The role of the Fanconi anemia pathway in sporadic head and neck cancer
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SLX4, a coordinator of structure-specific endonucleases, is mutated in a new Fanconi anemia subtype.
DNA interstrand crosslink repair requires several classes of proteins, including structure-specific endonucleases and Fanconi anemia proteins. SLX4, which coordinates three separate endonucleases, was recently recognized as an important regulator of DNA repair. Here we report the first human individuals found to have biallelic mutations in \textit{SLX4}. These individuals, who were previously diagnosed as having Fanconi anemia, add SLX4 as an essential component to the FA-BRCA genome maintenance pathway.

Fanconi anemia is a rare, heterogeneous chromosomal instability syndrome characterized by bone marrow failure, congenital abnormalities, hypersensitivity to DNA crosslinking agents and an increased susceptibility to cancer. Studies to unravel the genetic basis of Fanconi anemia have led to the identification of a previously unidentified genome maintenance pathway which evolved relatively late during evolution and exists — in its fully developed form — only in vertebrates. Fourteen Fanconi anemia genes have been identified\textsuperscript{1,2}, but a small percentage of individuals diagnosed with Fanconi anemia have remained unclassified, as no pathogenic mutations could be detected in the currently known Fanconi anemia genes.

One of these individuals (EUFA1354), a Dutch male with growth retardation, microcephaly, small eyes, hypopigmentation, thumb abnormalities and hearing loss, was diagnosed with pancytopenia at the age of 9 and Fanconi anemia was suspected (Table 1). We confirmed the Fanconi anemia diagnosis by a chromosomal breakage assay on T lymphocyte cultures, which showed increased spontaneous and excessive mitomycin C (MMC)-induced chromosomal aberrations that were well within the range established for Fanconi anemia (Supplementary Fig. 1). An EBV-immortalized lymphoblastoid cell line from this individual was also hypersensitive to MMC in terms of chromosomal breakage (Fig. 1A) and growth inhibition (Fig. 1B and Supplementary Fig. 2A). Notably, these lymphoblasts were also hypersensitive to the topoisomerase I inhibitor camptothecin (Fig. 1C and Supplementary Fig. 2B), a feature that until now was considered specific for the Fanconi anemia subgroups D1, M, N and O and which is possibly associated with defects in homologous recombination repair\textsuperscript{2,3}. In further support of the Fanconi anemia diagnosis, we observed an elevated MMC-induced accumulation in the G2/M phase of the cell cycle, both in primary and in SV40-immortalized fibroblasts from this individual (Supplementary Fig. 2C,D). Somewhat surprisingly, fibroblasts were not very sensitive to the crosslinking drug when using growth inhibition or chromosomal breakage as a readout (Supplementary Fig. 2E–G).

Sequence analysis, MMC-induced FANCD2 monoubiquitination and normal formation of nuclear FANCD2 foci (Supplementary Fig. 3A,B) excluded a defect in the upstream part of the FA-BRCA pathway in this individual\textsuperscript{4}. The induction
Table 1 Clinical phenotypes of individuals with SLX4 mutations

<table>
<thead>
<tr>
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<th>EUFA1354</th>
<th>457-1</th>
<th>457-2</th>
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<tr>
<td><strong>Age at diagnosis</strong></td>
<td>9</td>
<td>9</td>
<td>7 (via 457-1)</td>
<td>7 (via 457-1)</td>
</tr>
<tr>
<td><strong>Growth retardation</strong></td>
<td>Short stature (-2.5 s.d. at age 9; -4.5 s.d. at age 18)</td>
<td>Prenatal</td>
<td>Prenatal</td>
<td>Pre- and post-natal</td>
</tr>
<tr>
<td><strong>Thumb abnormalities</strong></td>
<td>Hypoplastic right thumb</td>
<td>No</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td><strong>Facial features</strong></td>
<td>Almond-shaped and short palpebral fissures, bulbous nasal tip, micrognathia, microcephaly (-2.5 s.d.)</td>
<td>No</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td><strong>Skin abnormalities</strong></td>
<td>Hypopigmented spot on back</td>
<td>No</td>
<td>No</td>
<td>Café-au-lait spots</td>
</tr>
<tr>
<td><strong>Ear abnormalities</strong></td>
<td>Bilateral hearing loss, hypoplastic malleus, narrow external ear canals</td>
<td>No</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td><strong>Kidney abnormalities</strong></td>
<td>No</td>
<td>Horseshoe kidney</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td><strong>Hematology</strong>&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Pancytopenia, Hb 2.3 mM/l, leucocytes 3.4 x 10&lt;sup&gt;12&lt;/sup&gt;/l, neutrophils 0.7 x 10&lt;sup&gt;9&lt;/sup&gt;/l, thrombocytes 18 x 10&lt;sup&gt;9&lt;/sup&gt;/l. Individual transplanted at age ten with bone marrow from the mother and is currently (age 18) doing well.</td>
<td>Beginning bone marrow failure, erythrocytes 3.7 x 10&lt;sup&gt;12&lt;/sup&gt;/l, leucocytes 3.7 x 10&lt;sup&gt;9&lt;/sup&gt;/l, neutrophils 0.93 x 10&lt;sup&gt;9&lt;/sup&gt;/l, thrombocytes 107 x 10&lt;sup&gt;9&lt;/sup&gt;/l. Current age is 8.5.</td>
<td>Pancytopenia, Hb 5.7 mM/l, erythrocytes 2.8 x 10&lt;sup&gt;12&lt;/sup&gt;/l, leucocytes 2.5 x 10&lt;sup&gt;9&lt;/sup&gt;/l, neutrophils 0.27 x 10&lt;sup&gt;9&lt;/sup&gt;/l, thrombocytes 20 x 10&lt;sup&gt;9&lt;/sup&gt;/l. Individual received HSCT with BM from unrelated donor at the current age of 8.5.</td>
<td></td>
</tr>
</tbody>
</table>

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<sup>a</sup>Age at diagnosis in years. <sup>b</sup>Normal values: Hb 7.4-9.0 mM/l, erythrocytes (male) 4.7-6.1 x 10<sup>12</sup>/l, leucocytes 4.5-13.5 x 10<sup>9</sup>/l, neutrophils 1.8-8.0 x 10<sup>9</sup>/l, thrombocytes 150-450 x 10<sup>9</sup>/l.
Chapter 2

A
untreated
200 nM MMC

HSC93 (WT)
EUFA045-L (WT)
EUFA1354-L
HSC72 (FA-A)

break events per cell

B
Growth (%)
MMC (nM)

HSC93 (WT)
EUFA867-L (FA-M)
EUFA1354-L
457/1
457/3

C
Growth (%)
CPT (nM)

HSC93 (WT)
EUFA867-L (FA-M)
EUFA1354-L
457/1
457/3

D
HSC93 (WT) EUFA1354-L
457/1 457/3

SE
LE
SLX4
SLX4
XPF
MUS81
ERCC1

input
IP: SLX4 N-term

E
HSC93 (WT) EUFA1354-L
457/1 457/3

LE
SLX4
SLX4 trunc
MUS81
ERCC1

input
IP: SLX4 N-term

F
HSC93 EUFA1354-L

CE NE CB
CE NE CB

p300
HDAC1
Tubulin
Histone H3
XPF
ERCC1
MUS81
SLX1

LE
SLX4
SLX4 trunc
MUS81
ERCC1

input
IP: SLX4 N-term

LN9SV EUFA1354-F

SV40

LN9SV EUFA1354-F

SV40

p300
HDAC1
Tubulin
Histone H3
XPF
ERCC1
MUS81
SLX1
SLX4 is mutated in a new Fanconi anemia subtype

SLX4 is mutated in a new Fanconi anemia subtype of RAD51 and H2AX foci in this individual’s fibroblasts suggested a normal DNA damage response (Supplementary Fig. 3C–F). We ruled out an abnormality in the downstream Fanconi anemia proteins BRCA2, PALB2 and FANCJ by cell fusion experiments and sequence analysis of the corresponding genes (data not shown), indicating that this individual represented a new Fanconi anemia subtype with a defect in a new player of the FA-BRCA pathway.

Recently, four research groups identified the human SLX4 scaffold protein, which was proposed to function in the processing of DNA repair intermediates and crosslink repair through interaction with the structure-specific endonucleases SLX1, XPF-ERCC1 and MUS81-EME1 (refs. 5–8). SLX4-depleted cells are hypersensitive to crosslinking agents and camptothecin, similar to lymphoblasts from the affected individual EUFA1354. Therefore, we hypothesized that SLX4 might be defective in this individual. Sequence analysis of genomic DNA and complementary DNA (cDNA) from EUFA1354 indeed revealed biallelic mutations in the reading frame of SLX4 (Supplementary Fig. 4). We detected a homozygous, 1-bp deletion (c.286delA) in the first exon of SLX4 that results in a frameshift at codon 96 and a premature stop at codon 126 (p.Thr96LeufsX30). The consanguineous parents and healthy sister of the affected individual were all heterozygous for this sequence variant.

We obtained additional evidence for SLX4 deficiency in individuals with Fanconi anemia by the identification of a second Fanconi anemia family with pathogenic SLX4 mutations. Linkage analysis with a genome-wide SNP array showed a common 13.5 Mbp region around the SLX4 locus in three unclassified German siblings with mild manifestations of Fanconi anemia and bone marrow failure (Table 1 and Supplementary Fig. 5). The siblings all inherited a 1-bp deletion in SLX4 (c.1093delC, p.Gln365SerfsX31) from their father and a splice
Chapter 2

A) Bebu (WT) vs EUFA1354-F

B) LN9SV (WT) vs EUFA1354-F SV40

C) EUFA1354-F SV40 + pcDNA3 vs EUFA1354-F SV40 + FLAG-SLX4

D) EUFA1354-F SV40 + GFP vs EUFA1354-F SV40 + GFP-SLX4

E) Western blot analysis showing FLAG-Slx4△Sxl1 compared to HSC93 (WT)

F) Growth (% vs MMC (nM))

G) Break events per cell (%) for untreated, 200 nM MMC, EUFA1354-L, and EUFA1354-L + FLAG-Slx4△Sxl1
SLX4 is mutated in a new Fanconi anemia subtype

Site mutation (c.1163+3dupT) from their mother ([Supplementary Fig. 6A](#)). The maternal mutation lead to exon 5 skipping, an in-frame deletion that disrupts the UBZ4 domain in SLX4, which may be involved in targeting SLX4 to sites of DNA damage by binding to ubiquitinated proteins ([Supplementary Fig. 6B,C](#)). We detected the residual mutant SLX4 protein in cell lysates, and this protein was able to interact with XPF, ERCC1 and MUS81 ([Fig. 1D](#)). Notably, lymphoblasts from these individuals were sensitive to MMC ([Fig. 1B](#)) but not to camptothecin ([Fig. 1C](#)), indicating that the mutant protein is partially functional.

We also examined SLX4 protein expression in lymphoblast lysates from EUFA1354. Full-length SLX4 was undetectable by immunoprecipitation with antibodies against the N or C terminus of SLX4, but we detected very low levels of a truncated SLX4 protein with antibodies against the C terminus of SLX4 ([Fig. 1D and [Supplementary Fig. 7A](#)]). Consequently, the amounts of XPF-ERCC1 and MUS81 in SLX4 immunoprecipitates were reduced compared to wildtype cells ([Fig. 1D and [Supplementary Fig. 7A](#)]). The truncated SLX4 protein was more pronounced in SV40-immortalized fibroblasts from EUFA1354 ([Fig. 1E and [Supplementary Fig. 7B](#)]) and may be derived from an alternative translation initiation site present at codon 213. Reciprocal immunoprecipitations with antibodies against ERCC1, XPF or MUS81 readily co-precipitated full-length SLX4 from wildtype cells, but only some truncated SLX4 protein was co-precipitated from EUFA1354 cells ([Supplementary Fig. 7C,D](#)). When transiently expressed in human HEK293 cells, a truncated SLX4 protein starting from Met213 is able to interact with XPF-ERCC1, MUS81-EME1 and SLX1, similar to full-length SLX4 ([Supplementary Fig. 7E](#)). These data indicate that the defects seen in the EUFA1354 cells are due to very low concentrations of the truncated SLX4 protein and not because truncated SLX4 is defective in interacting with a specific nuclease.

Gel filtration experiments showed that XPF-ERCC1 and SLX1, which normally exist in two subcomplexes⁸, eluted only in the low molecular weight fractions from EUFA1354 fibroblast lysates, whereas the elution of MUS81 was not affected.
Subcellular fractionation studies revealed a reduced chromatin association of XPF-ERCC1 in EUFA1354, whereas MUS81 and SLX1 were hardly affected (Fig. 1F). These data strongly suggest that the SLX4 defect in EUFA1354 interferes with the function of the XPF-ERCC1 endonuclease, an important player in crosslink repair.

According to a recent study, SLX4 accumulates in nuclear foci, where it co-localizes with XPF. We investigated the nuclear localization of ERCC1 in primary and immortalized EUFA1354 fibroblasts. We detected nuclear ERCC1 foci in wildtype cells; however, EUFA1354 fibroblasts lacked these structures (Fig. 2A,B). We confirmed this striking result with another ERCC1 antibody (Supplementary Fig. 9). EUFA1354 fibroblasts, transiently transfected with SLX4 cDNA, regained their capacity to form ERCC1 foci, which co-localize with both FLAG-SLX4 and GFP-SLX4 (Fig. 2C,D). This confirms that the SLX4 defect in EUFA1354 affects the XPF-ERCC1 endonuclease.

To further strengthen the evidence that SLX4 deficiency causes the cellular phenotype in EUFA1354, we tried to correct the MMC hypersensitivity through SLX4 cDNA transfection in EUFA1354 lymphoblasts. Although we were unable to stably express full-length human SLX4, a truncated mouse Slx4 protein that corrects Slx4-deficient mouse embryonic fibroblasts partially restored MMC resistance in terms of cell growth (Fig. 2E,F). Chromosomal breakage analysis on individual metaphases showed that only part of the lymphoblasts (57%) had been fully complemented (Fig. 2G).

In conclusion, defective SLX4 is associated with a previously unknown subtype of Fanconi anemia, Fanconi anemia-P. The presence of a truncated SLX4-FANCP protein in the individuals reported here indicates that the mutations may be hypomorphic, as has been found for individuals with an alteration in ERCC1 or XPF. Because germline mutations in all the other downstream players of the FA-BRCA pathway predispose to breast and/or ovarian cancer, SLX4 could also be a cancer predisposition gene.

Methods

Ethics statement
The research was carried out after approval by the Institutional Review Board of the Vrije Universiteit Medical Center that adhered to local ethical standards and was initiated only after the relevant informed consents had been obtained. Information and consent of the German family used in this study was in agreement with national legal regulations and the Declaration of Helsinki.

Cell culture and transfection
HEK293 cells were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM, Gibco) with 10% FBS (Hyclone), 100 international units ml⁻¹ penicillin and 0.1 mg ml⁻¹ streptomycin and 1% L-glutamine (Invitrogen). Cells were transiently transfected by calcium phosphate precipitation and incubated for 24–30 h at 37 °C before lysis.

Skin fibroblasts, either primary or immortalized with SV40 large T antigen, were cultured in DMEM or Ham’s F-10 with 10% FBS. Fibroblasts were transiently transfected with Amaxa Nucleofector using a
SLX4 is mutated in a new Fanconi anemia subtype

human dermal fibroblasts nucleofector kit.

EBV-transformed lymphoblasts were cultured in RPMI1640 medium containing 10% FBS. Stably expressing lymphoblasts were generated by electroporation of pMEP4 constructs (Invitrogen) and selection on 100 μg/ml hygromycin.

cDNA constructs

The cDNA encoding full-length human SLX4 was generated by PCR on IMAGE clones 6527830 and 4340346 and was cloned into pcDNA5 FRT/TO-FLAG and pcDNA5 FRT/TO-GFP (Invitrogen, Flip-In T-Rex system). For GFP-SLX4 213-end, the pcDNA5 FRT/TO-GFP with full-length SLX4 was used to PCR amplify a fragment encoding SLX4 residues 213–1,834, which was shuttled into pcDNA5 FRT/TO-GFP. Constructs were verified by sequencing. A cDNA construct encoding mouse SLX4 that lacks the SLX1 binding site (amino acids 1,417–1,565 in mouse Slx4 corresponding to amino acids 1,685–1,834 in human SLX4) was a gift of K.J. Patel.

Chromosomal breakage analysis.

Heparinized blood (2 ml) was diluted with 18 ml blood culture medium (Ham’s F-10 + 15% FBS + phytohemagglutinin). Subsequently, 5 ml of this suspension was cultured in the presence of 0, 15 or 300 nM MMC. After 72 h, 100 μl demecolcin (10 μg/ml in Hank’s Balanced Salt Solution (HBSS)) was added, and cells were incubated for 20 min at 37 °C. Cells were centrifuged, resuspended in 0.075 M KCl and incubated for 20 min at 18–22 °C. The cells were spun down, resuspended in 10 ml fixative (75% methanol and 25% acetic acid), incubated for 30 min at room temperature and centrifuged. The pellet was resuspended in 10 ml fixative and incubated for 5 min at room temperature. This step was repeated and finally the pellet was resuspended in 0.5–1.0 ml fixative. The cell suspension was dropped on a slide and allowed to dry. Slides were incubated for 15 min in 0.1 M HNO₃ and rinsed in tap water and 70% ethanol, respectively. Next, slides were stained for 5 min in a 4% Giemsa solution, rinsed in tap water, allowed to dry and coded. From each coded culture, at least 50 metaphases were examined for chromosomal damage. The presence of chromatid breaks and interchanges was expressed as break events per cell, counting chromatid interchange figures as the minimum number of break events required for their reconstruction. After scoring, the slides were decoded and the results were analyzed.

Lymphoblasts were seeded in 25 cm² tissue culture flasks at a density of 2–3 × 10⁶ per 10 ml fresh Roswell Park Memorial Institute (RPMI) medium containing 10% FBS and cultured at 37 °C for 48 h in the presence of 0 nM or 200 nM MMC. Next, 200 μl of demecolcin (10 μg/ml in HBSS) was added to each culture flask, and the cells were incubated for an additional 20 min at 37 °C. Metaphase spreads were prepared and analyzed as described above.

Fibroblasts (1 × 10⁶ cells in 15 ml Ham’s F-10 + 10% FBS) were seeded in an 80 cm² tissue culture flask and cultured at 37 °C in the presence or absence of 50 nM mitomycin C. After 48 h, 300 μl of demecolcin (10 μg/ml in HBSS) was added to each culture flask, and cells were incubated for an additional 30 min at 37 °C. Cells were then trypsinized, and metaphase spreads were prepared and analyzed as described above.

Cell cycle analysis

Fibroblasts were cultured for 72 h either without or with MMC (50 nM or 100 nM) in Ham’s F-10 medium supplemented with 10% FBS. Cells were harvested by trypsinization and permeabilized in buffer containing 100 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.5 mM MgCl₂, 1 mM CaCl₂, 0.2% BSA and 0.1% NP-40 followed by staining of DNA with PI/RNase staining buffer (BD Pharmingen, BD Biosciences). Cell suspensions were analyzed by flow cytometry on a BD FACSCalibur (BD Biosciences) to determine G2/M accumulation as the percentage of cells present in the G2/M phase of the cell cycle.

Lymphoblasts were stained with 4′,6-diamidino-2-phenylindole (DAPI) at a final concentration of 1 μg/ml. Flow histograms were made on an analytical, triple-laser-equipped flow cytometer (LSRII, BD Biosciences). Results were quantified with MPLUS AV software (Phoenix Flow Systems).

Growth inhibition assays

The MMC- and camptothecin-induced growth inhibition assays on lymphoblasts and fibroblasts were performed as previously described.

Cell fractionation

Cytoplasmic, nuclear and chromatin fractions were isolated using a Subcellular Protein Fractionation Kit (Thermo Scientific) according to the manufacturer’s instructions. Controls used for the fractionation
were tubulin (cytoplasmic fraction), p300 (nuclear fraction), histone H3 (chromatin fraction) and HDAC1 (nuclear and chromatin fraction).

**Cell lysis, immunoprecipitation and protein blotting**

Cells were lysed on ice with ice-cold lysis buffer containing 50 mM Tris-HCl (pH 7.4), 1% Triton X-100, 0.1% (v/v) 2-mercaptoethanol, 0.27 M sucrose and protease inhibitors for immunoprecipitation. For pull-downs with GFP-Trap beads (ChromoTek), cells were lysed on ice with ice-cold lysis buffer consisting of 10 mM Tris-HCl, (pH 5.5), 150 mM NaCl, 0.5 mM EDTA, 0.5% NP40 and protease inhibitors. Lysates were precleared with 50 μl protein G-Sepharose (50% slurry) for 30 min. Immunoprecipitations and pull-downs were performed for 2 h at 4 °C.

The primary antibodies used for immunoprecipitation and protein blotting were as follows: anti-XPF (Thermo MS-1381), anti-ERCC1 (Thermo MS-671 or Santa Cruz FL297), anti-MUS81 (ImmuQuest IQ285 or Abcam ab14387), anti-EME1 (ImmuQuest IQ284), anti-GFP (Roche 11814460001), anti-GAPDH (Abcam ab8245), anti-p300 (Santa Cruz SC-585), anti-HDAC1 (Santa Cruz SC7872), anti-tubulin (Abcam ab7291), anti-histone H3 (Cell Signaling Technology 9715), anti-FANCD2 (Santa Cruz sc20022) and anti-RAD50 (GeneTex 13B3). Sheep polyclonal antibodies were raised against the first 300 amino acids (SLX4-N) or the last 300 amino acids (SLX4-C) of SLX4 fused to GST. Antibodies were also raised against full-length SLX1-GST expressed in bacteria. SLX4 and SLX1 antibodies were affinity purified before use.

**Immunofluorescence**

Fibroblasts (100,000 cells per well) were seeded in four-well chamber slides (Nunc). After overnight culture, cells were washed with PBS and pre-permeabilized with 0.25% Triton X-100 in PBS (1 min on ice). Cells were fixed with 4% paraformaldehyde (15 min at room temperature) and permeabilized with 0.5% Triton X-100 in PBS (10–20 min at room temperature). Unspecific binding sites were blocked with PBS + 10% BSA for γH2AX staining for 1 h at room temperature. Slides were incubated with specific antibodies in blocking buffer (2 h at room temperature). Excess antibody was removed by four washing steps with PBS + 0.2% Triton X-100. Slides were then incubated with secondary antibody labeled with Alexa 594 or Alexa 488 (Invitrogen, 1:1,000) and DAPI (1:1,000) for 1 h at room temperature. Finally, slides were washed four times with PBS + 0.2% Triton X-100 and embedded. Slides were analyzed with a fluorescent microscope (DM5000, Leica). ERCC1 foci were also analyzed 72 h after cDNA transfection.

Lymphoblasts were attached to glass slides by cytocentrifugation. The percentage of foci-positive cells (>10 foci per nucleus) was determined on a Zeiss Axio Imager A1 fluorescence microscope. For each experiment, 200–400 nuclei were analyzed.

The following antibodies were used: anti-FANCD2 (Novus NB100-182, 1:200), anti-RAD51 (gift of R. Kanaar, 1:2,500, or Abcam ab63801), anti-phospho-H2AX (Ser139) (Millipore clone JBW301, 1:400) and anti-ERCC1 (Santa Cruz FL297, 1:200).

**Size exclusion chromatography**

For gel filtration experiments, cell extracts (3 mg of protein) were loaded on a Superose 6 column (GE Healthcare) in buffer containing 50 mM Tris/HCl (pH 7.4), 1 mM EDTA, 0.2 M sodium chloride and 0.1% (v/v) 2-mercaptoethanol. Molecular weight markers (Bio-Rad) as follows: thyroglobulin (670 kDa) and bovine gamma globulin (158 kDa). Fraction was denatured and subjected to protein blot analysis.

**Sequence analysis**

For direct sequencing, PCR products were generated by SLX4-specific primers (Supplementary Table 1) PCR fragments were treated with shrimp alkaline phosphatase (30 min at 37 °C) and Exonuclease I (15 min at 80 °C). Sequencing reactions were carried out using 10 pM of primer and the Big Dye Terminator cycle sequencing kit (Applied Biosystems). Samples were analyzed in an ABI 3730 DNA analyzer (Applied Biosystems).

**Genome wide SNP array**

Genome wide SNP genotypes for the German family members were determined on a HumanHap300v2 Genotyping BeadChip (Illumina Inc.). After scanning the microarrays on a BeadStation 500G with the software BeadScan Ver. 3.7.5.23284, SNP genotypes were called using the software BeadStudio Ver. 3.1.3.0 with the Genotyping Module Ver. 3.2.32 (all from Illumina Inc.).
Acknowledgements
We thank the affected individuals and their families for contributing to this study. We also thank A. Raams, R. Friedl, B. Gottwald and S. Darchinger for expert technical assistance. We acknowledge I. Carr for Phaser software, R. Kanaar for RAD51 antiserum and K.J. Patel for mouse Slx4 cDNA. Financial support was from the Cancer Center Amsterdam-VU Medisch Centrum Institute for Cancer and Immunology (CCA/V-ICI) Amsterdam (to C.S.), the Dutch Cancer Society (to H.J.), Schroeder-Kurth-Fund (to D.S.) and the Medical Research Council UK (to J.R.).

Author contributions
The study was designed by J.P.d.W., J.R., D.S. and H.J. Clinical information of affected individuals and referral for Fanconi anemia diagnosis was coordinated by E.T.K. and Y.H.-H. Fanconi anemia diagnosis was confirmed by A.W.M.N. SNP array studies were coordinated by T.B. Mutational analysis and functional studies were carried out by C.S., K.H., M.A.R., J.S., A.B.O. and K.E. The ERCC1 focus formation assay was coordinated by N.G.J.J. The manuscript was written by C.S., J.P.d.W., J.R. and D.S., with help from the other authors.

Competing financial interests
The authors declare no competing financial interests.
**Supplementary data**

**Supplementary Figure 1.** Spontaneous and MMC-induced chromosomal breakage in PHA-stimulated lymphocyte cultures from a healthy control and the affected individual (EUFA1354).

Percentages of cells with up to ≥ 10 break events per cell are shown. In EUFA1354 lymphocytes treated with 15 nM MMC, all metaphases were aberrant and exhibited 1 to ≥ 10 break events per cell; at 300 nM MMC 100% of the metaphases had ≥ 10 break events per cell (results not shown).

**Right: Supplementary Figure 2.** DNA cross-linker and camptothecin sensitivity in EUFA1354 lymphoblasts and fibroblasts.

(A) Growth inhibition of EUFA1354 lymphoblasts upon exposure to mitomycin C (MMC) or (B) camptothecin (CPT). Lymphoblasts from a healthy individual (HSC93) and a FANCM-deficient individual (EUFA867-L) were analyzed as controls. Data represent means and standard errors of the mean from at least three independent experiments. (C) Cell cycle analysis of primary EUFA1354 fibroblasts reveals G2/M arrest after treatment with mitomycin C (MMC). Wild type fibroblasts (Bebu) and FANCJ-deficient fibroblasts (EUFA1333-F) were used as controls. (D) MMC-induced G2/M arrest in primary and SV40-immortalized EUFA1354 fibroblasts. Percentages of cells in G2/M arrest are shown. The results are the mean of two independent experiments, with standard errors of the mean. Wild type fibroblasts (Bebu and LN9SV) and FA deficient fibroblasts (EUFA1333-F (FA-J) and GM6914 (FA-A)) were used as controls. (E) MMC-induced growth inhibition in primary fibroblasts. Data represent means and standard errors of the mean for three experiments. (F) MMC-induced growth inhibition in SV40-immortalized fibroblasts. A representative result of 3 independent experiments is shown. (G) Spontaneous and MMC-induced chromosomal breakage in SV40-immortalized fibroblasts. Percentages of cells with up to ≥ 10 break events per cell are shown. The number of EUFA1354 fibroblasts with zero MMC-induced break events per cell is significantly different from wild type cells (p=0.05, two-sample Chi2 test).
SLX4 is mutated in a new Fanconi anemia subtype

A

HSC93 (WT)
EUFA867-L (FA-M)
EUFA1354-L

B

C

D

Primary fibroblasts

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<th>50 nM MMC</th>
<th>100 nM MMC</th>
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<td>Bebu (WT)</td>
<td>7.66±1.44</td>
<td>17.01±4.69</td>
<td>24.49±7.45</td>
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<tr>
<td>EUFA1333-F (FA-J)</td>
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<td>EUFA1354-F</td>
<td>8.34±1.82</td>
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<td>45.16±6.25</td>
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SV40 immortalized fibroblasts

<table>
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<th>100 nM MMC</th>
</tr>
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<tbody>
<tr>
<td>LN9SV (WT)</td>
<td>25.20±11.00</td>
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E

Bebu (WT)
EUFA1333-F (FA-J)
EUFA1354-F

G

untreated
50 nM MMC

LN9SV (WT)
GM6914 (FA-A)
EUFA1354-F SV40

break events per cell
percentage of cells
Supplementary Figure 3. Normal FANCD2 monoubiquitination and FANCD2, RAD51 and γ-H2AX focus formation in EUFA1354.

(A) Immunoblotting revealed induction of FANCD2 monoubiquitination after treatment with mitomycin C (MMC) or hydroxyurea (HU) in EUFA1354-L lymphoblasts. (B) Immunofluorescence demonstrated normal induction of FANCD2 foci (red) in SV40-immortalized EUFA1354 fibroblasts. Wild type (LN9SV) and FANCA-deficient (GM6914) fibroblasts were included as a positive and negative control, respectively. DAPI (blue) was used as a nuclear counterstaining. (C) MMC-induced RAD51 foci (red) in SV40-immortalized EUFA1354 fibroblasts and LN9SV wild type fibroblasts. (D) Cisplatin-induced γ-H2AX foci (green) in SV40-immortalized EUFA1354 fibroblasts and LN9SV wild type fibroblasts. (E+F) Quantification of the results in C and D. Data represent means and standard errors of the mean for two experiments.
Supplementary Figure 4. SLX4 mutations in EUFA1354.

(A) Sequence analysis of genomic blood DNA of EUFA1354 revealed a homozygous 1-bp deletion in exon 1 of SLX4 (c.286delA), resulting in a frameshift at codon 96 and premature stop at codon 126 (p.T96LfsX30). The parents (second cousins) and a healthy sister are all heterozygous for the mutation.

(B) Sequence analysis on cDNA from EUFA1354 lymphoblasts and fibroblasts detected the homozygous 1-bp deletion in SLX4 (c.286delA).
Supplementary Figure 5. Characteristics of the German SLX4 deficient family.

(A) G2 phase arrest in the oldest affected German sibling (457/1). Lymphoblast cultures were left untreated (grey) or exposed to 45 nM mitomycin C (MMC) for 48 h (transparent overlay). As a manifestation of FA, cells show exaggerated G2 phase blockage (arrow) in response to MMC. Quantitative analysis of the cell cycle distributions reveals 60.1% G1, 30.2% S and 9.7% G2 phase without MMC and 42.2% G1, 18.3% S and 39.5% G2 phase with MMC. (B) Normal FANCD2 monoubiquitination in lymphoblasts from the German FA siblings (457/1-3) suggests a downstream defect in the FA/BRCA pathway. RAD50 served as loading control. (C) Normal formation of RAD51 foci in lymphoblasts of the German FA siblings (457/1-3) suggests properly functioning BRCA2, PALB2 and RAD51C. This was confirmed by Western blotting (BRCA2 and PALB2) or sequencing (RAD51C). FANCJ protein levels were also normal. (D) Graphic presentation of linkage analysis in the German family by the program Phaser. The disease genotype of chromosome 16 in this family is defined by the first affected sibling (457/1, violet central bar) between the corresponding parental alleles (left paternal blue bar, right maternal red bar). Regions of common linkage (violet) are shown for the second affected sibling (457/2) compared to the first, and for the third affected sibling (457/3) compared to the first. A region common to all three of them extends from 0 to 13.5 K (box). SLX4 lies therein at 3.5-3.6 K (arrow). Regions where only one parental mutation allele has been inherited are shown in that colour. Gaps between the bars characterize regions where the non-mutated alleles have been transmitted. Gray is the centromeric region. This analysis also reveals that the twin siblings are dizygotic.
SLX4 is mutated in a new Fanconi anemia subtype

Supplementary Figure 6. SLX4 mutations in the unclassified German family.

(A) Sequence analysis of genomic DNA revealed a 1-bp deletion (c.1093delC) and a splice site mutation (c.1163+3dupT) in the three affected siblings. The deletion was detected in genomic DNA from the father; the splice site mutation was present in genomic DNA from the mother. Only reverse sequences identify the duplication unequivocally in the affected siblings as forward sequences have superimposed sequence resulting from the paternal deletion. “Splicefinder” software (http://www.splicefinder.net/form.php) predicts a decrease of the splice donor score from 20.8 (wild type) to 11.9 (c.1163+3dupT).

(B) Sequence analysis on cDNA of affected sibling 3 (457/3) shows that c.1163+3dupT causes exon 5 skipping. Underlying sequence without exon 5 skipping from the other mutant allele is minor, suggesting instability of the transcript containing c.1093delC by nonsense-mediated mRNA decay. This finding is confirmed by cDNA sequencing in the father. The transcript that lacks exon 5 sequence due to c.1163+3dupT is stable as indicated by cDNA sequencing in the mother.

(C) The exon 5 deletion removes amino acids 317-387 (indicated in red) from the SLX4 protein, which disrupts the conserved UBZ4 domain.
Supplementary Figure 7. SLX4 expression and interaction with structure-specific endonucleases in EUFA1354.

(A) Immunoprecipitation (IP) and Western blot analysis showing the absence of full-length SLX4 protein and reduced levels of ERCC1, XPF and MUS81 in SLX4 precipitates from lymphoblasts of the affected individual (EUFA1354-L). Immunoprecipitation was performed with antibodies against the SLX4 N-terminus (1-300) or C-terminus (1534-1834) and Western blotting with the antibody against the SLX4 C-terminus. SE and LE are short and long exposures of the blot. (B) Immunoprecipitation (IP) and Western blot analysis revealing absence of full-length SLX4 protein and reduced levels of XPF, ERCC1, MUS81, EME1 and SLX1 in SLX4 precipitates of SV40-immortalized EUFA1354-F fibroblasts. A truncated SLX4 protein is detected. Immunoprecipitation and Western blotting were performed with antibodies against the N-terminus (1-300) or C-terminus (1534-1834) of SLX4. Ig indicates the immunoglobulin light chain. (C) Reciprocal immunoprecipitations showing the absence of full-length SLX4 in MUS81, ERCC1 or XPF precipitates from SV40-immortalized EUFA1354 fibroblasts. The truncated SLX4 protein does coprecipitate. SV40-immortalized fibroblasts from a healthy individual (LN9SV) were analyzed as a control. (D) Reciprocal immunoprecipitations showing undetectable SLX4 protein in ERCC1 and MUS81 precipitates from EUFA1354-L lymphoblasts. (E) A truncated SLX4 protein starting at methionine 213 is able to interact with structure specific endonucleases. HEK293 cells were transiently transfected with
SLX4 is mutated in a new Fanconi anemia subtype

pcDNA5.1 encoding GFP, GFP–SLX4 full–length or GFP–SLX4 (213–end) or with empty vector (−). After 48 hr, cells were lysed and extracts were subjected to immunoprecipitation with anti–GFP antibodies. Precipitates were analysed by Western blotting with the antibodies indicated. “Input” represents cell extracts.

Supplementary Figure 8. XPF/ERCC1 and SLX1 elute only in the low molecular weight fractions from EUFA1354 fibroblast lysates.
Extracts of wild type fibroblasts (LN9SV) or fibroblasts from individual EUFA1354 were analyzed by size exclusion chromatography on a Superose 6 10/300 GL column in buffer containing 0.2 M NaCl. The elution positions of Dextran blue (2 MDa), thyroglobulin (670 kDa) and bovine γ–globulin (158 kDa) are shown.

Supplementary Figure 9. Nuclear ERCC1 foci in SV40-immortalized wild type fibroblasts, but not in SV40- immortalized EUFA1354 fibroblasts.
These analyses were carried out with highly specific affinity-purified ERCC1 polyclonal antibody².
### Supplementary Table 1A. SLX4 specific primers used for sequencing genomic DNA

| SLX4ex1F       | TGTTTAACCACAGGCCCAGTAC  |
| SLX4ex1R       | GCCCTTTCCAGGAATTTTACAGTAC |
| SLX4ex2F       | ACCAACAAGCAGAGCCAGTAC   |
| SLX4ex2R       | ATCCAGTCAGAGGTCCAGTAC   |
| SLX4ex3F       | TTCCCGAGATGACTTCTACAGTAC |
| SLX4ex3R       | ACAAAAAGAGGTCCAGTAC     |
| SLX4ex4AF      | GCCCAAACTTTGACTTCTACAGTAC |
| SLX4ex4AR      | AGGGCCTTTTTTTCCTTACAGTAC |
| SLX4ex4BF      | TCCAGGCTGCGAGGCTACAGTAC |
| SLX4ex4BR      | TGCTCCGATTACAGGTTCACTTACAGTAC |
| SLX4ex5F       | AACTTCTGGCCTCGGATTGACAGTAC |
| SLX4ex5R       | ATACCGGGGTTTTCTTCTACAGTAC |
| SLX4ex6F       | CCAGAACGAGGTCTTCTACAGTAC |
| SLX4ex6R       | CCTTCTGGACTTCTACAGTAC   |
| SLX4ex7F       | ATGTGATGGACTTCTACAGTAC |
| SLX4ex7R       | AGAGGATTCAGTTCTACAGTAC |
| SLX4ex8F       | TCTCTACCTCCCTTGCTACAGTAC |
| SLX4ex8R       | GGGTCACTCAGAGGTTACAGTAC |
| SLX4ex9R       | AGGCTGCGAGTAAGGATCAGTAC |
| SLX4ex10F      | CTGCTGACGTACCTACAGGCTACAGTAC |
| SLX4ex10R      | CATCCACCTTGCTCCATACAGTAC |
| SLX4ex11AF     | TGGGCTGCTTGACCTACAGGCTACAGTAC |
| SLX4ex11AR     | TGGTGGCCTGGGAGGTACAGTAC |
| SLX4ex11BF     | TGGGCTGCTTGACCTACAGGCTAC |
| SLX4ex11BR     | TGGGCTGCTTGACCTACAGGCTAC |
| SLX4ex11CF     | CATCACACAGGCTGACCTACAGGCTAC |
| SLX4ex11CR     | CAGGTTTTGGCTGACCTACAGGCTAC |
| SLX4ex11DF     | GCTTTTGCTTGACCTACAGGCTAC |
| SLX4ex11DR     | GCCGTGGTCTGGGACCTACAGGCTAC |
| SLX4ex11ER     | GCCGTGGTCTGGGACCTACAGGCTAC |
| SLX4ex12F      | GTCGCCTGTGAGGTACCTACAGGCTAC |
| SLX4ex12R      | ACCAGGCTGGAGGTACCTACAGGCTAC |
| SLX4ex13F      | ATAGGGAAGGTGAGGTGACCTACAGGCTAC |
| SLX4ex13R      | GACGGGCTGGGAGGTGACCTACAGGCTAC |
| SLX4ex14F      | GCCGTGGGACCTACAGGCTACAGGCTAC |
| SLX4ex14R      | CACAGGCTGGGAGGTGACCTACAGGCTAC |
**Supplementary Table 1B. SLX4 specific primers used for sequencing cDNA**

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Chapter 2

References
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