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The involvement of SLX4 in interstrand cross-link repair is regulated by the *Fanconi anemia* pathway.

Kimiyo N. Yamamoto^{1,2}, Shunsuke Kobayashi¹, Masataka Tsuda¹, Hitoshi Kurumizaka³, Minoru Takata⁴, Koichi Kono², Josef Jiricny⁵, Shunichi Takeda¹, Kouji Hirota^{1*}

¹*Department of Radiation Genetics, Graduate School of Medicine, Kyoto University, Yoshidakonoe, Sakyo-ku, Kyoto 606-8501, Japan*

²*Public Health and Hygiene, Graduate School of Medicine, Osaka Medical College, Takatsuki, Osaka, Japan*

³*Graduate School of Advanced Science and Engineering, Waseda University, 2-2 Wakamatsu-cho, Shinjuku-ku, Tokyo 162-8480, Japan.*

⁴*Laboratory of DNA Damage Signaling, Department of Late Effect Studies, Radiation Biology Center, Kyoto University, Yoshidakonoe-cho, Sakyo-ku, Kyoto 606-8501, Japan.*

⁵*Institute of Molecular Cancer Research, University of Zurich, Winterthurerstrasse 190, 8057 Zurich, Switzerland*

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*Corresponding author: Kouji Hirota; Department of Radiation Genetics, Graduate School of Medicine, Kyoto University, Yoshidakonoe, Sakyo-ku, Kyoto 606-8501, Japan

E-mail: khirota@rg.med.kyoto-u.ac.jp;

Phone: +81-75-753-4410; Fax: +81-75-753-4419

Interstrand cross-links (ICLs) block replication and transcription and are thus extremely cytotoxic. In higher eukaryotes, ICLs processing involves the *Fanconi anemia* (FA) pathway and homologous recombination (HR). Stalled replication forks activate the eight-subunit FA core complex, which ubiquitylates FANCD2-FANCI. Once post-translationally modified, this heterodimer recruits downstream members of the ICL repairosome, including the FAN1 nuclease. However, ICL processing has been shown to involve also MUS81-EME1 and XPF-ERCC1, nucleases known to interact with SLX4, a docking protein that can bind also another nuclease, SLX1. To investigate the role of SLX4 more closely, we disrupted the *SLX4* gene in avian DT40 cells. SLX4 deficiency caused cell death associated with extensive chromosomal aberrations including a significant fraction of isochromatid-type breaks, where sister chromatids were broken at the same site. SLX4 thus appears to play an essential role in cell proliferation, probably by promoting the resolution of inter-chromatid HR intermediates. Because ubiquitylation plays a key role in the FA pathway, and because the N-terminal region of SLX4 contains a ubiquitin-binding zinc finger (UBZ) domain, we asked whether this domain is required for ICL processing. We show that *SLX4*^{-/-} cells expressing UBZ-deficient SLX4 were sensitive selectively to ICL-inducing agents, and that the UBZ domain was required for interaction of SLX4 with ubiquitylated FANCD2 and for its recruitment to DNA-damage foci generated by ICL-inducing agents. Our findings thus suggest that ubiquitylated FANCD2 recruits SLX4 to DNA damage sites, where it mediates the resolution of recombination intermediates generated during the processing of ICLs.

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INTRODUCTION

Interstrand cross-links (ICLs) inhibit transcription and replication. Their considerable cytotoxicity has been ascribed primarily to their blocking of replication forks, and it is this phenomenon that is believed to be responsible for the success of ICL-inducing agents such as cisplatin and mitomycin-C (MMC) in cancer chemotherapy (1). ICL processing is complex, inasmuch as it involves **proteins from** several distinct pathways of DNA metabolism. In higher organisms, it is orchestrated by the *Fanconi anemia* (FA) pathway (2, 3). Collision of replication forks with ICLs activates the ATR kinase, which in turn licenses the FANCL ubiquitin ligase subunit of the FA core complex (composed of FANCA, B, C, E, F, G, L and M proteins) to modify the FANCD2-FANCI heterodimer (2, 4-6). The mono-ubiquitylated FANCD2-FANCI complex is then targeted to chromatin (7, 8), where it recruits downstream components of the reairosome including the structure-specific nuclease FAN1 (9-12). However, ICL processing requires also other enzymes, such as the nucleases MUS81-EME1 and XPF-ERCC1 and it is currently not known how these are recruited to sites of damage in ICL repair.

The structure-specific endonucleases XPF-ERCC1 and MUS81-EME1, which are implicated in ICL repair and in the resolution of homologous recombination (HR) intermediates (13-22), interact with SLX4. The latter polypeptide interacts also with SLX1 both in yeast (23, 24) and in mammalian cells (21, 22, 25, 26) and the resulting complex displays 5'-flap endonuclease and Holliday junction resolvase activities. Based on this evidence, SLX4 has been assigned the role of a docking platform for structure-specific endonucleases and its pivotal role in regulating their activities is underscored by the finding that SLX4 downregulation sensitizes human cells to a wide variety of DNA-damaging agents (21, 22, 25, 26). However, although SLX4 has been implicated also in ICL repair, its role in this process, as well as its possible link to the FA pathway remains to be elucidated.

In this study, we set out to characterize the biological role of SLX4 by conditionally disrupting the *SLX4* gene in chicken DT40 cells (27). We now report that loss of SLX4 induces cell death associated with extensive chromosome aberrations, which shows that SLX4 plays a key role in the repair of spontaneous DNA damage. Many proteins interacting with ubiquitylated polypeptides contain so-called

ubiquitin-binding zinc finger (UBZ) domains (28). Because ubiquitylation plays an important role in ICL repair, we asked whether the UBZ domain of SLX4 is required for DNA damage processing. We show here that this domain is essential for the recruitment of SLX4 to sites of DNA damage upon exposure of cells to cross-linking agents, and that this recruitment is dependent on FANCD2 ubiquitylation. Consistently, the UBZ domain of SLX4 is required for interaction with mono-ubiquitylated FANCD2. Our data thus indicate that SLX4 involvement in ICL repair is controlled by the FA pathway.

RESULTS

SLX4 is essential for cell proliferation. To disrupt the *SLX4* gene, we used gene-targeting constructs designed to delete the five exons that encode residues 124 to 518 of the protein (Fig. S1A). However, we failed to generate *SLX4*^{-/-} cells, which suggested that SLX4 is essential for DT40 cell survival. We therefore first disrupted one *SLX4* allele and stably transfected the *SLX4*^{+/-} cells with constructs expressing the tTA repressor, and the chicken *SLX4* transgene under the control of doxycycline-repressible promoter (*tetSLX4*) (29). We then disrupted the second *SLX4* allele and confirmed the successful disruption by Southern blotting (Fig. S1B). RT-PCR analysis showed that the *SLX4*^{-/-}*tetSLX4* cells expressed *SLX4* mRNA, and that this transcript was undetectable in doxycycline-treated cells after 72 h (Fig. 1A). The *SLX4*^{-/-}*tetSLX4* cells proliferated slightly slower than wild type (WT) cells, possibly due to nonphysiological levels of expression of *SLX4* (Fig. 1B). 24 h after the addition of doxycycline, the *SLX4* transcript was barely-detectable (Fig. 1A), and the proliferation of the *SLX4*^{-/-}*tetSLX4* cells slowed down considerably (Fig. 1B). After 48 h doxycycline treatment, the *SLX4*^{-/-}*tetSLX4* cells accumulated in the G₂ phase of the cell cycle, with many cells dying (Fig. 1C), indicating that SLX4 is essential for cell proliferation at least in DT40 cells. This result is marked contrast with the viability of *SLX4* knockout mice (30). This difference might possibly be attributable to the absence of the *MUS81* gene in the chicken genome. Chromosome analysis of mitotic cells showed an increased number of spontaneous chromosomal aberrations, as seen in HR-deficient cells (Table S1). The above-described phenotypic traits were observed in two independent *SLX4*^{-/-}*tetSLX4* clones, which argues against clone-specific effects and demonstrates that SLX4 plays a pivotal role in the repair of spontaneous DNA damage (Fig. 1D, Table S1).

SLX4 is required for HR-dependent DSB repair. To assess the role of SLX4 in HR-dependent double strand break (DSB) repair, we exposed the *SLX4*^{-/-}*tetSLX4* cells to γ -rays (2 Gy) 48 h after the addition of doxycycline, harvested mitotic cells 3 h later and counted the number of chromosomal breaks. Using this protocol, we were able to selectively evaluate HR-dependent DSB repair in the G₂ phase, because only cells irradiated in G₂, and not in S, enter mitosis within 3 h, and also because cells in the G₂ phase preferentially use HR for DSB repair (31, 32). In WT cells, ionizing radiation induced predominantly chromatid-type breaks (breaks in one sister chromatid). In SLX4-depleted cells, a marked increase in the total number of chromosomal breaks was observed, coupled to a shift from chromatid-type to isochromatid-type breaks (breaks at the same site of both sister chromatids) (Fig. 1D, Table S1). The latter type of break is probably caused by HR-dependent repair between the broken and the intact sister chromatids, followed by defective resolution of recombination intermediates (33). Taking a role of SLX1(21, 26), XPF-ERCC1 (34-36), and MUS81-EME1 (37, 38) in HR into account, our findings thus support the hypothesis that SLX4 deficiency causes impaired resolution of HR intermediates, possibly through the disruption of functional SLX4 complex including these structure-specific endonucleases.

The UBZ domain of SLX4 plays a key role in ICL repair. To elucidate the role of the UBZ domain of SLX4, we generated mutant SLX4 cDNA lacking the UBZ domain, and stably transfected *SLX4*^{-/-}*tetSLX4* cells with vectors carrying either the mutant (*SLX4-UBZΔ*) or WT cDNA (*SLX4-wt*). We then treated the *SLX4-wt* and *SLX4-UBZΔ* cells with doxycycline for 5 d to repress the *tetSLX4* transgene. We confirmed that the protein levels of SLX4-wt and SLX4-UBZΔ were similar (Fig. S2), which showed that deletion of the UBZ domain did not affect SLX4 stability. As expected, the *SLX4-wt* cells displayed essentially the same phenotype as doxycycline-untreated *SLX4*^{-/-}*tetSLX4* cells, including cellular sensitivity to DNA damage (Fig. S3). Interestingly, constitutive expression of *SLX4-UBZΔ* in doxycycline-treated *SLX4*^{-/-}*tetSLX4* cells allowed the cells to proliferate similarly to doxycycline-untreated *SLX4-UBZΔ* and the parental *SLX4*^{-/-}*tetSLX4* cells, indicating that the UBZ domain is dispensable for cellular proliferation (Fig. S4). We next asked whether cells expressing SLX4-UBZΔ were sensitized to DNA damage. As shown in Fig. 2, the *SLX4-UBZΔ* cells were sensitive to

all ICL-inducing agents tested (cisplatin, melphalan, and MMC), but not to the other genotoxic agents (methylmethane sulfonate, UV light, ionizing radiation, topoisomerase I poison [camptothecin] and topoisomerase II inhibitors [VP-16 and ICR193]) (Fig. 2 and Fig. S5). Given that sensitivity to ICL-inducing agents is a common phenotypic trait of FA cells (2, 39), we hypothesized that the UBZ domain might link SLX4 to the FA pathway.

Recruitment of SLX4 to DNA damage sites requires the SLX4 UBZ domain and mono-ubiquitylated FANCD2. Disruption of the FA core complex abolishes DNA damage-induced ubiquitylation of FANCD2, as exemplified for *FANCC*⁻ cells (Fig. S6). As MMC treatment did not affect FANCD2 modification in *SLX4-UBZΔ* or *SLX4*^{-/-}*tetSLX4* cells treated with doxycycline for 48 h (Fig. S6), we concluded that SLX4 is not required for the activation of the ubiquitin ligase activity of the FA core complex.

Following treatment with DNA damaging agents, FANCD2 and other repair proteins accumulate in subnuclear foci (7-9). In order to find out whether SLX4 also accumulated in these foci, we stably transfected DT40 cells with a vector expressing an SLX4-GFP fusion protein. After treatment of the transfected cells with MMC for 6 h, SLX4-GFP foci became detectable and co-localized with those containing FANCD2 (Fig. 3A). That MMC-induced SLX4-GFP foci failed to form in similarly transfected *FANCC*⁻ and *FANCD2-K563R* (40) DT40 lines, in which mono-ubiquitylation of FANCD2 is impaired (40, 41), indicated that FANCD2 mono-ubiquitylation is required for SLX4 recruitment to damage sites (Fig. 3A).

We next asked whether the above recruitment required the UBZ domain of SLX4. As shown in Fig. 3A, the SLX4-UBZΔ-GFP mutant protein failed to form MMC-induced foci in WT DT40 cells, even though FANCD2 foci formed normally (Fig. 3A). The recruitment of SLX4 to MMC-induced DNA damage sites is thus dependent on both the SLX4 UBZ domain of and mono-ubiquitylated FANCD2.

The above co-localization suggested that SLX4 and FANCD2 interacted. In order to document this interaction, we immunoprecipitated the SLX4-GFP or SLX4-UBZΔ-GFP proteins from extracts of stably-transfected DT40 WT cells with an anti-GFP antibody and probed the precipitates for the presence of FANCD2. As shown in Fig. 3B, mono-ubiquitylated FANCD2 was present in immunoprecipitates isolated

from extracts of MMC-treated cells expressing SLX4-GFP, but not SLX4-UBZΔ-GFP. These data indicate that SLX4 forms a complex with mono-ubiquitylated FANCD2 and that this interaction requires the SLX4 UBZ domain. It remains investigated whether or not this interaction is direct.

The FA pathway and SLX4 are not fully epistatic in ICL processing. Having established that SLX4 interacts with mono-ubiquitylated FANCD2, we set out to test whether the involvement of SLX4 in ICL processing is fully dependent on this interaction. To this end, we deleted the *FANCC* gene in *SLX4-UBZΔ* as well as in *SLX4^{-/-}tetSLX4* cells. We then treated two independently isolated *FANCC^{-/-}/SLX4-UBZΔ* clones with doxycycline for 5 d and analyzed their phenotype. We also analyzed a *FANCC^{-/-}/SLX4^{-/-}tetSLX4* clone that was not treated with doxycycline. Surprisingly, the *FANCC^{-/-}/SLX4-UBZΔ* cells displayed higher sensitivity to MMC and cisplatin than either single mutant (Fig. 4A, and Fig. S7). Thus, in spite of its association with FANCD2, SLX4 appears to participate in the processing of ICLs also independently of the FA pathway.

We next analyzed chromosomal aberrations in MMC-treated mitotic cells. *SLX4-UBZΔ* as well as *FANCC^{-/-}/SLX4^{-/-}tetSLX4* cells showed increased numbers of chromosomal aberrations compared to WT DT40 cells. That *FANCC^{-/-}/SLX4-UBZΔ* cells displayed a larger number of chromosomal aberrations than either *SLX4-UBZΔ* or *FANCC^{-/-}/SLX4^{-/-}tetSLX4* cells (Fig. 4B, Table S2) faithfully reflects the greater sensitivity of the former cells to MMC (Fig. 4A and Fig. S7). Thus, SLX4 and the FA pathway appear to contribute to ICL repair also independently of each other.

DISCUSSION

In this study, we demonstrate that SLX4 is required for HR-dependent DSB repair, possibly for resolution of recombination intermediates, at least as implied by the substrate specificity of its complex with SLX1 (21, 26). We also show that SLX4 is recruited to sites of DNA damage by mono-ubiquitylated FANCD2 and that this recruitment requires the SLX4 UBZ domain. Thus, ubiquitylation of FANCD2 by the FA core complex has at least two functions: the recently-described recruitment of FANL1 (9-12) and the recruitment of SLX4 documented in the present study.

The following lines of evidence support the conclusion that SLX4 participates

in ICL repair downstream of mono-ubiquitylated FANCD2. (i) Deletion of SLX4 UBZ domain sensitized cells selectively to ICL-inducing agents, but not to other DNA damaging agents tested (Fig. 2). (ii) SLX4 recruitment to DNA-damage sites was dependent on FANCD2 mono-ubiquitylation (Fig. 3). (iii) This recruitment required the ubiquitin-binding domain of SLX4 (Fig. 3). (iv) SLX4 interaction with ubiquitylated FANCD2 required the SLX4 UBZ domain (Fig. 3). (v) Recent reports show FA patients carrying mutations in the *SLX4* gene including deletion of the UBZ domain (42, 43).

However, *FANCC/SLX4-UBZΔ* cells displayed a more severe phenotype than *SLX4-UBZΔ* cells, which indicated that the FA pathway regulates repair enzymes other than those associated with SLX4. The most likely candidate is the recently-identified FAN1 nuclease (9-12). Similarly to cells lacking both FANCC and SLX4, *FANCC/FAN1^{-/-}* DT40 cells were also more sensitive to ICL-inducing agents than cells lacking solely FAN1 (44). Thus, the FA pathway appears to control, and possibly co-ordinate, the action of the nucleases SLX1, XPF-ERCC1 and MUS81-EME1 that are known to associate with SLX4, as well as FAN1. Moreover, the *FANCC/SLX4-UBZΔ* cells displayed a more severe phenotype than *FANCC⁻* cells, which indicated that the UBZ domain of SLX4 might interact also with targets other than FANCD2, possibly proteins ubiquitylated by UBC13 (45).

Interestingly, ICL-induced chromosomal exchanges, a hallmark of FA cells (39) observed in *FANCC/SLX4^{-/-}tetSLX4* cells was significantly suppressed by deletion UBZ domain of SLX4 (2/50 in *FANCC/SLX4-UBZΔ* cells vs 6/50 in *FANCC/SLX4^{-/-}tetSLX4* cells, Fisher's exact test $p=0.0374$, Fig. 4, Table S2). Moreover, loss of FAN1 also suppresses chromosomal exchanges in FA cells following treatment with ICL-inducing agents (44). These data suggest that exchanges in FA deficient cells are attributable to the inappropriate resolution of HR intermediates by FAN1 and SLX4 associating nucleases. Taken together, the FA pathway appears to direct SLX4 to a subset of HR intermediates, where it prevents a specific type of chromosomal aberrations. In the absence of FA-mediated recruitment, the UBZ domain of SLX4 may interact with other ubiquitylated protein(s), resolve these HR intermediates differently and thus give rise to different types of chromosomal aberrations. The loss of the SLX4 UBZ domain would abolish also the FA-independent interactions and the pattern of chromosomal aberrations would alter yet again (Fig. S8).

The role of SLX4 in DNA repair is believed to be that of a docking platform

for SLX1, MUS81-EME1 and XPF-ERCC1 structure-specific nucleases, as well as other proteins (21, 26). The viability of null mutants in genes encoding these nucleases (19, 46, 47) contrasts with the lethality of SLX4 disruption. It suggests that the nucleases are largely redundant in the processing of spontaneous DNA damage and the SLX4 deletion results in a loss of function of more than one of these enzymes. Should this be the case, then cells in which two or more endonuclease genes were disrupted would be anticipated to display a more severe phenotype than the single mutants. This is indeed the case, as our preliminary finding shows that disruption of the *XPF* gene in *MUS81*-deficient DT40 cells is lethal (manuscript in preparation). Given that these endonucleases share a preference for 3'-flap substrates, their inactivation might result in a total loss of this activity from the reairosome responsible for the resolution of HR intermediates and thus in lethality. Similarly, it might be anticipated that cells lacking FAN1 and SLX1 would also be non-viable due to the loss of 5'-flap endonuclease activity of the complex responsible for resolution of HR intermediates.

Our present work extends the studies with the yeast and human SLX4 homologues (21, 22, 24, 26) and confirms that SLX4 fulfills a key role as an interacting platform for enzymes involved in the processing of HR intermediates and links SLX4 also to the repair of ICLs, a process co-ordinated by the FA pathway.

METHODS

Additional details are provided in SI Materials and Methods.

Chicken SLX4 cloning and gene-targeting vectors. Full-length chicken Slx4 cDNA was amplified with PCR using the primers 5'-CCAATGGATGAACAGGACAATGA-3' and 5'-GAAACGATACTGAATGGATCCC-3' and cloned using a TOPO cloning kit (Invitrogen). SLX4 targeting vector was constructed to replace 5 exons (5th-9th) with a resistance (*Puro* and *HisD*) gene cassette flanked by loxP signals at both ends. The primers used to amplify the left arm were 5'-AGGGACAAGTTGTGGTGAGG-3' and 5'-GGACAAGGTGTTTCAGCCATT-3', and the primers for the right arm were 5'-GCAAGGCGTAATTTGTTGGT-3' and 5'-CCAAGCCCAAATTCTTTTCA-3'.

Generation of SLX4 conditional mutant cells. To generate *SLX4*^{-/-} cells, *SLX4*-targeting vectors (*Puro*^R and *HisD*^R) linearized with *EcoRV* were transfected sequentially by electroporation (Bio-Rad). The genomic DNA of the transfectants was digested with *Bam*HI, and the targeted clones were confirmed by Southern blot analysis.

The probe used was 613 bp in length, a PCR-amplified fragment derived from chicken DT40 genomic DNA using the primer set 5'-CTCTGCTTTAATTCCAAGC-3' and 5'-AATCAGTACTAAATGTGAGC-3'. The probe was labeled using the Alkphos Direct Labelling Module (GE Healthcare, Little Chalfont, UK). To generate *SLX4*^{+/-} and *SLX4*^{-/-} cells, *wild-type* DT40 cells were transfected sequentially with the *SLX4* targeting vectors with *Puro*^R and then *HisD*^R. Although we identified 8 *SLX4*^{+/-} clones from 39 *Puro*^R clones analyzed, we obtained no *SLX4*^{-/-} clones out of 268 *HisD*^R clones tested. To obtain *SLX4*^{+/-}*tetSLX4* cells, one of the *SLX4*^{+/-} clones was transfected with both the tetracycline-controlled trans-activator (tTA) gene (Invitrogen) and the conditional *SLX4* expression construct. To generate *SLX4*^{-/-}*tetSLX4* cells, *SLX4*^{+/-}*tetSLX4* cells were transfected with the *SLX4* targeting vector (*HisD*^R). Expression of chicken *SLX4* in the *SLX4*^{-/-}*tetSLX4* cells was measured by Reverse Transcription Polymerase Chain Reaction (RT-PCR) using the SuperScript™ III First-Strand system (Invitrogen, CA, USA) and the 5'-TGCAGGTGTCCACGCTTGGT-3' and 5'-CCTATTCCAAGCTGGTGCTGG-3' primers.

Generation of *SLX4-wt* and *SLX4-UBZΔ* cells. We removed the *Puro*^R and *Bsr*^R cassettes from the *SLX4*^{-/-}*tetSLX4* cells by transiently expressing the Cre-recombinase. The resultant *SLX4*^{-/-}*tetSLX4* cells were transfected with a transgene, *GgSLX4-wt* or *GgSLX4-UBZΔ*. To construct the *GgSLX4-UBZΔ* gene, cloned *GgSLX4* cDNA was amplified using the primers 5'-GAAAGCCTGGAGGATGCAGGC-3' and 5'-GTTCTCCTAAGATGCTTCT-3', which resulted in the deletion of two UBZ motifs at the N-terminal region of SLX4. The expression vector was constructed by ligating together a β-actin promoter, *GgSLX4-wt* or *GgSLX4-UBZΔ*, and the drug-resistance marker gene (*Bsr*^R), in that order. We independently analyzed three clones, each of which grew with the same kinetics as the parent *SLX4*^{-/-}*tetSLX4* cells.

Immunoprecipitation of SLX4-GFP. DT40 cells carrying the *SLX4-GFP* or *SLX4-UBZΔ-GFP* transgene were treated with 1% formaldehyde for 5min at room temperature and 62.5mM glycine was added to stop the reaction. The cells were harvested and the cell extracts were prepared by sonicating the cell pellet in SDS lysis buffer (50mM Tris-HCl, pH 8.0, 10mM EDTA, 1% SDS) supplemented with a protease inhibitor cocktail (Roche). The cell extracts were diluted with dilution buffer (50mM Tris-HCl, pH 8.0, 167mM NaCl, 1.1% Triton X-100, 0.1% sodium deoxycholate) 20 fold. Whole cell extracts and immunoprecipitates were incubated with SDS elution

buffer (10mM Tris-HCl, pH 8.0, 300mM NaCl, 5mM EDTA, 0.5% SDS) at 65° C for 4 h.

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Figure legends

Figure 1 SLX4 is essential for cellular proliferation

(A) RT-PCR analysis of *SLX4* transcripts in *SLX4*^{-/-}*tetSLX4* cells using primers hybridizing with exon 14 and 18. β -actin served as loading control. (B) Growth curves of the indicated cells. The *tetSLX4* transcription was active without doxycyclin (ON) and inhibited upon addition of doxycyclin (OFF). (C) Representative cell-cycle profiles of indicated cells. Cells were pulse-treated with BrdU for 10 min, then subjected to flow-cytometric analysis. PI-staining is displayed on the x-axis (linear scale), whereas BrdU incorporation is displayed on the y-axis (logarithmic scale). The square on the left-hand side indicates apoptotic cells (sub-G₁ fraction), the rectangle at the bottom left represents G₁ phase cells, the arch represents S phase cells and the square at the bottom right represents G₂/M phase cells. Numbers indicate the relative percentage of cells falling into each gate, including dead cells in the sub-G₁ fraction. The experiment was repeated three times, and means $\pm$ standard deviations are shown. (D) Number of chromosome aberrations per 50 metaphase nuclei of indicated cells.

Figure 2 The UBZ domain of SLX4 is required for cellular tolerance to ICL inducing agents. Cells were exposed to the indicated genotoxic agents for 72 h, a period during which *wild-type* cells were able to divide nine times in the absence of exogenous DNA damage. The x-axis represents the concentration of the genotoxic agents and the y-axis represents the relative number of surviving cells at 72 h (logarithmic scale). Error bars represent standard deviation from independent experiments.

Figure 3 Recruitment of SLX4 to DNA-damage sites thorough interaction between SLX4-UBZ domain and ubiquitylated FANCD2.

(A) Localization of FANCD2, SLX4-GFP, and SLX4-UBZ Δ -GFP in cells treated with MMC. Indicated cells were exposed to 500 ng/ml MMC for 6 h. Fixed cells were stained with anti-FANCD2 antibody and visualized by fluorescence microscopy. Percentage of co-localization of SLX4-GFP and FANCD2 foci is shown in the bar graph. Cells displaying more than four co-localized foci were defined as foci-positive. (B) DT40 cells expressing *SLX4-GFP* or *SLX4-UBZ Δ -GFP* transgene were treated with 500ng/ml MMC for 6 h. Extracts of formaldehyde-fixed cells were incubated with an anti-GFP antibody and the immunoprecipitates were analyzed with antibodies against FANCD2. SLX4-GFP interacts preferentially with the mono-ubiquitylated form of

FANCD2.

Figure 4 Genetic relationship between *FANCC* and *SLX4-UBZΔ*

(A) Sensitivity of cells with the indicated genotype to cisplatin and MMC. The drug concentrations are displayed on the x-axes (linear scale), whereas the relative number of surviving cells at 72 h is displayed on the y-axes (logarithmic scale). Error bars represent standard deviation from 3 independent experiments. (B) Number of chromosomal aberrations per 50 metaphase nuclei from the indicated cells. Cells were treated with MMC (40ng/ml, 24 h). *FANCC/SLX4-UBZΔ* cells displayed a larger number of chromosomal aberrations than *SLX4-UBZΔ* or *FANCC/SLX4^{-/-}tetSLX4* cells ($P < 0.0001$ for each).

Figure 1

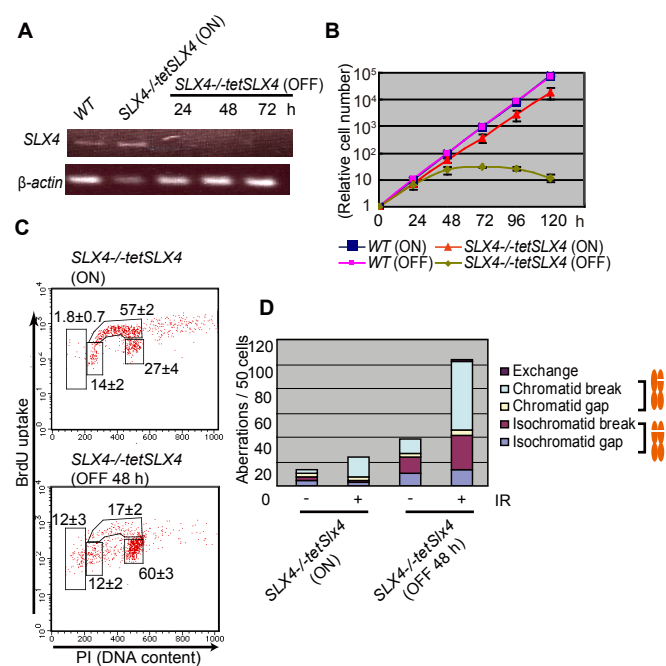


Figure 2

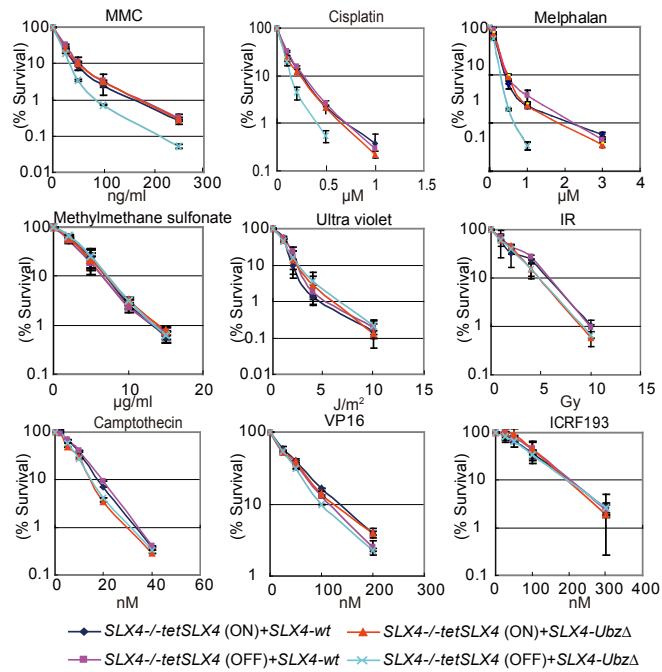
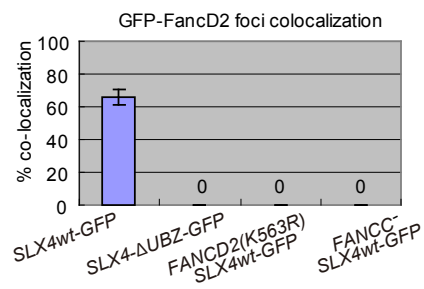
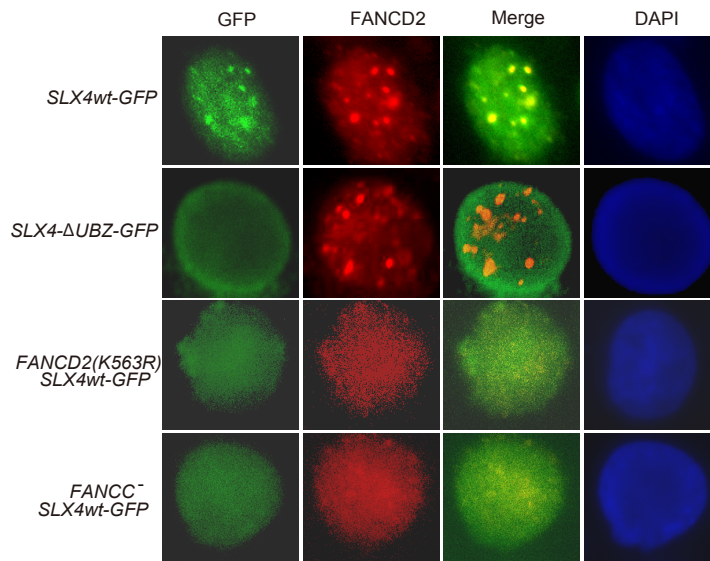


Figure 3

A



B

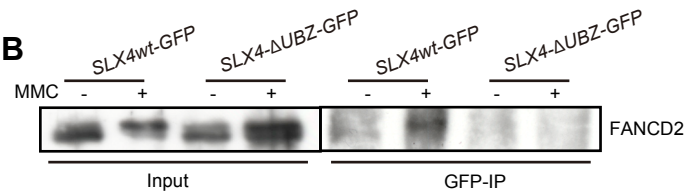
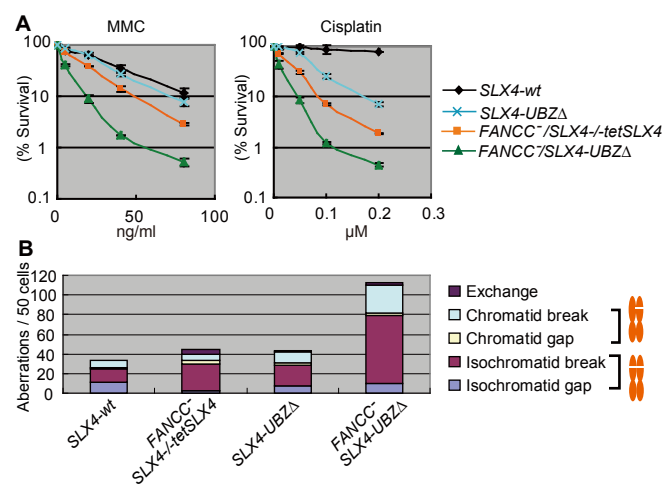
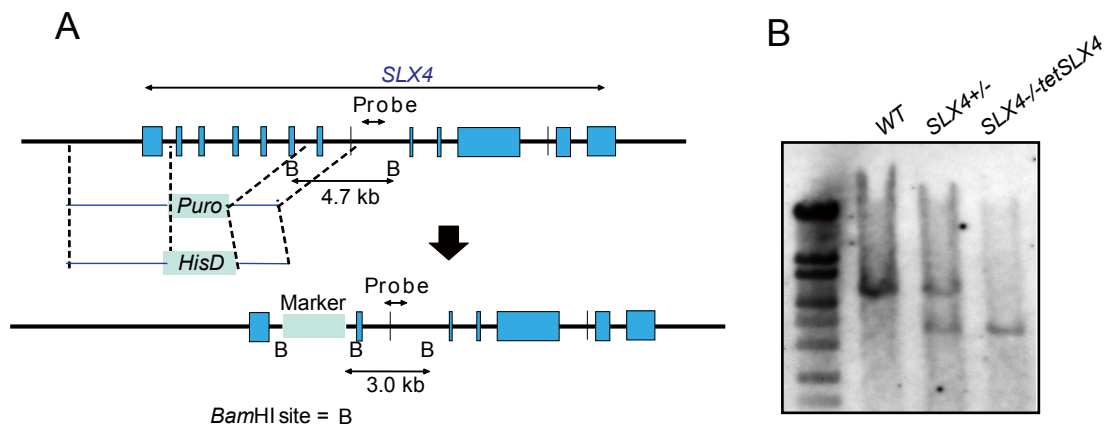
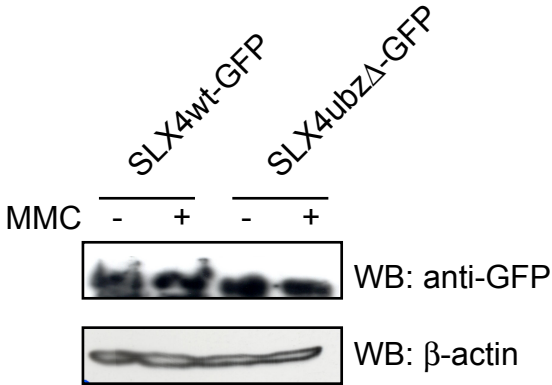


Figure 4

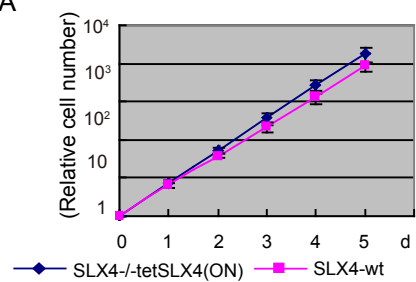


Supplemental Figure S1

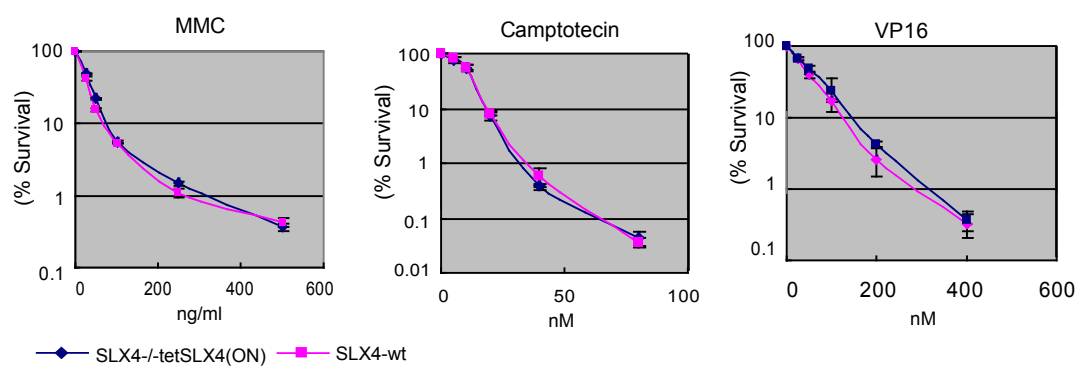




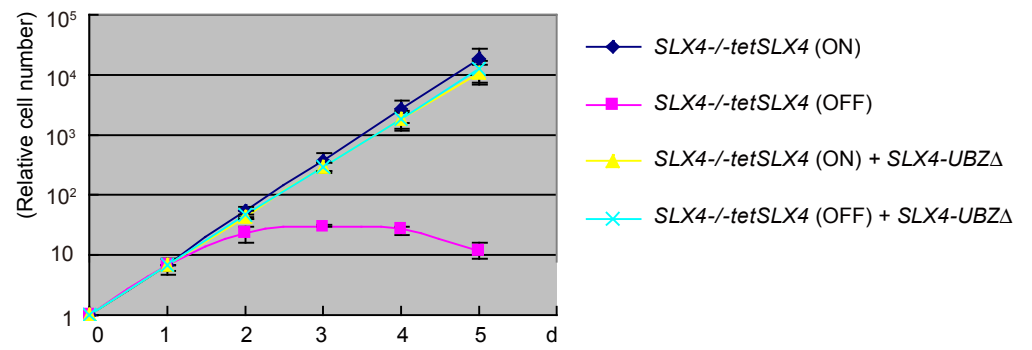
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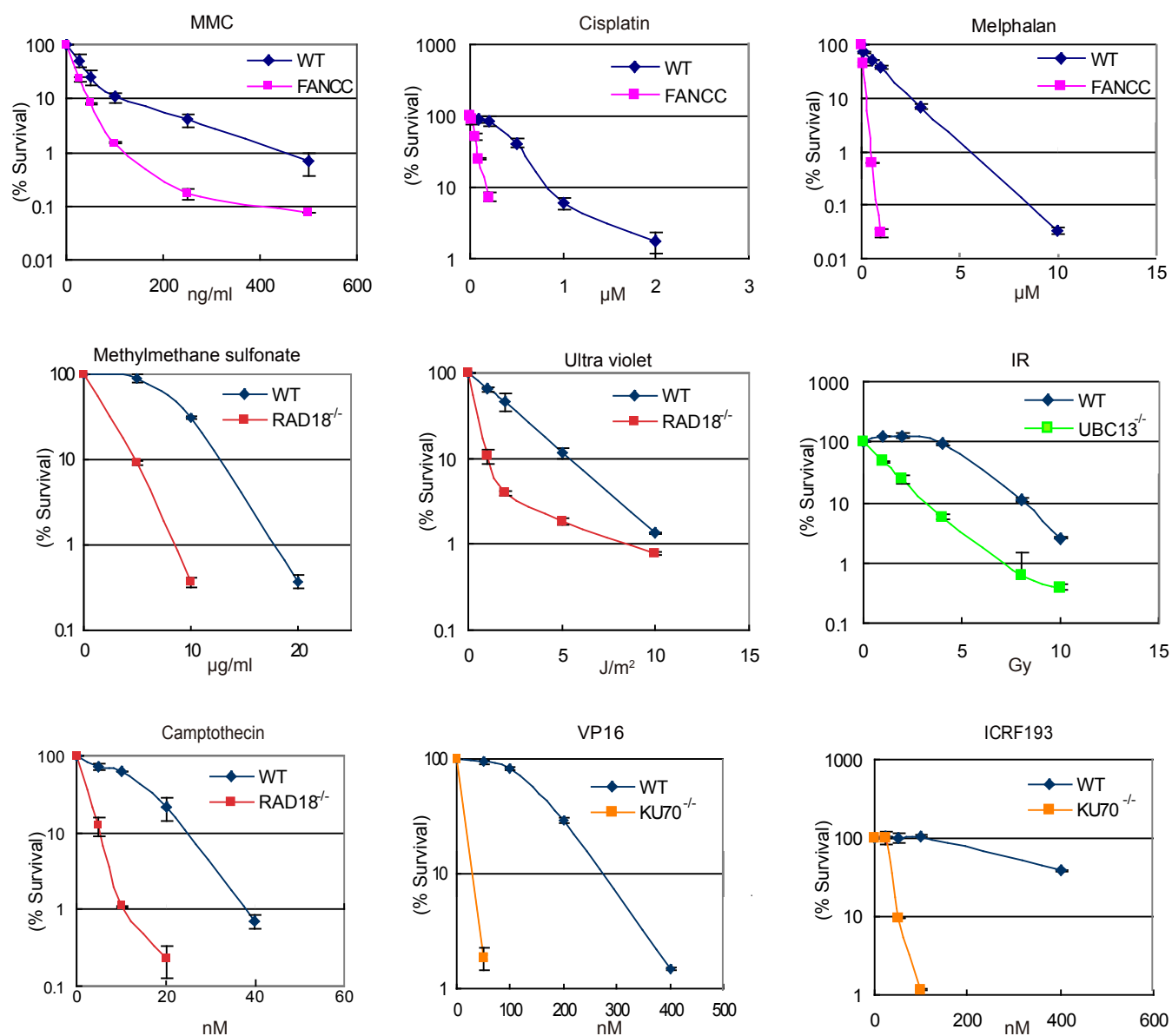


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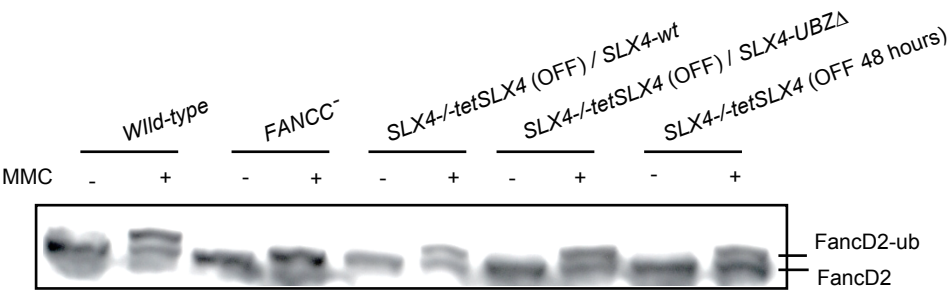


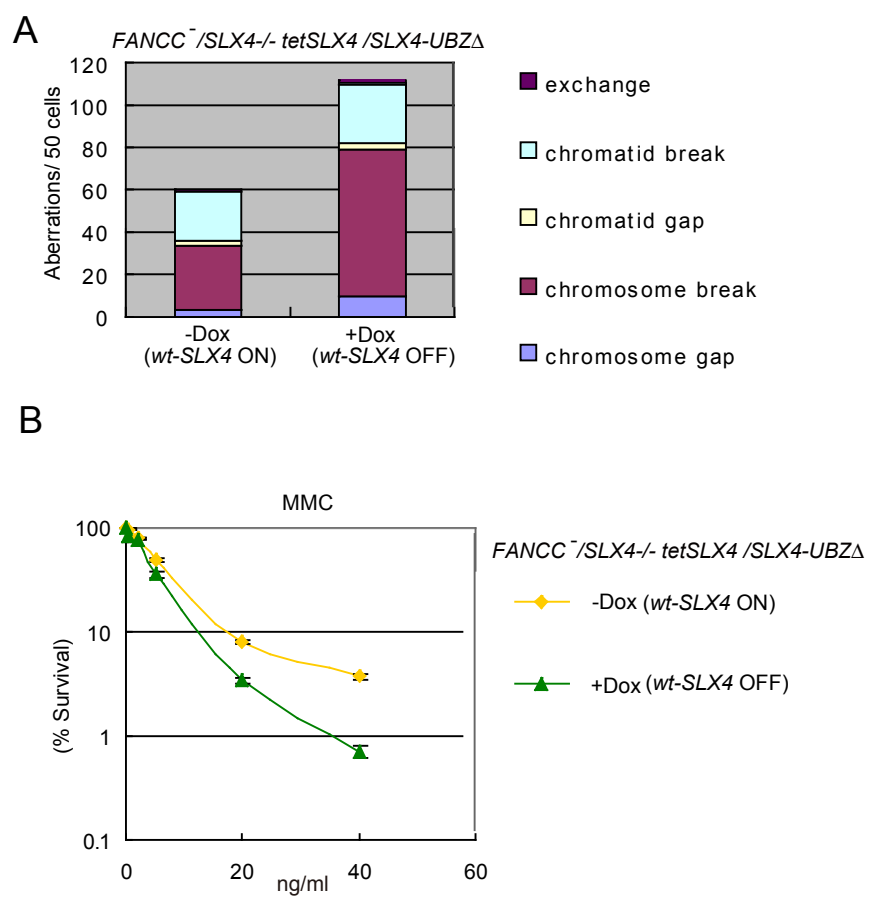
Supplemental Figure S4





Supplemental Figure S6





Supplemental Figure S8

