Localization-Dependent and -Independent Roles of SLX4 in Regulating Telomeres

Jamie S.J. Wilson,1 Agueda M. Tejera,2 Dennis Castor,1 Rachel Toth,1 Maria A. Blasco,2 and John Rouse1,*

1 MRC Protein Phosphorylation and Ubiquitylation Unit, College of Life Sciences, University of Dundee, Dundee DD1 5EH, Scotland, UK
2 Spanish National Cancer Research Centre (CNIO), Molecular Oncology Program, Madrid E-28029, Spain

*Correspondence: j.rouse@dundee.ac.uk
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SUMMARY

SLX4, a scaffold for structure-specific DNA repair nucleases, is important for several types of DNA repair. Many repair proteins bind to sites of DNA damage, resulting in subnuclear “foci,” but SLX4 forms foci in human cells even without DNA damage. Using several approaches, we show that most, but not all, SLX4 foci localize to telomeres in a range of human cell lines irrespective of the mechanisms used to maintain telomere length. The SLX1 Holliday-junction-processing enzyme is recruited to telomeres by SLX4, and SLX4, in turn, is recruited by a motif that binds to the shelterin subunit TRF2 directly. We also show that TRF2-dependent recruitment of SLX4 prevents telomere damage. Furthermore, SLX4 prevents telomere lengthening and fragility in a manner that appears to be independent of telomere association. These findings reveal that SLX4 plays multiple roles in regulating telomere homeostasis.

INTRODUCTION

SLX4 is a scaffold protein that binds to three DNA repair endonucleases, MUS81-EME1, XPF-ERCC1, and SLX1 (Andersen et al., 2009; Fekairi et al., 2009; Muñoz et al., 2009; Saito et al., 2009; Svendsen et al., 2009). The SLX4 complex is required for the efficient repair of DNA interstrand crosslinks (ICLs), (Fekairi et al., 2009; Muñoz et al., 2009; Svendsen et al., 2009), and the available evidence strongly suggests a role in processing DNA recombination intermediates during ICL repair. The importance of SLX4 for ICL repair was underscored by the findings that biallelic mutations in SLX4 in humans causes Fanconi anemia (FA) (Kım et al., 2011; Stoepker et al., 2011).

Many DNA repair proteins form subnuclear “foci” at sites of DNA damage, but SLX4 overexpressed in epitope-tagged form localizes to subnuclear foci even without DNA damage (Svendsen et al., 2009). It was suggested that these foci correspond to telomeres, regions of repetitive DNA at chromosome ends, which protect the ends from degradation (Svendsen et al., 2009). Telomeres terminate in an overhang that is thought to invade adjacent duplex telomeric repeats to form a telomeric (T) loop so that the chromosome ends are not perceived as DNA breaks. An additional layer of telomere protection is afforded by a multiprotein complex called shelterin, that binds to telomeric DNA (Palm and de Lange, 2008). In normal somatic cells, telomeres shorten with every cell division, and telomere shortening contributes to organismal aging by limiting the proliferative capacity of adult stem cells (Blasco, 2007). In immortalized cells and in cancers, telomere length is maintained by telomerase, a reverse transcriptase that can add telomere repeats to telomeres by SLX4, and SLX4, in turn, is recruited by a motif that binds to the shelterin subunit TRF2 directly. We also show that TRF2-dependent recruitment of SLX4 prevents telomere damage. Furthermore, SLX4 prevents telomere lengthening and fragility in a manner that appears to be independent of telomere association. These findings reveal that SLX4 plays multiple roles in regulating telomere homeostasis.

RESULTS

The Endogenous SLX4 Complex Localizes at Telomeres

We first tested if endogenous SLX4 localizes at telomeres. Using antibodies raised in-house, we found that endogenous SLX4 localizes...
forms subnuclear foci in U2OS cells without addition of genotoxins (Figure 1A). These foci are specific for SLX4 because they disappeared when cells were transfected with SLX4-specific small interfering RNAs (siRNAs) (Figure S1A). Endogenous SLX4 foci did not appear to be restricted to any particular cell-cycle stage, and they did not increase in number or intensity after exposure of cells to genotoxins (data not shown). To investigate if endogenous SLX4 foci correspond to telomeres, we tested colocalization with subunits of shelterin. Most (around 80%), but not all, endogenous SLX4 foci colocalized with TRF2 in U2OS cells (Figure 1A), and also with TRF1 and with a peptide-nucleic acid (PNA) probe specific for telomeric DNA (Figure 1B). When signal intensity was measured along a straight line in a single Z-section through the nucleus, the peaks corresponding to SLX4 overlap with TRF2 (Figure 1C). It is important to note that, whereas all TRF2 foci in U2OS cells contained SLX4, a proportion of SLX4 foci did not overlap with TRF2 (Figure 1A). These observations indicate that SLX4 is found at all telomeres in U2OS cells, but a proportion of the SLX4 foci do not correspond to telomeres.

SLX4 associates with three separate structure-specific nucleases, XPF-ERCC1, MUS81-EME1, and SLX1. XPF-ERCC1 was previously shown to localize at telomeres (Zhu et al., 2003), and in this light we found that all of the XPF-ERCC1 foci in cells colocalize with SLX4 (Figures S1B and S1D). MUS81-EME1 was also shown to localize at telomeres (Zeng et al., 2009), but only around 5% of U2OS cells show MUS81 foci colocalizing with SLX4 (Figures S1C and S1D). Endogenous SLX1 foci were only barely detectable with our anti-SLX1 antibodies (data not shown). SLX4 controls the stability of SLX1 (Muñoz et al., 2009), and overexpression of a GFP-tagged form of SLX4 increased the levels of endogenous SLX1 (data not shown). Under these conditions, endogenous SLX1 foci could be detected with anti-SLX1 antibodies (Figure 1D), and most of these foci colocalize with TRF2 (data not shown). In contrast, SLX1 foci could not be detected when we overexpressed a mutant form of SLX4 (C1805R) that is incapable of interacting with SLX1 (Figure 1D) (D.C., N. Nair, A.C. Declais, C. Lachaud, R.T., T.J. Macartney, D.M.J. Lilley, J.S.C. Arthur, and J.R., unpublished data). We also found that a GFP-tagged form of SLX1 formed foci that colocalized with TRF2 (Figure 1E). Taken together, these data show that the endogenous SLX4 complex localizes at telomeres.

SLX4 Binds to Telomeres in Cells Differing in Mechanisms of Telomere Length Maintenance

To confirm the association of SLX4 with telomeres, we carried out chromatin immunoprecipitation (ChIP) experiments. Telomeric DNA, but not Alu repeat DNA, was detected in SLX4 immunoprecipitates of U2OS cells using two separate anti-SLX4 antibodies raised in sheep, but not when nonspecific sheep immunoglobulin (Ig) G or anti-CENPA antibodies were used. The ChIP signal in SLX4 precipitates was comparable to the signal in TRF2 precipitates (Figure 2A). We also detected telomeric DNA, but not Alu DNA, in anti-SLX1 immunoprecipitates in U2OS cells (Figure 2B).

Telomere length is maintained by the ALT pathway in U2OS cells, whereas in other cell lines telomere length can be maintained by telomerase (Cesare and Reddel, 2010). ChIP experiments showed that SLX4 associates with telomeric DNA, but not Alu DNA, in A549 and HeLa epithelial cells, two non-ALT cell lines that are telomerase positive (Figures 2C and 2D, left panel). This is in contrast to the behavior of SLX1, which can be maintained in ALT cells independently of telomerase (Figure 2D, right panel).
panels). The ChIP signal in anti-SLX4 immunoprecipitates from these cells was weaker than in U2OS cells, probably because A549 and HeLa cell telomeres are shorter than in U2OS cells (Lee et al., 2008). Nonetheless, the ChIP signal in SLX4 precipitates from A549 and HeLa cells was comparable to the signal in TRF2 precipitates (Figures 2C and 2D, left panels). Furthermore, SLX4 foci in A549 cells and HeLa cells are considerably less intense than in U2OS cells, but a significant proportion of the foci still colocalized with TRF2 (Figures 2C and 2D, middle panels). When signal intensity along an arbitrary track in a single Z-section of the nucleus was quantitated, the peaks corresponding to SLX4 and TRF2 in A549 and HeLa cells demonstrated good overlap (Figures 2C and 2D, right panels). We also observed SLX4 foci in CCL-211 primary lung fibroblasts, and some of these colocalize with TRF1 similar to HeLa cells (Figure S1E). Although a significant proportion of telomeric foci in these cells do not contain SLX4, when signal intensity along an arbitrary track in a single Z-section of the nucleus was quantitated, the peaks corresponding to SLX4 demonstrated reasonably good overlap with TRF1 (Figure S1E). Taken together, these data show that the SLX4 complex localizes at telomeres in a range of human cell lines that differ in the mechanisms used to maintain telomere length.

**SLX4 Has a TRF2-Binding Motif**

Both subunits of the TRF2-RAP1 shelterin subcomplex were found previously in SLX4 immunoprecipitates (Mun˜oz et al., 2009; Svendsen et al., 2009). We next sought to determine whether SLX4 is recruited to telomeres by either of these two proteins. Yeast two-hybrid analysis, using XPF as a positive control, revealed that SLX4 binds to TRF2, not RAP1 (Figure 3A), and it seemed likely therefore that SLX4 is recruited to telomeres by TRF2. To investigate this possibility, we sought mutations in SLX4 that abolish the interaction with TRF2, and our search was aided by a previous report describing a motif found in a range of proteins that interact with TRF2 (F/Y-X-L-X-P; Figure 3B) (Chen et al., 2008). Inspection of the primary amino acid sequence of human SLX4 failed to identify a classical TRF2-binding motif (TBM), but we found a TBM-like motif (H 1020-X-L1022-X-P1024) in which the first residue (F/Y) is replaced by a histidine residue, which like F and Y contains a planar aromatic ring structure (Figure 3B). This motif in SLX4 lies after the BTB domain in an unstructured region of the protein (Figure 3B, data not shown).

We next tested the effect of mutating the key residues in the putative TBM in human SLX4 on its interaction with TRF2. To this end, U2OS cells were cotransfected with RFP-tagged TRF2 and HA-tagged SLX4 wild-type or HA-SLX4 in which H1020, L1022, or P1024 were mutated to alanine. Whereas...
wild-type HA-SLX4 coprecipitated with RFP-TRF2, mutation of H1020, L1022, or P1024 caused a major reduction in the amount of HA-SLX4 coprecipitating with RFP-TRF2 (Figure 3C). Similar results were obtained when GFP-tagged SLX4 was immunoprecipitated from cells coexpressing RFP-TRF2 (Figure 3D). Importantly, mutating H1020, L1022, or P1024 had no effect on the ability of SLX4 to interact with XPF-ERCC1, MUS81, or SLX1 (Figure S2A). Taken together, these data show that a TBM in human SLX4 mediates interaction with TRF2. Intriguingly, a clear TBM is found in SLX4 in primates but not in mammals lower down the evolutionary tree (Figure S2B). SLX4 orthologs in some mammals have a motif that is vaguely similar to the TBM in primates (Figure S2B), but none of the mammalian motifs would be expected to interact with TRF2 based on previous work (Chen et al., 2008).

**Figure 3. A TRF2-Binding Motif in SLX4**

(A) Yeast two hybrid analysis was performed with a GAL4 activation domain (GAD) fusion of SLX4 and GAL4 DNA binding domain (GBD) fusion of TRF2, RAP1, or XPF to detect interaction between these proteins. Cells grown on medium lacking LEU and TRP (to select for bait and prey plasmids) were replica plated to medium lacking LEU, TRP, and HIS to test for activation of the HIS3 reporter gene. (B) Schematic diagram of the modular domain organization of SLX4. The putative TBM is highlighted in red, and the sequence of the putative TBM from SLX4 is compared with the classical TRF2-binding motif (TBM). (C) U2OS cells were cotransfected with RFP-tagged TRF2 and HA-tagged SLX4 wild-type (WT), or HA-SLX4 bearing alanine substitutions at H1020, L1022, or P1024. Vector expressing HA tag only was used as control. Cells were lysed and subjected to immunoprecipitation with anti-RFP antibodies, and precipitates were probed with the antibodies indicated. (D) Same as (C) except that SLX4 was GFP tagged, and extracts were subjected to immunoprecipitation with anti-GFP antibodies. (E) Indirect immunofluorescence analysis of U2OS cells stably expressing GFP-SLX4-L1022A. GFP-SLX4 or GFP-SLX4-L1022A that cococalize with TRF2, that are adjacent to but not overlapping with TRF2, or that do not colocalize with TRF2 was quantitated. (G) Telomere-ChIP analysis of GFP-SLX4 or GFP-SLX4-L1022A, or GFP only, stably expressed in U2OS cells. DNA from immunoprecipitates was subjected to 3-fold serial dilutions, before spotting onto Hybond N+ and hybridization with a radiolabeled telomeric probe (upper panel) or an Alu DNA probe (lower panel). Input DNA prepared from cell extracts before immunoprecipitation was subjected to similar analysis. The “input DNA” lane shows 10%, 3%, and 1% of the total DNA in cell extract, respectively. (H) The ChIP signal in the dots corresponding to each serial dilution for each immunoprecipitate in (G) was quantitated and added together. To normalize the hybridization signals, the resulting totals for each precipitate were divided by the total input signal (left panels).

**The SLX4 Complex Is Recruited to Telomeres by TRF2 in Human Cells**

We next tested if mutating the TBM in SLX4 affects its localization at telomeres. To this end, GFP-tagged wild-type SLX4, or GFP-SLX4-L1022A, was stably expressed in U2OS cells and foci were analyzed. Surprisingly, the overall number of foci formed by the SLX4 L1022A mutant was similar to wild-type SLX4 (Figures 3E and 3F). However, whereas almost 65% of wild-type GFP-tagged SLX4 foci colocalized with TRF2, only around 10% of foci formed by the SLX4 L1022A mutant...
coincided with TRF2, and more than 40% of the foci clearly did not (Figures 3E and 3F). The remaining 40% of SLX4-L1022A foci did not colocalize with TRF2, but were found adjacent to, but distinct from TRF2 foci (Figures 3E and 3F). To further test the impact of mutating the SLX4 TBM on telomere binding, we used ChIP analysis, which revealed that mutating SLX4 L1022 to alanine caused an almost 80% reduction in the amount of telomeric DNA associated with SLX4 (Figures 3G and 3H). These data suggest that TRF2 is required for localizing SLX4 at telomeres. We tested this idea further using a dominant-negative form of TRF2 (TRF2ΔBΔM) that heterodimerizes with endogenous TRF2 blocking its binding to DNA (van Steensel et al., 1998). As shown in Figure S2C, overexpression of an GFP-tagged form of TRF2ΔBΔM, but not wild-type TRF2, caused a substantial reduction in the number of SLX4 foci in U2OS cells (Figure S2C). Some of the cells transfected with TRF2ΔBΔM did not express this protein, and SLX4 foci were normal in these cells (Figure S2C). Taken together, these data indicate that TRF2 recruits SLX4 to telomeres in human cells.

We also tested if the SLX4-TRF2 interaction is required for association of SLX1 with telomeres. We showed earlier that endogenous SLX1 forms TRF2-coincident foci in cells overexpressing wild-type SLX4 (Figure 1D). SLX1 also forms foci in cells overexpressing SLX4-L1022A, but, although these foci colocalize with SLX4-L1022A, they do not colocalize with TRF2 (Figure S3A). This experiment shows that SLX1 is recruited to telomeres by SLX4.

SLX4 Localizes at and Repairs DNA Damage Independently of TRF2

SLX4 is required for efficient DNA repair. Because TRF2 has been shown to localize at sites of DNA damage (Huda et al., 2012), and to promote DNA repair (Huda et al., 2009), we tested the possibility that TRF2 might recruit SLX4 to DNA damage sites as well as to telomeres. To this end, we induced DNA damage using local laser microirradiation and then checked by confocal microscopy for the ability of SLX4 to form “laser stripes.” Time-lapse experiments revealed that GFP-tagged SLX4 formed subnuclear stripes along the track of DNA damage induced by laser irradiation visible within 12 min of irradiation, reaching a maximum intensity after 1 hr (Figure S3B). The SLX4-L1022A mutant localized at tracks of laser-induced DNA damage with similar kinetics and intensity to wild-type SLX4 (Figure S3B). We conclude from these data that TRF2 is not required to localize SLX4 at sites of DNA damage.

We also tested if the association of SLX4 with TRF2 is required for DNA repair using genotoxic hypersensitivity as readout. To this end, we stably expressed untagged forms of SLX4 and SLX4-L1022A in cells from Fanconi anemia patient EUFA-1354 that express abnormally low levels of an N-terminally truncated form of SLX4 (Stoeckper et al., 2011). Empty vector was used as control. As shown in Figure S3C, wild-type SLX4 and SLX4-L1022A are indistinguishable in their ability to rescue the mitomycin-C hypersensitivity of EUFA-1354 cells. Taken together, the data above indicate that neither the localization of SLX4 at DNA damage sites, nor the ability of SLX4 to promote DNA repair, requires binding to TRF2.

Localization-Dependent and -Independent Roles for SLX4 in Promoting Telomere Homeostasis

We next tested if preventing localization of SLX4 at telomeres affects telomere function. Because the L1022A SLX4 mutant interacts with XPF-ERCC1, MUS81-EME1, and SLX1 (Figure S2A) but does not localize at telomeres (Figures 3E–3H), we reasoned that it might function as a dominant negative by sequestering the associated nucleases away from telomeres. In this light, we made U2OS cells that stably express GFP-tagged SLX4, or GFP-SLX4-L1022A in a tetracycline-inducible manner. Upon induction of SLX4-L1022A, we noticed an increase in the proportion of cells with “TIFs” (telomere dysfunction-induced foci) (Takai et al., 2003), defined as 53BP1 foci that colocalize with TRF1, compared with cells expressing wild-type SLX4 (Figure 4A). In contrast, there was little difference between wild-type SLX4 and the SLX4-L1022A mutant in terms of the induction of 53BP1 foci that did not colocalize with TRF1 (Figure 4A). This experiment argues that failure to properly target SLX4 to telomeres causes telomere damage. Consistent with these data, siRNA-mediated depletion of SLX4 from U2OS cells increased the proportion of cells with TIFs (Figures S4A and S4B). SLX4-specific siRNAs 3 and 4 resulted in more efficient depletion of SLX4 than siRNAs 1 and 2, and consequently they were more potent at inducing TIFs.

Intriguingly, the lack of an obvious TRF2 binding motif in nonprimate mammalian orthologs of SLX4 suggests that SLX4 should not localize at telomeres in mouse cells, for example, and we next investigated this possibility. As shown in Figure S4C, whereas human SLX4 forms foci that colocalize with TRF2 when ectopically expressed in mouse embryonic fibroblasts (MEFs), mouse SLX4 forms foci but they do not colocalize with TRF2. This is reminiscent of the human SLX4-L1022A mutant that forms foci that do not colocalize with telomeres (Figure 3E–3H). These observations indicate that SLX4 does not associate stably with telomeres in mouse cells, at least in fibroblasts. Nonetheless, we reasoned that SLX4 might have roles at telomeres that do not require stable association with chromosome ends. With this in mind, we analyzed telomeres in Slx4−/− mice generated in this laboratory, which will be described in detail elsewhere (D.C., N. Nair, A.C. Declais, C. Lachaud, R.T., T.J. Macartney, D.M.J. Lilley, J.S.C. Arthur, and J.R., unpublished data). Analysis of organs and cells from these mice revealed a number of telomere defects. First, we observed increased incidence of TIFs in mouse embryonic fibroblasts (MEFs) from Slx4−/− mice (Figure S4D). Second, telomeres in livers from Slx4−/− mice were longer than in Slx4+/− mice or wild-type mice (Figure 4B). Telomeres were also longer in MEFs from Slx4−/− mice (Figure 4D). These data suggest that SLX4 might have a role in trimming telomeres to prevent overlengthening. We also found that telomeres from Slx4−/− MEFs show increased fragility assessed by the frequency of multitelomeric signals (MTSs) compared with Slx4+/− MEFs and wild-type cells (Figure 4D). The data above indicate that localizing of SLX4 to telomeres prevents DNA damage in human cells, and that SLX4 also plays a role in telomere homeostasis in mice in a manner that does not appear to require the stable retention of the SLX4 complex at telomeres.
In this study, we showed SLX4 is recruited to telomeres in human cells by TRF2. We identified a motif in human SLX4 required for direct interaction with TRF2 that is similar but not identical to the TRF2-binding motif (TBM; F/Y-X-L-X-P) identified in proteins such as Apollo (Chen et al., 2008). In the SLX4 motif, however, the F/Y residue is replaced by a histidine residue. This observation extends the consensus TRF2-binding motif and raises the possibility that other proteins might interact with TRF2 through TBM’s similar to that of SLX4. Although SLX4 is a rapidly evolving protein, it is somewhat surprising that the SLX4 TBM-like motif is conserved in primates but not in mammals lower down the evolutionary tree (Figure S2B). The absence of a TBM, however, does not exclude the possibility that SLX4 (or an SLX4-associated protein) in nonprimate species associates with TRF2 and/or telomeres by alternative mechanisms. However, we have been unable to detect endogenous SLX4 at telomeres in mouse embryonic fibroblasts, and, whereas human SLX4 overexpressed in MEFs forms telomeric foci, mouse SLX4 does not (Figure S4C). Therefore, SLX4 associates with TRF2 and telomeres in primates but not in other mammals. In human cells, preventing localization of SLX4 at telomeres caused signs of DNA damage (Figure 4A). The precise nature of this damage is not yet clear, but it might result from difficulties in one of the many processing steps during and after DNA replication necessary for establishing the correct structure at telomeres.

It appears that not all of the telomere-related functions of the SLX4 complex require stable association with telomeres, because telomeres are longer and more fragile in organs and cells from Slx4−−− mice and telomere shortening in Slx4−−− MEFls can be reversed by expression of wild-type SLX4 (Figures 4B and 4C). From this perspective, SLX4 might mediate “telomere trimming,” a mechanism that has been proposed to prevent overlengthening of telomeres (Pickett and Reddel, 2012). It is not yet clear which of the SLX4-associated nucleases are required to prevent telomere shortening. It is unlikely that XPF–ERCC1 is the relevant nuclease because telomere length is not affected in Ercc1−−− mice (Zhu et al., 2003). The nuclease that promotes SLX4-dependent telomere shortening could be MUS81–EME1 and/or SLX1; it will be interesting to test the relevant knockout mice for abnormally long telomeres. SLX1 is a good candidate considering the Boulton lab has shown that it is required for the production of T circles in cells lacking RTEL1 (Vannier et al., 2012). The consequences of overlong telomeres for cell function and organism function are not clear, but this will be interesting to study.

**EXPERIMENTAL PROCEDURES**

**Immunofluorescence Analysis**

U2OS cells expressing GFP-SLX4 were preextracted in CSK buffer: 10 mM PIPES (pH 6.8) containing 100 mM NaCl, 300 mM sucrose, 3 mM magnesium chloride, 1 mM EGTA, and 0.5% [w/v] Triton X-100, fixed with 2% paraformaldehyde (PFA) (pH 7.0), permeabilized with PBS containing 0.5% [w/v] Triton X-100, blocked with BSA (3% w/v) in PBS, and probed with primary antibodies for 1 hr at room temperature. After extensive washing, cells were incubated with secondary Alexa antibodies for 2 hr at room temperature. Cells were
stained with DAPI before mounting on glass slides. Wide-field image stacks of cells were acquired using a DeltaVision microscope (Applied Precision) and subjected to iterative deconvolution to remove out-of-focus light and were further processed in OMERO (Allan et al., 2012). For endogenous SLX4 immunofluorescence, cells grown on poly-L-lysine-coated coverslips were pre-extracted in CSK buffer or PBS containing 0.5% Triton X-100 prior to fixation in 3% PFA in CSK (pH 7.0). Cells were permeabilized in PBS-containing 0.5% (v/v) Triton X-100 and then blocked in antibody dilution buffer (AbDil): PBS containing 5% normal donkey serum, 0.1% fish skin gelatin, 0.1% Triton X-100, 0.05% Tween-20, and 0.05% sodium azide, before incubation with primary antibodies in AbDil for 1 hr at room temperature or overnight at 4°C. After washing, cells were incubated with the relevant secondary antibodies for 2 hr at room temperature, stained with DAPI, and mounted on glass slides.

**Supplemental Information**

Full details of all other experimental procedures are given in the Extended Experimental Procedures.

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Zeng, S., Xiang, T., Pandita, T.K., Gonzalez-Suarez, I., Gonzalo, S., Harris, C.C., and Yang, Q. (2009). Telomere recombination requires the MUS81 endonuclease. Nat. Cell Biol. 11, 616–623.
**Cell Culture, Fractionation, and Lysis**

U2OS cells were cultured in DMEM growth medium (GibCo) supplemented with 10% (v/v) fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin and 1% L-glutamate (GIBCO, Invitrogen). U2OS cells stably expressing GFP-SLX4 were generated using the Flp-In T-REx system (Invitrogen) as described previously (Muñoz et al., 2009). Cells were maintained in selective media containing 100 μg/ml hygromycin and 15 μg/ml blasticidin. Protein expression was induced by adding tetracycline to 0.1 μg/ml for 24 hr. For immunoprecipitations and Western blotting, cells were lysed in 50 mM Tris-HCl pH 7.4 containing 150 mM NaCl, 1 mM EDTA pH 8, 1 mM EGTA pH 8, 1% (v/v) Triton X-100, 270 mM sucrose supplemented with β-mercaptoethanol (0.1%v/v), benzonase (50mU/μl) and 1 Roche protease inhibitor tablet per 50 ml of buffer.

**Slx4−/− Mice**

The generation of Slx4−/− mice will be described elsewhere (D.C. and J.R, unpublished data). 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.

**Isolation and Immortalization of MEFs**

Day 13.5 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. MEFs were 3T3-immortalized.

**siRNA**

Cells were transfected with the relevant siRNA duplex (30 nM) using the calcium phosphate precipitation method or HiPerFect (QIAGEN) according to the manufacturer’s protocol. siRNA oligonucleotides were from MWG Biotech, Germany. The SLX4 mRNA target sequences used in Figures S4A and S4B were:

- SLX4-1 GAGAAGAACCCTAATGAAA dTdT
- SLX4-2 GCACAAGGGCCCAGAACAA dTdT
- SLX4-3 GGAGAAGGAAGCAGAGAAT dTdT
- SLX4-4 AAACGTGAATGAAGCAGAA dTdT
- SLX4-4 was used in Figure S1 A. The final concentration of siRNA added to cells was 20 nM.

**Antibodies**

The following primary antibodies were used in this study.

**Western Blotting**

Mouse anti-human XPF diluted 1:5000 (Thermoscientific MS1381); rabbit anti-TRF2 diluted 1:1000 (CST #2645). Sheep polyclonal SLX4 antibodies were raised against a bacterially-expressed protein fragment corresponding to anti-human SLX4 (amino acids 1535-1834) fused to GST. The antibodies were purified from sheep serum by affinity chromatography on CH-Sepharose to which the peptide immunocon had been covalently coupled. Sheep polyclonal antibody recognizing GFP was produced following the same protocol. Anti-SLX4 and anti-GFP antibodies were diluted 1:5000. Sheep polyclonal SLX1 antibodies were raised against bacterially-expressed human SLX1 (full-length) fused to GST. The antibodies were purified from sheep serum by affinity chromatography on CH-Sepharose to which the peptide immunogen had been covalently coupled.

**Immunoprecipitation**

Sheep anti-human SLX4 (1535-1834) as above; sheep anti-GFP (as sheep IgG control, raised in the DSTT); mouse anti-TRF2 (Millipore 41794); rabbit anti-RAP1 (Bethyl A300-306A); normal mouse IgG (CST #5415); normal rabbit IgG (CST #2729). All antibodies were used at 4 μg antibody per 2 mg of cell lysate.

**Immunofluorescence**

Mouse anti-TRF1 diluted 1:200 (Abcam Ab10579); rabbit anti-S5BP1 diluted 1:500 (A300-272A); mouse anti-human XPF diluted 1:500 (Thermoscientific MS1381); mouse anti-TRF2 diluted 1:400 (Millipore 4A794); anti-SLX1 diluted 1:100. All secondary antibodies were from Invitrogen: donkey anti-mouse Alexa 594 (diluted 1:800); donkey anti-sheep Alexa 594 (diluted 1:800); donkey anti-mouse Alexa 647 (diluted 1:500); donkey anti-rabbit Alexa 647 diluted 1:500. Sheep polyclonal SLX4 antibodies for immunofluorescence were raised against a bacterially-expressed protein fragment corresponding to anti-human SLX4 (amino acids 381-680) fused to GST. The antibodies were purified from sheep serum by affinity chromatography on CH-Sepharose to which the peptide immunogen had been covalently-coupled. These antibodies were diluted 1:200.
**ChIP**

Sheep anti-human SLX4 (381-680) as above; sheep anti-human SLX4 (1535-1834) as above; mouse anti-TRF2 (Imgenex IMG124A); mouse anti-CENPA (Abcam ab13939); sheep anti-GFP; rabbit anti-GFP (Abcam ab290); normal mouse IgG (CST #5415); normal rabbit IgG (CST #2729). All antibodies used at 3 μg per ChIP.

**Dot Blotting and Hybridization**

ChIP DNA was blotted onto Hybond H+ membrane (GE Healthcare) and cross-linked with 1200 J/m² of UV-C light. Membranes were pre-hybridized by incubation in Church buffer (0.5 M sodium phosphate buffer pH7.2, 1 mM EDTA, 1% BSA) for 1 hr. Membranes were then incubated with 800bp (TTAGGGn) telomere repeat probe radio-labeled with [32P]-dCTP using Klenow fragment, boiled before incubation in Church buffer. Alternatively, membranes were incubated with a poly-nucleotide kinase (PNK) gamma 32P end labeled Alu repeat probe (CGGAGTCTCGCTCTGTCGCCCAGGCTGGAGTGCAGTGGCGGA) in Church Buffer. Membranes were hybridized overnight at 65 °C. Membranes were washed in 2 X SSC (300 mM NaCl, 15 mM sodium citrate) four times for 5 min each at 65 °C, and exposed to photographic film overnight at –80 °C.

**Coupled Immunofluorescence-FISH**

In coupled immunofluorescence (IF)-FISH experiments, immunofluorescence was carried out as normal with cells grown on coverslips. Cells were then washed twice in PBS and fixed again with 3% formaldehyde (5 min) before further washing. Cells were then serially dehydrated in 70%, 95% and then 100% ethanol before drying in air for 2 min. Cells were incubated with FITC labeled telomeric FISH probe (DAKO) and dishes were gently covered with SaranWrap to aid distribution of the probe. After 10 min at 80 °C, the coverslips were transferred to 4 °C and incubated overnight. After two 15 min washes in buffer (10 mM Tris-HCl, pH7.4) containing 70% (v/v) formamide at 50 °C, coverslips were washed three times for 5 min each with PBS; the second PBS wash included DAPI at 1 μg/ml. Cells were then mounted for microscopic analysis.

**Telomere Fluorescence Analyses of Liver Sections**

Paraffin-embedded liver sections were hybridized with a PNA-telomeric probe, and fluorescence intensity of telomeres was determined as previously described (Zijlmans et al., 1997). Quantitative image analysis was performed on images obtained using a confocal microscope (Leica TCS-SP5 DOBS) and the Definiens Developer Cell software (version XD 1.2; Definiens AG).

**Telomere Length and Cytogenetic Analysis Using Telomere Q-FISH on Metaphase Spreads**

Q-FISH on metaphases from primary MEF was performed as described previously (Herrera et al., 1999). Where indicated, MEFs were treated with aphidicolin at 0.5 μM for 18 hr followed by 6 hr recovery. Images were captured at 100X magnification using a COHU CCD camera on a Leica DMRA (Leica, Heidelberg, Germany) microscope. Telomere fluorescence signals were quantified by using the TFL-TELO program (gift from Peter Lansdorp, Vancouver, Canada) (Zijlmans et al., 1997).

**Laser Microirradiation**

Cells were plated on glass-bottom dishes and pre-sensitized with 10 mM 5-bromo-2′-deoxyuridine (BrdU, Sigma-Aldrich) for 24 h at 37 °C. DNA damaged was induced using a 405 nm CW 30 mW diode laser scanned 50x at 100% power over the selected ROI (region of interest) in one optical section corresponding to the middle of the nucleus. Live cell imaging was carried out on a Zeiss 710 confocal microscope equipped with a 37 °C environmental chamber and supplied with humidified 5% CO₂. Cells were imaged with a Zeiss Plan-Apochromat 63x 1.4NA oil immersion objective lens. Subsequent imaging involved acquiring stacks of ten optical sections at 0.63 μm intervals. GFP excitation was performed using the 488 nm line from an Argon laser.

**SUPPLEMENTAL REFERENCES**

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Zijlmans, J.M., Martens, U.M., Poon, S.S., Raap, A.K., Tanke, H.J., Ward, R.K., and Lanordorp, P.M. (1997). Telomeres in the mouse have large inter-chromosomal variations in the number of T2AG3 repeats. Proc. Natl. Acad. Sci. USA 94, 7423–7428.
Figure S1. Foci Formed by SLX4-Associated Nucleases, Related to Figure 1
(A) U2OS cells transfected with an SLX4-specific siRNA (SLX4-4) were fixed and endogenous SLX4 foci were visualized by indirect immunofluorescence using antibodies raised against amino acids 381-680 of human SLX4. TRF1 foci were also visualized.
(B and C) Indirect immunofluorescence analysis of SLX4 and XPF (B), or SLX4 and MUS81 (C), in U2OS cells. Cells were fixed and the relevant foci were visualized.
(D) Quantitation of the proportion of U2OS cells in which MUS81 or XPF foci colocalize with SLX4.
(E) CCL-211 primary lung fibroblasts were fixed and SLX4 or TRF1 foci were visualized by indirect immunofluorescence. A straight line was drawn using OMERO Insight (Allan et al., 2012) through a single Z-section of the nucleus and the intensity of the SLX4 or TRF1 signals were quantitated along the length of the line. a.u.f, arbitrary units of fluorescence.
Figure S2. Effect of the SLX4 L1022A TBM Mutation on SLX4-Associated Nucleases, Related to Figure 3

(A) HEK293 cells were transiently transfected with vectors expressing GFP only, GFP-SLX4 wild-type (WT) or GFP-SLX4 bearing alanine mutations at H1020, L1022 or P1024. Extracts were subjected to Western blot analysis (right panels), or immunoprecipitation with GFP-Trap beads (left panels) followed by Western blotting.

(B) Dendrogram of SLX4 orthologs from the species indicated. The diagram shows the TRF2-binding motifs (TBM) in SLX4 orthologs in primate species (boxed with a red dotted line), and the most similar motifs in a selection of other mammals.

(C) U2OS cells stably expressing GFP-SLX4 were transiently transfected with plasmids encoding RFP-tagged TRF2 or TRF2ΔBΔM. After 48 hr, cells were fixed and RFP-TRF2 and GFP-SLX4 foci were visualized (left panels). Quantitation of these data are shown in the right panel.
Figure S3. Effect of the SLX4 L1022A TBM Mutation on DNA Repair and on SLX4 Localization at Sites of DNA Damage, Related to **Figure 4**

(A) Indirect immunofluorescence analysis of fixed U2OS cells stably expressing GFP-SLX4 wild-type or GFP-SLX4-L1022A. GFP, SLX1 or TRF2 foci were visualized.

(B) Live-cell fluorescent images of U2OS cells stably expressing GFP-SLX4 or GFP-SLX4-L1022A. Images were obtained at the indicated times after laser-micro-irradiation. The extreme right-hand panels show immunofluorescence images of γ-H2AX in cells fixed 60 min after irradiation.

(C) Cells from Fanconi anemia patient EUFA-1354 were stably transfected with constructs expressing SLX4, SLX4-L1022A or empty vector. Cells were exposed to the indicated doses of mitomycin-C (MMC) for 18 hr, and after 72 hr cell viability was assessed by the MTS assay. Viability of untreated cells was defined as 100%. Data are represented as mean ± s.e.m, n = 3.
Figure S4. Depletion or Deletion of SLX4 from Mammalian Cells Causes Telomeric DNA Damage, Related to Figure 4

(A) U2OS cells were transfected with four different SLX4-specific siRNA duplexes and after 48 hr, SLX4 expression was tested by Western blotting. A scrambled version of siRNA SLX4-1 was used as control.

(B) U2OS Cells treated with the indicated siRNAs were fixed and subjected to immunofluorescence analysis of 53BP1 foci and TRF1 foci. The proportion of cells with greater than three 53BP1 foci that co-localized with TRF1 (TIFs) was counted in at least 200 cells, in two independent experiments.

(C) Mouse SLX4 or human SLX4, tagged at the N-terminus with GFP, were expressed in MEFs from Slx4+/− mice. Cells were fixed and GFP-SLX4 and TRF2 foci were visualized by indirect immunofluorescence.

(D) MEFs of the genotypes indicated were fixed and processed as described in (B). The proportion of cells with greater than 3 TIFs in at least 200 cells in two independent experiments.