The structure-specific endonuclease complex SLX4/XPF regulates Tus/Ter-induced homologous recombination

Ralph Scully (✉ rscully@bidmc.harvard.edu)

Beth Israel Deaconess Medical Cntr, Harvard Medical School <https://orcid.org/0000-0002-5064-0175>

Rajula Elango

Beth Israel Deaconess Medical Center/Harvard Medical School

Arvind Panday

Beth Israel Deaconess Medical Center/Harvard Medical School

Francis Lach

The Rockefeller Univeristy

Nicholas Willis

Beth Israel Deaconess Medical Center/Harvard Medical School

Erin Duffey

Beth Israel Deaconess Medical Center/Harvard Medical School

Agata Smogorzewska

The Rockefeller University <https://orcid.org/0000-0001-6285-1562>

Article

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5 **homologous recombination**
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9 Rajula Elango¹, Arvind Panday¹, Francis P. Lach², Nicholas A. Willis¹, Erin E. Duffey¹, Agata
10 Smogorzewska² and Ralph Scully^{1,*}
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15
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17 1. Department of Medicine, Division of Hematology-Oncology and Cancer Research Institute,
18 Beth Israel Deaconess Medical Center and Harvard Medical School, Boston, MA 02215, USA.

19 2. Laboratory of Genome Maintenance, The Rockefeller University, New York, NY 10065,
20 USA.

21 *. Correspondence: rscully@bidmc.harvard.edu
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Abstract

Vertebrate replication forks arrested at an interstrand DNA crosslink (ICL) can engage the Fanconi anemia (FA) pathway of ICL repair. The *FANCP* product, SLX4, binds the FANCD1/XPF/ERCC1-ERCC1 endonuclease, which incises bidirectionally arrested forks to ‘unhook’ the ICL. The resulting double strand break (DSB) is repaired by homologous recombination (HR). Whether this mechanism operates at replication blocks other than ICLs is unknown. Here, we study the role of mammalian SLX4 in HR triggered by a site-specific, chromosomal DNA-protein replication fork barrier formed by the *Escherichia coli*-derived Tus/*Ter* complex. We identify an SLX4-XPF-mediated step that is required for Tus/*Ter*-induced HR but not for HR induced by a replication-independent DSB. We additionally identify a requirement for SLX4-XPF in DSB-induced ‘long tract’ gene conversion, a replicative HR pathway related to break-induced replication. Our work suggests that Tus/*Ter*-induced HR recapitulates the incision step of replication-coupled ICL repair, and that the full FA mechanism can process DNA-protein barriers for HR.

Keywords: Fanconi anemia, homologous recombination, SLX4, XPF, replication fork stalling, Tus/*Ter*, DSB repair, long tract gene conversion, break-induced replication.

Introduction

DNA replication is frequently challenged by DNA template alterations that impede progression of the replication fork. Replication fork stalling ('replication stress') can be triggered by specific types of DNA damage or DNA repair intermediates, by unusual secondary structures in undamaged DNA, by collisions with transcription complexes, or by depletion of the nucleotide pool¹⁻⁴. Interstrand crosslinks (ICLs) covalently bind the two parental strands, preventing passage of the replicative CMG helicase. Unless the ICL can be disrupted or bypassed, it is an absolute block to replication fork progression⁵⁻⁷. In higher eukaryotes, the Fanconi anemia (FA) pathway can process the ICL-blocked fork for repair by homologous recombination (HR)⁸. Fanconi anemia is a rare, hereditary syndrome characterized by progressive bone marrow failure, developmental anomalies and cancer predisposition^{9,10}. Cells from FA patients display characteristic hypersensitivity to ICL-inducing agents, including aldehydes, which are an endogenous source of both ICLs and DNA-protein crosslinks^{6,11-14}. FA mutant cells reveal elevated frequencies of ICL-induced 'chromatid-type' aberrations, reflecting a defect in replication-coupled ICL repair. To date, 23 FA genes have been identified.

Studies of repair of ICL-containing plasmids replicating *in vitro* in *Xenopus laevis* egg extracts have begun to reveal the fundamental steps of the FA pathway^{8,15}. In this setting, FA pathway-mediated repair requires stalling of two opposing replication forks at the ICL. This bidirectional fork stalling triggers replisome disassembly ('fork collapse'), orchestrated by the E3 ubiquitin ligase TRAIP, which polyubiquitinates CMG helicase components *in trans*, targeting them for extraction by the p97/VCP ATPase and for proteasomal degradation¹⁶. Association of the DNA

translocase and scaffolding protein FANCM and its associated proteins with the stalled fork recruits the FA core complex—a large multi-subunit E3 ubiquitin ligase that monoubiquitinates and thereby activates the FANCD2-FANCI heterodimer^{8,17-19}. Monoubiquitinated FANCD2-I is a sliding clamp that encircles dsDNA at the stall site^{20,21}; in parallel, the stalled fork may undergo asymmetric fork reversal²². The motor proteins that mediate fork reversal during FA pathway activation are unknown; FANCM is one candidate, since it can reverse model fork structures *in vitro* and its motor activity is required for ICL resistance^{19,23,24}. The combination of fork reversal and FANCD2-I activation at the ICL promotes recruitment of the endonuclease scaffold SLX4/FANCP and its associated endonuclease XPF (xeroderma pigmentosum group F)/FANCG in complex with ERCC1 (excision repair cross complementation group 1)²⁵. XPF makes incisions either side of the ICL on one sister chromatid, generating a two-ended double strand break (DSB) opposite an ‘unhooked’ ICL on the unbroken sister chromatid. The single stranded (ss)DNA gap opposite the unhooked ICL is filled by translesion synthesis (TLS) DNA polymerases, and the DSB is repaired by conservative, two-ended HR. Indeed, several core HR genes are also FA genes. These include the hereditary breast and ovarian cancer predisposition genes *BRCA1/FANCS* and *BRCA2/FANCD1*, as well as the central mitotic recombinase *RAD51/FANCR* and certain *RAD51* paralogs⁹. Despite these advances, it is unclear whether the full FA mechanism is restricted to ICL repair, or whether FA-mediated incisions can induce HR at other types of fork-stalling lesions, such as DNA-protein complexes²⁶. In this regard, a molecularly defined system that recapitulates the FA mechanism *in vivo* on a mammalian chromosome has been lacking.

To study HR triggered by replication fork stalling at a defined chromosomal locus of cycling mammalian cells, we previously adapted the *Escherichia coli* Tus/*Ter* replication fork barrier (RFB) for use in mammalian cells²⁷. We found that an array of six tandem 23 bp *Ter* repeats, when targeted to a specific genomic locus and bound by the Tus protein, triggers bidirectional fork stalling and HR at the Tus/*Ter* RFB. The Tus/*Ter* RFB is a DNA-protein barrier lacking covalent DNA-protein crosslinks or ICLs. Fork stalling events at Tus/*Ter* do not map to a single defined nucleotide, but are distributed over the ~200 bp 6x*Ter* array²⁸. Thus, Tus/*Ter*-stalled forks do not present a ‘classical’ ICL substrate for the FA pathway. It is therefore important to determine the mechanisms underlying Tus/*Ter*-induced HR from first principles. Does it entail formation of a DSB intermediate, analogous to the DSB intermediate generated by SLX4-XPF during replication-coupled ICL repair by the FA pathway? Alternatively, does Tus/*Ter*-induced HR resemble HR induced at the *Schizosaccharomyces pombe* RTS1 RFB, which does not proceed *via* a DSB intermediate²⁹?

The major conservative HR outcome at the Tus/*Ter* RFB, ‘short tract’ gene conversion (STGC), is a product of two-ended HR, implicating bidirectional fork stalling in its initiation. Tus/*Ter*-induced STGC is a non-crossover outcome mediated by the canonical BRCA-Rad51 HR pathway^{27,28,30}. These findings implicate the non-crossover ‘synthesis-dependent strand annealing’ (SDSA) pathway as the underlying HR mechanism^{30,31}. We found that FANCM motor protein function and FANCM-mediated recruitment of the FA core complex are specifically required for efficient Tus/*Ter*-induced STGC²⁴. Indeed, *FANCM* and FA core genes act epistatically in this function. Thus, Tus/*Ter*-induced STGC is mediated by early components of the FA pathway and by canonical HR proteins. Whether Tus/*Ter*-induced STGC also entails

108 an SLX4-mediated incision step, analogous to the unhooking step of FA pathway-mediated ICL
109 repair, is currently unknown.

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111 *SLX4/FANCP* encodes a scaffolding protein that binds multiple endonuclease complexes in a cell
112 cycle-dependent manner to cleave branched DNA structures³²⁻³⁸. In S phase cells, SLX4
113 interacts with the XPF–ERCC1 heterodimer and SLX1 (synthetic lethal of unknown function 1)
114 *via* the SLX4 MLR (MEI9- Interaction-Like Region) and SBD (SLX1-binding) domains,
115 respectively, as well as with MutS β *via* the SLX4 N-terminus. The SLX4-XPF interaction is
116 required for cellular resistance to ICLs³⁹. XPF-ERCC1 binds and cleaves the 3' arm of
117 replication fork-like substrates containing an ICL, but can also cleave dsDNA internal to the
118 branch junction, leading to ICL unhooking^{25,40-42}. SLX4 binding enhances XPF nuclease activity
119 and increases its specificity for replication fork substrates. In mitosis, SLX4 additionally binds
120 nucleases MUS81 (methyl methanesulfonate and UV sensitive 81)–EME1 (essential meiotic
121 endonuclease 1) *via* the SAP (SAF-A/B, Acinus and PIAS) domain. These interactions enable
122 the SLX4 'trinuclease' complex to act on a variety of branched DNA substrates in mitosis,
123 cleaving stalled replication forks at sites of incomplete DNA synthesis and resolving post-
124 replicative Holliday junctions³²⁻³⁶. MUS81-EME1 preferentially cleaves 3' flap structures and
125 nicked Holliday junctions⁴³⁻⁴⁵. The preferred substrate for SLX1 is a 5' flap, which it cuts at the
126 ssDNA–dsDNA junction⁴⁶. SLX4 binding enhances SLX1 substrate promiscuity, allowing
127 SLX1 to cleave splayed arms, 5' flaps, 3' flaps and Holliday junctions. SLX4 contains at its N-
128 terminus two conserved Ubiquitin Binding Domain 4 (UBZ) motifs that facilitate its localization
129 to laser-induced damage sites, implicating tethering of the UBZ motifs to as yet unidentified

K63-linked poly-ubiquitinated substrates at sites of laser damage^{47,48}. The role of the SLX4 tandem UBZ domain in SLX4 function at stalled mammalian replication forks is unknown.

Biallelic *SLX4/FANCP* mutations have been identified in several FA patients^{47,49}. Two independent FA kindreds were found to carry germ line *SLX4* mutations that delete in-frame one or both UBZ motifs. Another FA patient was found to carry a 1-bp deletion (c.286delA) in exon 1 of *SLX4*, resulting in an early frameshift, and a premature stop at codon 126 (p.Thr96LeufsX30) in the second allele. Despite this disruption of the *SLX4* open reading frame (ORF), translation initiation at an alternative start site produced low levels of truncated protein in cells from the affected individual⁴⁹. Thus, FA-associated *SLX4* mutations described to date are hypomorphic alleles, encoding polypeptides with disrupted UBZ motifs. Possibly, more severe *SLX4* mutations are incompatible with human development. Indeed, mice carrying biallelic *Slx4* germ line mutations are born in sub-mendelian ratios and reveal prominent growth defects⁵⁰, while biallelic deletion of *SLX4* in chicken DT40 lymphoblastoid cells is cell lethal⁵¹.

Pathogenic mutations in *XPF/FANCDQ* also cause FA^{52,53}. Whether and how XPF contributes to mammalian HR remains to be determined.

SLX4, together with MUS81, has been implicated in mitotic DNA synthesis (MiDAS) at chromosomal ‘fragile sites’—a form of aberrant replication restart that occurs when cells enter mitosis with incompletely replicated chromosomes^{54,55}. MiDAS is mediated by RAD52, SLX4, MUS81-EME1, RTEL1 and POL δ . Current models propose that SLX4-MUS81 initiates MiDAS by breaking isolated stalled replication forks in mitotic cells, provoking break-induced replication (BIR) at the resulting one-ended breaks. Aberrant replication restart is also observed

in response to a Tus/*Ter* RFB, leading to ‘long tract’ gene conversion (LTGC) and, in *BRCA1*-defective cells, the formation of non-homologous tandem duplications (TDs)²⁸. Tus/*Ter*-induced LTGC and TD formation are suppressed by FANCM and the Bloom’s syndrome helicase BLM, suggesting an underlying BIR-like migrating bubble mechanism^{24,30}. The cell cycle phases in which Tus/*Ter*-induced STGC, LTGC and TD formation occur, and thus their relationships to MiDAS, are unknown.

It is also unknown whether SLX4 and its associated nucleases have roles in HR downstream of DSB formation. In wild type cells, the majority of HR events triggered by a nuclease-induced site-specific DSB resolve as STGC—a conservative HR outcome mediated by SDSA³⁰. A minority (~5%) resolve as LTGC—an aberrant replicative ‘gap repair’ outcome of HR, characterized by gene conversions of >3 kb⁵⁶⁻⁵⁹. Work in yeast has emphasized similarities between LTGC and BIR, and recent work in mammalian cells supports this connection⁶⁰. Although DSB-induced STGC and LTGC are both Rad51-dependent, mutation of certain core HR genes disproportionately impairs STGC, resulting in a bias in favor of LTGC^{27,57,58}. The identification of genes that specifically support LTGC is important for understanding mechanisms of genomic instability in human disease. In this regard, a number of different experimental models implicate DNA polymerase δ in LTGC⁶⁰⁻⁶². Recent studies additionally identified the MCM8-MCM9-MCM8IP/HROB helicase complex and the PIF1 helicase as mediators of mammalian DSB-induced LTGC^{60,63,64}. It is unknown whether DSB-induced LTGC is primarily S phase-restricted or, alternatively, a G2/M process potentially related to MiDAS. SLX4 may be a useful tool with which to study this question.

In this study, we use CRISPR/Cas9 genome editing to engineer precise, defined mutations in the *Slx4* gene of mouse embryonic stem (mES) cells that carry a reporter of stalled fork- and DSB-induced HR. We systematically examine the role of individual nuclease-interacting domains of SLX4 in stalled fork HR. Our results identify a critical role for the SLX4-XPF complex in Tus/*Ter*-induced STGC. We define a requirement for the SLX4 UBZ motifs in the recruitment of SLX4 to stalled forks, providing important insight into the mechanisms that connect clinically-described *SLX4/FANCP* mutations to genomic instability. We also identify an unexpected role for SLX4 in supporting LTGC in the repair of a replication-independent DSB.

Results

***Slx4* regulates error-free HR at Tus/*Ter*-stalled replication forks.**

To study the role of *Slx4* in mammalian stalled fork repair, we used dual CRISPR/Cas9-mediated incisions to introduce defined mutations into the endogenous *Slx4* gene of mouse embryonic stem (ES) cells that contain a single copy of a 6x*Ter*-HR reporter targeted to the *Rosa26* locus on chromosome VI²⁷ (**Fig. 1a**). In this reporter, HR can be triggered by either Tus/*Ter*-induced fork stalling or by a site-specific DSB induced by the rare-cutting homing endonuclease I-SceI. The reporter enables simultaneous flow cytometric quantitation of error-free HR as “short tract” gene conversion (STGC; GFP⁺RFP⁻) outcomes, and aberrant replicative HR responses as “long tract” gene conversion (LTGC; GFP⁺RFP⁺) products. We engineered a 125 bp frame-shift deletion in exon 2 (*Slx4*^{Δ125}), thereby disrupting *Slx4* early in the ORF (**Fig. 1b**), and likely generating a larger undefined deletion (*Slx4*^Δ) within the second allele^{24,65}. We assayed two independent *Slx4* mutant clones (*Slx4*^{Δ125/Δ} #40, 42) and two isogenic clones that received the same CRISPR/Cas9 treatment but retained wild type *Slx4* (*Slx4*^{+/+} #3, 13) (**Fig. 1b**). Both *Slx4*^{Δ125/Δ} clones expressed

normal levels of *Slx4* mRNA (**Supplementary Fig. 1**), suggesting that *Slx4*^{Δ125} may not be functionally a true null allele. To determine the sensitivity of *Slx4*^{Δ125/Δ} clones to the ICL-inducing agent mitomycin C (MMC), we mixed ~80% uncolored (GFP⁻) *Slx4*^{Δ125/Δ} mutants (or isogenic *Slx4*^{+/+} GFP⁻ controls) with ~20% GFP⁺ wild type cells and exposed them to titrated concentrations of MMC for 48 hours (**Fig. 1c**). We observed a significant enrichment of GFP⁺ cells in *Slx4*^{Δ125/Δ} mixed populations, implying loss of viability of *Slx4*^{Δ125/Δ} cells in comparison to GFP⁺ wild type cells (**Fig. 1c**). Thus, as expected, *Slx4*^{Δ125/Δ} mutants are sensitive to MMC.

To measure stalled fork HR and DSB-induced HR, we transfected *Ter*-HR reporter cells, in parallel, with *Tus* or *I-SceI* expression vectors and assayed repair products 72 hours post-transfection (see methods). Each *Slx4*^{Δ125/Δ} clone revealed a ~4-fold reduction in *Tus/Ter* induced STGC compared to the two isogenic wild type clones, whereas *Tus/Ter*-induced LTGC was unaffected by *Slx4* disruption (**Fig. 1d, 1e and 1f**). Hence, HR at *Tus/Ter* was skewed in favor of LTGC (**Supplementary Fig. 1**). In contrast, *I-SceI*-induced STGC was unaltered in *Slx4*^{Δ125/Δ} cells, whereas *I-SceI*-mediated LTGC was reduced (**Fig. 1d, 1g and 1h**). Hence, in DSB-induced HR, the ratio of LTGC:Total HR was reduced 2-fold in *Slx4*^{Δ125/Δ} cells (**Supplementary Fig. 1**). These results identify distinct roles for mammalian *Slx4* in stalled fork HR and in conventional DSB-induced HR. Specifically, the data suggest that *Slx4* mediates error-free HR (i.e., STGC) at stalled replication forks but not at a replication-independent DSB. Conversely, *Slx4* specifically mediates DSB-induced LTGC, but is dispensable for *Tus/Ter*-induced LTGC.

***Slx4* hemizygous cells phenocopy wild type cells**

To facilitate analysis of individual SLX4 domains in stalled fork repair, we used dual CRISPR/Cas9 incisions at exon 2 and exon 15 of *Slx4* to delete one copy of the entire 19.3 kb gene (**Supplementary Fig. 1**). We retrieved 202/262 *Slx4*^{+/+} clones from this experiment, 60/262 *Slx4*^{+/-} clones but no *Slx4*^{-/-} clones. The expected *Slx4*^{-/-} frequency was $(60/262)^2 = \sim 13/262$. This result therefore suggests that *Slx4*^{-/-} is cell lethal. We used direct sequencing of breakpoint PCR products to identify the deletion breakpoint in *Slx4*^{+/-} cells and to confirm that the retained allele is wild type at the sgRNA target sites (**Supplementary Fig. 1**). *Slx4* expression in the *Slx4*^{+/-} clone was at wild type levels, suggesting that transcription is upregulated in compensation for hemizyosity (**Supplementary Fig. 1**). Importantly, *Slx4*^{+/-} cells revealed wild type HR repair frequencies (**Supplementary Fig. 1**). Thus, *Slx4*^{+/-} mES cells are phenotypically wild type for stalled fork- and DSB-induced HR.

The SLX4 UBZ domain recruits SLX4 to stalled forks to mediate error-free HR

To study the role of the SLX4 UBZ domain in Tus/*Ter*-mediated HR, we used dual CRISPR/Cas9-mediated incisions to delete in-frame the UBZ1- and UBZ2-coding regions in *Slx4*^{+/-} *Ter*-HR reporter cells (**Fig. 2a, Supplementary Fig. 2**). UBZ-encoding mRNA sequences were undetectable in *Slx4*^{ΔUBZ/-} cells, whereas sequences downstream (encoding MLR, SAP and SBD domains) were expressed at normal levels (**Supplementary Fig. 2**). We performed competitive growth assays using three independent *Slx4*^{ΔUBZ/-} clones (#1, 2, 5) and three independent sgRNA/Cas9 treated, yet unaltered, *Slx4*^{+/-} clones (#7, 25, 44), in the presence of MMC or Olaparib. All *Slx4*^{ΔUBZ/-} clones were hypersensitive to MMC (**Supplementary Fig. 2**) and were resistant to Olaparib (**Supplementary Fig. 2**). A characteristic cellular feature of FA is increased late S/G2 phase enrichment following exposure to MMC⁹. Untreated *Slx4*^{ΔUBZ/-} cells

revealed cell cycle distribution patterns similar to *Slx4*^{+/-} cells (**Fig. 2b**). However, in response to titrated doses of MMC, *Slx4*^{ΔUBZ/-} cells accumulated in G2/M more readily than *Slx4*^{+/-} controls, and revealed a corresponding reduction in the S-phase fraction (**Fig. 2b**).

Next, we analyzed six independent *Slx4*^{ΔUBZ/-} clones and six similarly Cas9/sgRNA-exposed independent *Slx4*^{+/-} clones in stalled fork repair and DSB repair. Strikingly, Tus/*Ter*-induced STGC was reduced ~6-fold in *Slx4*^{ΔUBZ/-} clones in comparison to *Slx4*^{+/-} controls (**Fig. 2c**). In contrast, the frequency of I-SceI induced STGC was only modestly reduced (**Fig. 2d**). Transient expression of wild type human *SLX4* restored Tus/*Ter*-induced STGC to wild type levels in *Slx4*^{ΔUBZ/-} clones (#2 and #5), but had no impact on *Slx4*^{+/-} controls (#25 and #44) (**Fig. 2e and Supplementary Fig. 2**). A UBZ 4C>A mutant that specifically disrupts UBZ domain function³⁹ failed to complement the STGC defect of *Slx4*^{ΔUBZ/-} cells, despite equivalent levels of expression. In the same experiment, expression of wild type *SLX4* or 4C>A had no statistically significant impact on I-SceI-induced STGC in either *Slx4*^{ΔUBZ/-} or *Slx4*^{+/-} clones (**Fig. 2f**). The ability of wild type *SLX4* but not the 4C>A mutant to complement the defect in Tus/*Ter*-induced STGC in *Slx4*^{ΔUBZ/-} cells indicates a specific requirement for the SLX4 UBZ domain in error-free HR (i.e., STGC) at stalled forks. In contrast, the SLX4 UBZ domain has no clear role in DSB-induced STGC. Thus, *Slx4*^{ΔUBZ} is a separation-of-function allele that distinguishes control of STGC at stalled forks from STGC at replication-independent DSBs.

The same experiments revealed different effects on LTGC. Tus/*Ter*-induced LTGC was only minimally reduced in the six *Slx4*^{ΔUBZ/-} clones; hence, the ratio of LTGC:Total HR was elevated, reflecting the severe defect in STGC (**Supplementary Fig. 2**). Tus/*Ter*-induced LTGC was

unaffected by expression of either wild type *SLX4* or the 4C>A mutant (**Supplementary Fig. 2**). Thus, there is no clear evidence of a role for the SLX4 UBZ domain in Tus/*Ter*-induced LTGC. In contrast, I-SceI-induced LTGC was reduced >2-fold in the six *Slx4*^{ΔUBZ/-} clones in comparison to *Slx4*^{+/-} controls; as a result, the ratio of LTGC:Total HR was reduced~2-fold, reflecting the specific reduction in I-SceI-induced LTGC (**Supplementary Fig. 2**). The LTGC defect was complemented by wild type *SLX4* but not by the 4C>A mutant (**Supplementary Fig. 2**). Thus, *Slx4*^{ΔUBZ} is a separation-of-function allele that distinguishes control of LTGC at replication-independent DSBs from LTGC at stalled forks.

To facilitate detection of the endogenous *Slx4* gene product in mouse ES cells, we targeted the 3' end of the wild type or ΔUBZ endogenous *Slx4* allele in *Slx4*^{+/-} and *Slx4*^{ΔUBZ/-} cells with a dual minimal auxin-inducible and SMASh degron containing an 8x HA epitope tag positioned N-terminal of the SMASh degron cleavage site^{24,66-68}, to generate *Slx4*^{deg/-} and *Slx4*^{ΔUBZdeg/-} clones (**Fig. 2g**). *Slx4*^{deg/-} and *Slx4*^{ΔUBZdeg/-} clones expressed comparable levels of *Slx4* mRNA (**Fig. 2h**); HA-tagged gene products were detected in the chromatin fraction but not in whole cell extracts. Unexpectedly, the HA-tagged *Slx4*^{ΔUBZdeg} gene product was detected at higher levels in the chromatin fraction than the wild type protein (**Fig. 2i**). Activation of the dual degron triggered partial degradation of endogenous SLX4, confirming the specificity of the anti-HA western blot signal. We used these tools, in combination with chromatin immunoprecipitation (ChIP), to study SLX4 recruitment to Tus/*Ter*-stalled forks. We noted robust recruitment of wild type SLX4-deg to the Tus/*Ter* RFB (**Fig. 2j**). Strikingly, despite its greater abundance in chromatin fractions, SLX4-ΔUBZdeg was detected at much lower levels at Tus/*Ter* by ChIP. Thus, the

SLX4 UBZ domain mediates Tus/*Ter*-induced STGC by promoting SLX4 accumulation at sites of fork stalling.

Interaction of SLX4 with MUS81 or SLX1 is dispensable for Tus/*Ter*-induced and DSB-induced HR

Interactions of SLX4 with the endonucleases MUS81 and SLX1 in mitosis facilitate cleavage of branched DNA structures, including stalled replication forks and Holliday junctions (HJs)³². To study the SLX4-MUS81 interaction in Tus/*Ter*-induced repair, we used Cas9-dual sgRNA incisions to generate in-frame deletions of *Slx4* exons 11-13, encoding the SAP domain, in *Slx4*^{+/-} *Ter*-HR reporter cells (**Fig. 3a** and **Supplementary Fig. 3**). The mRNA sequence encoding the SAP domain was undetectable in *Slx4*^{ΔSAP/-} cells, whereas expression of other *Slx4* elements was at wild type levels (**Supplementary Fig. 3**). Consistent with a previous study³⁹, we found that three independent *Slx4*^{ΔSAP/-} clones (#21, #28, #51) are modestly sensitive to MMC and Olaparib (**Fig. 3b** and **3c**) as compared to three *Slx4*^{+/-} clones (#5, #8, #17) that had been similarly treated with Cas9/sgRNA. We analyzed stalled fork and DSB repair functions in five independent *Slx4*^{ΔSAP/-} clones and five isogenic *Slx4*^{+/-} clones. Tus/*Ter* induced and I-SceI-induced STGC and LTGC were indistinguishable between *Slx4*^{ΔSAP/-} and *Slx4*^{+/-} clones (**Fig. 3d, 3e** and **Supplementary Fig. 3**). Thus, the SLX4 SAP domain and, hence, the interaction of SLX4 with MUS81, is dispensable for these repair functions.

To study the SLX4-SLX1 interaction in Tus/*Ter* induced repair, we used Cas9-dual sgRNA incisions to generate in-frame deletions of *Slx4* exons 13-15, encoding the SBD domain, in *Slx4*^{+/-} *Ter*-HR reporter cells (**Fig. 3f** and **Supplementary Fig. 3**). The mRNA sequence

encoding the SBD domain was almost undetectable in the *Slx4*^{ΔSBD/-} cells, whereas expression of other elements of *Slx4* was at normal levels (**Supplementary Fig. 3**). Competitive growth assays revealed that *Slx4*^{ΔSBD/-} mutants (#15, #29, #31) are not sensitive to MMC or Olaparib (**Fig. 3g** and **3h**), as compared to *Slx4*^{+/-} clones (#2, #16, #22) that had been similarly treated with Cas9/sgRNA. We analyzed stalled fork and DSB repair functions in six independent *Slx4*^{ΔSBD/-} clones and six isogenic *Slx4*^{+/-} clones. No alterations in Tus/*Ter*-induced or I-SceI-induced STGC and LTGC were detected (**Fig. 3i, 3j**, and **Supplementary Fig. 3**). Thus, the SLX4 SBD domain and, hence, the interaction of SLX4 with SLX1 is dispensable for these repair functions.

The SLX4-XPF interaction is required for STGC at stalled forks

To study the SLX4-XPF interaction in Tus/*Ter* stalled fork repair, we used dual sgRNAs to generate in-frame deletion of the MLR-encoding exons 5 and 6 in *Slx4*^{+/-} Ter-HR reporter cells (**Fig. 4a, Supplementary Fig. 4**). *Slx4* mRNA transcripts in *Slx4*^{ΔMLR/-} clones lacked the deleted region but were otherwise detected at normal levels (**Supplementary Fig. 4**). *Slx4*^{ΔMLR/-} clones were slow-growing compared to isogenic *Slx4*^{+/-} controls that had been similarly exposed to Cas9/sgRNA but had retained wild type *Slx4*. In untreated cells, the proportion of cells in late S/G2/M was elevated in *Slx4*^{ΔMLR/-} cells (24%) in comparison to *Slx4*^{+/-} cells (12%; **Fig. 4b**). The S-phase fraction was correspondingly reduced. Similarly, following exposure of cells to titrated doses of MMC, *Slx4*^{ΔMLR/-} cells revealed more dramatic late S/G2/M arrest and S phase depletion than *Slx4*^{+/-} controls (**Fig. 4b**). Competitive growth assays confirmed that *Slx4*^{ΔMLR/-} cells (#5, #20, #31) are hypersensitive to MMC but not to Olaparib in comparison to *Slx4*^{+/-} controls (#13, #17, #28) (**Supplementary Fig. 4**). Slow growth and the increased late S/G2/M arrest is consistent with the idea that *Slx4*^{ΔMLR/-} cells experience high levels of spontaneous replication

stress. To explore this effect further, we analyzed metaphase spreads from *Slx4*^{+/-}, *Slx4*^{ΔUBZ/-} and *Slx4*^{ΔMLR/-} cultures that were either untreated or exposed to 20 ng/ml of MMC for 12 hours. Both *Slx4*^{ΔUBZ/-} and *Slx4*^{ΔMLR/-} cells revealed elevated levels of breaks and radial chromosomes in comparison to *Slx4*^{+/-} controls, but *Slx4*^{ΔMLR/-} cells revealed a more severe defect than *Slx4*^{ΔUBZ/-} cells (**Fig. 4c, 4d and 4e**). Thus, both *Slx4*^{ΔUBZ/-} and *Slx4*^{ΔMLR/-} cells display a classical Fanconi anemia chromosome breakage phenotype. In a second experiment, we additionally analyzed *Slx4*^{ΔSAP/-} and *Slx4*^{ΔSBD/-} cells (**Supplementary Fig. 4**). *Slx4*^{ΔSAP/-} cells were indistinguishable from *Slx4*^{+/-} cells. *Slx4*^{ΔSBD/-} cells revealed minimally elevated frequencies of breaks and radials compared to *Slx4*^{+/-} cells.

We analyzed stalled fork- and DSB-induced HR in six independent *Slx4*^{ΔMLR/-} clones and six independent *Slx4*^{+/-} isogenic clones. Tus/*Ter*-induced STGC was almost undetectable in *Slx4*^{ΔMLR/-} cells, whereas LTGC was detected at wild type levels (**Fig. 5a and Supplementary Fig. 5**). We noted reduced I-SceI induced STGC in *Slx4*^{ΔMLR/-} clones, while I-SceI induced LTGC was marginally reduced (**Fig. 5b and Supplementary Fig. 5**). These findings suggest that the SLX4 MLR domain and, hence the interaction of SLX4 with XPF, is required for Tus/*Ter*-induced STGC and for efficient DSB-induced STGC. A caveat of attempts to quantify repair directly in *Slx4*^{ΔMLR/-} cells is the possibility that the high levels of replication stress in these cells might artifactually alter the measured HR frequencies⁶⁹. To address this concern, we performed complementation experiments in *Slx4*^{ΔUBZ/-} *Ter*-HR reporter cells, which exhibit no proliferation defect. In addition to testing full-length hSLX4 expression vectors, we generated ‘mini-SLX4’ constructs that encode hSLX4 polypeptides that are truncated C-terminal to the BTB domain and therefore lack the MUS81-binding SAP or SLX1-binding SBD domains⁴⁰. These mini-SLX4

constructs included wild type, 4C>A and Δ MLR variants (**Fig. 5c**). Following transient transfection, hSLX4 was expressed equivalently from each construct (**Fig. 5d**). Consistent with our above-noted findings that the SLX4 SAP and SBD domains are dispensable for Tus/*Ter*-induced STGC, expression of mini-SLX4 complemented the defect in Tus/*Ter*-induced STGC in *Slx4* ^{Δ UBZ/-} cells as efficiently as wild type SLX4 (**Fig. 5f**). Importantly, both full length SLX4 Δ MLR and mini-SLX4 Δ MLR failed to complement the defect in Tus/*Ter*-induced STGC in *Slx4* ^{Δ UBZ/-} cells, behaving similarly to SLX44C>A vectors. No significant effects of any of these constructs were observed on Tus/*Ter*-induced STGC in *Slx4*^{+/-} cells, and I-SceI-induced STGC was unaffected by any SLX4 construct in either cell type (**Fig. 5f**). These results show that intact SLX4 UBZ and MLR domains are independently required for Tus/*Ter*-induced STGC. The absence of interallelic complementation between the endogenous *Slx4* ^{Δ UBZ} and exogenous SLX4 Δ MLR alleles suggests that the UBZ and MLR domains must each be intact on a single SLX4 molecule in order for SLX4 to mediate Tus/*Ter*-induced STGC.

In the same set of experiments, Tus/*Ter*-induced LTGC was unaffected by any of the above-noted hSLX4 constructs in either *Slx4* ^{Δ UBZ/-} or *Slx4*^{+/-} cells (**Supplementary Fig. 5**). In contrast, I-SceI-induced LTGC, which is specifically impaired in *Slx4* ^{Δ UBZ/-} cells, was complemented by expression of full-length wild type SLX4 (consistent with our previous experiments), but not by the Δ MLR mutant (**Supplementary Fig. 5**). Similarly, wild type mini-SLX4 complemented the defect in I-SceI-induced LTGC, whereas mini-SLX4 mutants 4C>A and Δ MLR failed to do so. These results suggest that I-SceI-induced LTGC requires both the UBZ and MLR domains of SLX4, but is independent of domains distal to the BTB dimerization domain.

The XPF nuclease domain is required for Tus/*Ter*- and I-SceI-induced STGC

The above findings implicate the SLX4-XPF interaction in Tus/*Ter*-induced STGC. We therefore used CRISPR/Cas9 with dual sgRNA targeting to delete the one entire ~42.3 kb *Xpf* allele in *Ter*-HR reporter cells (**Supplementary Fig. 6**). *Xpf* expression was reduced by ~50% in *Xpf*^{Δ/-} cells compared to an isogenic *Xpf*^{Δ/+} clone, implying an absence of transcriptional compensation for hemizyosity (**Supplementary Fig. 6**). Nonetheless, *Xpf*^{Δ/-} *Ter*-HR reporter cells revealed wild type repair frequencies (**Supplementary Fig. 6**). To determine whether the XPF nuclease domain contributes to stalled fork HR, we used Cas9 with dual sgRNAs to delete in-frame the nuclease domain-encoding region of *Xpf* in *Xpf*^{Δ/-} cells (**Fig. 6a and 6b**). We obtained one *Xpf*^{Nuc/-} clone containing an in-frame deletion (#1) and three that contained a frameshift (#2, #11, #13). Analysis of *Xpf* expression in each mutant clone revealed the absence of nuclease domain-encoding sequences but normal levels of expression of regions encoding the upstream helicase domain and the downstream HhH2 domain (**Supplementary Fig. 6**). This finding suggests that there was no nonsense-mediated decay of frame-shifted transcripts. We were unable to detect the endogenous XPF protein in mES cells. Western blot analysis of the HhH2-binding XPF heterodimeric partner ERCC1 in whole cell extracts revealed variably reduced abundance of ERCC1 in *Xpf*^{Nuc/-}-clones (**Supplementary Fig. 6**). Chromatin association of ERCC1 was detected in *Xpf*^{Nuc/-}-clone #1 but not in the three *Xpf*^{Nuc/-} frameshift clones, suggesting that interaction of XPF with ERCC1 is required for chromatin association of ERCC1 (**Supplementary Fig. 6**). All four *Xpf*^{Nuc/-} clones displayed a proliferative defect similar to that of *Slx4*^{ΔMLR/-} cells, associated with an increase in the G2/M fraction and a reduction in the S-phase fraction in unperturbed cells (**Supplementary Fig. 6**). Similarly, following exposure of cells to titrated doses of MMC, *Xpf*^{Nuc/-} cells revealed more dramatic G2/M arrest and S phase

depletion than $Xpf^{+/-}$ controls. Competitive growth assays corroborated these findings, revealing that $Xpf^{Nuc/-}$ cells are hypersensitive to MMC, but not to Olaparib, in comparison to $Xpf^{+/-}$ controls (**Fig. 6c** and **6d**). We prepared metaphase spreads from $Xpf^{Nuc/-}$ or $Xpf^{+/-}$ cells that were either untreated or exposed to low dose MMC. Strikingly, $Xpf^{Nuc/-}$ cells revealed dramatically elevated frequencies of MMC-induced chromatid breaks and radials, consistent with a classical FA phenotype (**Fig. 6e, 6f** and **6g**). These findings underscore the importance of *XPB/FANCDQ* as a *bona fide* FA gene.

We analyzed stalled fork and DSB repair functions in each of the four $Xpf^{Nuc/-}$ *Ter*-HR reporter clones and four isogenic $Xpf^{+/-}$ clones. Similar to $Slx4^{\Delta MLR/-}$ cells, $Xpf^{Nuc/-}$ cells revealed near-complete loss of Tus/*Ter*-induced STGC (**Fig. 6h**) but no detectable alteration in Tus/*Ter* induced LTGC (**Supplementary Fig. 6**). We also noted a significant reduction in I-SceI induced STGC (**Fig. 6i**) and a modest reduction in I-SceI induced LTGC (**Supplementary Fig. 6**). Thus, $Xpf^{Nuc/-}$ cells phenocopy $Slx4^{\Delta MLR/-}$ cells in stalled fork repair. The slow growth phenotype of $Xpf^{Nuc/-}$ cells suggests that these repair frequencies should be interpreted with caution. However, it is notable that all the experimental systems described herein point to SLX4-XPB as a critical mediator Tus/*Ter*-induced STGC. Further, the *Slx4* and *Xpf* mutants that revealed the most severe FA phenotype, in classical assays of MMC sensitivity and MMC-induced chromatid-type aberrations, also exhibited the most profound impairment of Tus/*Ter*-induced STGC.

Discussion

We show here that the structure-specific endonuclease scaffold SLX4 regulates distinct HR pathways at stalled replication forks and at replication-independent DSBs. In response to fork

stalling, two-ended, conservative HR (i.e., STGC) is dependent on *Slx4*, while DSB-induced STGC is either unaffected by or less sensitive to *Slx4* mutation. Conversely, DSB-induced LTGC, a replicative HR outcome possibly related to BIR, is *Slx4*-dependent, while LTGC at stalled forks is unaffected by *Slx4* mutation.

Tus/*Ter*-induced STGC is a product of the Fanconi anemia pathway

Our previous work showed that Tus/*Ter*-induced STGC is dependent on the early steps of the FA pathway of ICL repair. Similar to FA-mediated ICL repair, Tus/*Ter*-induced STGC is a product of two-ended HR, implicating bidirectional fork stalling as an initiating event²⁷. The stalled fork scaffold and motor protein FANCM regulates Tus/*Ter*-induced STGC by recruiting the FA core complex to stalled forks through direct interaction with FANCF^{24,70}. A *Fancm* mutation that disrupts FANCF binding is specifically defective for Tus/*Ter*-induced STGC, and this mutation is epistatic with loss of FA core complex proteins FANCA and FANCL. Experiments reported here suggest that Tus/*Ter*-induced STGC is almost completely dependent on a later step of the FA pathway, involving SLX4 and the XPF nuclease. We propose that SLX4-XPF controls Tus/*Ter*-induced STGC by introducing one or more incisions at the site of bidirectional fork arrest (**Fig. 7**). The resulting two-ended DSB intermediate is then repaired by canonical HR/SDSA. In other words, the role of SLX4-XPF in Tus/*Ter*-induced STGC recapitulates its role in the ‘unhooking’ step of ICL repair^{8,25,42}. However, in the absence of an ICL at the stall site, only one XPF-mediated incision of a stalled leading parental strand would be required to generate a two-ended DSB at Tus/*Ter* (**Fig. 7**). Thus, the minimal requirements for two-ended DSB formation at Tus/*Ter* may be less stringent than at an ICL. The proposed model would explain why SLX4 plays a major role in Tus/*Ter*-induced STGC but a much less significant role

in I-SceI-induced STGC. In the former case, if SLX4-XPF coordination at the stalled fork is impaired, production of a DSB at the stall site is suppressed, resulting in failure of FA-mediated HR and low levels of Tus/*Ter*-induced STGC. In the latter case, the DSB is induced by the I-SceI endonuclease, bypassing any requirement for SLX4-XPF in DSB formation.

This model raises additional important questions regarding mechanism. In FA-mediated ICL repair, close proximity of the two opposing replisomes is required to support TRAIP-mediated ubiquitination of opposing CMG complex *in trans*, leading to FA pathway activation¹⁶. It might appear that the ~200 bp Tus/*Ter* RFB would not allow the two opposing replisomes to approach sufficiently closely to one another to allow TRAIP-mediated CMG ubiquitination *in trans*. However, the Tus/*Ter* RFB is not an absolute barrier to the replicative helicase. Our previous study using high throughput genomewide translocation sequencing showed that fork stalling events are distributed throughout the 6x*Ter* array²⁸. Thus, the Tus/*Ter* RFB might be compatible with productive TRAIP activation. These considerations suggest that the full FA mechanism might indeed be activated at Tus/*Ter*-stalled forks, consistent with the findings presented here. In light of these findings, it will be important in future experiments to determine whether Tus/*Ter*-induced STGC requires TRAIP-mediated CMG ubiquitination, monoubiquitination of FANCD2-FANCI and/or a fork reversal step²².

Our findings extend the scope of the FA pathway beyond ICL repair and answer in the affirmative the longstanding question of whether the FA pathway can process a DNA-protein replication barrier for HR²⁶. Notably, aldehydes, a class of chemicals to which FA mutant cells are hypersensitive, can form both ICLs and DNA-protein crosslinks (DPCs)^{6,11-14}. Conceivably,

some aldehyde-induced DPCs encountered during replication might escape proteolytic mechanisms of DPC bypass and be processed by the FA pathway for HR^{26,71-74}. This hypothesis predicts that the sensitivity of FA mutant cells to aldehydes is, in part, a reflection of the inability of these cells to process aldehyde-induced DPCs for HR.

Our genetic analysis of SLX4 shows that the UBZ and MLR domains are each independently required for Tus/*Ter*-induced STGC. The absence of inter-allelic complementation between the endogenous *Slx4*^{ΔUBZ} and exogenous *SLX4*^{ΔMLR} alleles (**Fig. 5e**) suggests that each of the UBZ and MLR domains must be intact on a single SLX4 molecule for this function to be supported. In contrast, the SAP and SBD domains are fully dispensable for Tus/*Ter*-induced STGC. This result shows that Tus/*Ter*-induced STGC does not require assembly of the mitosis-associated SLX4 trinuclease complex. Rather, Tus/*Ter*-induced STGC appears to be coupled to S phase, when SLX4-XPF interactions dominate.

We show that a key function of the SLX4 UBZ domain is to support efficient retention of SLX4 at the stall site. This finding is important in light of recent structural studies, which showed that activated (monoubiquitinated) FANCD2-FANCI forms a sliding clamp on dsDNA^{20,75}.

Importantly, the ubiquitin marks associated with FANCD2-FANCI are buried within the barrel of the heterodimer and are unavailable for binding to SLX4²¹. Our findings underscore the importance of ubiquitin binding by SLX4 in stalled fork HR; the identity of the ubiquitinated substrate that anchors SLX4 at replication stall sites remains to be discovered. The *Slx4*^{ΔUBZ/-} phenotype also sheds light on clinical cases of Fanconi anemia associated with *SXL4/FANCP* mutation. As noted earlier, FA-associated *SLX4* mutations are hypomorphs, implicating

inactivation of UBZ domain function as a common defect in *FANCP* patients^{47,49}. Our data suggest that a key defect in *FANCP* mutant FA patient cells is ineffective retention of SLX4 at stalled forks. Unlike the true *SLX4* null, which our data suggests is cell lethal when homozygous, germ line homozygous *SLX4* Δ UBZ mutations may be compatible with human development because the SLX4 retention defect is quantitative rather than absolute.

Role of SLX4 in DSB-induced HR

Our work unexpectedly identified SLX4 as a mediator of mammalian DSB-induced LTGC. This finding might appear to connect DSB-induced LTGC to MiDAS, which is also mediated by SLX4. However, unlike the role of SLX4 in MiDAS, where SLX4-MUS81 interaction is thought to initiate the process by cleaving solitary arrested forks, we find that interaction of SLX4 with either MUS81 or SLX1 is dispensable for DSB-induced LTGC, i.e., there is no indication of a role for the mitotic SLX4 trinuclease complex. Instead, DSB-induced LTGC requires the SLX4 UBZ and MLR domains, implicating ubiquitin chain binding and the SLX4-XPF interaction in a mechanism that operates subsequent to DSB induction. We show here that this *Slx4*-dependent step, presumably involving XPF-mediated incisions, differentiates DSB-induced LTGC from DSB-induced STGC. The non-involvement of SLX4 in Tus/*Ter*-induced LTGC further underscores the idea that LTGC at stalled forks, which is Rad51-independent²⁷, is the product of a mechanism distinct from that of DSB-induced LTGC.

How SLX4 might promote DSB-induced LTGC is a matter of speculation. Both STGC and LTGC are initiated by Rad51-mediated strand invasion by one end of the DSB, forming a D-loop as a precursor to repair by SDSA^{30,31}. Both pathways will require a 3' flap endonuclease to

cleave non-homologous sequences from the invading strand. Perhaps the defects in I-SceI-induced STGC we observed in *Slx4*^{ΔMLR/-} and *Xpf*^{Nuc/-} cells, but not in *Slx4*^{ΔUBZ/-} cells, reflect a ubiquitin-binding-independent role for SLX4-XPF in this 3' flap-processing activity. DSB-induced LTGC might be specifically favored if synapsis or processing of the two ends of the DSB were impaired, or if normal maturation of the D-loop were disturbed⁵⁷. Efficient branch migration of the lagging edge of the D loop might normally promote nascent strand displacement, thereby supporting timely HR termination and STGC. Conceivably, SLX4-XPF-mediated incision of the trailing edge of the D-loop might disable the branch migration mechanism that normally promotes STGC. This disruption of D-loop processing might stabilize the nascent strand and thereby promote LTGC. We assume that such a role of SLX4-XPF in DSB-induced LTGC would be opportunistic and pathological, favoring chromosome rearrangements driven by LTGC and, potentially, BIR.

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Author Contributions

R.E., A.P., F.P.L., N.A.W., and E.E.D. conducted the experiments; R.E., A.S. and R.S. designed the experiments. R.E. and R.S wrote the manuscript.

Declaration of Interests

The authors declare no competing interests.

Figure Legends

Fig. 1. *Slx4* regulates error-free HR at Tus/*Ter* stalled forks. a. Schematic of 6x*Ter*-HR reporter and repair products of Tus/*Ter* induced fork stalling. Light blue boxes: mutant *GFP* alleles. Green box: wild type *GFP*. Open circles A and B: 5' and 3' artificial *RFP* exons, respectively. 5' Tr-*GFP*: 5'-truncated *GFP*. Red triangle: 6x*Ter* array. Black line: I-SceI restriction site. STGC/LTGC, short/long tract gene conversion outcomes. LTGC generates wild-type *RFP* through RNA splicing (red filled circles). **b.** Strategy for generation of *Slx4*^{Δ125} frameshift allele. Gel shows products of PCR of gDNA from *Slx4*^{+/+} and *Slx4*^{Δ125/Δ} clones. Red letters above sequencing chromatogram indicate frame shift. **c.** Proliferative competition assay in presence of Mitomycin C (MMC), measuring enrichment of GFP⁺ wild type vs. GFP⁻ test culture. Data, normalized to 0 ng/mL MMC, shows mean (n=3) and standard deviation. **d.** Representative raw FACS data (uncorrected for transfection efficiency) for one *Slx4*^{+/+} (#3) and one *Slx4*^{Δ125/Δ} (#40) 6x *Ter*-HR reporter clone co-transfected with either empty vector (EV), I-SceI or Tus expression vectors as shown. FACS plots produced from pooled technical duplicates, n=6. Numbers indicate percentages. **e** and **f.** Tus/*Ter*-induced STGC (**e**) and LTGC (**f**) in *Slx4*^{+/+} clones and *Slx4*^{Δ125/Δ} clones. **g** and **h.** I-SceI-induced STGC (**g**) and LTGC (**h**) in *Slx4*^{+/+} vs. *Slx4*^{Δ125/Δ} clones. Data shows mean values, n=6. Error bars: standard error of the mean (s.e.m). In this and all subsequent figures: *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001; ns, not significant.

Fig. 2. The SLX4 UBZ domain mediates Tus/Ter-induced STGC by retaining SLX4 at stalled forks. **a.** Schematic of SLX4 protein. MLR: XPF-interacting domain. SAP: MUS81-interacting domain. SBD: SLX1-interacting domain. **b.** Cell cycle distribution of *Slx4*^{+/-} and *Slx4*^{ΔUBZ/-} cells either untreated or treated with 20, 30, 40 or 50 ng/mL of MMC. Data shows mean values, n=3. Error bars: s.e.m. Untreated samples were compared using the Student's *t*-test; MMC-treated groups were compared by one-way ANOVA. **c** and **d.** Tus/*Ter*-induced STGC (**c**) and I-SceI induced STGC (**d**) in *Slx4*^{+/-} vs. *Slx4*^{ΔUBZ/-} clones. Analysis by ANOVA, n=5. Error bars: s.e.m. **e** and **f.** Impact of exogenous hSLX4 on Tus/*Ter* induced STGC (**e**) and I-SceI induced STGC (**f**) in two independent *Slx4*^{+/-} and two independent *Slx4*^{ΔUBZ/-} clones. EV: empty vector. 4C>A: hSLX4 with inactivated UBZ motifs. Analysis by Student's *t*-test, n=6. Error bars: s.e.m. **g.** Schematic of *Slx4*^{+/-} and *Slx4*^{ΔUBZ/-} tagged with a dual degron containing a C-terminal 8xHA tag. **h.** RT-qPCR analysis of *Slx4* mRNA in *Slx4*^{deg/-} and *Slx4*^{ΔUBZdeg/-} clones. Data normalized to *Gapdh* mRNA using the 2^{-ΔCT} method. Expression of *Slx4*^{ΔUBZdeg/-} normalized to *Slx4*^{deg/-}. Analysis by Student's *t*-test (n=3). Error bars: standard deviation (s.d.). **i.** Anti-HA immunoblot of HA-degron tagged SLX4, 24 hours after addition of degron-activating drugs 5-IAA and/or Asv. Asv: Asunaprevir. 5-IAA: 5-adamantyl-IAA. H3: Histone H3 loading control. **j.** Anti-HA ChIP analysis of SLX4-HA at Tus/*Ter* RFB in *Slx4*^{deg/-} and *Slx4*^{ΔUBZdeg/-} cells. Analysis by Student's *t*-test (n=3). Error bars: s.d.

Fig. 3. Interactions of SLX4 with MUS81 and with SLX1 are dispensable for Tus/Ter-induced HR. **a.** Schematic of SLX4 protein lacking the MUS81-interacting SAP domain. **b** and **c.** Proliferative competition assay in presence of MMC (**b**), Olaparib (**c**), showing enrichment of wild type GFP⁺ cells against the genotypes shown. Data show mean of values normalized to

untreated samples (n=3). Error bars: s.d. **d** and **e**. Tus/*Ter*-induced STGC (**d**) and I-SceI induced STGC (**e**) in *Slx4*^{+/-} vs. *Slx4*^{ΔSAP/-} clones. Analysis by ANOVA, n=5. Error bars: s.e.m. **f**. Schematic of SLX4 protein lacking the SLX1-interacting SBD domain. **g** and **h**. Proliferative competition assay in presence of MMC (**g**), Olaparib (**h**), showing enrichment of wild type GFP⁺ cells against the genotypes shown. Data show mean of values normalized to untreated samples (n=3). Error bars: s.d. (**i** and **j**). Tus/*Ter*-induced STGC (**i**) and I-SceI induced STGC (**j**) in *Slx4*^{+/-} vs. *Slx4*^{ΔSBD/-} clones. Analysis by ANOVA, n=5. Error bars: s.e.m.

Fig. 4. Loss of the SLX4-XPF interaction causes a characteristic FA phenotype **a**. Schematic of SLX4 protein lacking the XPF-interacting MLR domain. **b**. Cell cycle distribution of *Slx4*^{+/-} and *Slx4*^{ΔMLR/-} cells either untreated or treated with 20, 30, 40 or 50 ng/mL of MMC. Data shows mean values, n=3. Error bars: s.e.m. Untreated samples were compared using the Student's *t*-test; MMC-treated groups were compared by one-way ANOVA. **c**. Representative images of metaphase spreads from *Slx4* mutants indicated, showing chromatid breaks (black arrows) and radial chromosomes (black arrowheads) in untreated cells and cells treated with 20 ng/mL MMC for 12h (**d**) and (**e**). Quantitation of the number of breaks (**d**) and radial chromosomes (**e**) per metaphase nucleus in *Slx4*^{+/-}, *Slx4*^{ΔUBZ/-} and *Slx4*^{ΔMLR/-} cell lines.

Fig. 5. The SLX4-XPF interaction is required for Tus/*Ter*-induced STGC. **a** and **b** Tus/*Ter*-induced STGC (**a**) and I-SceI induced STGC (**b**) in *Slx4*^{+/-} vs. *Slx4*^{ΔMLR/-} clones. Analysis by ANOVA, n=4. Error bars: s.e.m. **c**. Schematic showing mutants of human *SLX4* used for transient expression experiments. **d**. RT-qPCR analysis of expression of human *SLX4* variants in mES cells. *SLX4* expression normalized to *Gapdh* and displayed as fold difference compared to

empty vector (EV) of the same experiment, n=3. **e** and **f**. Impact of hSLX4 variants on Tus/Ter-induced STGC (**e**) and I-SceI-induced STGC (**f**), following transient *SLX4* expression in *Slx4*^{+/-} and *Slx4*^{ΔUBZ/-} Ter-HR reporter clones.

Fig. 6. The XPF nuclease domain is required for Tus/Ter- and I-SceI-induced STGC. a. Schematic of wild type XPF protein and XPF mutant lacking the nuclease domain. **b.** Strategy for generating *Xpf*^{ΔNuc} allele. Red half-arrow heads: genotyping primers. Gel shows PCR products on gDNA from *Xpf*^{+/-} and *Xpf*^{ΔNuc/-} clones and sequencing chromatogram showing the breakpoints in the in-frame mutant (#6) **c** and **d**. Proliferative competition assay in presence of MMC (**c**) or Olaparib (**d**), showing enrichment of wild type GFP⁺ cells against the genotypes shown. Data show mean of values normalized to untreated samples (n=3). Error bars: s.d. **e.** Representative images of metaphase spreads showing chromatid breaks (black arrows) and radial chromosomes (black arrowheads) from indicated *Xpf* mutant cells, either untreated or treated with 20 ng/mL MMC for 12h. **f** and **g**. Quantitation of the number of chromatid breaks (**f**) radial chromosomes (**g**) per metaphase in *Xpf*^{+/-} (#6) and *Xpf*^{ΔNuc/-} (#1) clones. **h** and **i**. Tus/Ter-induced STGC (**h**) and I-SceI induced STGC (**i**) in *Xpf*^{+/-} vs. *Xpf*^{ΔNuc/-} clones. Analysis by ANOVA, n=6. Error bars: s.e.m.

Fig. 7. Model of SLX4 action in Tus/Ter-induced HR. a. In wild type cells, FA pathway activation and asymmetric fork reversal at forks bidirectionally stalled at Tus/Ter RFB enables SLX4 recruitment and its retention at the stall site, mediated by interactions of the SLX4 UBZ domain with as yet unidentified ubiquitinated components at the stall site. SLX4 MLR domain enables recruitment of XPF-ERCC1, which incises the stalled leading strand of at least one sister

chromatid (incision *a*, red triangle). Note that the second incision (red triangle *b*), which would be required for ICL unhooking, may be redundant for generation of a DSB at Tus/*Ter*, since there is no ICL. The two-ended DSB generated by SLX4-XPF is repaired by conservative HR (STGC). **b.** In cells lacking the SLX4 UBZ domain, retention of SLX4-XPF at the stall site is impaired, leading to inefficient incision of the stalled forks and reduced DSB formation. There is a corresponding defect in Tus/*Ter*-induced STGC. **c.** In cells lacking the SLX4 MLR domain, or lacking the XPF nuclease domain, XPF-mediated incision of the stalled forks is abolished, resulting in a severe defect in Tus/*Ter*-induced STGC.

Methods

Molecular biology and sgRNA oligos

The 6x*Ter*-HR reporters used were assembled using standard cloning methods described previously for the 6x*Ter*-HR reporter^{27,28}. Derivation of Tus and I-SceI expression plasmid are described in^{27,59}. *Ter*-containing plasmids were amplified in JJC33 (Tus⁻) strains of *E. coli*. All plasmids used for transfection were prepared by endotoxin-free maxiprep (QIAGEN Sciences). All primers used for genotyping, RT-qPCR, sgRNA synthesis, and chromatin immunoprecipitation were purchased from Life Technologies.

Generation of mouse *Slx4* and *Xpf* mutant cell lines and cell culture

Slx4 mutant cell lines were derived from a conditional mouse *Brca1*^{fl/exon11} *Ter*-HR reporter ES cell line²⁷. This founder cell line contains a single copy of the 6x *Ter*-HR reporter cassette, targeted to the *Rosa26* locus and verified by southern blot. Mouse ES cells are routinely thawed

657 onto plates coated with mouse embryonic fibroblast (MEF) feeders, maintained in ES medium on
 658 gelatinized plates, and regularly tested for mycoplasma infection by Myco-Alert assay (Lonza).
 659 *Slx4* mutant cell lines were generated using CRISPR/Cas9 mediated mutation of the *Slx4* locus.
 660 sgRNAs targeting Cas9 to the *Slx4* locus were transcribed *in vitro* using the Engen sgRNA
 661 Synthesis kit (New England Biolabs E3322S), purified using the RNA Clean and Concentrator
 662 Kit (Zymo Research, R1017), and verified by denaturing 10% TBE-urea PAGE. Cas9-sgRNA
 663 RNP was pre-assembled *in vitro* in OptiMEM by mixing Spy NLS Cas9 (New England Biolabs,
 664 M0646T) and purified sgRNA. Cells were co-transfected with either 0.45 µg Cas9(1.1)
 665 expression plasmid and 0.05 µg of each purified sgRNA using Lipofectamine 2000 (Invitrogen).
 666 After 72 hr, transfected cells were plated onto 6-cm dishes containing feeder MEFs without
 667 selection. Individual clones were picked for expansion between 9 and 14 days later and *Slx4*
 668 mutant clones were identified by PCR and confirmed by sequencing. *Slx4* targeting sgRNA
 669 sequences including PAM: *Slx4*^{Δ125/Δ} 5' exon 2 (5' CCT TCC TGT TTC ACG ACA ACT GG
 670 3'), *Slx4*^{Δ125/Δ} 3' exon 2 (5' ATC CCC CCA AAA GAC TGC ACT GG 3'), *Slx4* hemizygote
 671 exon 2 (5' ATC CCC CCA AAA GAC TGC ACT GG 3'), *Slx4* hemizygote exon 15 (5' ATC
 672 CTT TTT CTT TCG GC CTG AGG 3'); *Slx4* ΔUBZ 5' (5' ATG ACA GCC TGG AGG AGA
 673 AGG GG 3'), *Slx4* ΔUBZ 3' (5' ACA GCC TGA AGC AGG AGT TGG GG 3'); *Slx4* ΔMLR 5'
 674 (5' CCA AAA GGG AGC CAA GGA GGA GG 3'), *Slx4* ΔMLR 3' (5' GGG CAT TGG GAC
 675 ACA ATT GGA GG 3'); *Slx4* ΔSAP 5' (5' CCA GAA CTC GGA AGC CAG ACA GG 3'), *Slx4*
 676 ΔSAP 3' (5' GGT AGT TCT GCG GGT ATC TGG GG 3'); *Slx4* ΔSBD 5' (5' CGC AGA ACT
 677 ACC TTG TCG CCA GG 3'), *Slx4* ΔSBD 3' (5' GAA AAA GGA TCA GAA ATG ACA GG
 678 3'); sgRNA targeting *Slx4* dual degron (5' ATC CTT TTT CTT TCG GCC TGA GG 3'); *Xpf*
 679 hemizygote 5' UTR (5' GTG CGG CCG CGG TCC GAA GGG 3'), *Xpf* hemizygote exon 11 (5'

CAA ACA GCT CGG CTG TGG CGT GG 3'); *Xpf*^{Nuc/-} 5' (5' CTC TGC CTT CCC GTT CTT
CGG GG 3'), *Xpf*^{Nuc/-} 3' (5' ACC AGA GCA GCC GCA AAC GCG GG 3').

Recombination assays

These assays were performed as described in detail in ⁷⁶. Briefly, 1.6 x 10⁵ cells were co-transfected in suspension with 0.5 mg empty vector, pcDNA3b-myc NLS-Tus, or pcDNA3b-myc NLS-I-SceI using Lipofectamine 2000 (Invitrogen). GFP+RFP-, GFP+RFP+ and GFP-RFP+ frequencies were scored 72 hours after transfection by flow cytometry Beckman Coulter CytoFlex LX. For each duplicate sample condition, 3-6 x 10⁵ total events were scored. Repair frequencies presented are corrected for background events and for transfection efficiency (50-85%). Transfection efficiency was measured by parallel transfection with 0.05 mg wild type GFP expression vector and 0.45 mg empty vector. For transient hSLX4 rescue experiments, 1.6 x 10⁵ cells were co-transfected in suspension with 0.4 mg empty vector, pcDNA3b-myc NLS-Tus ²⁷, or pcDNA3b-myc NLS-I-SceI ⁵⁹, and either 0.1 mg empty vector and pHIV- 3xFLAG-AttB1-hSlx4-AttB2- NatR-T2A-hCD52 and all other Slx4 domain mutant derivatives using Lipofectamine 2000.

Competition assays

For all competition experiments, 1.6 x 10⁵ cells were co-transfected in suspension with 0.45 mg empty vector and either 0.05 mg empty vector or 0.05 mg GFP-expression plasmid using Lipofectamine 2000 (Invitrogen). 18 hours after transfection, cells were counted, mixed 4:1,

uncolored vs. GFP+ marked cells, and 5×10^4 cells plated in triplicate. 6 hours after cell plating growth medium was replaced with media containing MMC (Millipore Sigma Cat#475820-10MG). After two days incubation, GFP+ frequencies were scored on a Beckman Coulter CytoFlex LX. Fold enrichment of cultures transiently co-transfected with GFP-expression plasmid normalized to 0 mg/mL MMC control. Plots represent the mean of triplicate samples from three independent experiments (n=3).

RT-qPCR

RNA extraction was performed using QIAGEN RNeasy Mini Kit (QIAGEN Sciences,). All analyses of *Gapdh* and genes of interest were performed using an Applied Biosystems 7300 Real time PCR System using Power SYBR Green RNA-to CTTM 1-Step Kit (Applied Biosystems, Foster City, CA). Primers for RT-PCR: *Gapdh* sense (5' CGT CCC GTA GAC AAA ATG GT 3'); *Gapdh* antisense (5' TCG TTG ATG GCA ACA ATC TC 3'); *Slx4 exon2 frameshift* sense (5' CAG AGA CAC CAA AAG GTG CTA ACC 3); *Slx4 exon2 frameshift* antisense (5' GCA CGG ACT CCA AAC CTG TCC AGT 3'); *Slx4 hemizygote* sense (5' CAG AGA CAC CAA AAG GTG CTA ACC 3'); *Slx4 hemizygote* antisense (5' GCA CGG ACT CCA AAC CTG TCC AGT 3'); *Slx4-UBZ* sense (5' GCA ACA TGT GAA CAG ATG CCT); *Slx4-UBZ* antisense (5' TTG GTG GTG AGG AAC GGT TT 3'); *Slx4-MLR* sense (5' ACC ACC AAA AGG GAG CCA AG 3'); *Slx4-MLR* antisense (5' CTT GCG ACT TTT CTT CTC TGC T 3'); *Slx4-SAP* sense (5' CAG AGA CAC CAA AAG GTG CTA ACC 3'); *Slx4-SAP* antisense (5' GCA CGG ACT CCA AAC CTG TCC AGT 3'); *Slx4-SBD* sense (5' CTG CAA CCC TTC AAG GTT GCC AAC 3'); *Slx4-SBD* antisense (5' GAG GAG CTT TTA GAG CTA AAG GAG 3'), *Xpf* hemizygote sense (5' GGG AAA CCT CTG AGG GTT TAC TTT 3'), *Xpf* hemizygote antisense

726 (5' CTA GCT TTT TCT CTT ATG AGC TTT 3'), *Xpf* helicase sense (5' GTC AGA ATT CAG
 727 GTT GGC TTT TCC 3'); *Xpf* helicase antisense-(5' TTG TTT CTT GAA CTT CTG GAC TCG
 728 3'), *Xpf* nuclease sense (5' GAA AAG CTC ATA AGA GAA AAA GCT 3'); *Xpf* nuclease
 729 antisense-(5' CTC CTG GCC ACC GGC TTT CCG AGT 3'); *Xpf* Hhh2 sense (5' GAA GAT
 730 GCC TGG GGT CAA CGC CAA 3'); *Xpf* Hhh2 antisense-(5' AGG AAG TCG TGC AGC
 731 TGC TTG GCA T 3'). N-terminal FLAG tagged human SLX4 sense (5' ATG TGA ACA GGT
 732 GCT TGG ATG AAG 3'), antisense (5' ATC ACT GAA GCT GAA CAT GGG TGG 3').
 733 mRNA levels were measured in technical triplicates. Target gene expression level was
 734 normalized to *Gapdh* using the $2^{-\Delta CT}$ method and the mutants were normalized to the
 735 corresponding wild-type.

736

737 **Isolation of Chromatin-bound Slx4 and ERCC1 western blot**

738 ~10x10⁶ cells were freshly harvested and washed with 1x PBS and lysed in 5 volumes of chilled
 739 A1 buffer (50mM HEPES, 140mM NaCl, 1mMEDTA, 10%glycerol, 0.5% NP-40, 0.25% TritonX-
 740 100, 1mM DTT, and 1x protease inhibitor). Each lysate was centrifuged at 1,100 x g at 4 °C for 2
 741 min and the supernatant was discarded. Pellets were resuspended by gentle pipetting in A1 buffer
 742 and samples were incubated for 10 minutes on ice. Each suspension was centrifuged at 1,100 x g
 743 at 4 °C for 2 min and the supernatant again discarded. Pellets were resuspended by gentle
 744 pipetting in 2 volumes of ice cold E2 buffer (10mM Tris-HCl pH-8, 200mMNaCl, 1mM EDTA
 745 and 0.5mM EGTA, and 1x Protease inhibitor). Each suspension was centrifuged at 1,100 x g at 4
 746 °C for 2 min and the supernatant discarded. Pellets were resuspended by gentle pipetting with A1
 747 buffer and incubated for 10 minutes on ice. Each suspension was centrifuged at 1,100 x g at 4 °C
 748 for 2 min and the supernatant discarded. Pellets were resuspended by gentle pipetting in 2

volumes of ice cold E3 buffer (500mM Tris-HCl pH-6.8, 500mMNacl and 1x Protease inhibitor. Each sample was sonicated in a water bath using Diagenode Bioruptor 300 with attached 4°C chiller cycling 30 sec on and 30 sec off on high setting for 5 min. Sonicated samples were centrifuged at 16,000xg for 15 min at 4°C. The supernatant corresponding to the chromatin fraction was transferred and subjected for western blot analysis using following antibodies; anti-HA (Abcam ab9110, 1:1000), anti-ERCC1 (Santacruz sc-17809, 1:250), Histone H3 (Abcam ab1791, 1:3000).

Chromatin immunoprecipitation Assay

Chromatin immunoprecipitation was performed as described previously ⁷⁷. Transfection of 2.0 x 10⁵ *Ter*-HR reporter cells of the genotypes noted was performed with 0.5 µg empty vector, pcDNA3β-MYC NLS-I-SceI or pcDNA3β-MYC NLS-Tus-F140A-3xFlag using Lipofectamine 2000 (Invitrogen). 10 million cells were collected 24 hours after transfection and fixed in 1% formaldehyde supplemented serum free media. Fixation was quenched by addition of glycine to 125 mM. Cells were lysed in lysis buffer (0.1% SDS, 20 mM EDTA, 50 mM Tris pH 8.1) containing protease inhibitor (Roche protease inhibitor, Roche 13539320). Chromatin shearing to ~500 bp was accomplished using Diagenode Bioruptor 300 with attached 4°C chiller for 20 cycles, 15 seconds on and 30 seconds off at medium setting. To avoid non-specific binding to protein A/G beads, 100 µl lysates for each ChIP sample were precleared by the addition of 10 µl activated Magna ChIP magnetic beads (Millipore Sigma, 16-663) in ChIP dilution buffer (1% Triton-X-100, 2mM EDTA, 150mM NaCl, 20mM Tris pH 8.1) and incubation for 1 hr at 4°C with gentle mixing. After removal of beads by magnet, for each immunoprecipitation, 7 µl of anti-HA (Abcam ab9110) was added and mixed for 12 hours at 4°C followed by addition of 10 µl

activated Magna ChIP beads and mixing for 16 hours at 4°C. Beads were washed six times in ice-cold ChIP RIPA buffer (50mM HEPES pH 7.6, 1mM EDTA, 7 mg/mL sodium deoxycholate, 1% NP-40) followed by two washes in ice-cold TE (10 mM Tris pH8.0, 1 mM EDTA). Crosslinks were reversed and DNA was eluted by incubation in 100 µL Elution buffer (1% SDS, 200mM sodium bicarbonate, 5.6 µg/mL RNase A) overnight at 65°C. Protein was removed by proteinase K digest for 30 min at 55°C. Released DNA was purified by Qiagen PCR Purification column (Qiagen, 28106) and analyzed by qPCR on an ABI Prism 7300 or QuantStudio3 using SYBR Green (Applied Biosystems, 4368702). Primers for qPCR: +109 bp sense (TCC GGA TAG GGA TAA CAG GGT A); +109 bp antisense (GTC GGC CAT GAT ATA GAC GTT G); 128 bp sense (GAG CGC ACC ATC TTC TTC A); 128 bp antisense (TCC CTA CGA TGC CCT TCA). Data are presented as the mean calculated from three independent experiments (n = 3) normalized against untreated controls (empty vector) and control locus (beta-actin) using the $2^{-\Delta CT}$ method ⁷⁸.

Cell Cycle Analysis

ES cells were grown on a 6-well plate until ~70% confluent. Cells were treated with varying concentrations of MMC for 12 h and compared with untreated controls. After 12h, cells were pulsed with 10 µM BrdU for 15 min. Harvested cells were then washed with pre-chilled 1x PBS pH 7.0 by gently pipetting it and spun at 1000 rpm for 5 mins. Supernatant was discarded and the pellet was gently resuspended in 50µl PBS. Cells were fixed by adding 5mL of chilled 70% Ethanol in a dropwise manner while gently but constantly vortexing to prevent clumping. Fixed cells are stored at 4°C in the dark. Before immunostaining for BrdU, cells were spun at 1000rpm for 5 mins to remove the ethanol. While gently vortexing, 0.5mL of 2M HCl, 0.5% Triton-X-100

795 solution was added in a dropwise manner to denature DNA. Cells were incubated at RT for 30
796 mins, with intermittently vortexing approximately every 10 mins. Excess acid was removed by
797 spinning and cells were neutralized using 1mL 0.1M Sodium Borate Decahydrate. Once the
798 neutralizing solution is removed, resuspend pellet in 50-100µl of 1xPBS containing 1%BSA.
799 BrdU was counterstained using anti-BrdU antibody (Abcam ab8039, 1:100) and cells were
800 incubated at RT for 30 mins in the dark. Cells were then washed with 5mL chilled 1xPBS
801 containing 20mM HEPES pH 7.4, 0.5% tween-20 to remove excess antibody. A secondary
802 FITC-conjugated rabbit anti-mouse antibody (Jackson Immunoresearch, 115-095-146-1:50) was
803 used and cells were incubated at RT for 30 mins in the dark. Cells were then washed with 5mL
804 chilled 1x PBS containing 20mM HEPES pH 7.4, 0.5% tween-20 to remove secondary antibody.
805 Cells were resuspended with 300µl of solution containing 38mM sodium citrate, 69µM
806 propidium iodide, 5µl/mL RNaseA in the dark for 15 mins at 37°C. Approximately 10⁴ events
807 were acquired using Cytoflex LX and the results were analyzed using FloJo software.

808

809 **Chromosomal breakage assay**

810 ~ 3x10⁶ adherent cells were either untreated or treated with 0.02µg/mL MMC for 12h. Cells
811 were arrested with colcemid (0.17 µg per ml of media) for 20 min. Cells were harvested and
812 incubated at 37°C in 0.075 M KCl for 10 mins and fixed in freshly prepared methanol:glacial
813 acidic acid (3:1 vol/vol). Cells were stored at 4°C at least overnight. Cells were dropped onto wet
814 slides and air dried at 40°C for 60 min before staining with KaryoMAX Giemsa (Invitrogen)
815 Gurr Buffer for 3 min. After rinsing with fresh Gurr Buffer followed by distilled water, the slides
816 were fully dried at 40 °C for 60 min and scanned using the Metasystems Metafer application.

The experiments were randomized and breaks, and radial chromosomes were scored by a person blinded to the identity of the samples.

Statistical methods

Data shown represents the arithmetic mean and error bars represent either the standard error of the mean (s.e.m.) or standard deviation (s.d.) of between three ($n = 3$) and nine ($n = 9$) independent experiments (as noted in each Figure Legend). Data points for each independent experiment were typically collected as the mean of technical duplicates. This mean value was taken as the solitary data point for that individual experiment. The arithmetic mean of samples collected for groups of independent experiments for repair frequency statistical analysis, was calculated and data points for each independent experiment used to calculate the mean and s.e.m., calculated as standard deviation/ $\sqrt{n}$, (n indicates the number of independent experiments). Differences between sample pairs repair frequencies and fold enrichment for ChIP were analyzed by Student's two-tailed unpaired t -test, assuming unequal variance. One-way ANOVA statistical analysis of greater than three samples was performed when indicated. P-values are indicated in the figure and the P-value code for each figure is as written in the **Fig. 1** legend. No statistical methods were used to predetermine sample size. The experiments were not randomized, and investigators were not blinded to allocation during experiments and outcome assessment. All statistics were performed using GraphPad Prism v7.0d software.

Supplementary Figure Legends

Supplementary Fig. 1. Characterization of *Slx4*^{Δ125/Δ} cells. **a.** RT qPCR analysis of *Slx4* mRNA normalized to *Gapdh* mRNA using the 2^{-ΔCT} method in three independent experiments (n=3). Error bars, standard deviation (s.d.). **b** and **c.** Ratio of Tus/Ter-induced (**b**) and I-SceI-induced (**c**) LTGC: total HR in *Slx4*^{+/+} clones and *Slx4*^{Δ125/Δ} clones. **d.** CRISPR/Cas9 with dual sgRNA targeting to generate *Slx4*^{+/-} hemizygous cells. The *Slx4*⁻ allele contains a 19.3kb deletion between exons 2 and 15. Red half arrowheads: PCR and sequencing primers specific to Exons 2 and 15. Predicted PCR product sizes for *Slx4*⁺ (wild type) allele shown. **e.** Gel shows PCR products detected using gDNA from *Slx4*^{+/+} and *Slx4*^{+/-} clones. Note the 444 bp PCR product formed across the 19.3kb deletion between exons 2 and 15 in *Slx4*^{+/-} cells. **f.** DNA sequencing chromatogram of PCR products from (**e**). **g.** RT qPCR analysis of *Slx4* mRNA in *Slx4*^{+/+} and *Slx4*^{+/-} cells. Data shows mean of three independent experiments (n=3) normalized to *Gapdh* mRNA using the 2^{-ΔΔCT} method and analyzed by Student's *t*-test. Error bars: s.d.. **h.** Tus/Ter-induced and I-SceI-induced repair frequencies in *Slx4*^{+/+} and *Slx4*^{+/-} cells. In this and all subsequent Supplemental Figures: **p* < 0.05; ***p* < 0.01; ****p* < 0.001; *****p* < 0.0001; ns, not significant.

Supplementary Fig. 2. The SLX4 UBZ domain mediates I-SceI-induced LTGC. **a.** Strategy for in-frame deletion of UBZ domain-encoding regions of *Slx4*. Red half-arrow heads: genotyping PCR primers. Gel shows PCR products using gDNA from *Slx4*^{+/-} and *Slx4*^{ΔUBZ/-} clones. Sequencing chromatogram shows in-frame breakpoint of *Slx4*^{ΔUBZ} allele. **b.** RT-qPCR analysis of mRNA encoding UBZ, MLR, SAP and SBD domains in *Slx4*^{+/-} and *Slx4*^{ΔUBZ/-} clones. Data normalized to *Gapdh* mRNA using the 2^{-ΔCT} method, mRNA expression in *Slx4*^{ΔUBZ/-} samples were normalized to *Slx4*^{+/-} of the same experiment and analyzed by Student's *t*-test

862 (n=3). Error bars: standard deviation. P=0.0125 for mRNA expression of the UBZ region in
 863 *Slx4*^{+/-} compared to *Slx4*^{ΔUBZ/-}. No significant differences were observed in expression levels of
 864 all other domains. **c** and **d**. Proliferative competition assay in presence of MMC (**c**) or Olaparib
 865 (**d**), showing enrichment of wild type GFP⁺ cells against the genotypes shown. Data show mean
 866 of values normalized to untreated samples (n=3). Error bars: s.d. **e**. RT-qPCR analysis of N-
 867 terminal Flag-tagged wild-type full-length human SLX4 and UBZ 4C>A expression plasmids
 868 48h after transfection. Data normalized to *Gapdh* mRNA using the 2^{-ΔCT} method and compared
 869 to empty vector (EV) control. Error bar: s.d. **f** and **g**. Tus/*Ter*-induced LTGC (**f**) and LTGC/Total
 870 HR ratio (**g**) in *Slx4*^{+/-} and *Slx4*^{ΔUBZ/-} clones. Analysis by ANOVA, n=5. Error bars: s.e.m. **h**.
 871 Impact of exogenous hSLX4 on Tus/*Ter*-induced LTGC in two independent *Slx4*^{+/-} and two
 872 independent *Slx4*^{ΔUBZ/-} clones. EV: empty vector. 4C>A: hSLX4 with inactivated UBZ motifs.
 873 Analysis by Student's *t*-test, n=6. Error bars: s.e.m. **i** and **j**. I-SceI-induced LTGC (**i**) and
 874 LTGC/Total HR ratio (**j**) in *Slx4*^{+/-} and *Slx4*^{ΔUBZ/-} clones. Analysis by ANOVA, n=5. Error bars:
 875 s.e.m. **k**. Impact of exogenous hSLX4 on I-SceI-induced LTGC in two independent *Slx4*^{+/-} and
 876 two independent *Slx4*^{ΔUBZ/-} clones. EV: empty vector. 4C>A: hSLX4 with inactivated UBZ
 877 motifs. Analysis by Student's *t*-test, n=6. Error bars: s.e.m.
 878 .

879 **Supplementary Fig. 3. Characterization of *Slx4*^{ΔSAP/-} and *Slx4*^{ΔSBD/-} mutant clones. a.**

880 Strategy for in-frame deletion of SAP domain-encoding region of *Slx4*. Red half-arrow heads:
 881 genotyping PCR primers. Gels show PCR products using gDNA from *Slx4*^{+/-} and *Slx4*^{ΔSAP/-}
 882 clones. Sequencing chromatogram shows in-frame breakpoint of *Slx4*^{ΔSAP} allele. **b**. RT-qPCR
 883 analysis of UBZ, MLR, SAP and SBD domains encoding mRNA in *Slx4*^{+/-} and *Slx4*^{ΔSAP/-} clones.
 884 Data normalized to *Gapdh* mRNA using the 2^{-ΔCT} method, mRNA expression in *Slx4*^{ΔSAP/-} were

885 normalized to *Slx4*^{+/-} (n=3) of the same experiment and analyzed by Student's *t*-test. Error bars:
886 s.d. P=0.0478 for mRNA expression of the SAP region in *Slx4*^{+/-} compared to *Slx4*^{ΔSAP/-}. No
887 significant differences were observed in expression levels of all other domains. **c** and **d**. Tus/*Ter*-
888 induced LTGC (**c**) and I-SceI-induced LTGC (**d**) in *Slx4*^{+/-} and *Slx4*^{ΔSAP/-} clones. Analysis by
889 ANOVA, n=5. Error bars: s.e.m. **e**. Strategy for in-frame deletion of SBD domain-encoding
890 region of *Slx4*. Red half-arrow heads: genotyping PCR primers. Gels show PCR products using
891 gDNA from *Slx4*^{+/-} and *Slx4*^{ΔSBD/-} clones. Sequencing chromatogram shows in-frame breakpoint
892 of *Slx4*^{ΔSBD} allele. **f**. RT-qPCR analysis of UBZ, MLR, SAP and SBD domains encoding mRNA
893 in *Slx4*^{+/-} and *Slx4*^{ΔSBD/-} clones. Data normalized to *Gapdh* mRNA using the 2^{-ΔCT} method,
894 mRNA expression in *Slx4*^{ΔSBD/-} was normalized to *Slx4*^{+/-} of the same experiment and analyzed
895 by Student's *t*-test (n=3). Error bars: standard deviation. P=0.048 for mRNA expression of the
896 SAP region in *Slx4*^{+/-} compared to *Slx4*^{ΔSBD/-}. No significant differences were observed in
897 expression levels of all other domains. **g** and **h**. Tus/*Ter*-induced LTGC (**c**) and I-SceI-induced
898 LTGC (**d**) in *Slx4*^{+/-} and *Slx4*^{ΔSBD/-} clones. Analysis by ANOVA, n=5. Error bars: s.e.m.

900 **Supplementary Fig. 4. Characterization of *Slx4*^{ΔMLR/-} mutant clones.** **a**. Strategy for in-frame
901 deletion of MLR domain-encoding region of *Slx4*. Red half-arrow heads: genotyping PCR
902 primers. Gels show PCR products using gDNA from *Slx4*^{+/-} and *Slx4*^{ΔMLR/-} clones. Sequencing
903 chromatogram shows in-frame breakpoint of *Slx4*^{ΔMLR} allele. **b**. RT-qPCR analysis of UBZ,
904 MLR, SAP and SBD domains encoding mRNA in *Slx4*^{+/-} and *Slx4*^{ΔMLR/-} clones. Data normalized
905 to *Gapdh* mRNA using the 2^{-ΔCT} method, mRNA expression in *Slx4*^{ΔMLR/-} were normalized to
906 *Slx4*^{+/-} of the same experiment and analyzed by Student's *t*-test (n=3). Error bars: standard
907 deviation (s.d.). P=0.01 for mRNA expression of the MLR region in *Slx4*^{+/-} compared to

Slx4^{ΔMLR/-}. No significant differences were observed in expression levels of all other domains. **c** and **d**. Proliferative competition assay in presence of MMC (**c**) or Olaparib (**d**), showing enrichment of wild type GFP⁺ cells against the genotypes shown. Data show mean of values normalized to untreated samples (n=3). Error bars: s.d. **e**. Representative images of metaphase spreads showing chromatid breaks (black arrows) and radial chromosomes (black arrowheads) in cells either untreated or treated with 20 ng/mL MMC for 12h in *Slx4*^{ΔSAP/-} and *Slx4*^{ΔSBD/-} cells. **f** and **g**. Quantitation of the number of breaks (**f**) and radial chromosomes (**g**) per metaphase nucleus in *Slx4* mutant cells shown.

Supplementary Fig. 5. The SLX4 MLR domain contributes to I-SceI-induced LTGC. **a** and **b**. Tus/*Ter*-induced LTGC (**a**) and I-SceI-induced LTGC (**b**) in *Slx4*^{+/-} and *Slx4*^{ΔMLR/-} clones. Analysis by ANOVA, n=5. Error bars: s.e.m. **c** and **d**. Impact of exogenous hSLX4 alleles (see **Fig. 5c**) on Tus/*Ter*-induced LTGC (**c**) and on I-SceI-induced LTGC (**d**) in *Slx4*^{+/-} and *Slx4*^{ΔUBZ/-} clones. EV: empty vector. 4C>A: hSLX4 with inactivated UBZ motifs. Analysis by Student's *t*-test, n=6. Error bars: s.e.m.

Supplementary Fig. 6. Characterization of *Xpf*^{+/-} and *Xpf*^{ΔNuc/-} cells. **a**. CRISPR/Cas9 with dual sgRNA strategy for generation of *Xpf*^{+/-} hemizygous cells. The *Xpf*⁺ allele contains a 42.3 kb deletion between the 5'UTR and exon 11. Red half arrowheads: PCR and sequencing primers specific to 5'UTR and Exon 11. Predicted PCR product sizes for *Xpf*⁺ (wild type) allele shown. **b**. Gel shows PCR products using gDNA from *Xpf*^{+/+} and *Xpf*^{+/-} clones. Note the 440 bp PCR product formed across the 42.3kb deletion between the 5'UTR and exon 11 in *Xpf*^{+/-} cells. **c**. Sequencing chromatogram of PCR products from (**b**), showing breakpoint sequence of *Xpf*⁺

allele and intact sgRNA-targeted sequences of *Xpf⁺* allele. Underlined base in the *Xpf⁺* sequence denotes a single base-pair insertion at the breakpoint generated during CRISPR/Cas9 treatment.

d. RT qPCR analysis of mRNA in *Xpf^{+/+}* and *Xpf^{+/-}* cells. Data shows mean of three independent experiments (n=3) normalized to *Gapdh* mRNA using the $2^{-\Delta\Delta CT}$ method and analyzed by Student's *t*-test. Error bars: s.d. **e.** Tus/*Ter*-induced and I-SceI-induced HR frequencies in *Xpf^{+/+}* or *Xpf^{+/-}* clones. Analysis by Student's *t*-test, n=4. Error bars: s.e.m. **f.** RT-qPCR analysis of mRNA encoding the XPF helicase, nuclease and Hh2h domains in 4 isogenic *Xpf^{+/-}* clones and 4 *Xpf^{ΔNuc/-}* clones. Data normalized to *Gapdh* mRNA using the $2^{-\Delta CT}$ method, mRNA expression in *Xpf^{ΔNuc/-}* was normalized to *Xpf^{+/-}* of the same experiment and analyzed by Student's *t*-test (n=3). Error bars: s.d. P=0.0153 for mRNA expression of the Nuclease region in *Xpf^{+/-}* compared to *Xpf^{ΔNuc/-}*. No significant differences were observed in expression levels of all other domains. **g.** Western blot analysis of ERCC1 in whole cell extract (WCE). *: non-specific band. β-tubulin: Loading control. **h.** Western blot analysis of ERCC1 in chromatin fraction of *Xpf^{+/-}* and *Xpf^{ΔNuc/-}* clones. H3: Histone H3 loading control. Note presence of ERCC1 signal in in-frame deleted *Xpf^{ΔNuc/-}* clone #1, and absence of ERCC1 signal in frame-shifted *Xpf^{ΔNuc/-}* clones #2, 11 and 13 **i.** Cell cycle distribution of *Xpf^{+/-}* clone #6 and *Xpf^{ΔNuc/-}* clone #1, either untreated or treated with 20, 30, 40 or 50 ng/mL of MMC. Data shows mean values, n=3. Error bars: s.e.m. Untreated samples were compared using the Student's *t*-test; MMC-treated groups were compared by one-way ANOVA. **j** and **k.** Tus/*Ter*-induced LTGC (**j**) and I-SceI induced LTGC (**k**) in *Xpf^{+/-}* and *Xpf^{ΔNuc/-}* clones. Analysis by ANOVA, n=6. Error bars: s.e.m.

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