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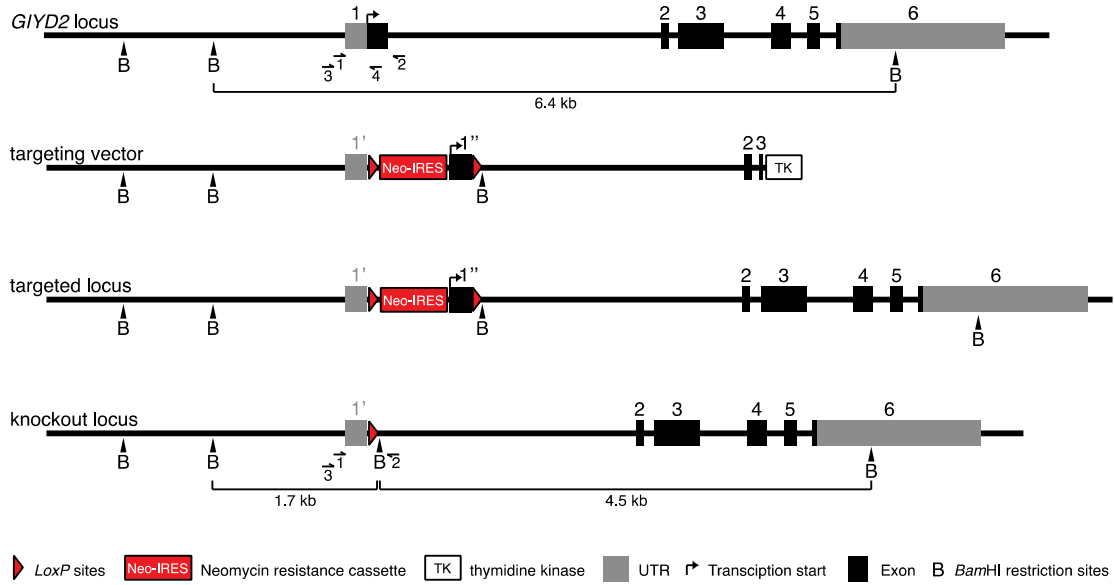
Supplemental Information

Cooperative Control of Holliday Junction Resolution and DNA Repair by the SLX1 and MUS81-EME1 Nucleases

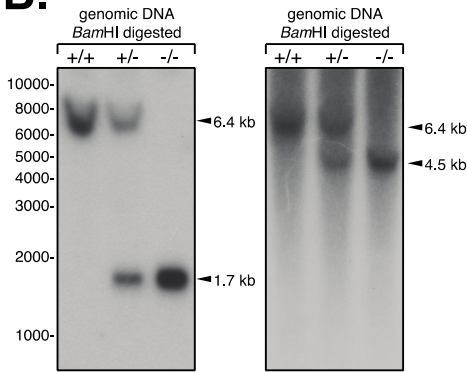
Dennis Castor, Nidhi Nair, Anne-Cécile Déclais, Christophe Lachaud, Rachel Toth, Thomas J. Macartney, David M.J. Lilley, J. Simon C. Arthur, and John Rouse

Castor_Fig.S1

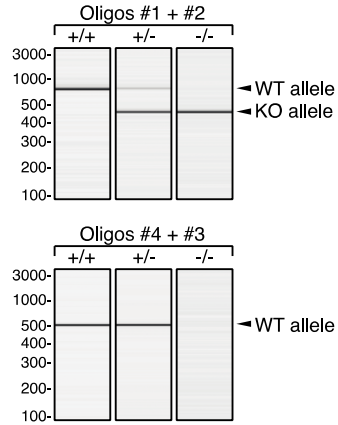
A.



B.



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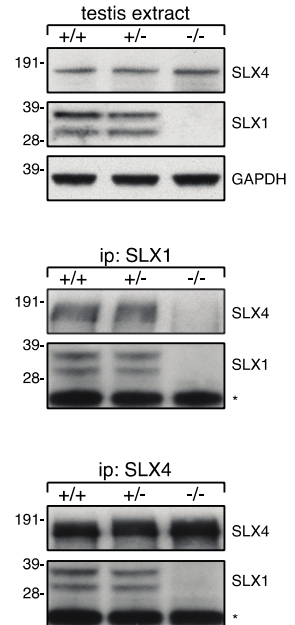
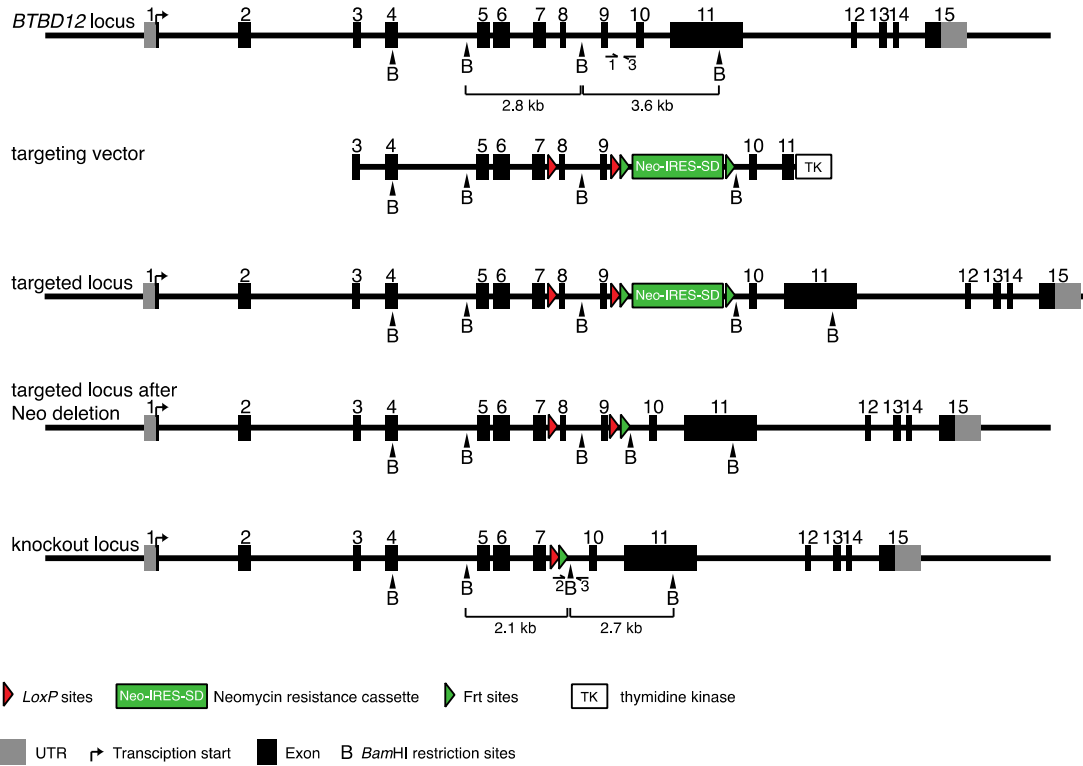


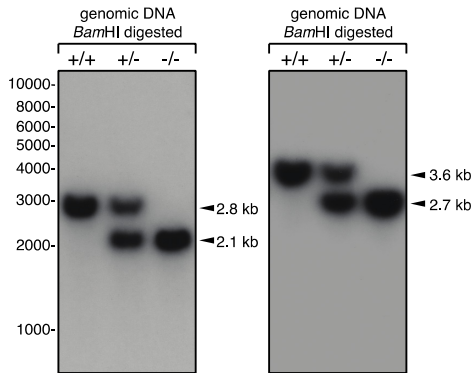
Figure S1: Disruption of the murine *Giyd2/Slx1* gene

(A) Disruption strategy. Exon 1 of *Giyd2/Slx1* comprises 5' UTR, the start ATG and some coding sequence. A neomycin resistance marker was inserted between the 5'UTR and the start codon with *LoxP* sites 5' of the neomycin marker and 3' of exon 1. The neomycin marker was deleted by crossing mice to mice expressing CRE recombinase in all tissues to generate *Slx1*^{+/-} mice. (See also Table S4) **(B)** Southern blot analysis of genomic DNA from wild-type, heterozygous and knockout MEFs confirming the deletion of the coding sequence of exon 1. **(C)** Biopsies for genotyping were analyzed by PCR with the oligonucleotides indicated in (A). **(D)** Western blot and co-immunoprecipitation analysis of testis extracts from mice of the genotypes indicated. *, IgG light chain.

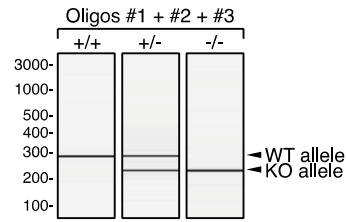
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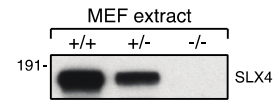


Figure S2: Disruption of the murine *Btbd12/Slx4* gene

(A) Disruption strategy. In the targeting vector, exons 8 and 9 of *Slx4* were flanked by *LoxP* sites followed immediately by a neomycin cassette flanked by *Frt* sites. After targeting the first *Slx4* allele, the neomycin marker was deleted by crossing mice to mice expressing *Flpe* in all tissues. This resulted in the generation of *Slx4*^{+/*fl*ox} mice. These mice were subsequently crossed with mice expressing CRE recombinase in all tissues resulting in the generation of *Slx4*^{+/-}. (See also Table S4) **(B)** Southern blot analysis of genomic DNA from wild-type, heterozygous and knockout MEFs confirming the deletion of exon 8 and 9. **(C)** Biopsies for genotyping were analyzed by PCR with the oligonucleotides indicated in (A). **(D)** Western blot analysis of MEF extracts of the genotypes indicated.

Castor_Fig.S3

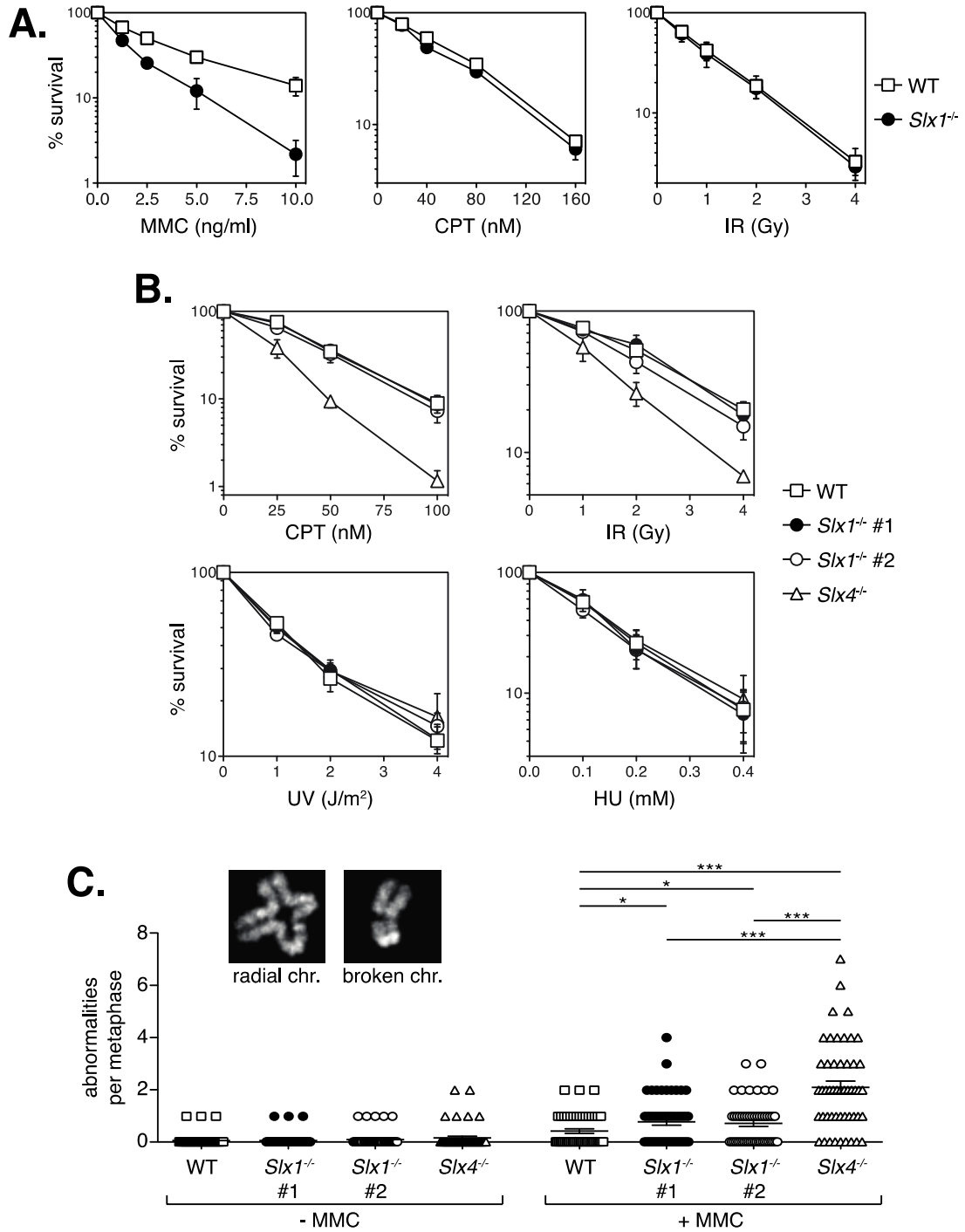


Figure S3: Genotoxin sensitivity of cells lacking SLX1 or SLX4

(A, B) Clonogenic survival analysis of ES cells (A) or MEFs (B) exposed to the genotoxins indicated. For each genotype, cell viability of untreated cells was defined as 100%. Data are represented as mean \pm SEM, $n=3$. **(C)** Metaphase spreads of MEFs treated with MMC (20 ng/ml) were stained with DAPI and analysed for the presence of radial and broken chromosomes. 50 metaphase spreads were analysed for each cell line either untreated or treated with MMC and the number of abnormalities per metaphase was determined. Significance was calculated using an unpaired T-test with; *, $p < 0.05$; ***, $p < 0.0001$. Error bars, \pm SD. Representative images of a radial chromosome and a broken chromosome are shown.

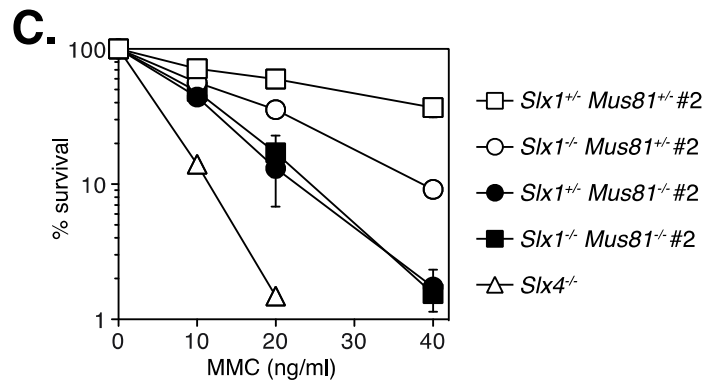
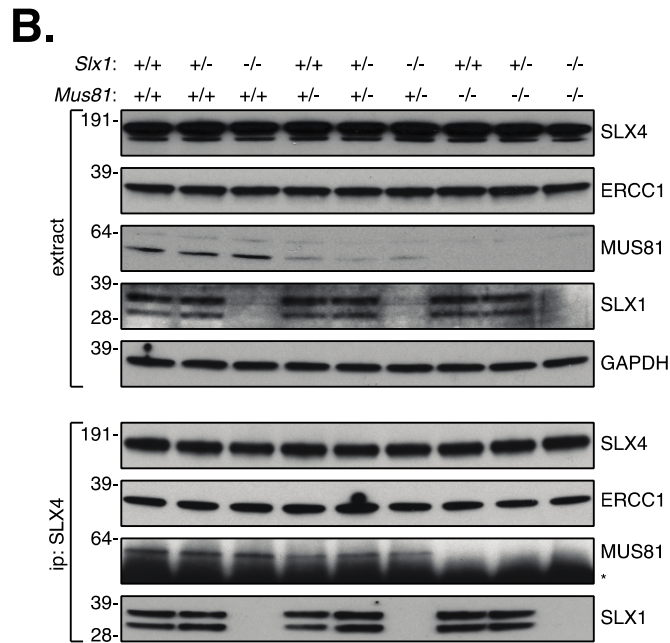
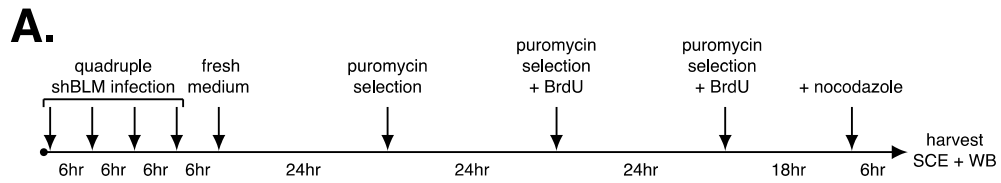


Figure S4: Characterisation of *Slx1*^{-/-} *Mus81*^{-/-} MEFs

(A) Schematic describing the protocol for SCE analysis after BLM knockdown using BLM-specific shRNA retroviruses and puromycin selection to ensure virus uptake. **(B)** Western blot and co-immunoprecipitation analysis of MEF extracts from littermates resulting from crossing *Slx1*^{+/-} *Mus81*^{+/-} mice. **(C)** Clonogenic survival analysis of MEFs from the genotypes indicated exposed to MMC. For each genotype, cell viability of untreated cells was defined as 100%. Data are represented as mean ± SEM, *n*=3. These data represent alternative MEF clones to those shown in Fig. 3B.

Castor_Fig.S5

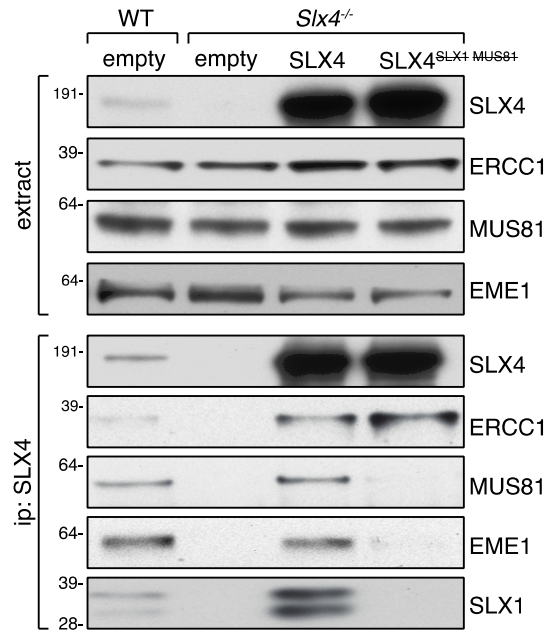


Figure S5: An SLX4 mutant incapable of interacting with SLX1 and MUS81
Slx4^{-/-} MEFs were infected with viruses expressing SLX4 wild-type (SLX4), or SLX4 in which the C1536R mutation, which abolishes interaction with SLX1 was combined with the E1351A+L1352A mutations that abolish interaction with MUS81 (SLX4^{SLX1⁻MUS81⁻}). Extracts were subjected to Western blot analysis before (upper panels) or after immunoprecipitation with anti-SLX4 antibodies (lower panels).

Castor_Fig.S6

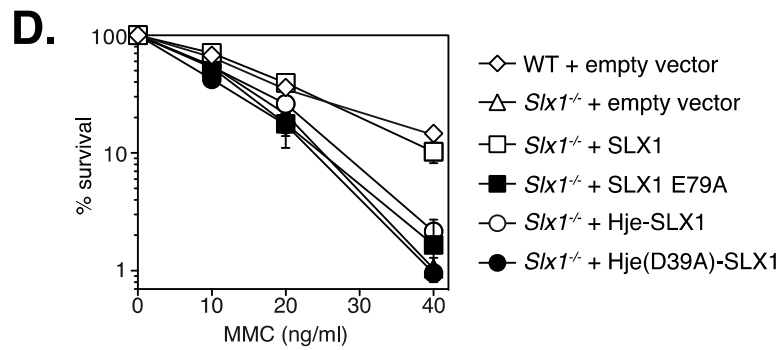
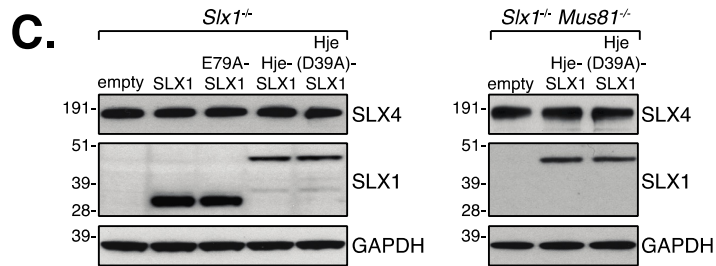
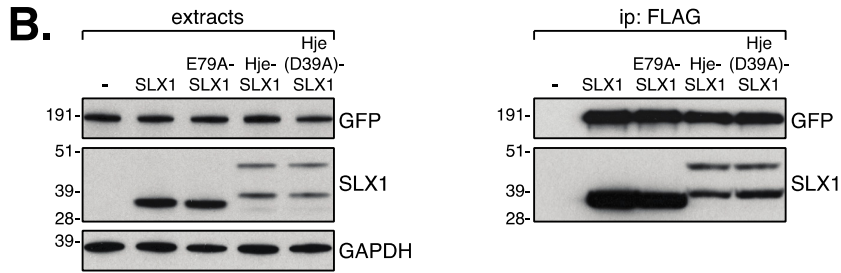
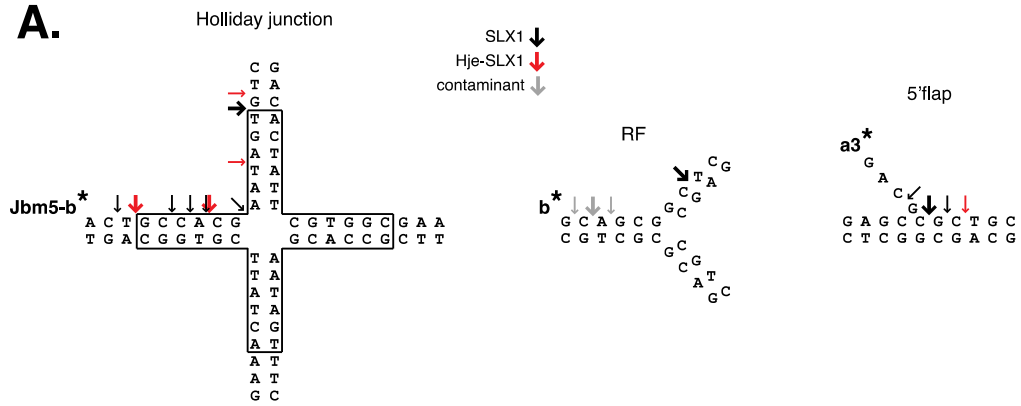


Figure S6: Characterization of an Hje-SLX1 fusion protein

(A) Schematic diagram of the DNA substrates used in Fig. 6B. Sites of specific DNA cleavage, mediated by SLX1 or Hje-SLX1, or contaminant nucleases in the immunoprecipitates are indicated by arrows. **(B)** HEK239 cells stably expressing GFP-tagged mouse SLX4 were transfected with mouse SLX1 wild-type, SLX1 E79A, Hje-SLX1 or Hje(D39A)-SLX1. Extracts were subjected to western blotting before (left panels) or after (right panels) immunoprecipitation with anti-FLAG antibodies and probed with the antibodies indicated. **(C)** *Slx1*^{-/-} MEFs were infected with viruses expressing untagged forms of SLX1: SLX1 wild-type, SLX1 E79A, Hje-SLX1 or Hje(D39A)-SLX1. Extracts were subjected to western blotting (left panels) with the antibodies indicated. Viruses expressing Hje-SLX1 or Hje(D39A)-SLX1 were also used to complement *Slx1*^{-/-} *Mus81*^{-/-} MEFs. Extracts were subjected to Western blotting with the antibodies indicated (right panels). **(D)** Clonogenic survival analysis of MEFs from (B, left panels) exposed to MMC. For each genotype, cell viability of untreated cells was defined as 100%. Data are represented as mean ± SEM, *n*=3. The slight rescue of MMC sensitivity that is apparent with Hje-SLX1 compared with Hje(D39A)-SLX1 is not statistically significant, as judged by the student t-test (data not shown).

Castor_Table S1

P:	<i>Slx1^{+/+}</i> x <i>Slx1^{-/-}</i>		
F1:	<i>Slx1^{+/+}</i>	<i>Slx1^{+/-}</i>	<i>Slx1^{-/-}</i>
expected	25%	50%	25%
embryos 13.5 (n=19)	21.1%	52.6%	26.3%
day 21 (n=365)	21.9%	54.5%	23.6%

Chi-square test
Two-tailed P values

0.9241
0.2038

Table S1. Frequencies of genotypes obtained from $Slx1^{+/-}$ x $Slx1^{+/-}$ crosses.
Statistical significance was measured with a Chi-square test. Two-tailed P-values are shown.

Castor_Table S2

P:	<i>Slx4^{+/+}</i> x <i>Slx4^{+/+}</i>		
F1:	<i>Slx4^{+/+}</i>	<i>Slx4^{+/-}</i>	<i>Slx4^{-/-}</i>
expected	25%	50%	25%
embryos 13.5 (n=104)	31.7%	48.1%	20.2%
day 21 (n=167)	24.0%	62.3%	13.8%

Chi-square test
Two-tailed P values

0.2319
0.0012

Table S2. Frequencies of genotypes obtained from $Slx4^{+/-}$ x $Slx4^{+/-}$ crosses.
Statistical significance was measured with a Chi-square test. Two-tailed P-values are shown.

Castor_Table S3

P:	<i>Slx1(S)^{+/-}Mus81(M)^{+/-} x Slx1(S)^{+/-}Mus81(M)^{+/-}</i>								
F1:	<i>S^{+/+}M^{+/+}</i>	<i>S^{+/+}M^{+/-}</i>	<i>S^{+/+}M^{-/-}</i>	<i>S^{+/-}M^{+/+}</i>	<i>S^{+/-}M^{+/-}</i>	<i>S^{+/-}M^{-/-}</i>	<i>S^{-/-}M^{+/+}</i>	<i>S^{-/-}M^{+/-}</i>	<i>S^{-/-}M^{-/-}</i>
expected	6.25%	12.5%	6.25%	12.5%	25%	12.5%	6.25%	12.5%	6.25%
embryos 13.5 (n=46)	4.3%	8.7%	6.5%	15.2%	17.4%	21.7%	6.5%	10.9%	8.7%
day 21 (n=95)	9.5%	8.4%	3.2%	17.9%	26.3%	9.5%	6.3%	12.6%	6.3%

Chi-square test
Two-tailed P values

0.6667
0.5074

Table S3. Frequencies of genotypes obtained from *Slx1*^{+/-} *Mus81*^{+/-} x *Slx1*^{+/-} *Mus81*^{+/-} crosses.

Statistical significance was measured with a Chi-square test. Two-tailed P-values are shown.

Castor_Table S4

<i>Giyd2/Slx1</i>	
arm1 sense	tgc ggc cgc ggt ggc aca cgc ctt taa tct cag
arm1 antisense	agg ccg gcc ata act tcg tat agc ata cat tat acg aag tta ttt att ctg ggt tga agg gcc aga gtt ggg ggt g
arm2 sense	tgg cgc gcc ata act tcg tat aat gta tgc tat acg aag tta tgg atc ctt ttt ttt tag atc tgg gcc ggt gct gag tcc ctg
arm2 antisense	aaa tat tga gga tgt tgc cag gcc cat tca aac
SD arm sense	aac cgg tgt gcc cat gga cca tgc agc aag acc c
SD arm antisense	aac cgg tcc cgc ctc tgt cac tcc ttt gc
<i>Btbd12/Slx4</i>	
arm1 sense	gcg gcc gca tcc tca cac cat gac agc ctg gag
arm1 antisense	act agt ggg cta act tta gga atg tga aat cgt taa gac
Floxed region sense	cac cgg cgc ata tga gga cag agc ttc cta gcc taa tc
Floxed region antisense	agc gat cgc tgg ctc aag cct ata atg tga gac tg
arm2 sense	tgg cgc gcc gga tcc aca cag gaa tcc agc aca ctg
arm2 antisense	act cga gaa gct tcc tct gcg tag cag caa at
SD arm sense	aac cgg tgt gcc atg gtc aat aat cca cac ctg agt g
SD arm antisense	aac cgg tgg ctc aag cct ata atg tga gac

Table S4. Primers used in generating arms for targeting vectors for murine *Giyd2/Slx1* and *Btbd12/Slx4*.

All oligonucleotide sequences are shown 5'-3'.

Supplemental Experimental Procedures

Mouse strains and husbandry

C57BL/6 mice were obtained from Charles River Laboratories. Flpe transgenic and CRE recombinase transgenic mice were obtained from Taconic-Artemis. Animals were housed under specific pathogen free conditions in accordance with UK and EU regulations. All procedures were carried out in accordance with University of Dundee and United Kingdom Home Office regulations.

To generate a knockout of the *Giyd2/Slx1* gene, a targeting vector was constructed to introduce a selectable marker upstream of the start codon in exon 1 (Fig. S1A). *LoxP* sites were introduced 5' to the selectable marker and in intron 1 so that CRE mediated excision resulted in a deletion of both the marker and the coding region of exon1, which encodes the first 60 amino acids of SLX1, as well as the splice donor sequence. The selectable marker consisted of a neomycin resistance gene under the control of a constitutive promoter but no polyadenylation sequence. An IRES was placed 3' to the *neo* coding region that was followed by the remainder of exon 1. This created a poly-A trap and the *neo* gene would only be expressed if inserted in the genome in a position where it could splice onto an endogenous exon containing a polyadenylation sequence. The arms for the targeting vector were cloned by PCR using an appropriate BAC clone as template. All clones were fully sequenced to ensure that they contained no PCR generated errors before assembly into the final targeting vector (Fig. S1A). Primer sequences used for cloning are shown in Table S2. The linearized targeting vector was electroporated into E14 (129P2) embryonic stem (ES) cells, and cells were subjected to positive (G418) and negative (ganciclovir) selection using standard procedures. Resistant clones were screened for targeted integration using an initial RT-PCR based method that detected an mRNA corresponding to the *neo* genes spliced onto the downstream exons that were 3' to the sequence used in the targeting vector. ES cell clones with a targeted *Slx1* allele were injected into C57BL/6 mouse blastocysts, which were then re-implanted into recipient female mice. Male chimeric mice showing a high

percentage of ES cell contribution were bred to C57BL/6 female mice. Germline transmission was determined by coat colour and the genotypes of these mice were confirmed by PCR on ear notch biopsies. To create the deletion in exon 1, mice with one targeted *Slx1* allele were crossed with mice ubiquitously expressing the CRE recombinase. The offspring that were heterozygous for the deleted allele were then backcrossed with wild type C57BL/6. Offspring from this cross that no longer carried CRE recombinase were used for further breeding. *Slx1*^{-/-} mice were generated by crossing *Slx1*^{+/-} mice. The correct targeting and deletion of exon 1 was confirmed by Southern blot analysis (Fig. S1B). Mice were routinely genotyped from ear notch biopsies according to standard procedures. Genotyping with primers GIYD2_1 (5'-agt tag cca atc cga gcc gga ct-3') and GIYD2_2 (5'-ccc tgg tag tgg agt tac aaa gca g-3') gives rise to a 690 bp band in wild type alleles and 449 bp in knockout alleles. GIYD2_3 (5'-gct aca gcc cag cgt gca aag aac-3') and GIYD2_4 (5'-cag tga atc caa cgt aaa cgc ggc-3') gives rise to a 514 bp product in wild type alleles only. Representative genotyping results are shown in Fig. S1C.

Btbd12/Slx4 target alleles were generated by inserting *LoxP* sites upstream of exon 8 and downstream of exon 9 followed by a poly-A trap neomycin-IRES-SD cassette flanked by Frt sites between exon 9 and 10. The splice donor (SD) used was the SD sequence from exon 9 of *Btbd12/Slx4*. The sequences required for the targeting vector were generated by PCR from an appropriate BAC clone and sequenced to ensure that there were no PCR generated errors. Primers used for the vector construction are shown in Table S2. ES cell targeting was carried out as above and cells were screened by an RT-PCR strategy based on the poly-A trap. Positive ES cells were used to generate chimeric mice as for *Giyd2/Slx1*. Chimeric mice were crossed to homozygous Flpe transgenic mice on a C57BL/6 background and germline transmission identified by coat colour and then confirmed by PCR based genotyping. The cross to the Flpe mice resulted in excision of the Frt flanked neo selectable marker. This gave a conditional *Slx4* allele in which exons 8 and 9 were flanked by *LoxP* sites. Expression of SLX4 in

these *Slx4*^{+/*flox*} mice was normal (data not shown). To excise exon 8 and 9 from the floxed *Slx4* allele, mice were crossed with mice ubiquitously expressing the CRE recombinase. Following deletion of exons 8 and 9 in the germline, the allele was bred away from the CRE recombinase. Knockout mice were then obtained from heterozygous crosses. Deletion of exon 8 and 9 of *Slx4* was confirmed by Southern blot analysis (Fig. S2B). Mice were then routinely genotyped from ear notch biopsies according to standard procedures. Genotyping with primers BTBD12_1 (5'-tgc atc act gtg aga ctc ctg atc acc ctc-3'), BTBD12_2 (5'-tct cta gaa agt ata gga act tcg gcg cgc-3') and BTBD12_3 (5'-cca gtg tta ggt ggg tgg aag aaa gaa ccc-3') gave rise to a 281 bp band for wild type alleles and a 230 bp product for knockout alleles. Representative genotyping results are shown in Fig. S2C.

Isolation and immortalization of MEFs

13.5 dpc embryos were derived from timed matings between heterozygous parents. After decapitation, the heads were used for genotyping. The red organs were removed, the embryo was minced and resuspended in 1 ml trypsin and incubated at 37°C for 15 min before the addition of 10 ml growth medium. Cells were plated and allowed to attach over night before cells were washed with fresh medium to remove debris. When cells reached confluency they were split and re-plated and this was considered passage 1. All MEFs, except those in Fig. 3 derived from matings between *Slx1*^{+/-} *Mus81*^{+/-} double heterozygous animals, were immortalized using the 3T3 protocol (Xu, 2005). MEFs derived from littermates of matings between *Slx1*^{+/-} *Mus81*^{+/-} double heterozygous animals were immortalized using SV40 large T antigen.

Virus-based rescue experiments in MEFs

Viruses were produced by co-transfecting 293T cells with the relevant open reading frames cloned into pBABE vector, and with pCMV-VSV-G and pCMV-Gag-Pol expression vectors to direct viral packaging. After 48 hours the virus-containing supernatant was filtered through a 45 µm filter, mixed with polybrene

(final concentration of 8 $\mu\text{g/ml}$) and added to MEFs. Three hours later the infection medium was replaced with fresh growth medium. The infection protocol was repeated 24 hours later. MEFs were stably selected with the respective antibiotics 24 hours after the second infection. Protein expression was tested by western blotting.

ES cell generation

Embryos at day 2.5 post coitum derived from matings between wild type and/or *S/x1* knockout mice, respectively, were cultured overnight in M16 medium covered with mineral oil before plating the embryos on EMFI feeder cells in ES cell medium supplemented with 25% FBS and leukemia inhibitory factor (LIF, 2000 U/ml). Two days after the embryos hatched out of the zona pellucida, the inner cell mass was isolated and subjected to trypsinization. With every second passage, the concentrations of FBS and LIF were reduced by 5% and 500 U/ml, respectively.

Cell culture

All cells were kept at 37°C under humidified conditions with 5% CO₂. HEK293 and 293T cells were grown in DMEM, 10% FBS, L-glutamine and penicillin/streptomycin. For MEFs, medium was supplemented with Na-pyruvate and non-essential amino acids. Puromycin was used at 3 $\mu\text{g/ml}$ and G418 was used at 400 $\mu\text{g/ml}$ for selection of complemented MEFs. ES cells were kept in DMEM, 14% heat-inactivated FBS, L-glutamine, Na-pyruvate, non-essential amino acids, penicillin/streptomycin and 0.0001 M β -mercaptoethanol supplemented with LIF (1000 U/ml). Cells were initially kept on gamma irradiated MEF feeder layers before adapting them to 0.1% gelatin-coated dishes. Stemness of the cells was confirmed by the expression of OCT4 (data not shown).

Clonogenic survival analysis

MEFs were seeded in triplicates in 10 cm dishes and were allowed to attach before treatment. For UV-irradiation cells were washed with PBS and exposed to a UV-C source, and for IR treatment cells were exposed to a ¹³⁷Cs radiation source. In the case of HU, CPT or MMC treatment, genotoxin was added to cells for 24 hours before medium was replaced with fresh growth medium except in the case of HN-2 which was only added for 2 h. ES cells were seeded in triplicates on 0.1% gelatin-coated six-well plates and were allowed to attach prior to treatment. After 10 days (MEFs) or 5 days (ES cells) cells were washed, fixed and stained with Giemsa. The number of colonies with >100 cells were counted. Results were normalized to plating efficiency. For each genotype, cell viability of untreated cells was defined as 100%. Data are represented as mean ± SEM from three independent experiments.

Cell lysis and immunoprecipitation

Cells were lysed in ice-cold buffer: (50 mM Tris-HCl [pH 7.4], 150 mM NaCl, 270 mM sucrose, 1% (v/v) Triton X-100, 1 mM EDTA, 1 mM EGTA, 0.1% β-mercaptoethanol) with protease inhibitors (Roche) and 50 U/ml of benzonase (Novagen). Lysate was pre-cleared on empty protein G sepharose beads for 30 min at 4°C. All immunoprecipitations were carried out in lysis buffer for 1.5 h at 4°C. Immunoprecipitations were carried out using 2 µg of primary antibody coupled to 10 µl protein G sepharose per 2 mg of whole cell extract. For FLAG-tagged proteins FLAG-M2 agarose beads (Sigma) were used.

Nuclease assays

The oligonucleotides used to assemble the branched DNA substrates had the following sequences (5' to 3'): Jbm5-a (gcg tta caa tgg aaa cta ttc gtg gca gtt gca tcc aac g), Jbm5-b (cgt tgg atg caa ctg cca cga ata gtg tca gtt cca gac g), Jbm5-c (cgt ctg gaa ctg aca cta ttc gtg gcg aat ggt cgt aag c), Jbm5-d (gct tac gac cat tcg cca cga ata gtt tcc att gta acg c), a3 (cct cga tcc tac caa cca gat gac gcg ctg cta cgt gct acc gga agt cg), b (cga ctt ccg gta gca cgt agc agc ggc tcg cca cga act

gca ctc tag gc), c (gcc tag agt gca gtt cgt ggc gag c), d3 (cgt cat ctg gtt ggt agg atc gag g). For each substrate, a single oligonucleotide was 5'-³²P-labeled and annealed with the other component DNA strands by slow-cooling. The annealed substrates were then purified by native PAGE, eluted by the crush and soak method and ethanol-precipitated. HJ was assembled with Jbm5-a, Jbm5-b*, Jbm5-c and Jbm5-d; RF with a3, b*, c and d3; 5' flap with a3*, b and c where * denotes the radioactively-labelled strand.

HEK293 cells stably expressing tetracycline-inducible GFP-tagged mouse SLX4 were transfected with different FLAG-tagged mouse SLX1 constructs. After 12 h, medium was replaced with fresh medium supplemented with 1 µg/ml tetracycline. Cells were harvested 36 h later. Lysis and immunoprecipitation with FLAG-M2 agarose beads was performed as described above except that benzonase was omitted from the lysis buffer. After immunoprecipitation, FLAG-M2 beads were washed extensively including a final wash with high-salt lysis buffer (lysis buffer same as above with 500 mM NaCl). Immunoprecipitates were equilibrated in nuclease buffer (20 mM Tris-HCl [pH 7.5], 50 mM NaCl, 10 mM MgCl₂, 0.1 mg/ml BSA) and assay was performed as described in (Munoz et al., 2009).

Southern blotting

Southern Blot was performed according to standard procedures. 12 µg of genomic DNA extracted from either tail biopsies in case of SLX1 or MEFs in case of SLX4 was digested with *Bam*HI overnight. The probes were generated by PCR using the targeting plasmids as template and the following oligos: GIYD2_left arm (fwd, 5'-ctt tgg gaa tga gtt ggt cc-3'; rev, 5'-gaa acc tcc caa gtg ctg g-3'), GIYD2_right arm (fwd, 5'-gga cag gaa ctc aaa caa gag c-3'; rev, 5'-ctg cca gtc tca ggc tcc-3'), BTBD12_left arm (fwd, 5'-cgt tgg agg ttt gaa acg gaa agg-3'; rev, 5'-cat cca ctt atg tac ttt aag g-3') and BTBD12_right arm (fwd, 5'-gta ctt tcc atc cac tcc ac-3'; rev, 5'-ctt gtt cac aca gct gaa caa gg-3').

Antibodies

Antibodies against mouse SLX1 were raised in sheep against full-length mouse SLX1 fused to GST. Antibodies against mouse SLX4 were raised against a GST-tagged fragment of mouse SLX4 corresponding to the C-terminal 300 amino acids. Antibodies against mouse MUS81, ERCC1 and EME1 were raised against GST-tagged fragments of the respective mouse proteins corresponding to the first 200 amino acids of each protein. Anti-GFP antibodies were raised in sheep against GST-tagged full-length GFP. All antibodies were affinity purified using immobilized antigen. Anti-GAPDH (14C10) antibodies were purchased from Cell Signalling and anti-BLM antibodies were from Bethyl (A300-572A). All secondary antibodies were purchased from Pierce.

DNA constructs

The full-length coding regions for the relevant proteins were generated by PCR using IMAGE consortium EST clones. Point mutations were introduced by quickchange mutagenesis. The sequence integrity was confirmed by sequencing analysis. To generate the Hje-SLX1 fusion protein, the sequence of *Solfolobus solfataricus* Hje was fused to the SLX1 open reading frame separated by a polyglycine linker.

Analysis of chromosomal aberrations

For the analysis of chromosomal abnormalities MEFs were treated for 48 hours with 20 ng/ml MMC or left untreated before the addition of colcemid for 30 min. Cells were then trypsinized, washed and incubated with 75 mM KCl for 20 min at 37°C. Cells were subsequently fixed with 3:1 methanol:acetic acid at 4°C O/N before spreading. Spreads were stained with DAPI and 50 spreads were analysed for chromatid breaks and radial chromosomes.

Supplemental References

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- Xu, J. (2005). Preparation, culture, and immortalization of mouse embryonic fibroblasts. *Curr Protoc Mol Biol Chapter 28*, Unit 28 21.