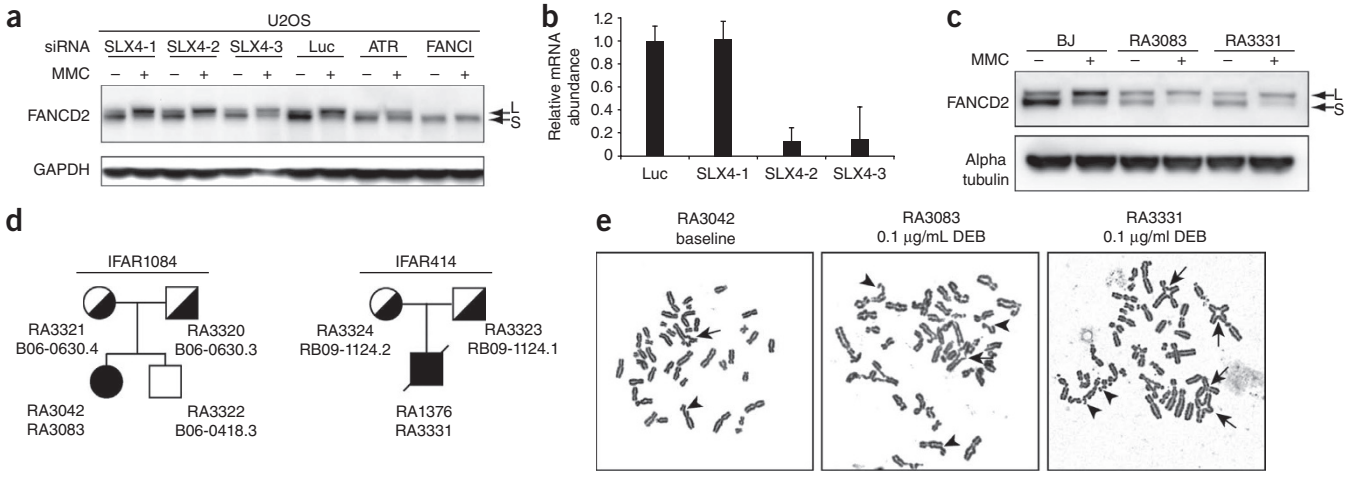


Figure 1 Characterization of cell lines from individuals with Fanconi anemia with *SLX4* mutations. (**a**) Protein blot analysis with a FANCD2 antibody of U2OS cells transfected with the indicated siRNAs and treated with 1 μM MMC for 24 h. L (long) indicates a monoubiquitinated form and S (short) indicates the non-monoubiquitinated form of FANCD2. (**b**) RT-quantitative PCR in U2OS cells transfected with various siRNAs against SLX4 used in the experiment shown in **a**. Error bars indicate the standard deviation (s.d.) of three replicates. (**c**) Protein blot analysis with a FANCD2 antibody of BJ, RA3083 and RA3331 fibroblasts. Cells were left untreated or were treated with 1 μM MMC for 24 h. (**d**) Pedigrees of the two families described in this study showing accession numbers for cell lines (RA) and peripheral blood samples (B, RB). The two probands are indicated with filled symbols. Mutation carriers are indicated by half-filled symbols. (**e**) Examples of metaphases of the LCL RA3042 (no drug treatment) and fibroblast RA3083 cell lines from individual 1084/1 and the fibroblasts RA3331 from individual 414/1 (treatment with diepoxybutane (DEB)).

Immunoprecipitation of SLX4 from the cell line RA3083 confirmed the presence of a slightly shorter protein product (**Fig. 2b** lane 5 and **Supplementary Fig. 2d**).

In individual 414/1, we detected a heterozygous frameshift mutation in exon 2 (c.514delC) by sequencing of the full length RT-PCR product (**Supplementary Fig. 3a**) and confirmed it in the genomic DNA of this individual and his father (**Supplementary Fig. 3b**). The predicted protein effect of this frameshift mutation is a truncated protein with N-terminal 171 amino acids of SLX4 followed by 22 non-SLX4 amino acids (p.Leu172Phe*fs*X22) (**Fig. 2a**). The second allele of SLX4 in the individual 414/1, identified as described in the Online Methods, showed a large genomic deletion from intron 9 to exon 12 resulting in c.2013+225_3147delinsCC (**Supplementary Fig. 3c–e**). The predicted effect of this mutation is a truncated protein with N-terminal 671 amino acids of SLX4 followed by 119 non-SLX4 amino acids due to a frameshift (p.Leu672Val*fs*X119) (**Fig. 2a**). Consequently, immunoprecipitation with the antibody against SLX4 failed to identify the full-length protein in the individual's fibroblasts (RA3331) (**Fig. 2b** lane 6).

To prove that the mutations identified in *SLX4* were causal for the Fanconi anemia phenotype of both affected individuals, we introduced the wildtype or the mutant *SLX4* cDNAs into these individuals' fibroblasts (RA3083 and RA3331) and performed functional complementation assays (**Fig. 3** and **Supplementary Fig. 4**). Expression of wild-type SLX4 in both cell lines almost fully rescued the MMC sensitivity (**Fig. 3a** and **Supplementary Fig. 4a,b**), the late S/G2 arrest with MMC treatment (**Fig. 3b** and **Supplementary Fig. 4c–e**) and the chromosomal instability after treatment with DEB (**Supplementary Fig. 4f**). Some residual MMC sensitivity, cell cycle arrest and chromosomal breakage is most likely due to some cells losing expression of SLX4, as evident by immunofluorescence analysis (data not shown). Introduction of the mutant proteins did not rescue the Fanconi anemia phenotypes of these individuals' cells, although we noted a slight improvement in the various assays, possibly due to overexpression of the mutant proteins, which might have residual function. These experiments demonstrate that biallelic *SLX4* mutations cause a new subtype of Fanconi anemia, Fanconi anemia-P, and that *FANCP* becomes an alias for *SLX4*.

SLX4 interacts with multiple factors; two of which, ERCC4/XPF and MUS81, have been previously implicated in crosslink repair[8]. We therefore tested whether the mutant SLX4 proteins from both affected individuals still interacted with the ERCC4/XPF and MUS81 complexes. We found that ERCC4/XPF, MUS81 and ERCC1 coimmunoprecipitated with endogenous mutant SLX4 (p.Arg317_Phe387del) from RA3083 fibroblasts (**Fig. 4a** lane 5 and **Supplementary Fig. 5a** lane 4), although the levels of the mutant SLX4 protein were consistently lower in multiple experiments, leading to diminished immunoprecipitation of the interacting factors. The SLX4 p.Leu672Val*fs*X119 mutant protein, overexpressed in RA3331 fibroblasts, showed diminished but present interaction with ERCC4/XPF

Table 1 Characteristics of individuals with Fanconi anemia and mutations in *SLX4*

Individual	Maternal allele	Paternal allele	Ethnicity	Phenotypic and hematologic abnormalities
1084/1	c.1163+2T>A, p.Arg317_Phe387del[a]	c.1163+2T>A, p.Arg317_Phe387del[a]	South Indian	Fifteen-year-old female, short stature (height –2.1 s.d., 1st percentile); vitiligo; presented at 9 years of age with isolated thrombocytopenia.
414/1	c.2013+225_3147 del4890insCC, p.Leu672Val*fs*X119[b]	c.514delC, p.Leu172Phe*fs*X22[c]	American of European descent	Bilateral absent thumbs and right radial aplasia, undescended left testicle, pelvic kidney, malformed auricle and short stature; squamous cell carcinoma of the tongue at 21 years of age; platelets, 35,000 cells/μl; Hb, 10 g/dL; MCV, 105.5 fL; died at 22 years of age from complications of metastatic disease.

[a]The predicted protein has an internal deletion from amino acids 317 to 387. [b]The predicted protein has 671 N-terminal amino acids of SLX4 followed by 119 non-SLX4 amino acids due to a frameshift. [c]The predicted protein has 172 N-terminal amino acids of SLX4 followed by 22 non-SLX4 amino acids due to a frameshift. s.d., standard deviation.

Table 2 Chromosome breakage analysis in the indicated cell lines with and without diepoxybutane treatment

	RA3042 (LCL)		RA3083 E6E7		RA3331 E6E7		BJ E6E7[a]	
DEB concentration (μg/ml)	0	0.1	0	0.1	0	0.1	0	0.1
Metaphases	56	29	53	32	50	31	63	51
Total breaks[b]	41	221	8	140	7	217	8	8
Chromatid breaks	29	123	6	92	7	123	6	8
Triradials	5	44	1	16	0	36	1	0
Quadriradials	1	5	0	8	0	11	0	0
% of metaphases with breaks	30	90	13	81	14	100	11	16
Breaks per metaphase	0.73	7.6	0.15	4.4	0.14	7.0	0.11	0.16

[a]BJ foreskin fibroblasts from a healthy donor were used as a normal control. [b]Total number of breaks includes chromatid breaks and radial chromosomes. DEB, diepoxybutane.

and ERCC1 but not with MUS81 (**Fig. 4b** lane 3). This result is consistent with the previous findings that MUS81 interacts with the amino acid 684–1,834 fragment of the SLX4 protein[7], which is deleted in the p.Leu672Val*fs*X119 mutant protein. Immunoprecipitation with an antibody recognizing the N terminus of SLX4 from RA3331 cells showed greatly diminished interaction with ERCC4/XPF, ERCC1 and MUS81 (**Supplementary Fig. 5b** lane 6).

As UBZ domains are known to interact with ubiquitin[17], we hypothesized that the absence of the tandem UBZ domains in the mutant SLX4 from individual 1084/1 might disrupt the binding of the SLX4 complex to ubiquitin chains of repair proteins at the sites of DNA damage, as shown for the tandem UBZ domains of RAP80 (ref. 18). We therefore performed *in vitro* ubiquitin binding assays (**Fig. 4c**) that showed binding of the isolated UBZ domains of SLX4 to the K63 chains of ubiquitin (**Fig. 4c** lane 8). When the two conserved cysteines from each UBZ domain were mutated to alanines (**Supplementary Fig. 2c**), the binding was reduced to background levels seen with GST alone (**Fig. 4c**, compare lane 7 and 9), suggesting the possibility that SLX4 may localize to the sites of damage through binding to K63 ubiquitinated substrates. As SLX4 would localize other proteins, including ERCC4/XPF, MUS81 and SLX1, to sites of DNA damage, the SLX4-deficient cell lines described here are important tools to understand

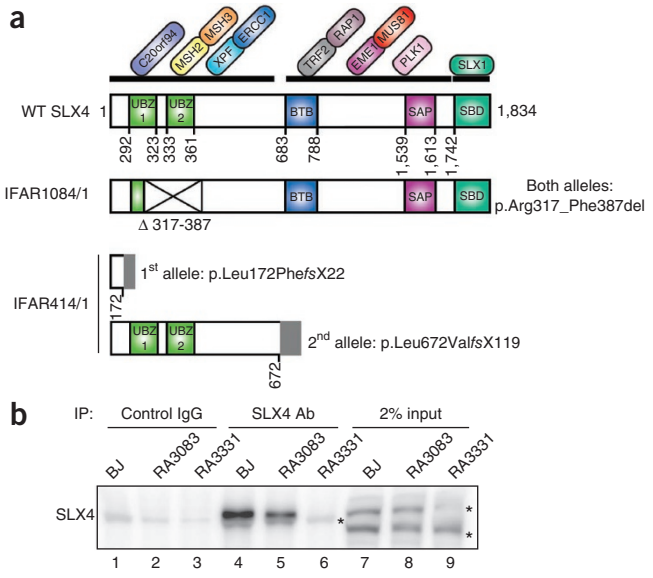
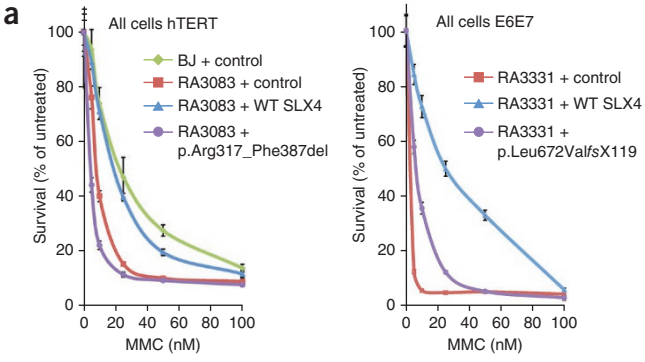


Figure 2 *SLX4* is defective in two individuals with Fanconi anemia. (**a**) Schematic of SLX4 (based on ref. 7) showing the domain architecture, the interacting proteins and the predicted protein effect of *SLX4* mutations in IFAR1084/1 and IFAR414/1 individuals. (**b**) Analysis of the mutant SLX4 protein in the cell lines. We subjected cell extracts of primary BJ, RA3083 and RA3331 fibroblasts to immunoprecipitation using a control rabbit antibody (control IgG) or the SLX4 antibody. Asterisks indicate the crossreacting bands. Note that the antibody does not identify SLX4 in straight protein blotting (lanes 7 to 9). WT, wildtype.

which interactions of SLX4 are essential for the repair of cross-linked DNA and ultimately to define the importance of the SLX4 (FANCP) function in the Fanconi anemia pathway. Phenotypes of the affected individuals also provide an important clue. Individuals with Fanconi anemia with mutations in *PALB2* (*FANCN*) or *BRCA2* (*FANCD1*), which are essential for homologous recombination, have very early onset of childhood solid tumors and acute myelogenous leukemia[19,20].



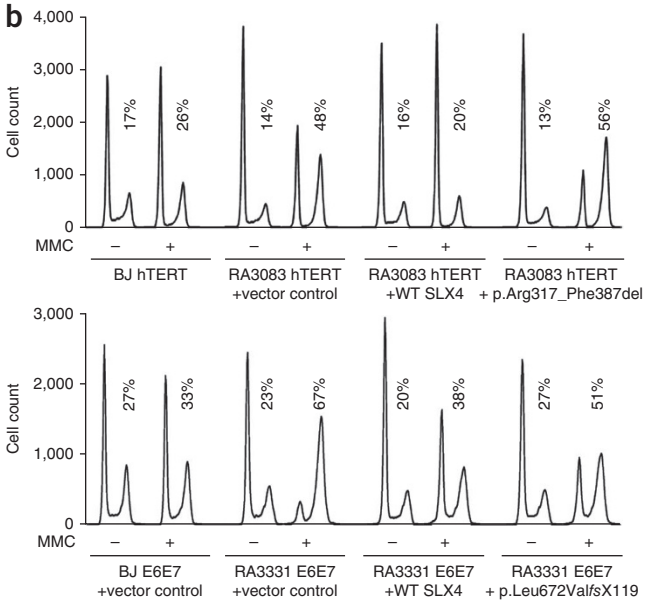


Figure 3 Complementation of RA3083 and RA3331 cells with the *SLX4* cDNA. (**a**) Complementation of MMC sensitivity. We exposed fibroblasts stably transduced with empty vector (control) or the vector expressing wildtype SLX4 or the mutant SLX4 cDNAs to different levels of MMC ranging from 0–100 nM. After 8 days, the cell number was determined using a coulter counter. Total cell numbers at each dose were divided by the number of cells in the untreated sample to arrive at percent survival. Error bars indicate s.d. (**b**) Complementation of the cell cycle defect after MMC treatment. Indicated cells were treated with 100 nM MMC, and the cell cycle was analyzed 48 h later. Untreated samples were analyzed in parallel. Expression levels of the exogenous proteins are shown in **Supplementary Figure 4a–c**. Quantification of the data is shown in **Supplementary Figure 4d,e**. WT, wildtype.

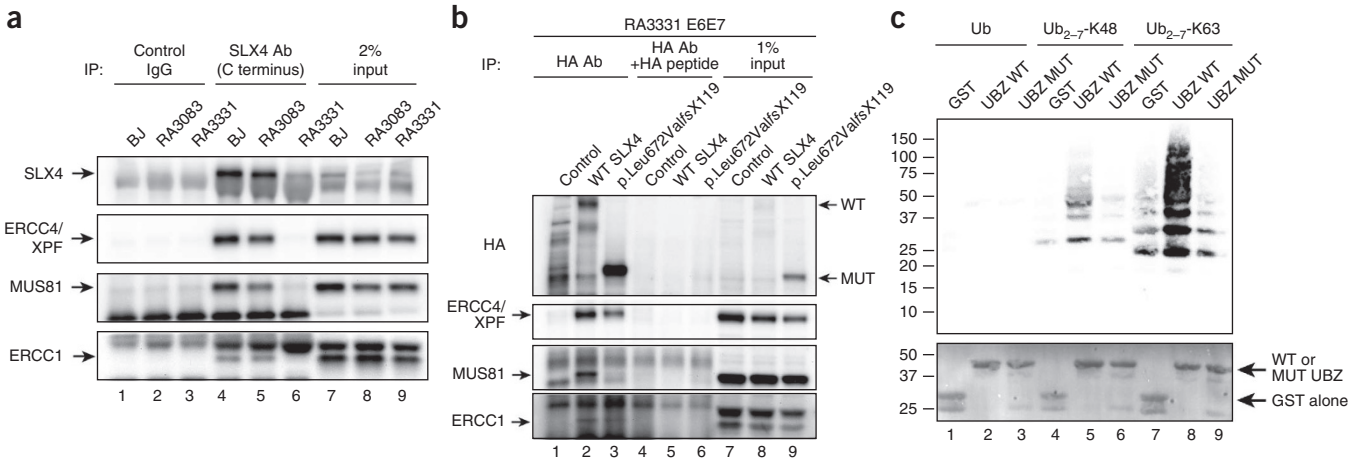


Figure 4 Interaction of mutant forms of SLX4 with its partners and with ubiquitin. (**a**) Analysis of SLX4-interacting partners in SLX4 mutant cells. We subjected cell extracts of primary fibroblasts (BJ, RA3083 and RA3331) to immunoprecipitation using the SLX4 antibody. We identified interacting proteins by immunoblotting with the indicated antibodies. (**b**) Analysis of SLX4-interacting partners in RA3331 cells. We subjected cell extracts of RA3331 E6E7 cells expressing HA-tagged control vector, wild-type SLX4 or the p.Leu672Val*fs*X119 SLX4 alteration to immunoprecipitation using HA antibody or HA antibody in the presence of 30 μg of HA peptide. We identified interacting proteins by immunoblotting with the indicated antibodies. (**c**) Interaction of the UBZ domains with ubiquitin. We incubated GST-purified GST control, wild-type UBZ domains (SLX4 amino acids 251–402) and UBZ domains with four cysteines mutated to alanines (SLX4 amino acids 251–402 p.Cys296Ala, p.Cys299Ala, p.Cys336Ala and p.Cys339Ala) with the indicated forms of ubiquitin, purified by binding to GST beads, separated on a PAGE gel and immunoblotted with ubiquitin antibody. The bottom panel shows Ponceau staining of the GST proteins. WT, wild type; MUT, mutated.

Individuals with Fanconi anemia having *SLX4* (*FANCP*) mutations show a milder phenotype more akin to that seen in individuals with mutations in the Fanconi anemia core or the FANCI-FANCD2 complex components. This suggests that the Holliday junction resolution, an integral step of homologous recombination, might not be the essential function of SLX4 in the somatic compartment during crosslink repair and that the repair depends on the other nucleases, ERCC4/XPF and MUS81, that interact with SLX4.

SLX4 (FANCP) represents a second protein (besides FANCM) that is conserved in lower eukaryotes, which do not have any other Fanconi anemia pathway components. Yeast Slx4, like human SLX4, interacts with orthologs of ERCC4/XPF and SLX1, and the work in this model organism will provide insight into the function of the Fanconi anemia pathway in human cells. Because germ-line mutations in three Fanconi anemia genes (*BRCA1* (*FANCD1*), *PALB2* (*FANCN*) and *BRIP1* (*FANCJ*)) and *RAD51C*, mutated in an Fanconi anemia–like disorder, are associated with a high risk of developing familial breast and ovarian cancers[21–24], *SLX4* should also be sequenced in individuals from pedigrees where no other predisposing mutations could be identified.

METHODS

Methods and any associated references are available in the online version of the paper at http://www.nature.com/naturegenetics/.

Accession codes. The *SLX4* reference sequences are deposited in NCBI with the following reference sequences: NM_032444.2 and NP_115820.2.

Note: Supplementary information is available on the Nature Genetics website.

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AUTHOR CONTRIBUTIONS

The study was designed by A.S., Y.K. and F.P.L. Subject recruitment and sample collection was done by A.D.A., F.P.L. and A.S. Characterization with respect to Fanconi anemia subgroups was performed by A.S., F.P.L., H.H. and A.D.A. Mutation analysis and functional studies were performed by A.S., Y.K., F.P.L. and R.D. The manuscript was written by A.S. with help from other authors.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

Subjects. Cell lines and genomic DNA samples were derived from individuals with Fanconi anemia and their family members registered in the International Fanconi Anemia Registry after obtaining informed written consent. The Institutional Review Board of The Rockefeller University, New York, New York, USA approved the studies.

Cell Culture. U2OS cells were grown in Dulbecco Modified Eagle medium (DMEM) supplemented with 10% (v/v) FBS, 100 units of penicillin per ml and 0.1 mg streptomycin per ml (all from Invitrogen). Lymphoblast cell lines were grown in Roswell Park Memorial Institute medium (RPMI) supplemented as above except with 20% FBS. Fibroblasts were grown as above except with 15% FBS and non-essential amino acids. Fibroblasts were grown in 3% oxygen. BJ cells are normal foreskin fibroblasts and were obtained from ATCC. Subjects' fibroblasts were immortalized using a catalytic subunit of telomerase (hTERT) or were transformed using HPVE6 and E7 proteins.

cDNA and genomic sequencing. RNA was extracted from the cell lines using QIAGEN RNeasy Plus Mini Kit. First-strand cDNAs were then synthesized using the Invitrogen SuperScript III Reverse Transcriptase kit. SLX4 RT-PCR was done using primers 621 and 624 (**Supplementary Table 1**) and Invitrogen Platinum Pfx DNA Polymerase kit. PCR products were cleaned up using the USB Exo-Sapit kit before being set up for sequencing, done by Genewiz, Inc. (South Plainfield, New Jersey, USA). Primers 623, 627, 628, 629, 630, 633, 634, 639, 640 and 641 were used to sequence the full-length SLX4 (**Supplementary Table 1**). Genomic PCR was done using QIAGEN Taq Polymerase with primers shown in **Supplementary Table 1**. The PCR products were cleaned up using the USB Exo-Sapit kit before being set up for sequencing. In most cases, the PCR primers were also suitable for sequencing.

Identification of the second allele in IFAR414/1. In order to identify the second allele in the RA3331 cell line, full genomic sequencing of the coding exons was done on the subject's and the maternal DNA. A genomic deletion was found to be likely based on informative polymorphic markers in exon 12: c.3162G>A (p.Ser1054Ser, rs76488917) and c.3365C>T (p.Pro1122Leu, rs714181). Amplification of parts of the cDNA followed by a detailed sequence analysis revealed retention of IVS9 through c.2013+224, with deletion of c.2013+225_3147 and insertion of two cytosine nucleotides in its place: hence c.2013+225_3147del4890insCC (**Supplementary Fig. 2d**). To confirm this result, a genomic PCR assay was set up using primers flanking the deletion. The resulting wild-type amplicon (6,821 bp) failed to amplify under the PCR conditions we used, but the deleted allele resulted in a 1,931-bp amplicon from the subject's and the mother's DNA but not the father's DNA (**Supplementary Fig. 3e**). Direct sequencing of the mutant amplicon confirmed the result observed in the cDNA (**Supplementary Fig. 3c**). The sequencing result appears homozygous because the wildtype allele did not amplify.

Plasmids. The wild-type SLX4 cDNA was a kind gift from the Harper Lab, Harvard Medical School, Boston, Massachusetts, USA. Mutant alleles were amplified and recombined into pDONR223 (ref. 25). pDONR223 derivatives were recombined into pHAGE vectors or pDEST15 using LR clonase (Invitrogen). HPV16 E6E7 genes (gift from Howley Lab, Harvard Medical School, Boston, Massachusetts, USA) were subcloned into pMSCVneo (Clontech) and used to transform the primary cells. pWZLhTERT[26] was used to immortalize RA3083 and BJ cell lines.

Antibodies. The following antibodies were used: FANCD2 (Novus NB100-182), GFP (Roche 11814460001), HA (Covance MMS-101R), SLX4 (Bethyl A302-269A and A302-270A), ERCC4/XPF (Neomarkers Ab-1), ERCC1 (Neomarkers Ab-2), Mus81 (Abcam, MTA30 2G10/3), ubiquitin (Millipore, MAB1510) and an antibody raised against amino acids 251–402 of SLX4 (Bethyl).

Mutagenesis. Mutagenesis was performed using a multisite mutagenesis kit (Agilent) using primers shown in **Supplementary Table 2**.

RNAi. Short interfering RNA (siRNA) transfections were performed using Lipofectamine RNAiMAX as suggested by the manufacturer with a final siRNA concentration of 50 nM. siRNAs (Invitrogen) are shown in **Supplementary Table 3**.

RT quantitative PCR. Superscript III reverse transcriptase followed by Platinum cybergreen super mix (Invitrogen) was used according to the manufacturer's instructions. Actin was used as the control.

Ubiquitin binding assay. Five micrograms of purified GST SLX4_amino acids 251–402, GST SLX4 amino acids 251–402, ZNF1 and ZNF2 mutants, or GST alone as a control were added to 7.5 µl GST beads and 1 µg mono-Ub, Ub-K48 or Ub-K63 (Boston Biochem) in 100 µl binding buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 10% glycerol, 0.1% Triton, 2 mM NEM and 200 µg/ml BSA) and incubated for 3 h at 4 °C. After washing five times with binding buffer, the samples were boiled and loaded on a Bis-Tris gel (Invitrogen) and immunoblotted with ubiquitin antibody (Millipore, MAB1510).

Cell cycle studies. For cell cycle analysis, cells were left untreated or were treated with 100 nM MMC and were grown for 48 h. Collected cells were resuspended in 300 µl PBS. While vortexing, 700 µl of ice cold 100% (v/v) ethanol were added dropwise, and the suspension was stored at −20 °C at least overnight. Thirty minutes before fluorescence-activated cell sorting (FACS), cells were spun down, resuspended in propidium iodine mix (1 ml PBS, 10 µl RNase (of stock solution of 20 mg/ml), 10 ml propidium iodine (of stock solution of 1 mg/ml)) and analyzed using FACSCalibur (Becton Dickinson). Cell cycle analysis was performed using the FlowJo software (Tree Star, Inc).

Breakage analysis. Cells were treated with 0.1 µg DEB per ml of media for 72 h, arrested with colcemid (0.17 µg per ml of media) for 20 min (LCL) or 2 h for fibroblasts, harvested, incubated for 10 min at 37 °C in 0.075 M KCl and fixed in the freshly prepared methanol:glacial acidic acid (3:1 vol/vol). Cells were stored at 4 °C and, when needed, dropped onto wet slides and air dried at 40 °C for 60 min before staining with KaryoMAX Giemsa (Invitrogen) Gurr Buffer for 3 min. After rinsing with fresh Gurr Buffer followed by distilled water, the slides were fully dried at 40 °C for 60 min and scanned using the Metasystems Metafer application.

Immunoprecipitations. For immunoprecipitations, cells were lysed in MCLB (50 mM Tris, 150 mM NaCl and 0.5% NP-40) supplemented with protease Inhibitors (Roche) and phosphatase inhibitors (Calbiochem). One or two milligrams of protein extract was incubated with 5 µg of the indicated antibody and 10 µl of Protein A/G PLUS-Agarose (Santa Cruz). Following five washes in lysis buffer, the immunoprecipitates were eluted in tris-glycine SDS sample buffer and size fractionated on Novex 3%–8% Tris-Acetate gel (Invitrogen).

Mitomycin C sensitivity assay. Cells were plated in a six-well plate in triplicate at a density of 2.5×10^4 cells per well. Twenty-four hours later, MMC was added at final concentrations from 0–100 nM. After 8 days in culture, cell numbers were determined using a Z2 Coulter Counter (Beckman Coulter). The cell number after MMC treatment was normalized to the cell number in the untreated sample to arrive at the percent survival.

25. Lamesch, P. *et al.* hORFeome v3.1: a resource of human open reading frames representing over 10,000 human genes. *Genomics* **89**, 307–315 (2007).
26. Dolma, S., Lessnick, S.L., Hahn, W.C. & Stockwell, B.R. Identification of genotype-selective antitumor agents using synthetic lethal chemical screening in engineered human tumor cells. *Cancer Cell* **3**, 285–296 (2003).

Supplementary Figures and Tables

Figure S1. Damage sensitivity of patient cell lines. **A.** Mitomycin C (MMC) sensitivity of lymphoblasts from IFAR 1084 family. **B.** MMC sensitivity of lymphoblasts from IFAR 414 family. **C.** and **D.** UV sensitivity of the indicated cell lines. **E** and **F.** Camptothecin (CPT) sensitivity of the indicated cell lines. Cells were treated in triplicate with different levels of indicated damaging agents. After 8 days, cell number was determined using a coulter counter. Total cell numbers at each dose were divided by the number of cells in the untreated sample to arrive at percent survival. Error bars indicate standard deviation.

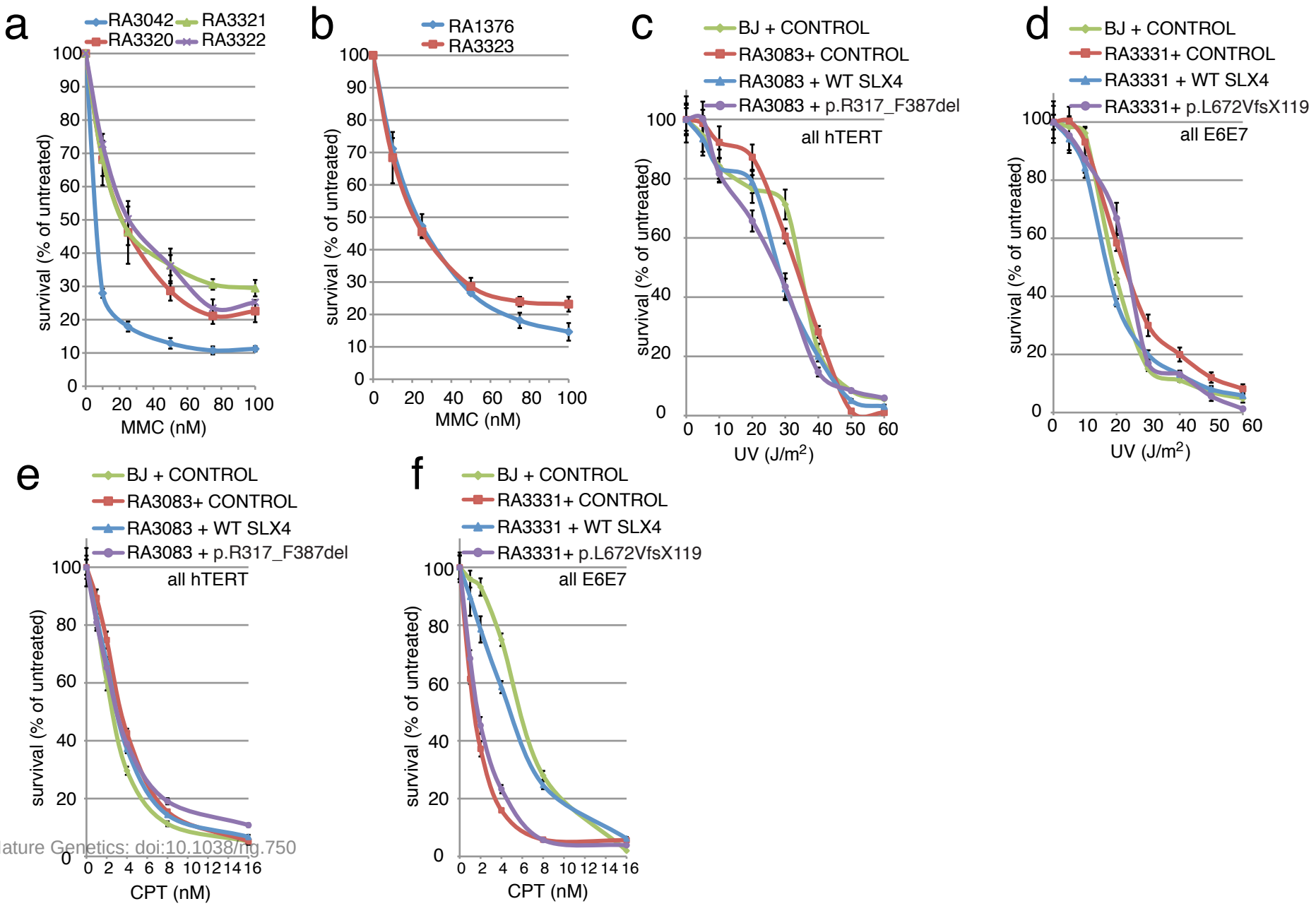
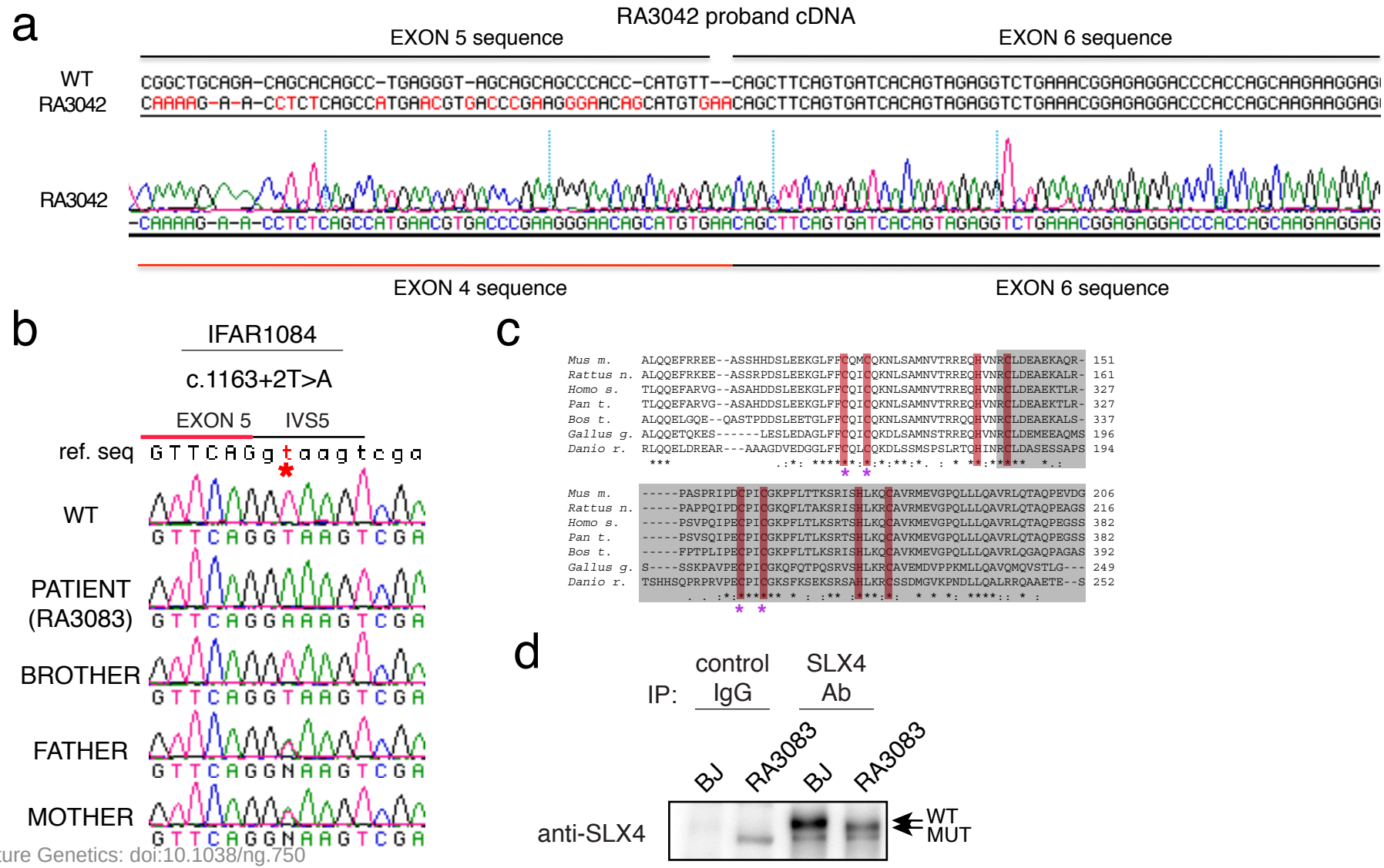
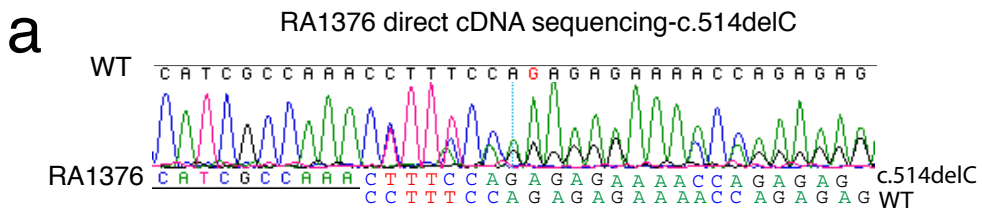


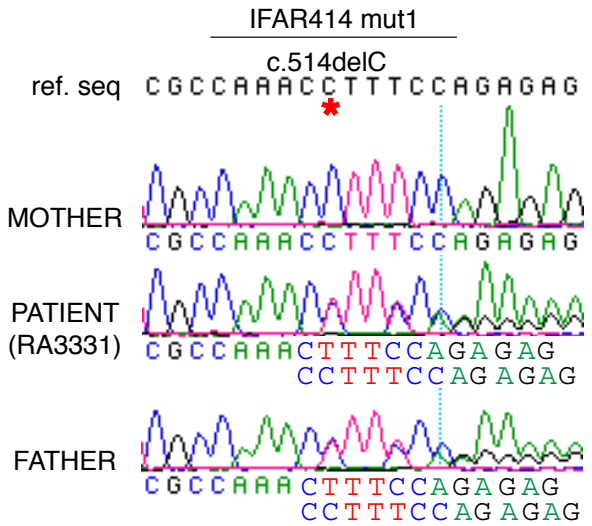
Figure S2. Sequencing of SLX4 in the IFAR1084. **A.** Chromatogram displaying skipping of the exon 5 as identified by direct sequencing of full length RT-PCR product from RA3042 cell line. **B.** Chromatogram displaying the splice donor site mutation and its segregation in the genomes of the IFAR1084 family. Genomic DNA from the fibroblast RA3083 cell line derived from the proband and the genomic DNA isolated from the peripheral blood (PB) of the other individuals were amplified and sequenced as described in Online Methods. **C.** Alignment of the UBZ domains of SLX4 from Mus musculus (Mus m.), Rattus norvegicus (Rattus.n), Homo sapiens (Homo s.), Pan troglodytes (Pan t.), Bos Taurus (Bos t.), Gallus gallus (Gallus g.), Danio Rerio (Danio r.). The residues that coordinate Zn are highlighted in red. Grey highlights the residues that are deleted in the patient from IFAR 1084 family. Magenta stars point to the Cys residues mutated for the experiment shown in Figure 4C (UBZ MUT). **D.** Cell extracts of primary BJ and RA3083 cells were subjected to immunoprecipitation using the SLX4 antibody and immunoprecipitates were bloted with another SLX4 antibody.

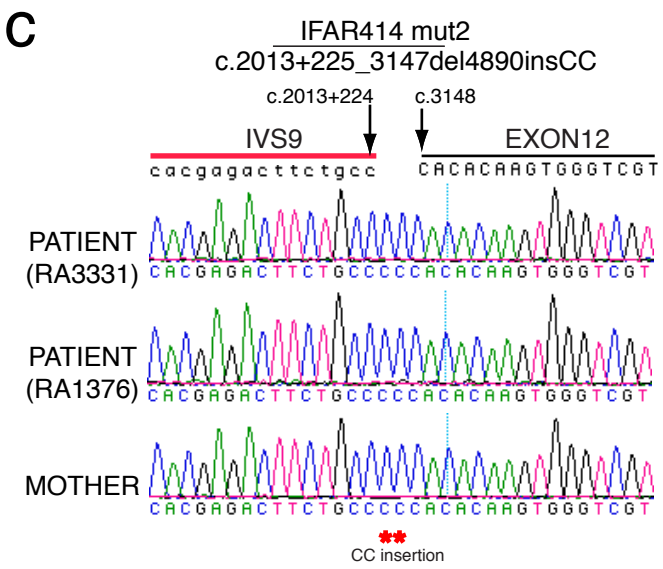


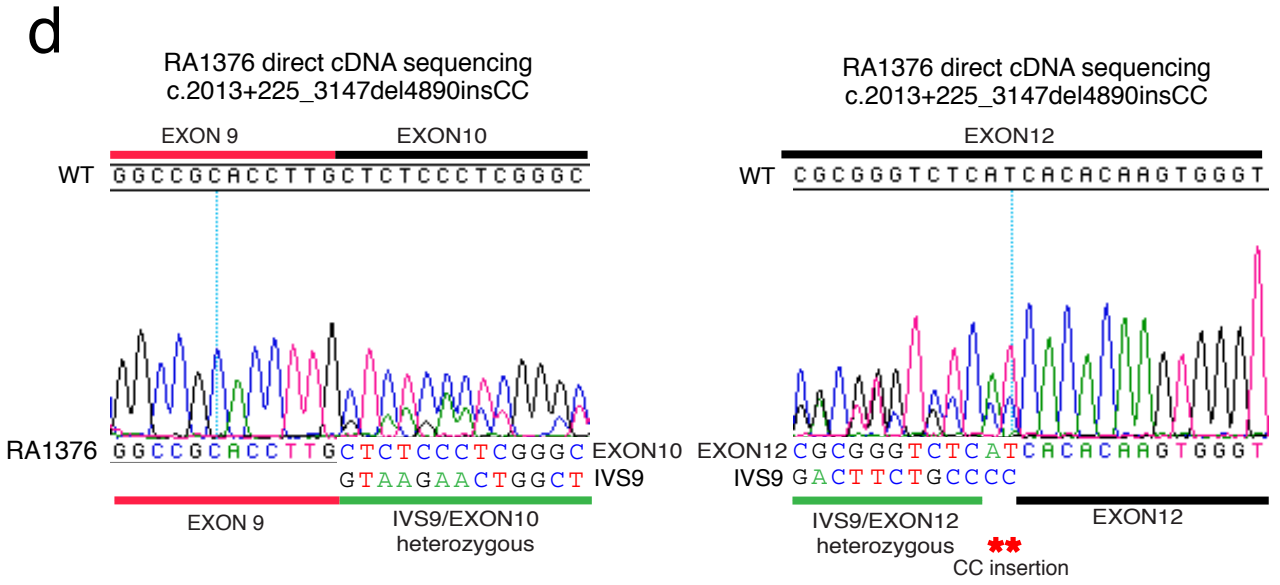
Nature Genetics: doi:10.1038/ng.750

Figure S3. Sequencing of SLX4 in the IFAR414. **A.** Chromatogram displaying heterozygosity for the c.514delC mutation in direct sequencing of the cDNA from RA1376 cell line. **B.** Chromatogram displaying the first heterozygous mutation in the genomic DNA from the fibroblast RA3331 cell line derived from the proband and the genomic DNA isolated from the PB of the other individuals. **C.** Chromatogram displaying the second heterozygous mutation in IFAR414 family. Genomic DNA from the fibroblast RA3331 and RA1376 cell lines derived from the proband and the genomic DNA isolated from the mother's peripheral blood were amplified and sequenced as described in Online Methods. Note that there is apparent homozygosity in the patient and the carrier due to sequencing of a PCR product detecting only the allele carrying a genomic deletion. **D.** Chromatogram displaying heterozygosity for the deletion/insertion mutation in RA1376. The 3' junction is shown on the left and the 5' junction is shown on the right. **E.** PCR to identify the mutant deletion/insertion in the SLX4 allele in the indicated cell line and peripheral blood samples. Father's blood (sample RB09-1124.1), Mother's blood (sample RB09-1124.2).









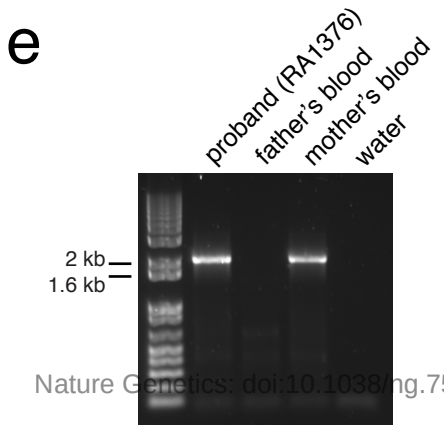


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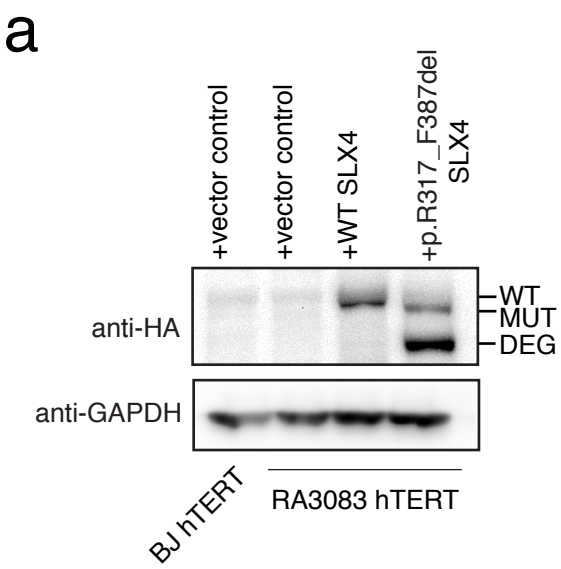
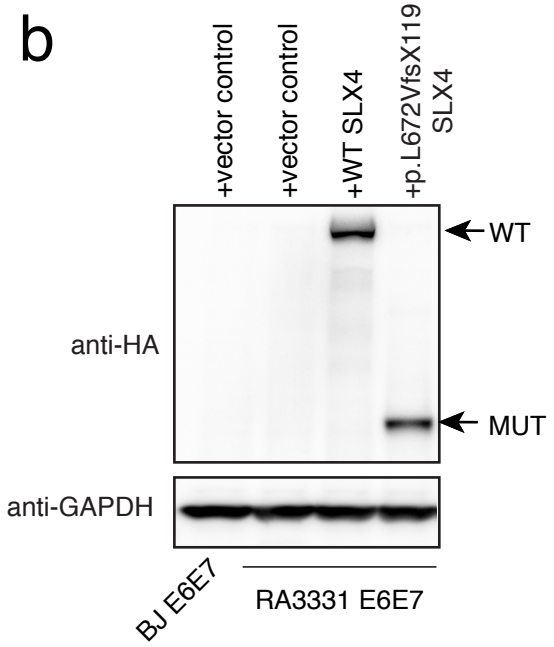
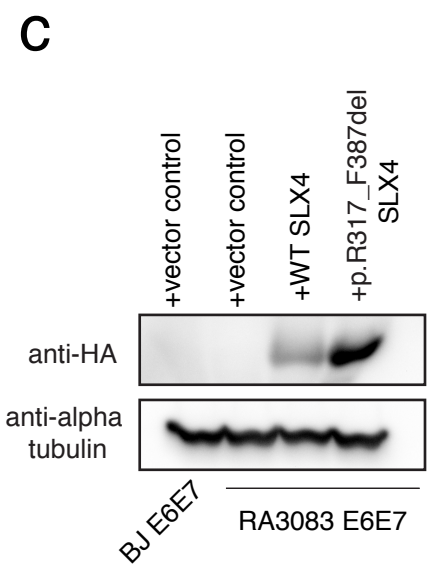
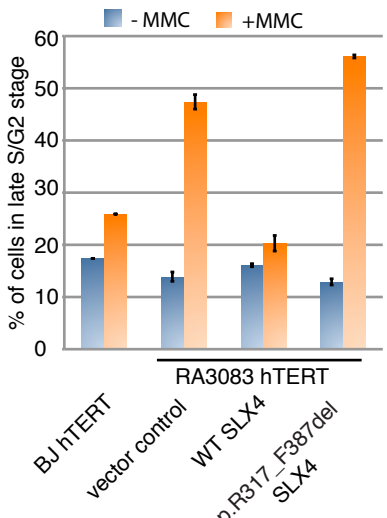
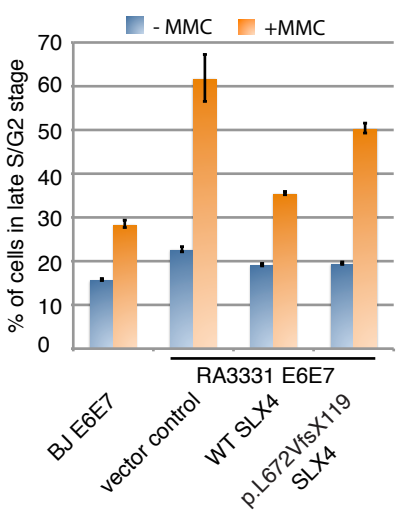
Figure S4. Complementation of RA3083 and RA3331 cells with SLX4 cDNAs. **A.** Expression of SLX4 alleles in RA3083 hTERT cells used in studies of MMC sensitivity and cell cycle analysis after MMC treatment. WT, mutant SLX4 (MUT) and a degradation (DEG) product are indicated. **B.** Expression of SLX4 alleles in RA3331 E6E7 cells used in MMC sensitivity, breakage and cell cycle studies. **C.** Expression of SLX4 alleles in RA3083 E6E7 cells used in breakage studies. **D.** Quantification of cell cycle data shown in Figure 3B top panel. The experiment was done in triplicate and error bars indicate standard deviation. **E.** Quantification of cell cycle data shown in Figure 3B bottom panel. The experiment was done in triplicate and error bars indicate standard deviation **F.** Complementation of the chromosome breakage phenotype. Indicated cells were treated with DEB at 0.1 µg/ml and chromosomal abnormalities were analyzed on metaphase spreads..











f

	RA3083 E6E7			RA3331 E6E7		
	CONTROL	WT SLX4	p.R317_F387del SLX4	CONTROL	WT SLX4	p.L672VfsX119 SLX4
DEB concentration g/ml	0.1	0.1	0.1	0.1	0.1	0.1
# Metaphases	43	53	33	32	50	57
#Total Breaks	156	49	78	401	175	306
#Chromatid breaks	100	35	46	311	169	232
#Triradial	20	6*	9	34	2	31
#Quadriradial	8	1*	7	11	1	6
% Metaphases with breaks	51	13	48	97	22	74
# Breaks per metaphase	3.6	0.9	2.4	12.5	3.5	5.4

*1 cell had 3 triradial and one quadriradial chromosomes

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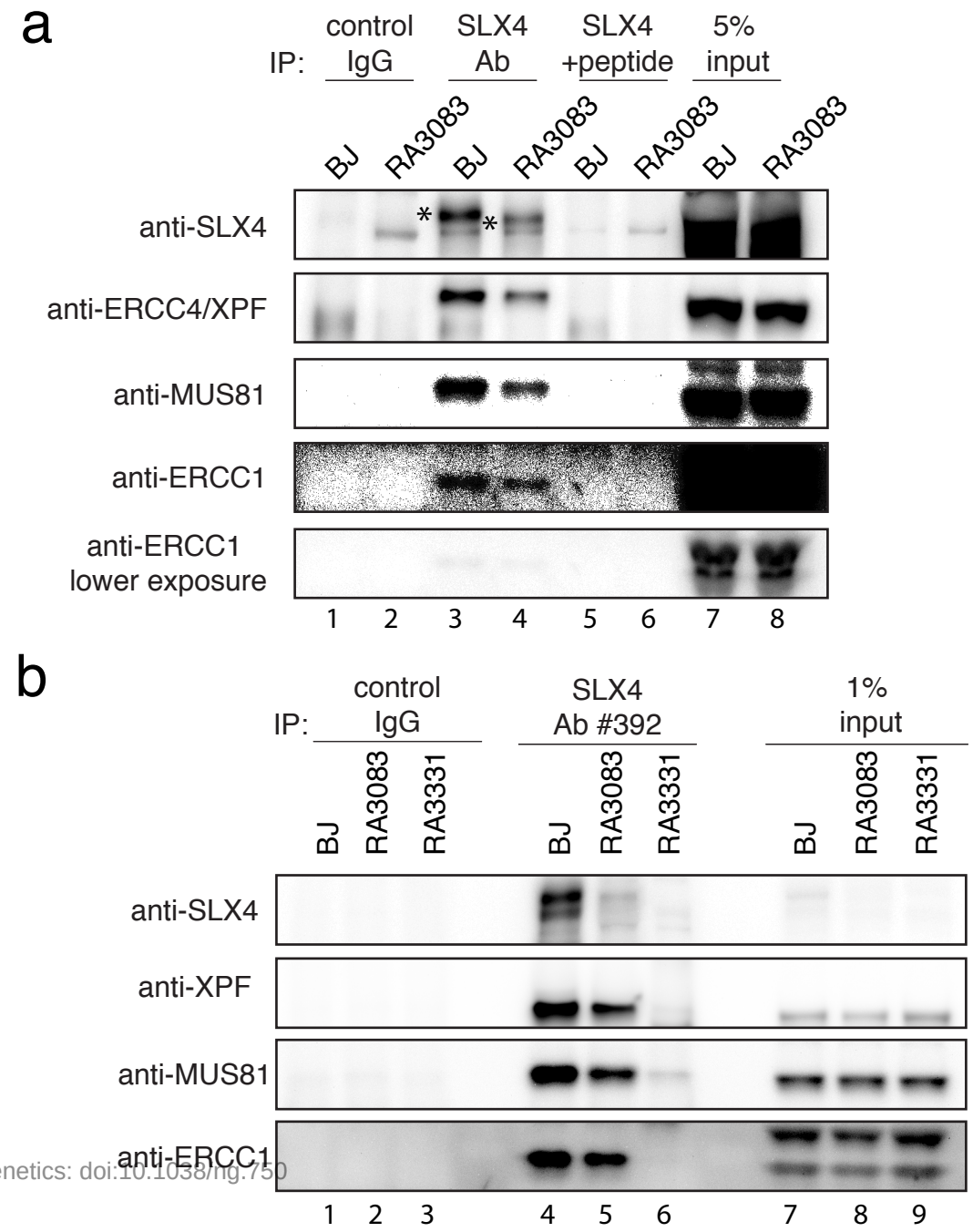
Figure S5. Analysis of SLX4 interacting partners in SLX4 mutant cells. **A.** Cell extracts of primary BJ and RA3083 cells were subjected to immunoprecipitation using the SLX4 antibody. Interacting proteins were identified by immunoblotting with the indicated antibodies. Asterisk indicates SLX4 proteins. **B.** BJ E6E7 and RA3331 E6E7 cells were subjected to immunoprecipitation using antibody #392, which recognizes the N-terminus of SLX4. Interacting proteins were identified by immunoblotting with the indicated antibodies.



Supplementary Table 1. Cloning and sequencing primers

Exon/ Intron (i)	Primer Name	Forward Primer	Primer Name	Reverse Primer	PCR Product (bp)
2	FL043 BTBD12-SLX4 ex2-1F	CCTCCCTGGAAGAACTGGCATAAA	FL044 BTBD12-SLX4 ex2-1R	TCTTCAGAGGAGCGAGGGTCAATC	768
2	FL045 BTBD12-SLX4 ex2-2F	AGGCTTCTACTTGGGTTCACTTTC	FL046 BTBD12-SLX4 ex2-2R	GCTGGCTGCCTGTTTTTGTAA	711
3	FL019 BTBD12-SLX4 ex3F	ACCACCACCACTACCTAACG	FL020 BTBD12-SLX4 ex3R	GACTCTTCATTCTCTGGCTGGAT	675
4	FL005 BTBD12-SLX4 ex4F	GTGGAGCTGGGTTCTGGTTTCTTT	FL006 BTBD12-SLX4 ex4R	TCACGGCAGCTCTATCCACAATCC	934
5	FL007 BTBD12-SLX4 ex5F	GGCGGACATTTGGAGCATTTCTAC	FL008 BTBD12-SLX4 ex5R	TGGCTCACTGCAACCTCTACCTTC	889
i5	FL009 BTBD12-SLX5 ivs5-1F	TGGACGCAGTAGTTCACACCT	FL010 BTBD12-SLX5 ivs5-1R	AGCAGTAGTCCCCAACCTTTTT	870
i5	FL011 BTBD12-SLX5 ivs5-2F	AAACCATTCCCCTACTCCTGTC	FL012 BTBD12-SLX5 ivs5-2R	CACCCCTGCCATGCTAAATAC	939
i5	FL013 BTBD12-SLX5 ivs5-3F	AAGGCGATAGCTGTTTGTCC	FL014 BTBD12-SLX5 ivs5-3R	GGGTCTGGGTTCCTTCACG	1105
6_7	FL015 BTBD12-SLX5 ex6_7F	TCGGAAGTATTGATGGGTGAAG	FL016 BTBD12-SLX5 ex6_7R	GTAGTTGCTGGACAGAGAAGAAAG	1171
i7	FL081 BTBD12-SLX4 ivs7F	CTCAGGCACATGGATAGGCTTTTG	FL082 BTBD12-SLX4 ivs7R	CCACGTGGTCACTGTCATTG	946
8_9	FL021 BTBD12-SLX4 ex8_9F	TTGGCCGTGACAGAGGAGGAGCAT	FL022 BTBD12-SLX4 ex8_9R	CCAGAGGCCAGCGGTGAGC	1099
i9	FL053 BTBD12-SLX4 ivs9-1F	CCGGCGGCCTTCCTCTGACTG	FL054 BTBD12-SLX4 ivs9-1R	GCTGCGGGATACCTGGGCTTTGA	763
i9	FL055 BTBD12-SLX4 ivs9-2F	AGCGCGGTTGCCCCATCCA	FL056 BTBD12-SLX4 ivs9-2R	GGCGCCAAAGTCAGCAACCAG	722
10	FL023 BTBD12-SLX4 ex10F	CATCGTGGGAAGCGTTTTGGAGAG	FL024 BTBD12-SLX4 ex10R	GTGGGGCAGGAAGTGAGGGAGAGT	428
i10	FL057 BTBD12-SLX4 ivs10-1F	CCAGTTTCAGACGGACAGC	FL058 BTBD12-SLX4 ivs10-1R	CCATGCCCCAGGAAAAG	998
i10	FL059 BTBD12-SLX4 ivs10-2F	GTGTGGGCAGGAACTCAG	FL060 BTBD12-SLX4 ivs10-2R	TCACCCAGCAGGACACG	939
11	FL025 BTBD12-SLX4 ex11F	GTAAGCCATGATCACACCACT	FL026 BTBD12-SLX4 ex11R	TGCAGCACAACCCTCCAG	413
i11	FL061 BTBD12-SLX4 ivs11-1F	CACTATCTCTACACTGCGGACACT	FL062 BTBD12-SLX4 ivs11-1R	AAAAGACATGGCATCAGACACAAT	759
i11	FL063 BTBD12-SLX4 ivs11-2F	AGGCAGCAGCTCAGAGGGACAAGT	FL064 BTBD12-SLX4 ivs11-2R	ACGCCAAACCTGCAACACGAAACA	944
12	FL047 BTBD12-SLX4 ex12-1F	TGGGTGTTTCTGGCAAGGAGTGTG	FL048 BTBD12-SLX4 ex12-1R	CCAGGGAGCCAGGCGATGAGAAAC	915
12	FL049 BTBD12-SLX4 ex12-2F	AGATGATGCCGGGGATTACGAACA	FL050 BTBD12-SLX4 ex12-2R	CCCGCTCCTGAGGCTGCTGATT	913
12	FL051 BTBD12-SLX4 ex12-3F	GGGCTCCCTGGCTGTTCTGT	FL052 BTBD12-SLX4 ex12-3R	GCCCGGCTCTCCTCCACTG	1111
i12	FL065 BTBD12-SLX4 ivs12-1F	GCCGGGCTGGTTCTCAT	FL066 BTBD12-SLX4 ivs12-1R	GACTTTGGTTCTTCTGCCTTTTAT	1344
i12	FL067 BTBD12-SLX4 ivs12-2F	TCGGGAGGGTGACTAAGAAT	FL068 BTBD12-SLX4 ivs12-2R	CTGGGAGGTCGAGGCTACAAT	1261
i12	FL083 BTBD12-SLX4 ivs12-3F	CAAGCTGGTCTCGAACTCCTAAC	FL084 BTBD12-SLX4 ivs12-3R	GTGAAACCCCGTCTCTATT	1217
i12	FL071 BTBD12-SLX4 ivs12-4F	GGCCACCACACCCTGCTAACTTTT	FL072 BTBD12-SLX4 ivs12-4R	TCTTCAGCACCGGCGTCTCCATAA	1068
13	FL031 BTBD12-SLX4 ex13F	CCAGACAGGGAAGGGGTGAC	FL032 BTBD12-SLX4 ex13R	TGGGGAATGGTACGCAGAGA	486
i13	FL073 BTBD12-SLX4 ivs13-1F	ACTGGATAGGTTGGCGGTCTTC	FL074 BTBD12-SLX4 ivs13-1R	CCAGCTACTAGGGAGGATGAGGT	893
i13	FL075 BTBD12-SLX4 ivs13-2F	TGGGAGCAGCATATCTTTTGTT	FL076 BTBD12-SLX4 ivs13-2R	AGCGGCTGTGAGGACTGG	645
14_15	FL033 BTBD12-SLX4 ex14_15F	TAAGATAGGGAACGTGGAGTGTGG	FL034 BTBD12-SLX4 ex14_15R	ATTGGGCTGTGGTCATCATCA	1399
i5	FL086 BTBD12-SLX4 i5-3R seq 2076_2096	TTGGGCTGTGGGGAAAGAATA			
i9	FL087 BTBD12-SLX4 i9-1R seq 509_527	ATGCAAATGGGAGGAGGTC			
i10	FL085 BTBD12-SLX4 i10-2R seq 1275_1295	CCTCCTCGGTTCCAGCGTATT			
2	621 slx4 413F	CCCCCGCCTCGTCCTCCAAAAG			
2	641 slx4_1051R	TGGAGCAGAGGCAAGCACACC			
5	640 slx4_1698R	GGGGGCCAACCTCCATCTTCACA			
7	627 slx4_2055F	TGAAACCACAGGCCGACAGATAGA			
8	639 slx4 _2386R	CAGTGGGGGTGCCGTGGAG			
10	628 slx4 2647F	CTCGGGCTGCTGGTTGCTGACTTT			
12	629 slx4 3310F	CAGGTGCAGAAACAGTGGGACAA			
12	623 slx4 3910F	CCAGGGCACCAGAAAGGCAAAGA			
12	630 slx4 4516F	GGAAACAGGGAAGGGAACGAAGTC			
15	634 slx4 5791R	CTCAAATGCCGCTCCAAACTCACA			
15	633 slx4 6354R	GGAAGGGGCTGGAGTCTGTAAATC			
15	624 slx4 6804R	GTGGGCTCAGAGGGTGGCAAATGT			

Supplementary Table 2. Mutagenesis primers

SLX4_C296A_C299A	GGAAAAGGGTTTGTTCTTCGCCCAGATTGCTCAAAAG
SLX4_C336A_C339A	CCTCAGATCCCTGAGGCCCCGATTGCTGGGAAACCGTT

Supplementary Table 3. siRNA sequences

SLX4_1	TTTGGATGAAGATTTCTGAGATCTG,
SLX4_2	TTCCGTGGCTCCTTCTTGCTGGTGG
SLX4_3	AAGAGTTCCTGGAAATTCTCGGCCC
FANCI (used as a combination of three at total concentration of 50 nM)	
	TCTCCTCAGTTTGTGCAGATGTTAT,
	GGCAGCTGTGTGGACACCTTGTTAA
	GCTGGTGAAGCTGTCTGGTTCTCAT
ATR (used as a combination of three at total concentration of 50 nM)	
	GGGAAATAGTAGAACCTCATCTAAA
	GGTCTGGAGTAAAGAAGCCAATTTA
	CCACCTGAGGGTAAGAACATGTTAA