Involvement of SLX4 in interstrand cross-link repair is regulated by the Fanconi anemia pathway

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Interstrand cross-links (ICLs) block replication and transcription and thus are highly cytotoxic. In higher eukaryotes, ICLs processing involves the Fanconi anemia (FA) pathway and homologous recombination. Stalled replication forks activate the eight-subunit FA core complex, which ubiquitylates FANCD2-FANCI. Once it is posttranslationally modified, this heterodimer recruits downstream members of the ICL repairosome, including the FAN1 nuclease. However, ICL processing has been shown to also involve MUS81-EME1 and XPF-ERCC1, nucleases known to interact with SLX4, a docking protein that also can bind 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, with sister chromatids broken at the same site. *SLX4* thus appears to play an essential role in cell proliferation, probably by promoting the resolution of interchromatid homologous recombination 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 found that *SLX4*^{-/-} cells expressing UBZ-deficient SLX4 were selectively sensitive 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.

endonuclease | mitomycin C | cisplatin | DNA repair

Interstrand cross-links (ICLs) inhibit transcription and replication. Their considerable cytotoxicity has been ascribed primarily to their blockage of replication forks, and this phenomenon 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, involving proteins from several distinct pathways of DNA metabolism. In higher organisms, ICL processing 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 monoubiquitylated FANCD2-FANCI complex is then targeted to chromatin (7, 8), where it recruits downstream components of the repairosome, including the structure-specific nuclease FAN1 (9–12). However, ICL processing also requires other enzymes, such as the nucleases MUS81-EME1 and XPF-ERCC1, and how these are recruited to sites of damage in ICL repair is not known.

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 also interacts with SLX1 in both yeast (23, 24) and 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 down-regulation sensitizes human cells to a wide variety of DNA-damaging agents (21, 22, 25, 26). However, although SLX4 also has been implicated in ICL repair, its role in this process, as well as its possible link to the FA pathway, remain 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 the loss of SLX4 induces cell death associated with extensive chromosomal aberrations, which shows that SLX4 plays a key role in the repair of spontaneous DNA damage. Many proteins interacting with ubiquitylated polypeptides contain 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 on the 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 monoubiquitylated FANCD2. Thus, our data 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 124–518 of the protein (Fig. S1A). However, we failed to generate *SLX4*^{-/-} cells, suggesting that SLX4 is essential for DT40 cell survival. Thus, we 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 blot analysis (Fig. S1B). RT-PCR analysis showed that the *SLX4*^{-/-}*tetSLX4* cells expressed *SLX4* mRNA, and that this transcript was un-

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detectable in doxycycline-treated cells after 72 h (Fig. 1A). The *SLX4*^{-/-}*tetSLX4* cells proliferated slightly slower than the WT cells, possibly due to nonphysiological levels of *SLX4* expression (Fig. 1B). At 24 h after the addition of doxycycline, the *SLX4* transcript was barely detectable (Fig. 1A), and the proliferation of *SLX4*^{-/-}*tetSLX4* cells slowed considerably (Fig. 1B). After 48 h of 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 to the viability of *SLX4* knockout mice (30). This difference might be attributable to the absence of the *MUS81* gene in the chicken genome. Chromosomal analysis of mitotic cells showed an increased number of spontaneous chromosomal aberrations, as seen in HR-deficient cells (Fig. 1D and Table S1). The aforementioned phenotypic traits were observed in two independent *SLX4*^{-/-}*tetSLX4* clones, arguing against clone-specific effects and demonstrating that SLX4 plays a pivotal role in the repair of spontaneous DNA damage (Fig. 1D and Table S1).

SLX4 Is Required for HR-Dependent Double-Strand Break 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) at 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 irra-

diated 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 (i.e., breaks in one sister chromatid). In *SLX4*-depleted cells, a marked increase in the total number of chromosomal breaks was observed, coupled with a shift from chromatid-type to isochromatid-type breaks (i.e., breaks at the same site of both sister chromatids) (Fig. 1D and Table S1). The latter type of break is likely caused by HR-dependent repair between the broken and the intact sister chromatids, followed by defective resolution of recombination intermediates (33). Taking roles 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.

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 then 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), demonstrating that deletion of the UBZ domain did not affect SLX4 stability. As expected, the *SLX4-wt* cells displayed essentially the same phenotype as the 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 Monoubiquitylated FANCD2. Disruption of the FA core complex abolishes DNA damage-induced ubiquitylation of FANCD2, as exemplified for *FANCC*^{-/-} cells (Fig. S6). Because 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 activation of the ubiquitin ligase activity of the FA core complex.

After treatment with DNA damaging agents, FANCD2 and other repair proteins accumulate in subnuclear foci (7–9). To examine 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 colocalized with those containing FANCD2 (Fig. 3A). The finding that MMC-induced SLX4-GFP foci failed to form in similarly transfected *FANCC*^{-/-} and *FANCD2-K563R* (40) DT40 lines, in which monoubiquitylation of FANCD2 is impaired (40, 41), indicates that FANCD2 monoubiquitylation is required for SLX4 recruitment to damage sites (Fig. 3A).

We next asked whether the foregoing recruitment required the UBZ domain of SLX4. We found that the *SLX4-UBZΔ*-GFP mutant protein failed to form MMC-induced foci in WT DT40

Fig. 1. SLX4 is essential for cellular proliferation. (A) RT-PCR analysis of *SLX4* transcripts in *SLX4*^{-/-}*tetSLX4* cells using primers hybridizing with exons 14 and 18. β -actin served as a loading control. (B) Growth curves of the indicated cells. The *tetSLX4* transcription was active without doxycycline (ON) and was inhibited after the addition of doxycycline (OFF). (C) Representative cell cycle profiles of indicated cells. Cells were pulse-treated with BrdU for 10 min, then subjected to flow cytometric analysis. Propidium iodide staining is displayed on the x-axis (linear scale), and BrdU incorporation is displayed on the y-axis (logarithmic scale). The square on the left 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 in each gate, including dead cells in the sub-G₁ fraction. The experiment was repeated three times; values are mean $\pm$ SD. (D) Number of chromosomal aberrations per 50 metaphase nuclei of indicated cells.

Fig. 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 WT 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 SD from independent experiments.

cells, even though FANCD2 foci formed normally (Fig. 3A). This indicates that the recruitment of SLX4 to MMC-induced DNA damage sites is dependent on both the SLX4 UBZ domain and monoubiquitylated FANCD2.

The foregoing colocalization suggests an interaction between SLX4 and FANCD2. 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, monoubiquitylated FANCD2 was present in immunoprecipitates isolated from extracts of MMC-treated cells expressing SLX4-GFP, but not those expressing SLX4-UBZΔ-GFP. These data indicate that SLX4 forms a complex with monoubiquitylated FANCD2, and that this interaction requires the SLX4 UBZ domain. Whether or not this interaction is direct remains to be investigated.

FA Pathway and SLX4 Are Not Fully Epistatic in ICL Processing. Having established that SLX4 interacts with monoubiquitylated 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* cells 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 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, despite 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

Fig. 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-UBZΔ-GFP in cells treated with MMC. Indicated cells were exposed to 500 ng/mL of MMC for 6 h. Fixed cells were stained with anti-FANCD2 antibody and visualized by fluorescence microscopy. The bar graph shows the percentage of colocalization of SLX4-GFP and FANCD2 foci. Cells displaying more than four colocalized foci were defined as foci-positive. (B) DT40 cells expressing the *SLX4-GFP* or *SLX4-UBZΔ-GFP* transgene were treated with 500 ng/mL of 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 monoubiquitylated form of FANCD2.

compared with WT DT40 cells. The finding of a larger number of chromosomal aberrations in *FANCC^{-/-}/SLX4-UBZΔ* cells than in either *SLX4-UBZΔ* or *FANCC^{-/-}/SLX4^{-/-}tetSLX4* cells (Fig. 4B and 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 have demonstrated 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 monoubiquitylated 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

Fig. 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-axis (linear scale), and the relative number of surviving cells at 72 h is displayed on the y-axis (logarithmic scale). Error bars represent SD from three independent experiments. (B) Number of chromosomal aberrations per 50 metaphase nuclei from the indicated cells. Cells were treated with MMC (40 ng/mL for 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.

of FAN1 (9–12) and the recruitment of SLX4 documented in the present study.

The following lines of evidence support our conclusion that SLX4 participates in ICL repair downstream of monoubiquitylated 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 monoubiquitylation (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); and (v) recent reports describe 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, indicating 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). Similar to cells lacking both FANCC and SLX4, *FANCC*^{-/-}/*FAN1*^{-/-} DT40 cells also were more sensitive to ICL-inducing agents compared with cells lacking only FAN1 (44). Thus, the FA pathway appears to control, and possibly coordinate, the action of the nucleases SLX1, XPF-ERCC1, and MUS81-EME1 known to associate with SLX4, as well as FAN1. Moreover, the *FANCC*^{-/-}/*SLX4-UBZΔ* cells displayed a more severe phenotype than the *FANCC*^{-/-} cells, indicating that the UBZ domain of SLX4 also might interact 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 of the UBZ domain of SLX4 (2/50 in *FANCC*^{-/-}/*SLX4-UBZΔ* cells vs. 6/50 in *FANCC*^{-/-}/*SLX4*^{-/-}*tetSLX4* cells; *P* = 0.0374, Fisher's exact test) (Fig. 4 and Table S2). Moreover, loss of FAN1 also suppresses chromosomal exchanges in FA cells after 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, these findings indicate that 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 the different types of chromosomal aberrations. The loss of the SLX4 UBZ domain would abolish the FA-independent interactions, and thus the pattern of chromosomal aberrations would change 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 that *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 shown by our preliminary findings indicating that disruption of the *XPF* gene in *MUS81*-deficient DT40 cells is lethal (data not shown). Given that these endonucleases share a preference for 3'-flap substrates, their inactivation might result in the total loss of this activity from the repairosome responsible for the resolution of HR intermediates, and thus in lethality. Similarly, it might be anticipated that cells lacking FAN1 and SLX1 also will be nonviable due to the loss of 5'-flap endonuclease activity of the complex responsible for resolution of HR intermediates.

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

Methods

Additional details are provided in *SI 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'-GAAACGATAGTGAATGGATCCC-3' and then cloned using the TOPO Cloning Kit (Invitrogen). *SLX4* targeting vector was constructed to replace five exons (exons 5–9) 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'-GCAAGGCGTAATTTGTGGT-3' and 5'-CCAAGCCCAATTCTTTTCA-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 613-bp probe was a PCR-amplified fragment derived from chicken DT40 genomic DNA using the primer set 5'-CTCTGCTTTAATTCACAGC-3' and 5'-AATCAGTACTAAATGTGAGC-3'. The probe was labeled using the Alkphos Direct Labeling Module (GE Healthcare). To generate *SLX4*^{+/-} and *SLX4*^{-/-} cells, WT DT40 cells were transfected sequentially with the *SLX4* targeting vectors with *Puro*^R and then with *HisD*^R. Although we identified eight *SLX4*^{+/-} clones from the 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 transactivator (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 RT-PCR using the SuperScript III First-Strand System (Invitrogen) and the 5'-TGCAGGTGTCACGCTTGGT-3' and 5'-CCTATTCCAAGCTGGTGCTGG-3' primers.

Generation of SLX4-wt and SLX4-UBZΔ Cells. The *Puro*^R and *Bsr*^R cassettes were removed 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'-GAAA-GCCTGGAGGATGCAGGC-3' and 5'-GTTCTCTTAAGATGCTTCT-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 5 min at room temperature, and 62.5 mM 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 [50 mM Tris-HCl (pH 8.0), 10 mM EDTA, 1% SDS] supplemented with a protease inhibitor mixture (Roche). The cell extracts were diluted 20-fold with dilution buffer [50 mM Tris-HCl (pH 8.0), 167 mM NaCl, 1.1% Triton X-100, 0.1% sodium deoxycholate]. Whole cell extracts and immunoprecipitates were incubated with SDS elution buffer [10 mM Tris-HCl (pH 8.0), 300 mM NaCl, 5 mM EDTA, 0.5% SDS] at 65 °C for 4 h.

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