# SLX4 interacts with RTEL1 to prevent transcription-mediated DNA replication perturbations 

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## - To cite this version:

A. Takedachi, E. Despras, S. Scaglione, R. Guérois, J. Guervilly, et al.. SLX4 interacts with RTEL1 to prevent transcription-mediated DNA replication perturbations. Nature Structural and Molecular Biology, 2020, 27 (5), pp.438-449. 10.1038/s41594-020-0419-3 . hal-03079782

HAL Id: hal-03079782

## https://hal.science/hal-03079782

Submitted on 17 Dec 2020

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| Extended Data Fig. 1 | RTEL1 is a binding partner of SLX4 | NSMB A415 14B_Takedac hi_Extended Data_Fig_1_fi nal.jpg | (a) YFP-pull down from HeLa cells expressing YFP-SLX4. In lanes 3, 4 and 5 YFP-pull downs were washed in a high salt buffer $(\mathrm{NaCl})$ or carried out in the presence of Benzonase (Benzo) or Ethidium bromide ( EtBr ) (See Methods). MUS81 and XPF were used as positive controls for SLX4-binding. <br> (b) Endogenous SLX4 was immunoprecipitated from a HeLa celllysate with a combination of two different anti-SLX4 antibodies (See methods). <br> (c) Immunoprecipitation of Flag-HASLX4 from dox-inducible HeLa cells at various time points after release from a thymidine block. Cell cycle profiles of the samples (left). Immunoblot of a representative experiment (right) and the quantifications of three independent experiments (mean with SEM). <br> (d) RTEL1-binding domain of SLX4 and multiple SLX4 sequence alignment centred on the region 599-635 of human SLX4. Top two sequences report secondary structures (H: helix) and disorder status (D) predicted by PSIPRED and SPOTD algorithms, respectively (See Methods). NCBI RefSeq identifiers are given within brackets. UBZ4: ubiquitin-binding, MLR: primary XPF-binding domain, BTB: homodimerization domain (also contributes to XPF-binding), TBM: TRF2-binding motif, SIM: SUMOinteracting motifs, SAP: MUS81-binding region, CCD: SLX1-binding domain. <br> (e) YFP-pull downs from HeLa cells expressing WT or mutated YFP-SLX4. $\Delta \mathrm{BTB}: \mathrm{M}_{684} \mathrm{VNN}^{2}$ GLPP $_{764}$ was deleted from the BTB domain. BTB5A and SIM: point mutations in the BTB domain and SIM motifs, respectively, as described $\mathrm{in}^{2}$. D614G and L618P: cancer-associated |


|  |  |  | mutations (Fig. 1d). <br> (f) YFP-pull downs from HeLa cells expressing WT or mutated YFP-SLX4. L1022A: TRF2-binding defective mutant. All immunoblots were performed with antibodies against the indicated proteins. Uncropped images for panels $a, b, d, e, f$ and data for graphs in panel $b$ are available as source data. |
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| Extended Data Fig. 2 | List of known SLX4 interactors identified in YFP-SLX4 pull down | NSMB_A415 14B Takedac hi_Extended Data_Fig_2_fi nal_new.jpg | List of all known SLX4 interactors (light green) and RTEL1 (dark green) that were identified in the YFP-SLX4 pull down shown in Extended Data 1a. The table shows a spectral counting based on the number of peptide-to-spectrum matching (PSM) events. (see Supplementary Note for Mass spectrometry and data analysis methods and Supplementary Table 1 for the full data report). |
| Extended Data Fig. 3 | List of SLX4 partners impacted by the D614G and L618P SLX4 mutations | NSMB_A415 14B_Takedac hi_Extended Data_Fig_3_fi nal_new.jpg | List of all proteins identified in all three runs of the wild-type YFP-SLX4577-795 sample but in none of the runs of the D614G and L618P mutated samples or the HeLa "Fit0" (HeLa Flp-In TREX cells with no SLX4 cDNA integrated at the FRT site) negative control (see Supplementary Table 4 for the mass spectrometry data full report). The table shows a spectral counting based on the number of peptide-to-spectrum matching (PSM) events. (see Supplementary Note for Mass spectrometry and data analysis methods and Supplementary Table 1 for the data full report). |
| Extended Data Fig. 4 | SLX4 binds HD1 of RTEL1 | NSMB_A415 14B_Takedac hi Extended Data_Fig_4_fi nal_new.jpg | (a) Multiple sequence alignment of RTEL1 homologs focused on the region 888-1156 of human RTEL1. Top two sequences report the secondary structures (H for helix) and disorder status (D) predicted by PSIPRED and SPOTD algorithms, respectively (See Methods). Blue boxes indicate the delimitation of the canonical harmonin/PAH domains HD1 and HD2 and the red box spots out the extension required for interaction with SLX4. For species having diverged before the emergence of bony fishes, the |


|  |  |  | second harmonin domain is not present. NCBI RefSeq identifiers are given within brackets. <br> (b) E. coli produced 6 His-tagged HD1a (RTEL1 $1_{885-975}$ ), HD1b ( RTEL1 $_{885-990}$ ) and HD2a (RTEL1 ${ }_{1046-1142}$ ) fragments were used in a $\mathrm{Ni}++$-pull down in vitro assay to monitor their interaction with a GSTtagged SLX4 ${ }_{577-1042}$ (Helix+BTB) fragment. The first and last lanes represent the inputs of the $\mathrm{Ni}++$-pull down assays. B: Ni++-beads, Ft: Flow through. The pelleted beads were resuspended in a volume of Laemmli buffer equivalent to the initial volume of the binding assay. Identical volumes of the GST-tagged SLX4 ${ }_{577}$ - <br> 1042 (Helix+BTB) fragment (diluted to the final concentration used in the binding assay), the B and the Ft samples were loaded on the gel. <br> (c) Schematic representation of the RTEL1 fragment (Top) used in Y2H to assess direct binding to the SLX4 ${ }_{577}$ 1042 fragment. K897E: HoyeraalHreidarsson syndrome (HHS) associated mutation. Bottom panel shows Y2H to assess direct binding between the RTEL1 fragments and SLX4 ${ }_{577-1042}$ (Helix+BTB) fragment. <br> (d) Schematic representation of the YFPtagged RTEL1 fragments (Top) used in the YFP-pull down to assess binding to endogenous SLX4 (Bottom). All indicated RTEL1 point mutations are from Hoyeraal-Hreidarsson syndrome (HHS) patients ${ }^{31}$. <br> Uncropped images of the immunoblots in panels $\mathrm{b}, \mathrm{d}$ and Y 2 H in c are available as source data. |
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| Extended Data Fig. 5 | Interaction between SLX4 and RTEL1 is required for proper replication fork progression but not for ICL repair | NSMB A415 14B Takedac hi_Extended Data_Fig_5_fi nal_new.jpg | (a) Colony survival assay with mockdepleted (siLUC) and SLX4-depleted (siUTR) HeLa "Fit0" and HeLa Flp-In TREX cells expressing WT or mutated Flag-HA-SLX4 as indicated treated with MMC for 24 hrs. Values represent the means and SEM from three independent experiments. The Immunoblots were carried out with antibodies against SLX4 and XPF. |


|  |  |  | $\mathrm{n} / \mathrm{a}$ : lanes that are not relevant to the colony survival assay. A portion of the corresponding Ponceau stained membrane is shown under the immunoblots. SLX4 runs just above the 250 kDa mark while XPF runs slightly above the 100 kDa mark. <br> (b) Analysis of replication fork dynamics in HeLa cells depleted for SLX4 or RTEL1, as described in Fig. 3a. NT: nontargeting control siRNA. Data are shown in box-plots (median, first and third quartile) with $5^{\text {th }}-95^{\text {th }}$ percentile whiskers ( + : mean, n : number of unbroken signals analysed). Statistical significance was assessed with the Mann-Whitney test (ns: not significant, ${ }^{* * *}$ : $\mathrm{p}<0.001$, ****: $\mathrm{p}<0.0001$ ). The immunoblots were performed with antibodies against SLX4, RTEL1 and $\beta$-actin used as internal loading control. The arrow indicates the SLX4 band. <br> (c) as in b in U2OS "Fit0" cells depleted for SLX4 or RTEL1. LUC: control siRNA. <br> (d) Control immunoblots and the corresponding Ponceau stained membrane for Fig. 3b showing the relative levels of endogenous SLX4 (lane 1 before depletion; lanes 2 to 8 after depletion) and recombinant WT or mutated SLX4 proteins expressed in cells depleted for endogenous SLX4 (lanes 3 to 8 ). SLX4 runs just above the 250 kDa mark while XPF runs slightly above the 100 kDa mark. <br> $\mathrm{n} / \mathrm{a}$ : lanes that are not relevant to the data shown in Fig. 3b. <br> (e) as in b in U2OS "Fit0" and U2OS Flp-In TREX cells stably expressing DOX-inducible WT or mutated Flag-HASLX4 as indicated. siSLX4 ${ }^{\text {UTR }}$ was used to deplete endogenous SLX4. <br> Uncropped images of the immunoblots in panels a-d and data for graphs in panels $\mathrm{a}, \mathrm{b}, \mathrm{c}, \mathrm{e}$ are available as source data. |
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| Extended Data Fig. 6 | SLX4 promotes replication fork progression independently | NSMB_A415 14B_Takedac hi Extended Data Fig 6 fi | (a) PLA between SLX4 (HA) and RTEL1 was performed in HeLa Flp-In TREX cells expressing Flag-HA-SLX4 before HA counterstaining (in green and red, |

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|  |  |  | TREX cells expressing Flag-HA-SLX4 before HA counterstaining. Single-cell HA intensity ( $\mathrm{n}>152$, left panel) and PLA spots per HA-positive cells (right panel) are plotted ( $\mathrm{n}>109$, red bars: median with interquartile range). Parental U2OS "Fit0" cells were used as a negative control and PLA spots were counted in random nuclei for this condition (grey distribution with orange bars). KruskalWallis test (ns: not significant, ${ }^{* * * *: ~}$ $\mathrm{p}<0.0001$ ). <br> (e) PLA between endogenous RTEL1 and RNA pol II pS2 was performed in SV40immortalised patient fibroblasts expressing WT or R957W RTEL1. Reactions omitting one of the primary antibodies ( Ab ) were used as negative controls. Kruskal-Wallis test (ns: not significant, ${ }^{* * * *}$ : $\mathrm{p}<0.0001$ ). <br> Data for graphs in panels a,d,e are available as source data. |
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| Extended Data Fig. 8 | Transcription is toxic to replication in absence of SLX4-RTEL1 complex formation | NSMB A415 14B Takedac hi Extended Data_Fig_8_fi nal_new.jpg | a) Supporting data for the DNA fiber assay shown in Fig. 6a. <br> (b) HeLa "Fit0" cells were depleted for SLX4 or RTEL1. $1 \mu \mathrm{M}$ triptolide was added to the culture medium for 3 h before and during the IdU and CldU pulses to inhibit transcription initiation. Replication fork dynamics was analysed as in Fig. 3a. Mann-Whitney test, ns: not significant, ${ }^{* *}$ : $\mathrm{p}<0.01, * * * *: p<0.0001$ ). Uncropped images of the immunoblots and data for graphs in panels $a$ and $b$ are available as source data. |

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SLX4 Interacts With RTEL1 To Prevent Transcription-Mediated DNA Replication Perturbations

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[^0]The SLX4 tumour suppressor is a scaffold that plays a pivotal role in several aspects of genome protection including homologous recombination, interstrand DNA cross-link repair and the maintenance of common fragile sites and telomeres. Here we unravel an unexpected direct interaction between SLX4 and the DNA helicase RTEL1, which until now were viewed as having independent and antagonistic functions. We identify cancer and Hoyeraal-Hreidarsson syndrome-associated mutations in SLX4 and RTEL1, respectively, that abolish SLX4-RTEL1 complex formation. We show that both proteins get recruited to nascent DNA, tightly colocalize with active RNA pol II and that SLX4, in complex with RTEL1, promotes FANCD2/RNA pol II colocalization. Importantly, disrupting SLX4-RTEL1 interaction leads to DNA replication defects in unstressed cells which are rescued by inhibiting transcription. Our data demonstrate that SLX4 and RTEL1 interact to prevent replication-transcription conflicts and provide evidence that this is independent of the nuclease scaffold function of SLX4.

## Keywords

SLX4, RTEL1, FANCD2, DNA replication, transcription, cancer, Hoyeraal Hreidarsson syndrome, Fanconi anemia, replication stress, harmonin N -like domain, PAH domain, BTB domain

## INTRODUCTION

Multi-protein scaffolds fulfil pivotal functions in the maintenance of genome stability by orchestrating the action of their partners and coordinating mechanisms ranging from DNA damage signalling, cell-cycle control, DNA repair, chromosome segregation to cell division. Amongst these, the human SLX4 (FANCP) tumour suppressor has been in the spotlight since it was found to associate with the XPF-ERCC1, MUS81-EME1 and SLX1 structure-specific endonucleases (SSE) and control these enzymes in interstrand DNA cross-link (ICL) repair, homologous recombination and/or the maintenance of telomeres and common fragile sites ${ }^{1-11}$. SLX4 also associates with other factors involved in the maintenance of genome stability including MSH2, TRF2, TOPBP1 and the PLK1 kinase ${ }^{1,3,4,12}$. Interaction with both ubiquitin and SUMO contributes to the regulatory functions of SLX4 ${ }^{2,7,13}$, which itself has been shown to promote its own SUMOylation as well as that of its XPF partner ${ }^{2}$. In yeast, SIx4 fulfils additional functions ranging from checkpoint dampening to promoting DNA end resection(for review ${ }^{14}$ ). The importance of its contribution to such diverse aspects of genome maintenance is underscored by the fact that biallelic mutations in SLX4 are causative of the rare hereditary syndrome Fanconi anemia that is characterized by chromosomal instability, bone marrow failure, developmental defects and high cancer predisposition ${ }^{15,16}$. Despite the progress made on our understanding of some of SLX4 functions, in particular its well-established prominent role in ICL
repair ${ }^{1,3,4,13,14,17-19}$, much remains to be done before we can fully understand the various ways by which SLX4 contributes to the maintenance of genome stability.

Here we unravel an unexpected direct interaction between SLX4 and the RTEL1 helicase, which were viewed until now as having rather independent and antagonistic functions ${ }^{20}$. The RTEL1 helicase contributes to the maintenance of genome stability by facilitating telomere as well as genome-wide replication ${ }^{20-26}$. Its ability to unfold D-loops is believed to reduce crossover rates by promoting double-strand break repair through synthesisdependent strand annealing ${ }^{27}$. It interacts with $\mathrm{PCNA}^{22}$ and contributes to the replication of pericentromeric heterochromatin in complex with TRF2 ${ }^{28}$. In addition to these DNA-metabolism related functions, which are believed to primarily rely on its helicase activity, RTEL1 is also involved in the trafficking of ribonucleoproteins ${ }^{29}$. The functional importance of RTEL1 is underscored by the fact that biallelic RTEL1 mutations are associated with Dyskeratosis congenita (DC) and Hoyeraal-Hreidarsson syndrome (HHS), its severe form, characterized by developmental defects, bone marrow failure and immunodeficiency ${ }^{30,31}$ while heterozygous RTEL1 mutations cause pulmonary fibrosis ${ }^{32,33}$.

We demonstrate that SLX4 is necessary for optimal DNA replication in unstressed cells and that this relies on its interaction with RTEL1 but not its SSE partners. Importantly, we identify cancer-patient associated SLX4 and RTEL1 somatic mutations and HHS-associated RTEL1 germline mutations that abrogate SLX4-RTEL1 complex formation. We show that both SLX4 and RTEL1 get recruited to nascent DNA strands and that they can be found in the immediate vicinity of active RNA polymerase II (RNA pol II). SLX4 turns out to drive the recruitment and/or accumulation of FANCD2 at RNA pol II. In line with the recently described role of FANCD2 in preventing endogenous transcription-induced replication stress ${ }^{34,35}$, we demonstrate that SLX4 and RTEL1 interact to prevent replication-transcription conflicts in unstressed cells.

## RESULTS

## SLX4 interacts with RTEL1

While conducting tandem mass spectrometry analyses of proteins that co-purify with YFP-SLX4 stably produced in HeLa cells under the control of a doxycycline (Dox)-inducible promoter, we reproducibly and specifically found a small number of RTEL1 peptides in SLX4 complexes (Extended data Fig. 1a, Extended data Fig.2, Supplementary Table 1). Immunoprecipitation and Western blot analysis confirmed SLX4-RTEL1 complex formation and showed that it is not mediated by DNA and is sufficiently robust to be maintained in a high-salt buffer (Fig. 1a). Importantly, endogenous RTEL1 was also detected in pull downs of endogenous SLX4 (Fig. 1b). Furthermore, we found SLX4 and RTEL1 to partially colocalize in the nucleus (Extended data

Fig. 1b) and their interaction, while constitutive throughout the cell cycle, to be enhanced in lateS/G2 and mitosis suggesting a cell-cycle dependent control (Fig. 1c and Extended data Fig. 1c).

## SLX4 and RTEL1 are direct binding partners

To map the RTEL1 binding domain in SLX4, we assessed the ability of endogenous RTEL1 to co-immunoprecipitate with various recombinant YFP-tagged fragments of SLX4 produced in HeLa cells (Extended data Fig. 1d). As depicted in Fig. 1d, the RTEL1 binding domain corresponds to a region of SLX4 that encompasses both the BTB domain, which drives the homodimerization of SLX4 and is important for ICL-repair and telomere related functions of SLX4 ${ }^{2,36}$, and a short conserved amphipathic motif (residues 603 to 626) of unknown function located just upstream of the BTB domain (Fig. 1d and Extended data Fig. 1d). Interestingly, we have identified in biopsied metastases from two unrelated patients (lung metastasis of chondroblastic osteosarcoma and liver metastasis of gastric adenocarcinoma), two somatic mutations that alter conserved residues within that motif (Fig. 1d). Both D614G and L618P mutations abrogate interaction of SLX4 with RTEL1 (Fig. 1e) but not with XPF despite the nearby MLR XPF-binding domain (Fig. 1d). Deletion of the BTB or point mutations in that domain also strongly impairs interaction with RTEL1 (Fig. 1e). Our results indicate that both the short conserved motif upstream of the BTB and the BTB itself are required for optimal interaction with RTEL1. Accordingly, proteomic analyses confirmed that RTEL1 is the primary binding partner of an SLX4 fragment containing the conserved amphipathic motif and the BTB domain (Extended data Fig. 1e, Extended data Fig. 3, Supplementary Table 2). Most strikingly, the D614G and L618P mutations abrogated interaction only with RTEL1, amongst all functionally relevant potential interactors of that region of SLX4 (Extended data Fig. 1e, Extended data Fig. 3, Supplementary Table 2).
Noteworthy, SLX4 and RTEL1 can both interact with TRF2 ${ }^{1,4,8,9,37}$. However, SLX4 mutations that abrogate interaction with RTEL1 do not impact interaction with TRF2 and vice versa (Fig. 1f). This indicates that TRF2 does not contribute to SLX4-RTEL1 complex formation and suggests that RTEL1 and TRF2, which preferentially associate at the G1/S transition and in S phase ${ }^{37}$, bind SLX4 when not in complex with one another.
We next undertook the identification of the SLX4-binding domain in RTEL1. The important contribution of the amphipathic motif in SLX4 provided clues as to which part of RTEL1 might be involved in the SLX4-RTEL1 interaction. Indeed, RTEL1 contains two harmonin-N-like motifs that are related to the paired amphipathic helix (PAH) domain ${ }^{38}$. The PAH domain is a proteinprotein interaction module that folds into a helical bundle structure that forms a hydrophobic cleft for the binding of a short amphipathic helix. Such a helix is predicted to form between residues 604 and 620 within the conserved domain of SLX4 that is critical for binding to RTEL1 (Fig. 1d). Our modelling analyses suggested that both harmonins of RTEL1 could accommodate
this helix (Fig. 2a). In particular, residues D614 and L618 in SLX4 would lie at the interface of the helix and the harmonins-binding site. Supporting our prediction, an RTEL1 $1^{763-1164}$ fragment that contains both harmonin domains (HD) efficiently pulls down endogenous SLX4 (Fig. 2b). Direct interaction between SLX4 and RTEL1 and precise mapping of the SLX4-binding domain in RTEL1 were further monitored by yeast two hybrid (Y2H). Interestingly, the amphipathic helix and the BTB are jointly needed to interact with an RTEL1 fragment containing both HDs as no interaction was detected with just the helix (Fig. 2c). In contrast to our modelling predictions (Fig. 2a), interaction was detected only with the first HD (HD1) (Fig. 2c). This specificity turned out to rely on a short conserved sequence located just after the C-terminus of HD1 that is not found after the second harmonin domain (HD2) as an HD1 fragment lacking this short sequence, does not bind SLX4 (Fig. 2c and Extended data Fig. 4a). Both SLX4 D614G and L618P patientderived mutations totally abrogate interaction with RTEL1 in Y2H (Fig. 2d), confirming that the conserved amphipathic motif of SLX4 is essential for direct binding to RTEL1. The specificity of this direct interaction was further confirmed in vitro with bacterially produced recombinant SLX4 and RTEL1 fragments (Figure 2 e and Extended data Fig. 4b). Interestingly, amongst the RTEL1 germline mutations identified in HHS patients, four missense mutations have been mapped within HD1 and two nonsense mutations in the non-structured segment that links HD1 and HD2 ${ }^{38}$. Strikingly, all six mutations negatively impact interaction with SLX4 (Fig. 2f-h and Extended data Fig. 4c,d). Our findings demonstrate that SLX4 and RTEL1 are direct binding partners and suggest a complex mode of interaction that strictly relies on not only the docking of a conserved amphipathic helix of SLX4 with the first HD of RTEL1 but also on the BTB homodimerization domain of SLX4.

## SLX4 promotes replication fork progression and genome stability via interaction with RTEL1

Having established that SLX4 and RTEL1 are direct binding partners, we next sought to understand the functional relevance of this interaction. Overall, both proteins are required for many of the same genome maintenance aspects including control of telomere homeostasis, ICL repair and homologous recombination. However, it is not known whether they act within the same pathways and, at least in mice, they have rather independent and antagonistic functions at telomeres where RTEL1 unfolds T-loops to prevent their SLX4-driven endonucleolytic processing and telomere attrition ${ }^{20}$. Considering our findings, one explanation could have been that RTEL1 also prevents such unscheduled processing of secondary DNA structures at telomeres by directly interacting with SLX4 and negatively controlling its associated structurespecific endonucleases (SSEs). However, we observed no telomere attrition in human cells producing the RTEL1-binding defective SLX4 $4^{\text {D614G }}$ and SLX4 ${ }^{\text {L618P }}$ mutants, arguing against such a scenario (data not shown). We also did not observe any increased cellular sensitivity to
mitomycin C in those cells indicating that SLX4 and RTEL1 do not need to interact to fulfil their functions in ICL repair (Extended data Fig. 5a).
RTEL1 plays a role in genome wide replication through direct interaction with PCNA ${ }^{22}$. Accordingly, impaired replication is observed in mouse cells expressing a PCNA-binding defective mutant ${ }^{22}$ or following depletion of RTEL1 in human cells ${ }^{22,28}$. Since SLX4 can also be found associated with the replisome ${ }^{39}$, we first assessed in DNA fiber assays whether loss of SLX4 similarly impairs replication. Cells were transfected with different siRNAs targeting SLX4 or control siRNAs and nascent DNA was labelled in vivo by successive pulses of iododeoxyuridine (IdU) and chlorodeoxyuridine (CIdU). Analogues and total DNA were detected by immunofluorescence on spread DNA molecules. Depletion of SLX4 in HeLa and U2OS cells resulted in shorter nascent DNA tracks and increased fork ratio. This is indicative of impaired replication fork progression, as seen following depletion of RTEL1 ${ }^{28}$ (Fig. 3a and Extended data Fig. 5b,c). Importantly, the replication defects caused by depletion of SLX4 were fully rescued by the expression of Flag-HA-SLX4 ${ }^{\text {WT }}$ but not Flag-HA-SLX4 ${ }^{\text {D614G }}$ or Flag-HA-SLX4 ${ }^{\text {L618P }}$ (Fig. 3b and Extended data Fig. 5d,e). Using in situ proximity ligation assays (PLA) between SLX4 and neo-synthesized DNA or RTEL1, we found that SLX4 does not need to interact with RTEL1 to get recruited to the replication fork (Fig. 3c) and that both proteins do not need to interact to get recruited in the vicinity of one another (Extended data Fig. 6a). To confirm that SLX4-RTEL1 complex formation is nevertheless critical for proper replication fork progression in unchallenged cells, we used an HHS patient-derived immortalized cell line (P7) carrying a homozygous missense R957W mutation in HD1 ${ }^{31}$ that abrogates binding to SLX4 (Fig. 2f,g and Extended data Fig. 4d). Although RTEL1 ${ }^{\text {R957w }}$, which has an intact PCNA interacting motif (PIP), is recruited like RTEL1 ${ }^{\text {WT }}$ to neo-synthesized DNA (Extended data Fig. 6b,c), the P7 patientderived cell line presented short nascent DNA tracks and a high fork ratio (Fig. 3d), reminiscent of what we observed in cells producing SLX4 mutants that cannot bind RTEL1 (Fig. 3b and Extended data Fig. 5d,e).
Overall our results demonstrate that SLX4 is necessary for proper replication in unchallenged cells and that it must associate with RTEL1 to help the replisome overcome situations which impede replication fork progression during normal S-phase.

## SLX4 prevents replication perturbations independently of its associated structurespecific endonucleases

Both MUS81 and XPF-ERCC1 were shown to promote normal replication fork rates during unperturbed $S$ phase ${ }^{40}$. To assess whether the RTEL1-dependent function of SLX4 in DNA replication in unstressed cells relies or not on its interaction with its associated SSEs, we generated an SLX4-SMX mutant that is unable to interact with all three SSE partners (Fig. 4a). This mutant carries a combination of SLX4 mutations that were previously shown to each
abrogate interaction between SLX4 and one of its SSE partners ${ }^{2,41}$. Immunoprecipitation of DOX-inducible Flag-HA-SLX4 ${ }^{- \text {SmX }}$ stably expressed in HeLa Flp-In TREX cells confirmed severely impaired interactions with all three SSE partners (Fig. 4b). Remarkably though and in stark contrast to the RTEL1-binding defective mutants, the SLX4 ${ }^{-S M X}$ mutant fully rescued the replication defects caused by depletion of endogenous SLX4 (Fig. 4c). This demonstrates that in unstressed cells SLX4 acts with RTEL1 to promote replication fork progression independently of its associated SSEs.

## SLX4 promotes FANCD2 foci formation via interaction with RTEL1

The Fanconi anemia pathway protein FANCD2 ensures proper replication fork progression in response to various endogenous and exogenous sources of replication impediments (for review ${ }^{42}$ ). In unchallenged cells, FANCD2 is monoubiquitinated and can form spontaneous foci ${ }^{43}$. Interestingly, we noticed that depletion of SLX4 induced a drop in the amount of spontaneous FANCD2 foci (Fig. 5a) without altering the level of monoubiquitination of FANCD2 (data not shown). This was fully rescued by expression of Flag-HA-SLX4 ${ }^{\text {WT }}$ but not Flag-HA-SLX4 ${ }^{\text {D614G }}$ or Flag-HA-SLX4 ${ }^{\text {L618P }}$ (Fig. 5a and Extended data Fig. 7a,b). Our results demonstrate that SLX4 drives the formation of FANCD2 foci in unchallenged cells and that this relies on its interaction with RTEL1. FANCD2 was recently shown to colocalize with RNA polymerase II (RNA pol II) and contributes to preventing endogenous transcription-induced replication stress ${ }^{34,35}$. We thus assessed whether SLX4 may influence the recruitment and/or persistence of FANCD2 in the vicinity of active RNA pol II. We observed a reduction in proximity ligation assay (PLA) signals between FANCD2 and phosphorylated RNA pol II upon depletion of SLX4 (Fig. 5b). This was rescued by SLX4 ${ }^{\mathrm{WT}}$ but not the RTEL1-binding defective mutants (Fig. 5c and Extended data Fig. 7c), indicating that the accumulation of FANCD2 in the vicinity of active RNA pol II requires an interaction between SLX4 and RTEL1.

## SLX4 binds RTEL1 to prevent conflicts between replication and transcription

Since SLX4 appeared to drive the association of FANCD2 with RNA pol II we hypothesized that it might itself be found in the immediate vicinity of RNA pol II. As shown in Fig. 5d, SLX4 can be found in association with RNA pol II in PLA analyses. This was also the case for RTEL1 (Fig. 5e). However, as observed for the association of SLX4 and RTEL1 with nascent DNA, RTEL1binding defective SLX4 ${ }^{\mathrm{D} 614 \mathrm{G}}$ and SLX4 ${ }^{\mathrm{L618P}}$ mutants were also detected in the vicinity of RNA pol II indicating that SLX4 does not need to associate with RTEL1 to reach RNA pol II (Extended data Fig. 7d). Similarly, we also found the SLX4-binding defective RTEL1 ${ }^{\text {R957W }}$ mutant to be in tight vicinity with RNA Pol II in the P7 patient-derived cell line (Extended data Fig. 7e). Although we cannot exclude that the pools of SLX4 and RTEL1 that we find associated with nascent DNA strands (Fig. 3c and Extended data Fig. 6b,c) are different from those associated with active

RNA pol II (Fig. 5d,e and Extended data Fig. 7d,e), it is tempting to speculate that SLX4 and RTEL1 play a role at the interface of DNA replication and transcription where they help replication overcome transcription-mediated impediments. To test this hypothesis we used PLA to determine whether depletion of SLX4 or RTEL1 leads to increased colocalization between PCNA and active RNA pol II, which has been used as a readout of collisions between DNA replication and transcription ${ }^{44}$. In agreement, a significant increase of PCNA tightly colocalized with RNA pol II was detected in SLX4 and RTEL1-depleted cells compared to mock depleted cells (Fig. 5f).
Finally, to determine if transcription is responsible for the replication defects seen in cells lacking SLX4 and RTEL1 we tested the impact of transcription inhibition on replication fork dynamics. Strikingly, inhibiting transcription with cordycepin or triptolide rescued the replication defects caused by depletion of SLX4 or RTEL1 (Fig. 6a and Extended data Figure 8a,b). It also corrected those resulting from loss of SLX4-RTEL1 complex formation in cells producing the RTEL1-binding defective SLX4 ${ }^{\text {D614G }}$ or SLX4 ${ }^{\text {L618P }}$ mutants and in the P7 patient-derived cell line that produces the SLX4-binding defective RTEL1 ${ }^{\text {R957W }}$ mutant (Fig. 6b,c). Overall our results demonstrate that SLX4 and RTEL1 play a key role in preventing replication impediments caused by transcription which relies on their direct interaction.

## DISCUSSION

We have unravelled a direct interaction between SLX4 and RTEL1 and demonstrated that this interaction is critical to help DNA replication overcome transcription-mediated impediments. By showing that the SLX4-RTEL1 interaction is mediated through the association of a conserved amphipathic helix and the BTB domain of SLX4 with the HD1 of RTEL1, we assign a function to a highly conserved region of SLX4 that was of unknown function until now and identify the first partner of one of the HDs of RTEL1, which has important implications in terms of human disease as later discussed. We also assign a possibly novel function to the BTB domain, which was previously shown to mediate homodimerization of SLX4 ${ }^{2,36}$. The fact that the interaction between the amphipathic helix of SLX4 and the HD1 of RTEL1 is not sufficient for stable SLX4-RTEL1 interaction, which also relies on the BTB domain of SLX4, suggests a more elaborate mode of interaction than the canonical interaction that is established between a harmonin-like PAH domain in SIN3 and an amphipathic helix in MAD1 ${ }^{45,46}$. Instead, the contribution made by the BTB domain of SLX4 is closer to what has been described for the interaction between CCM2 and MEKK3 where the interaction between an amphipathic helix in MEKK3 with the harmonin homology domain of CCM2 is stabilized by the PB1 protein binding domain of MEKK3 ${ }^{47}$. Structural analyses will help to better characterize the SLX4-RTEL1 binding interface and determine whether dimerization per se is necessary for binding to RTEL1
and/or whether the BTB domain makes direct contacts with RTEL1. Such analyses should also provide insight into how the association between SLX4 and RTEL1 might be controlled.
Adding to the multiple roles that SLX4 fulfils in the maintenance of genome stability (for review see ${ }^{14}$ ), we demonstrate that SLX4 contributes to genome wide DNA replication in unstressed cells and that this relies on its direct interaction with RTEL1 (Fig. 3), since SLX4 ${ }^{\text {D614G }}$ and SLX4 ${ }^{\text {L618P }}$ mutants that cannot bind RTEL1 are unable to rescue the replication defects caused by depletion of endogenous SLX4. We cannot fully exclude that the D614G and L618P mutations might impact additional functionalities of SLX4. However, both mutations affect highly conserved residues within the amphipathic helix of SLX4 that are ideally positioned to engage in contacts with the harmonins of RTEL1 (Fig. 2) and they are not predicted to impact the overall fold of SLX4. Furthermore, we identified RTEL1 as the only functionally relevant interactor of SLX4 to be impacted by both mutations (Extended data Fig. 1e, Extended data Fig. 3, Supplementary Table 2) and, last but not least, an HHS patient-derived cell line producing an RTEL1 mutant that cannot bind SLX4 (Fig. 2f,g and Extended data Fig. 4d) phenocopies the replication defects of cells producing the SLX4 ${ }^{\text {D614G }}$ and SLX4 ${ }^{\text {L618P }}$ mutants (Fig. 3b and 3d, Extended data Fig. 5d,e). Therefore, all evidence points towards a need for SLX4 to interact with RTEL1 to facilitate replication genome wide. Quite remarkably though, we find that it does not need to interact with its SSE partners (Fig. 4), providing unprecedented evidence of a function of SLX4 in human cells that is totally independent of its established nuclease scaffold functions. Our findings further demonstrate that the SLX4-RTEL1 complex is necessary to prevent replication-transcription conflicts (Fig. 5, 6). Indeed, inhibiting transcription not only compensated for the loss of SLX4 or RTEL1, it alleviated to the same extent the replication defects that result from impaired SLX4-RTEL1 complex formation (Fig. 6 and Extended data Fig. 8). Noteworthy, those replication defects were monitored through unbiased DNA fiber analyses in unstressed cells. Therefore, the transcription-mediated impairments to DNA replication seen in absence of SLX4 or RTEL1 must be frequent enough to be detected by such genome wide analyses and not restricted to a limited number of loci.

Interestingly, RTEL1 was recently found to contribute to the removal of protein-DNA complexes that hinder the progression of replication forks ${ }^{48}$. Therefore, one way by which the SLX4-RTEL1 complex may circumvent replication-transcription conflicts could be by promoting the clearance of the RNA polymerase complex in the vicinity of replication forks. However, given the functional ties between both RTEL1 and SLX4 and the processing of secondary DNA structures, it is likely that the SLX4-RTEL1 complex is involved in non-nucleolytic processing of nucleic acid structures that form as a result of replication-transcription conflicts. Amongst these, R-loops that consist of a DNA:RNA hybrid and a displaced single-stranded DNA loop, which itself can form G-quadruplexes that stabilize the R -loop ${ }^{49}$, represent a major obstruction for replication fork progression ${ }^{50}$. In line with a role in R-loop processing, we find that SLX4 drives
the accumulation and/or stabilization of FANCD2 in the direct vicinity of RNA pol II (Fig. 5b,c). FANCD2, along with other components of the Fanconi anemia pathway, makes important contributions to the signalling of R-loops and their processing $34,35,44,51$. Intriguingly, we found that SLX4 and RTEL1 can be detected in close proximity to one another, even when they cannot interact, and are independently recruited to nascent DNA and RNA pol II (Fig. 3c and Extended data Fig. 6). Yet, SLX4-RTEL1 complex formation is required for the tight colocalization of FANCD2 and RNA pol II and is essential for proper replication fork progression (Fig. 3b,d, Extended data Fig. 5e and Fig. 7c). This suggests that both proteins get recruited independently from one another but that they need at one stage to make contact for replication to proceed normally. Such contact may constitute a molecular switch that allows SLX4 to control the catalytic activity of the RTEL1 helicase for the timely processing of secondary structures that impede replication fork progression.

Although we found the SLX4-RTEL1 interaction to be constitutive throughout the cell cycle, we noticed that it increased in late $S$ and G2/M phases (Fig. 1c). Noteworthy, both FANCD2 and SLX4 contribute to the maintenance of common fragile sites (CFS) in late G2 and mitosis $^{2,6,52,53}$. Furthermore, R-loops have been found to accumulate at CFS in absence of FANCD2 ${ }^{51,54}$. Therefore, the preferential interaction between SLX4 and RTEL1 in late S/G2 may reflect an additional and specific role related to the maintenance of CFS and/or other late replicating loci where SLX4 recruits RTEL1 for the processing of G4-associated R-loops as recently described ${ }^{55}$.

The relevance of our findings in terms of human disease is underscored by the identification of cancer-derived somatic SLX4 mutations and HHS-associated germline RTEL1 mutations that abrogate the SLX4-RTEL1 interaction (Fig. 1d,e and 2d-h). It is striking that de novo mutations, each impacting a different and highly conserved residue in the predicted amphipathic helix of SLX4 and that abrogate interaction with RTEL1, were identified in two unrelated patients presenting different disease profiles. Furthermore, the HHS-associated germline homozygote R957W mutation in the HD1 of RTEL1, is also reported in the COSMIC data base ${ }^{56}$ as the most represented cancer-associated somatic mutation identified in RTEL1. Considering that replication stress is an established hallmarks of tumorigenesis (for review ${ }^{57}$ and that loss of interaction between SLX4 and RTEL1 perturbs DNA replication (Fig. 3b and Extended data Fig. 5e), it is tempting to speculate that mutations in SLX4 and RTEL1 that abrogate complex formation are more than just passenger mutations. Replication-transcription conflicts, which have been linked to the fragility of both late and early-replicating fragile sites of the genome ${ }^{58-60}$, are emerging as a potentially much broader source of genome instability with the realization that transcription is a pervasive process that covers more than $80 \%$ of the human genome ${ }^{61,62}$. Moreover, oncogene activation during tumorigenesis drives premature entry into S phase and the firing of intragenic origins, which increases conflicts between replication and
transcription and genomic instability ${ }^{63}$. Thus, it will be important to assess what impact SLX4 and RTEL1 mutations that abrogate SLX4-RTEL1 complex formation have on the tumoral process. Importantly, we have also shown that all reported HHS-associated germline mutations mapped in the HD1 of RTEL1 negatively impact interaction with SLX4, suggesting that loss of SLX4-RTEL1 complex formation contributes to the aetiology of the disease. We cannot at this stage draw any conclusions as to whether SLX4 mutations that abrogate SLX4-RTEL1 complex formation could be associated with HHS, since both D614G and L618P SLX4 variants that we have identified in cancer patients were somatic mutations with low allelic frequencies. Furthermore, bi-allelic germline mutations in SLX4 have been associated until now with Fanconi anemia ${ }^{15,16}$. However, there are many cases where different mutations in the same gene are associated with different pathologic outcomes and diseases. Therefore, it will be important to determine what clinical phenotypes might be associated with germline SLX4 mutations that abrogate SLX4-RTEL1 complex formation and whether SLX4 should be considered as a possible candidate gene for HHS.

Our demonstration of a functionally relevant interaction between SLX4 and RTEL1 redefines the way we ought to think about how they contribute to the maintenance of genome stability and opens new lines of investigation to help better understand how they prevent the emergence of cancer and other human diseases.

## ACKNOWLEDGEMENTS

A.T. and P.-H.G. express their gratitude to Micaela Boiero Sanders and Christophe Machu for their help in the analysis of microscopy data. The authors thank Mauro Modesti for supplying the GFP nanobody and all members of the 3 n community of the CRCM for helpful discussions. They also thank Samuel Granjeaud for helpful discussions on statistical analyses.
Work in the laboratory of P.H.L.G. was funded by Institut National du Cancer (INCa-PLBio2016159), Siric-Cancéropôle PACA (AAP Projets émergents 2015). A.T. was supported by (INCa-PLBio2016-159), Z.H. was supported by (INCa-PLBio2016-159) and Fondation ARC. Work in the laboratory of D.B. was funded by SIRIC (INCa-DGOS-Inserm 6038), Label Ligue (EL2016 DB), Ruban Rose and Fondation Groupe EDF. The laboratory of P.K. was supported by INCa (INCa-PLBio2016-159) and INCa-DGOS-Inserm 12551, E.D. was supported by INCa (PLBio2016-144). V.N.'s research is supported by the ERC starting grant agreement \# 638898. Work in the laboratory of R.G. was funded by FRISBI (ANR-10-INSB-05-01) and ANR CHIPSET (ANR-15-CE11-0008-01). RTEL1 related work in ALV, PR and IC laboratories was partially supported by a joint grant from the Agence Nationale pour la Recherche (ANR-14-CE10-000601). The IBiSA Marseille Proteomic platform is funded by Institut Paoli-Calmettes, National Institute of Cancer and Aix-Marseille University. J.P.B. is a scholar of Institut Universitaire de France.

We wish to thank Dr Liu and Dr Hickson for sharing unpublished results.

## AUTHOR CONTRIBUTIONS

A.T. performed experiments for proteomic analyses, biochemical analyses of the SLX4-RTEL1 interaction, colony survival assays and the analysis of FANCD2 foci formation. A.T. generated all cell lines producing YPF- or Flag-HA-tagged recombinant proteins used in the current study. E.D. performed all DNA fiber analyses and PLA experiments. S.S. generated plasmids used in Y2H and designed and performed all Y2H experiments. R.G. performed all structural analyses with the help of I.C. and helped in the design of in vitro biochemical studies. J.H.G. worked on the detection of the endogenous SLX4-RTEL1 complex, generated reagents and helped with data analysis. M.B. performed the pulldowns and characterization of Flag-HA-SLX4 ${ }^{-S M X}$. S.A. and L.C. carried out all proteomic analyses, with insight and expertise from J.P.B.. A.G., M.C., F.B. and D.B. ran the NGS of biological samples and bioinformatic analyses of the SLX4 patient derived mutations. P.R. generated the fibroblast cell line from the RTEL1-deficient patient P7 and helped in the design and data interpretation of experiments with HHS-associated RTEL1 mutations. Z.H., D.C., and V.N. helped in the design of cellular studies and data analysis. M.S. and A.L-V. generated the RTEL1 antibody and contributed to the design of experiments and data interpretation. P.H.G. produced recombinant proteins and performed in vitro binding assays and wrote the manuscript. The manuscript was reviewed by all authors. A.T., E.D., P.K. and P.H.G. conceived and planned the study.
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## COMPETING INTEREST STATEMENT

The authors declare no competing interests.

## REFERENCES

1. Fekairi, S. et al. Human SLX4 is a Holliday junction resolvase subunit that binds multiple DNA repair/recombination endonucleases. Cell 138, 78-89 (2009).
2. Guervilly, J.-H. et al. The SLX4 complex is a SUMO E3 ligase that impacts on replication stress outcome and genome stability. Mol. Cell 57, 123-137 (2015).
3. Munoz, I. M. et al. Coordination of structure-specific nucleases by human SLX4/BTBD12 is required for DNA repair. Mol. Cell 35, 116-127 (2009).
4. Svendsen, J. M. et al. Mammalian BTBD12/SLX4 assembles a Holliday junction resolvase and is required for DNA repair. Cell 138, 63-77 (2009).
5. Andersen, S. L. et al. Drosophila MUS312 and the vertebrate ortholog BTBD12 interact with DNA structure-specific endonucleases in DNA repair and recombination. Mol. Cell 35, 128-135 (2009).
6. Minocherhomji, S. et al. Replication stress activates DNA repair synthesis in mitosis. Nature 528, 286-290 (2015).
7. Ouyang, J. et al. Noncovalent interactions with SUMO and ubiquitin orchestrate distinct functions of the SLX4 complex in genome maintenance. Mol. Cell 57, 108-122 (2015).
8. Wilson, J. S. J. et al. Localization-Dependent and -Independent Roles of SLX4 in Regulating Telomeres. Cell Rep 4, 853-860 (2013).
9. Wan, B. et al. SLX4 Assembles a Telomere Maintenance Toolkit by Bridging Multiple Endonucleases with Telomeres. Cell Rep 4, 861-869 (2013).
10. Wyatt, H. D. M., Sarbajna, S., Matos, J. \& West, S. C. Coordinated Actions of SLX1SLX4 and MUS81-EME1 for Holliday Junction Resolution in Human Cells. Mol. Cell 52, 1-14 (2013).
11. Duda, H. et al. A Mechanism for Controlled Breakage of Under- replicated Chromosomes during Mitosis. Dev. Cell 39, 740-755 (2016).
12. Gritenaite, D. et al. A cell cycle-regulated SIx4-Dpb11 complex promotes the resolution of DNA repair intermediates linked to stalled replication. Genes Dev 28, 1604-1619 (2014).
13. Kim, Y. et al. Regulation of multiple DNA repair pathways by the Fanconi anemia protein SLX4. Blood 121, 54-63 (2013).
14. Guervilly, J.-H. \& Gaillard, P.-H. SLX4: multitasking to maintain genome stability. Critical Reviews in Biochemistry and Molecular Biology 53, 475-514 (2018).
15. Stoepker, C. et al. SLX4, a coordinator of structure-specific endonucleases, is mutated in a new Fanconi anemia subtype. Nat Genet 43, 138-141 (2011).
16. Kim, Y. et al. Mutations of the SLX4 gene in Fanconi anemia. Nat Genet 43, 142-146 (2011).
17. Douwel, D. K. et al. XPF-ERCC1 Acts in Unhooking DNA Interstrand Crosslinks in Cooperation with FANCD2 and FANCP/SLX4. Mol. Cell 54, 1-12 (2014).
18. Hodskinson, M. R. G. et al. Mouse SLX4 is a tumor suppressor that stimulates the activity of the nuclease XPF-ERCC1 in DNA crosslink repair. Mol. Cell 54, 472-484 (2014).
19. Lachaud, C. et al. Distinct functional roles for the two SLX4 ubiquitin-binding UBZ domains mutated in Fanconi anemia. J Cell Sci 127, 2811-2817 (2014).
20. Vannier, J.-B., Pavicic-Kaltenbrunner, V., Petalcorin, M. I. R., Ding, H. \& Boulton, S. J. RTEL1 dismantles T loops and counteracts telomeric G4-DNA to maintain telomere integrity. Cell 149, 795-806 (2012).
21. Ding, H. et al. Regulation of murine telomere length by Rtel: an essential gene encoding a helicase-like protein. Cell 117, 873-886 (2004).
22. Vannier, J.-B. et al. RTEL1 is a replisome-associated helicase that promotes telomere and genome-wide replication. Science 342, 239-242 (2013).
23. Uringa, E.-J. et al. RTEL1 contributes to DNA replication and repair and telomere maintenance. Mol Biol Cell 23, 2782-2792 (2012).
24. Sfeir, A. et al. Mammalian telomeres resemble fragile sites and require TRF1 for efficient replication. Cell 138, 90-103 (2009).
25. Sarek, G., Vannier, J.-B., Panier, S., Petrini, J. H. J. \& Boulton, S. J. TRF2 Recruits RTEL1 to Telomeres in S Phase to Promote T-Loop Unwinding. Mol. Cell 61, 788-789 (2016).
26. Porreca, R. M. et al. Human RTEL1 stabilizes long G-overhangs allowing telomerasedependent over-extension. Nucleic Acids Res 46, 4533-4545 (2018).
27. Youds, J. L. et al. RTEL-1 enforces meiotic crossover interference and homeostasis. Science 327, 1254-1258 (2010).
28. Mendez-Bermudez, A. et al. Genome-wide Control of Heterochromatin Replication by the Telomere Capping Protein TRF2. Mol. Cell 70, 449-461.e5 (2018).
29. Schertzer, M. et al. Human regulator of telomere elongation helicase 1 (RTEL1) is required for the nuclear and cytoplasmic trafficking of pre-U2 RNA. Nucleic Acids Res 43, 1834-1847 (2015).
30. Ballew, B. J. et al. A recessive founder mutation in regulator of telomere elongation helicase 1, RTEL1, underlies severe immunodeficiency and features of Hoyeraal Hreidarsson syndrome. PLoS Genet 9, e1003695 (2013).
31. Touzot, F. et al. Extended clinical and genetic spectrum associated with biallelic RTEL1 mutations. Blood Adv 1, 36-46 (2016).
32. Cogan, J. D. et al. Rare variants in RTEL1 are associated with familial interstitial pneumonia. Am. J. Respir. Crit. Care Med. 191, 646-655 (2015).
33. Kannengiesser, C. et al. Heterozygous RTEL1 mutations are associated with familial pulmonary fibrosis. Eur. Respir. J. 46, 474-485 (2015).
34. Schwab, R. A. et al. The Fanconi Anemia Pathway Maintains Genome Stability by Coordinating Replication and Transcription. Mol. Cell 60, 351-361 (2015).
35. García-Rubio, M. L. et al. The Fanconi Anemia Pathway Protects Genome Integrity from R-loops. PLoS Genet 11, e1005674 (2015).
36. Yin, J. et al. Dimerization of SLX4 contributes to functioning of the SLX4-nuclease complex. Nucleic Acids Res 44, gkw354-4880 (2016).
37. Sarek, G., Vannier, J.-B., Panier, S., Petrini, J. H. J. \& Boulton, S. J. TRF2 recruits RTEL1 to telomeres in S phase to promote t-loop unwinding. Mol Cell 57, 622-635 (2015).
38. Faure, G., Revy, P., Schertzer, M., Londoño-Vallejo, A. \& Callebaut, I. The C-terminal extension of human RTEL1, mutated in Hoyeraal-Hreidarsson syndrome, contains harmonin-N-like domains. Proteins 82, 897-903 (2014).
39. Dungrawala, H. et al. The Replication Checkpoint Prevents Two Types of Fork Collapse without Regulating Replisome Stability. Mol Cell 59, 1-14 (2015).
40. Fu, H. et al. The DNA repair endonuclease Mus81 facilitates fast DNA replication in the absence of exogenous damage. Nat Commun 6, 6746-14 (2015).
41. Castor, D. et al. Cooperative Control of Holliday Junction Resolution and DNA Repair by the SLX1 and MUS81-EME1 Nucleases. 52, 1-13 (2013).
42. Federico, M. B., Campodónico, P., Paviolo, N. S. \& Gottifredi, V. Beyond interstrand crosslinks repair: contribution of FANCD2 and other Fanconi Anemia proteins to the replication of DNA. Mutat Res 808, 83-92 (2018).
43. Taniguchi, T. et al. S-phase-specific interaction of the Fanconi anemia protein, FANCD2, with BRCA1 and RAD51. Blood 100, 2414-2420 (2002).
44. Okamoto, Y. et al. FANCD2 protects genome stability by recruiting RNA processing enzymes to resolve R-loops during mild replication stress. FEBS J. 668, 4 (2018).
45. Brubaker, K. et al. Solution structure of the interacting domains of the Mad-Sin3 complex: implications for recruitment of a chromatin-modifying complex. Cell 103, 655-665 (2000).
46. Spronk, C. A. et al. The Mad1-Sin3B interaction involves a novel helical fold. Nat. Struct. Biol. 7, 1100-1104 (2000).
47. Fisher, O. S. et al. Structure and vascular function of MEKK3\–cerebral cavernous malformations 2 complex. Nat Commun 6, 1-11 (1AD).
48. Sparks, J. L. et al. The CMG Helicase Bypasses DNA-Protein Cross-Links to Facilitate Their Repair. Cell 176, 167-181.e21 (2019).
49. De Magis, A. et al. DNA damage and genome instability by G-quadruplex ligands are mediated by R loops in human cancer cells. Proceedings of the National Academy of Sciences 116, 816-825 (2019).
50. Bhatia, V. et al. BRCA2 prevents R-loop accumulation and associates with TREX-2 mRNA export factor PCID2. Nature 511, 362-365 (2014).
51. Okamoto, Y. et al. Replication stress induces accumulation of FANCD2 at central region of large fragile genes. Nucleic Acids Res 46, 2932-2944 (2018).
52. Chan, K. L., Palmai-Pallag, T., Ying, S. \& Hickson, I. D. Replication stress induces sisterchromatid bridging at fragile site loci in mitosis. Nat Cell Biol 11, 753-760 (2009).
53. Naim, V. \& Rosselli, F. The FANC pathway and BLM collaborate during mitosis to prevent micro-nucleation and chromosome abnormalities. Nat Cell Biol 11, 761-768 (2009).
54. Madireddy, A. et al. FANCD2 Facilitates Replication through Common Fragile Sites. Mol. Cell 64, 388-404 (2016).
55. Wu, W. RTEL1 suppresses G-quadruplex-associated R-loops at difficult-to-replicate loci in the human genome. Nature structural molecular biology 1-2 (2020).
56. Tate, J. G. et al. COSMIC: the Catalogue Of Somatic Mutations In Cancer. Nucleic Acids Res 47, D941-D947 (2018).
57. Macheret, M. \& Halazonetis, T. D. DNA replication stress as a hallmark of cancer. Annu Rev Pathol 10, 425-448 (2015).
58. Helmrich, A., Ballarino, M. \& Tora, L. Collisions between replication and transcription complexes cause common fragile site instability at the longest human genes. Mol. Cell 44, 966-977 (2011).
59. Blin, M. et al. Transcription-dependent regulation of replication dynamics modulates genome stability. Nat Struct Mol Biol 26, 58-66 (2019).
60. Barlow, J. H. et al. Identification of early replicating fragile sites that contribute to genome instability. Cell 152, 620-632 (2013).
61. Hangauer, M. J., Vaughn, I. W. \& McManus, M. T. Pervasive transcription of the human genome produces thousands of previously unidentified long intergenic noncoding RNAs. PLoS Genet 9, e1003569 (2013).
62. Djebali, S. et al. Landscape of transcription in human cells. Nature 489, 101-108 (2012).
63. Macheret, M. \& Halazonetis, T. D. Intragenic origins due to short G1 phases underlie oncogene-induced DNA replication stress. Nature 555, 112-116 (2018).

## FIGURE LEGENDS

## Fig. 1: SLX4 interacts with RTEL1

(a) YFP-pull down from HeLa cells expressing YFP-SLX4. In lanes 3-5, YFP-pull downs were washed in a high salt buffer ( NaCl ) or carried out in the presence of Benzonase (Benzo) or Ethidium bromide (EtBr) as indicated. MUS81 and XPF were used as positive controls for SLX4binding.
(b) Endogenous SLX4 was immunoprecipitated from a HeLa cell-lysate with a combination of two different anti-SLX4 and visualized by western blotting.
(c) Immunoprecipitation of Flag-HA-SLX4 from dox-inducible HeLa cells at the indicated time points after release from a thymidine block. Cell cycle profiles of the samples are shown at left. Immunoblot of a representative experiment (right) and the quantifications of $n=3$ independent experiments. Error bars $=$ mean $\pm$ s.e.m.
(d) Schematic diagram showing RTEL1-binding domain of SLX4, and multiple SLX4 sequence alignment centred on the region 599-635 of human SLX4. Top two sequences report secondary structures (H: helix) and disorder status (D) predicted by PSIPRED and SPOTD algorithms, respectively (See Methods). NCBI RefSeq identifiers are given within brackets. UBZ4: ubiquitinbinding, MLR: primary XPF-binding domain, BTB: homodimerization domain (also contributes to XPF-binding), TBM: TRF2-binding motif, SIM: SUMO-interacting motifs, SAP: MUS81-binding region, CCD: SLX1-binding domain.
(e) YFP-pull downs from HeLa cells expressing WT or mutated YFP-SLX4. $\triangle \mathrm{BTB}$ : $\mathrm{M}_{684}$ VNNGLPP $_{764}$ was deleted from the BTB domain. BTB5A and SIM: point mutations in the BTB domain and SIM motifs, respectively, as described $\mathrm{in}^{2}$. D614G and L618P: cancer-associated mutations (Fig. 1d).
(f) YFP-pull downs from HeLa cells expressing WT or mutated YFP-SLX4. L1022A: TRF2binding defective mutant.

All immunoblots were performed with antibodies against the indicated proteins.
Uncropped images for panels $a, b, d-f$ and data for graphs in panel $b$ are available as source data.

Fig. 2: Mapping the interaction domains of SLX4 and RTEL1
(a) Schematic of the RTEL1 protein (top) and 3D modelling (ribbon representation) of HD1 and HD2, on which the SLX4 peptide has been docked.
(b) Co-immunoprecipitation of endogenous SLX4 with Flag-tagged full-length RTEL1 or a fragment containing the two harmonins (763-1164) indicated in (a). Immunoblots were performed with antibodies against SLX4 or the Flag peptide. *Flag indicates the band corresponding to Flag-RTEL1 full-length and the Flag-RTEL1 ${ }_{763-1164}$ fragment.
(c) Schematic of RTEL1 fragments containing HD1 and/or HD2 (top panel) that were used to assess direct binding to the SLX4 577-685 (Helix) and SLX4 577-1042 (Helix+BTB) fragments by Y2H (bottom panel). SLX4-interacting fragments are in red. SD: media complemented with all amino acids except Leu and Trp; X-Gal: ß-galactosidase test; -URA: same as -Leu-Trp with no uracil; 5 -FOA: same as -Leu-Trp complemented with $0,2 \% 5$-Fluoroorotic acid; 3-AT: same as -LeuTrp with no histidine and complemented with 50 mM 3 -Amino-1,2,4-triazole.
(d) Y2H carried out with WT or mutated SLX4 $4_{577-1042}$ (Helix+BTB) fragment and the RTEL1 ${ }_{885-990}$ fragment (HD1b in Fig. 2c). All interaction assays are in duplicate. Media are indicated as in (c). (e) E.coli-produced 6His-tagged HD1b (RTEL1 ${ }_{885-990}$ ) and HD2a (RTEL1 $1_{1046-1422}$ ) fragments were used in a $\mathrm{Ni}^{++}$-pull down assay with a WT or a mutated GST-tagged SLX4 ${ }_{577-1042}$ (Helix+BTB) fragment. $\mathrm{Ni}^{++}$-agarose beads were resuspended in a volume of Laemmli buffer equivalent to the initial volume of the assay. Identical volumes of Helix+BTB fragments, diluted in Laemmli buffer to the final concentration used in the assay, and of the $\mathrm{Ni}^{++}$-agarose beads resuspended in Laemmli buffer were loaded on the gel.
(f) Schematic of RTEL1 fragments used in $\mathbf{g}$ and $\mathbf{h}$ with Hoyeraal-Hreidarsson syndrome (HHS) patient mutations mapped in HD1.
( $\mathbf{g}, \mathbf{h}$ ) Co-immunoprecipitation of endogenous SLX4 with transiently expressed WT or mutated YFP-RTEL1 fragments. Immunoblots were performed with antibodies against the indicated proteins.
Uncropped images for panels $b-e, g, h$ are available as source data.

Fig. 3: SLX4 promotes replication fork progression during S phase through interaction with RTEL1
(a) HeLa Flp-In TREX "Fit0" cells (empty FRT site) were transfected with siRNAs targeting SLX4 (siSLX4 ${ }^{\text {UTR }}$ and siSLX4 ${ }^{\text {SP }}$ ) or control siRNAs (siLUC). Nascent DNA was labelled with pulses of iododeoxyuridine (IdU) and chlorodeoxyuridine (CldU). Replication fork dynamics were analysed on stretched DNA after immunostaining of IdU, CIdU and DNA (red, green and blue, respectively). Distributions of replication track lengths and fork ratios (ratio between the longest tract over the shortest for each individual unbroken fork) are shown in box-plots (median, first and third quartiles) with $5^{\text {th }}-95^{\text {th }}$ percentile whiskers (+=mean; dots= outliers; $n=$ number of unbroken signals analysed by Mann-Whitney test, **: $p<0.01$, ****: $p<0.0001$ ). The immunoblot was performed with antibodies against SLX4 and $\beta$-catenin (internal loading control). Arrow: SLX4 band. *: prominent non-specific band. Scale bar $=10 \mu \mathrm{~m}$.
(b) HeLa "Fit0" and Hela cells stably expressing DOX-inducible WT or mutated Flag-HA-SLX4 as indicated. siSLX4 ${ }^{U T R}$ was used to deplete endogenous SLX4. ns: not significant; **: $\mathrm{p}<0.01$; ***: p<0.001; ****: p<0.0001 by Mann-Whitney test. Scale bar = $10 \mu \mathrm{~m}$.
(c) Nascent DNA strands were pulse-labelled with 5-ethynyl-2-deoxyuridine (EdU). Biotin was conjugated to EdU by click chemistry after cell fixation. In situ proximity ligation assay (PLA, green) was performed between SLX4 (anti-HA) and EdU (anti-biotin) before EdU counterstaining (red). Representative fields and zooms on single cells (white squares) are shown. The number of PLA spots per EdU-positive cells is plotted (red bars: median with interquartile range; $n>79$; Kruskal-Wallis test, ****: $p<0.0001$ ). In parallel, single-cell expression of the different Flag-HA-SLX4 constructs was assessed by HA immunostaining ( $n>99$ ). Parental HeLa "Fit0" cells (-) were used as negative control. Scale bar $=10 \mu \mathrm{~m}$.
(d) Replication fork dynamics analysed as in a in HHS-patient derived SV40-transformed fibroblasts (P7) bearing a homozygous RTEL1 ${ }^{\text {R957w }}$ mutation in HD1 that abrogates interaction with SLX4 (Fig. 2g,h and Extended data Fig 4). P7 cells were compared to SV40-immortalized fibroblasts expressing WT RTEL1. ${ }^{* * * *: ~} \mathrm{p}<0.0001$ by Mann-Whitney test. Scale bar $=10 \mu \mathrm{~m}$.
Uncropped images of the immunoblots in panel a and data for graphs in panels a-d are available as source data.

Fig. 4: SLX4 promotes replication fork progression independently of its associated SSEs
(a) Schematic of SLX4 showing the F529LW > ALA, E1577L > AA and C1805 > R point mutations introduced to generate the SLX4 ${ }^{- \text {SMX }}$ mutant that abrogate binding to XPF, MUS81 and SLX1, respectively.
(b) anti-Flag immunoprecipitations were performed on cell lysates from HeLa "Fit0" and HeLa Flp-In TREX cells stably expressing DOX-inducible WT or Flag-HA-SLX4 ${ }^{- \text {SMX }}$ as indicated. Immunoblots were performed with antibodies against SLX4, XPF, MUS81 and SLX1. The fraction of SLX1, MUS81 and XPF associated with SLX4 was normalized to the total immunoprecipitated SLX4. The relative ratio of SLX1, MUS81 and XPF associated with SLX4 ${ }^{-}$ ${ }^{\text {SMX }}$ was then normalized to the corresponding SLX $4{ }^{\mathrm{WT}}$ ratio.
(c) Replication fork dynamics were assessed as in Fig. 3 in HeLa "Fit0" and HeLa Flp-In TREX cells stably expressing DOX-inducible Flag-HA-SLX4 $4^{-S M X}$. siSLX4 ${ }^{U T R}$ was used to deplete endogenous SLX4. Box-plots show median, first and third quartiles with $5^{\text {th }}-95^{\text {th }}$ percentile whiskers (+=mean; dots= outliers; n=number of unbroken signals analysed). ns: not significant; *: $p<0.05 ; * *: p<0.01 ;{ }^{* * * *}: p<0.0001$ by Mann-Whitney test. Uncropped images of blots for panels $b$ and $c$ and data for co-IP ratios in $b$ and graphs in $c$ are available as source data.

Fig. 5: SLX4 and RTEL1 interaction promotes FANCD2:RNA pol II colocalization and prevents conflicts between replication and transcription
(a) Quantification of FANCD2 foci in unchallenged interphase U2OS "Fit0" cells or expressing WT or mutated Flag-HA-SLX4. The number of foci per cell with the mean $\pm$ s.e.m. of the distribution is plotted ( $\mathrm{n}=120$ cells). Cells were mock- (siLUC) or SLX4-depleted (siSLX4 ${ }^{\text {UTR }}$ ). Immunoblots at top were performed with anti-SLX4, anti-RTEL1, anti-XPF and anti-GRB2 (loading control).
(b) PLA between FANCD2 and RNA pol II phospho S2 in HeLa "Fit0" cells mock- or SLX4depleted. Reactions with single primary antibodies (Ab) were used as negative controls. Boxplots show number of PLA spots per nucleus ( $n>180$; median, first and third quartiles, $5-95^{\text {th }}$ percentile whiskers, $+:$ mean). Immunoblot at right was performed with anti-SLX4 or anti- $\beta$ catenin (loading control). The arrow indicates the SLX4 band.
(c) As in b, in U2OS cells expressing Flag-HA-SLX4 ( $n>180$ ). Endogenous SLX4 was depleted with siSLX4 ${ }^{U T R}$.
(d) PLA between SLX4 (HA) and RNA pol II phospho S2 in U2OS cells expressing Flag-HASLX4 ${ }^{\text {WT }}$ (left panel, $\mathrm{n}>139$, median with interquartile range). Parental U2OS "Fit0" cells (-) were used as a negative control. Right panel shows single-cell expression of the construct (HA staining, $\mathrm{n}>124$ ).
(e) PLA between endogenous RTEL1 and RNA pol II phospho S2 in HeLa "Fit0" cells ( $n>153$ ). PLA negative controls as in $\mathbf{b}$.
(f) PLA between endogenous PCNA and RNA pol II phospho S2 was performed in mockdepleted (siLUC), SLX4-depleted (siSLX4 ${ }^{\text {UTR }}$, siSLX4 ${ }^{\text {sp }}$ ) or RTEL1-depleted (siRTEL1) HeLa "Fit0" cells ( $n>175$ ). PLA negative controls as in $\mathbf{b}$.
Kruskal-Wallis (a, b, c, e, f) and Mann-Whitney (d) tests (ns: not significant, *: p<0.05, **: $p<0.01,{ }^{* * *}: p<0.001,{ }^{* * * *}: p<0.0001$ ). Scale bar of representative fields and cells $=10 \mu \mathrm{~m}$ in all panels.
Uncropped images of the immunoblots in panels $a, b$ and data for graphs in panels a-f are available as source data.

Fig. 6: Transcription inhibition rescues replication defects caused by loss of SLX4-RTEL1 complex formation
(a) HeLa "FitO" cells were depleted for SLX4 or RTEL1 and $50 \mu \mathrm{M}$ cordycepin was added to culture medium 3 h before and during the IdU and CldU pulses to inhibit transcription elongation. Replication fork dynamics were analysed as in Fig. 3a. The distribution of the fork ratio is shown in box-plots (median, first and third quartiles with $5^{\text {th }}-95^{\text {th }}$ percentile whiskers; +=mean; dots= outliers; $n=n u m b e r ~ o f ~ u n b r o k e n ~ s i g n a l s ~ a n a l y s e d ; ~ n s: ~ n o t ~ s i g n i f i c a n t, ~ * *: ~ p<0.01, ~ * * * *: ~ p<0.0001 ~$ by Mann-Whitney test). See also Extended Data Fig. 8a for the distributions of replication track lengths and western blot showing depletion efficiency.
(b) As in a in HeLa Flp-In TREX cells stably expressing DOX-inducible WT, D614G or L618P Flag-HA-SLX4 depleted for endogenous SLX4. The immunoblot was performed with antibodies against SLX4 and $\beta$-catenin used as internal loading control.
(c) As in a in RTEL1 ${ }^{\text {WT }}$ and RTEL1 ${ }^{\text {R957W }}$ SV-40 immortalized human fibroblasts.

Uncropped images of the immunoblots in panel $b$ and data for graphs in panels a-c are available as source data.

## METHODS

## DNA construction and mutagenesis

DNA constructions and cloning were performed using Gateway Technology (Invitrogen) as per manufacturer's instruction. A list of primers used in this study is available upon request. The BTB and SIM mutants of SLX4 were generated as previously described ${ }^{2}$ :
SLX4 ${ }^{\text {BTB }^{*}}\left(\mathrm{H}_{706} \mathrm{KFVL}_{710} \rightarrow\right.$ AAAAA)
SLX4 ${ }^{\text {SIM }}$ (SIM1: V $_{1151 \text { ILLL }_{1155}} \rightarrow$ AAAAA; SIM2: $I_{1194}$ IDV $\rightarrow$ AADV; SIM3: V $_{1392}$ VEV $\rightarrow$ AAEV) In addition, a deletion of the BTB domain was generated and used in the present study:
SLX4 ${ }^{\triangle B T B}$ (deletion $\mathrm{M}_{684}$ VNN-GLPP ${ }_{764}$ )
SLX4 $4^{-S M X}\left(\mathrm{~F}_{529} \mathrm{LW} \rightarrow \mathrm{ALA}, \mathrm{E}_{1577} \mathrm{~L} \rightarrow \mathrm{AA}, \mathrm{C}_{1805} \rightarrow \mathrm{R}\right)$

## Antibodies

Rabbit anti-SLX4 (A302-270A), rabbit anti-XPF (A301-315A-T), rabbit anti-biotin (A150-109A) and rabbit anti-TRF2 (A300-796A) antibodies were purchased from Bethyl. Rabbit anti-RTEL1 antibody was produced by the laboratory of Arturo Londoño-Vallejo's ${ }^{64}$. Mouse anti-MUS81 [MTA30 2G10/3] (ab14387), mouse anti-GAPDH (ab9484), rabbit anti-FANCD2 (ab2187) and rabbit anti-RNA polymerase II (ab5095 [phospho S2]) antibodies were purchased from Abcam. Mouse anti-RNA polymerase II (clone H5 MMS-129R [phospho S2]), mouse anti-HA [16B12] (901513) antibodies were purchased from BioLegend. Mouse anti-XPF [clone 219] (XPF Ab-1) antibody was from Thermo scientific. Mouse anti-ß-catenin (clone 14, 610153) and mouse antiGRB2 (610111) antibodies were from BD Biosciences. Mouse anti- $\beta$-actin antibody (AC-15, A5441) was purchased from Sigma. Mouse anti-laminA/C antibody was purchased from Santa Cruz (sc-7292). Mouse anti-BrdU antibody (clone B44, 347580) was from Becton Dickinson. Rat anti-BrdU antibody (clone BU1/75, AbC117-7513) was purchased from Abcys. Mouse antissDNA antibody (clone 16-19, MAB3034) was from Millipore. Mouse anti-biotin antibody (200-002-211) was purchased from Jackson Immunoresearch.
Duolink® In Situ PLA® Probe anti-Rabbit PLUS (DUO92002) anti-Mouse MINUS (DUO92004) were from Sigma.
Goat anti-rabbit IgG/HRP (2019-08) and goat anti-mouse IgG/HRP (2019-05) antibodies were purchased from Dako. Alexa Fluor 488 conjugated donkey anti-mouse (A-21202), goat antimouse (A-11001), goat anti-rat (A-11006) and Alexa Fluor 594 conjugated donkey anti-rabbit (A-21207), goat anti-mouse (A-11032 and A-11020), goat anti-rabbit (A-11072), rabbit antimouse (A-21062), goat anti-rabbit (A-11046) antibodies were from Molecular probes.

## Immunoblotting

SDS-PAGE and immunoblotting were done with Novex® NuPAGE® SDS-PAGE Gel System and XCell $I I^{T M}$ blot module (Invitrogen), respectively. Hybond-C Extra (RPN203e) was purchased from GE healthcare. Western lightning plus ECL (NEL105001eA) was from PerkinElmer, Hyperfilm ECL (28-9068-35) was purchased from GE healthcare. Chemidoc MP imaging system (Biorad), ImageQuant LAS 4000 system (GE Healthcare) or Amersham Imager 600 (GE health care) were also used for detection.

## Cell lines, cell culture and transfection

HeLa cells (ATCC) were cultured in DMEM with $10 \%$ FBS and Pen/Strept (Gibco). HeLa FIp-In TRex and U2OS Flp-In TRex cells (Fit0: parental cells with no cDNA integrated at the FRT site) (kindly provided by Stephen Taylor and Palm Silver, respectively) were maintained in DMEM containing 10\% FBS and Pen/Strept with $2 \mu \mathrm{~g} / \mathrm{mL}$ Blasticidine (Invivogen) and $100 \mu \mathrm{~g} / \mathrm{mL}$ Zeocin (Invitrogen) to maintain the genomic FRT site. For generating stable cell lines expressing YFP- or Flag-HA- tagged SLX4 in a doxycycline-inducible manner, pDEST-YFP-SLX4 or pDEST-Flag-HA-SLX4 were co-transfected with pOG44 (encoding the Flp recombinase) with 1:9 ratio, respectively, using JetPEI (Polyplus transfection) as per manufacturer's instruction. Recombinant clones were selected with the medium described above with $100 \mu \mathrm{~g} / \mathrm{mL}$ Hygromycin B (Invitrogen) instead of Zeocin. Selected clones were pooled to generate a population in order to minimize clonal heterogeneity. Fibroblasts from a severe combined immunodeficient patient with genetic defect in the lymphoid specific RAG1 gene that is not expressed in fibroblasts (WT RTEL1 control in Extended data Fig. 6c) and from an RTEL1deficient patient $P 7^{31}$ were obtained from skin biopsies. Informed and written consent was obtained from donors and patients. The study and protocols comply with the 1975 Declaration of Helsinki as well as with the local legislation and ethical guidelines from the Comité de Protection des Personnes de l'lle de France II and the French advisory committee on data processing in medical research. Fibroblasts were transformed by the large $T$ antigen from SV40T as previously described ${ }^{65}$. Fibroblasts were cultured in RPMI with $15 \%$ FBS, Pen/Strept and 25 mM Hepes (Gibco) under 3\% $\mathrm{O}_{2}$. All cell lines were checked for mycoplasma contamination.

## Immunoprecipitation

In YFP-pull downs, the SLX4 complex was purified from HeLa Fit0 cells transfected with pDEST-YFP-SLX4 or HeLa FIp-In TREX cells expressing YFP-SLX4 under $10 \mathrm{ng} / \mathrm{mL}$ doxycycline, as previously described ${ }^{2}$. Briefly, cells were harvested by Trypsin/EDTA, washed with PBS then lysed with NETN buffer ( 50 mM Tris-HCI [pH 8.0], $150 \mathrm{mM} \mathrm{NaCl}, 1 \mathrm{mM}$ EDTA, $1 \%$ NP-40, 1 mM DTT, 0.25 mM PMSF) containing proteasome inhibitor cocktail (Complete EDTA-free [Roche]) for 30 min at $4^{\circ} \mathrm{C}$. After centrifugation ( $15000 \mathrm{rpm}, 10 \mathrm{~min}$ at $4^{\circ} \mathrm{C}$ ),
supernatants were incubated with GFP nanobody (kindly provided by Mauro Modesty) for 2 hrs at $4^{\circ} \mathrm{C}$. The beads were washed 3 times with NETN buffer, twice with TBS, and 5 times with 50 mM Tris- HCl [pH 8.0] before samples were eluted with NuPAGE LDS sample buffer (Invitrogen) for 5 min at $95^{\circ} \mathrm{C}$. For Fig. 1a (lane 3), NETN buffer including 850 mM NaCl was added to the supernatant before immunoprecipitation to adjust the final concentration of NaCl to 500 mM . After the incubation, beads were washed with NETN including 500 mM NaCl for 3 times before TBS wash. For Fig. 1a (lane 4 and 5), beads were incubated with either $500 \mathrm{U} / \mathrm{mL}$ Benzonase (Sigma) in presence of 2 mM MgCl 2 or $50 \mu \mathrm{~g} / \mathrm{mL}$ EtBr for 16 hrs after TBS wash. Beads were washed 5 times with 50 mM Tris- HCl [pH 8.0] buffer then samples were eluted with NuPAGE LDS sample buffer. For anti-Flag immunoprecipitation samples were processed as described above using FLAG M2 Agarose beads (Sigma-Aldrich) instead of a GFP-nanobody.

## Co-immunoprecipitation of endogenous SLX4 and RTEL1

$10^{7} \mathrm{HeLa}$ (FitO) cells were lysed in NETN buffer ( 50 mM Tris- $\mathrm{HCl}[\mathrm{pH}=8.0], 150 \mathrm{mM} \mathrm{NaCl}, 1 \mathrm{mM}$ EDTA, $0.5 \%$ NP-40 supplemented with anti-proteases cocktail (Roche)) at $4^{\circ} \mathrm{C}$ before sonication and centrifugation. Half of the clarified lysate were incubated either with $2 \mu \mathrm{~g}$ of $\lg G$ rabbit (Cell Signaling \#2729S) ) or $2 \mu \mathrm{~g}$ of anti-SLX4 ( $1 \mu \mathrm{~g}$ of A302-270A $+1 \mu \mathrm{~g}$ A302-269A from Bethyl laboratories) and $15 \mu \mathrm{~L}$ dynabeads-protein G (invitrogen) overnight at $4^{\circ} \mathrm{C}$. Beads were washed 4 times in NETN buffer before elution in loading buffer. To probe for RTEL1 and SLX4 in WB and avoid the signal of the IgG used for immunoprecipitation, specific secondary antibodies were used (Veriblot, Abcam).

## Cell cycle synchronization

HeLa Flp-In TREX cells expressing Flag-HA-SLX4 were synchronized by double thymidine block. $2 \times 10^{6}$ cells were seeded the day before treatment then incubated with $10 \%$ FBS/DMEM containing 2 mM Thymidine (Sigma) and doxycycline ( $10 \mathrm{ng} / \mathrm{mL}$ ) for 18 hrs . After PBS wash, cells were incubated in the medium without Thymidine for 9 hrs then incubated in the medium containing 2 mM of Thymidine for 16 hrs . The cells synchronized at G1/S phase were released from Thymidine block and collected at several time points after release (described in the Fig.). Cell cycle stages of samples were analyzed with a Muse ${ }^{T M}$ cell analyser (Millipore) as per manufacturer's instructions.

## Yeast Two Hybrid

Yeast Two-Hybrid assay was performed using ProQuest Two-Hybrid systems as per manufacturer's instruction. Briefly, various Human SLX4 and RTEL1 cDNA fragments indicated in Fig. 2c,d and Extended data Fig. 4 were inserted in yeast two-hybrid vectors pDEST22 and pDEST32, respectively. Yeast two-hybrid vectors were transformed into the yeast strain

MaV203. Cells were cultured on plates with the selective media as indicated in Fig. 2c,d and Extended data Fig. 4.

## Protein Expression and Purification and in vitro binding assay

The 6His-tagged RTEL1 HD1a, HD1b and H2a fragments and the GST-SLX4 (Helix-BTB) WT and D614G mutant fragments were expressed in E.coli (Rosetta) induced with 0.1 mM IPTG at $16^{\circ} \mathrm{C}$ overnight. Bacterial pellets were frozen in PBS. The next day, an equivalent volume of 2 x lysis buffer ( 100 mM Tris [pH=8], 20\% [v/v] glycerol, 2\% [v/v] Triton X-100, 1 M NaCl, 1 mg/ml lysozyme (Sigma)) was added. A protease inhibitor cocktail (Complete EDTA-free, Roche) and PMSF were added to the thawed lysates just before sonication and clarification. $\mathrm{Ni}^{++}$-agarose was charged with the 6His-tagged RTEL1 HD fragments after incubation with the supernatant for 3 hrs at $4^{\circ} \mathrm{C}$. The Ni++-agarose resin was washed 5 times in wash buffer ( 50 mM Tris. $\mathrm{Cl} \mathrm{pH}=8,1 \mathrm{M} \mathrm{NaCl}, 0.01 \%[\mathrm{v} / \mathrm{v}]$ Triton $\mathrm{X}-100$ ) and stored at $4^{\circ} \mathrm{C}$ in binding buffer ( 150 mM NaCl , Tris.Cl pH=8, 0,01\% [v/v] Triton X100, 10\% [v/v] glycerol). The GST-SLX4 (Helix-BTB) WT and D614G mutant fragments were purified on Glutathione agarose (Molecular probes) following standard procedures
For in vitro binding assays the GST-SLX4 (Helix-BTB) fragments were incubated with $\mathrm{Ni}^{++}$agarose charged with the 6 His -tagged RTEL1 HD fragments for 2 hours at $22^{\circ} \mathrm{C}$. The flow through (Ft) was recovered after centrifugation. The pelleted $\mathrm{Ni}^{++}$-agarose was subsequently was washed 10 times with 10 bead volume of binding buffer before it was resuspended in a volume of Laemmli buffer equivalent to the total volume of the in vitro binding assay.

Proteins were analyzed by SDS-PAGE and detected following transfer to a nitrocellulose membrane (Amersham) with anti-6His or anti-GST antibodies.

## siRNA transfection and Complementation assays

The following siRNA sequences were used in this work.
siLUC (CGUACGCGGAAUACUUCGAdTdT) siSLX4 $4^{\text {UTR }}$ targeting the 5'UTR and 3'UTR of SLX4 consists of a mix of two siRNAs:
SLX4 UTR87 (GCACCAGGUUCAUAUGUAUdTdT)
SLX4 UTR7062 (GCACAAGGGCCCAGAACAAdTdT)
In addition, siRNAs for SLX4 (M-014895-01-0005) (siSLX4 ${ }^{\text {SP }}$ ) and RTEL1 (M-013379-00-0005) (siRTEL1 ${ }^{\text {SP }}$ ) were purchased from Dharmacon.
Fit0 cells or stable cell lines described in Cell lines section were seeded the day before transfection ( $1.5 \times 10^{5}$ cells/ 6 -well). The transfection was performed in $10 \%$ FBS/DMEM containing doxycycline to induce Flag-HA-SLX4 protein expression. siRNAs were transfected at 10 or 20 nM of final siRNA concentrations using INTERFERin (Polyplus transfection) as per manufacturer's instructions. The transfection was repeated 24 hrs later then cells were
reseeded 6 hrs after the $2 n d$ siRNA transfection. Cells were then incubated in 10\%FBS/DMEM containing doxycycline for 1 or 2 days in order to achieve optimal knockdown efficiency and to maintain Flag-HA-SLX4 expression. Cell biological and biochemical analysis were performed as described below.

## Colony survival assay

siRNA-treated HeLa FIp-In TREX cells expressing Flag-HA-SLX4 were reseeded at 900 cells/60 mm Petri dish 6 hrs after the 2nd siRNA transfection. 20 hrs later, cells were treated with the indicated concentrations of mitomycin C (MMC) in presence of doxycycline ( $1 \mathrm{ng} / \mathrm{mL}$ ) for 24 hrs . Cells were then subsequently washed with PBS and cultured with $10 \%$ FBS/DMEM including 1 $\mathrm{ng} / \mathrm{ml}$ of doxycycline. Colonies were fixed with fix solution ( $50 \% \mathrm{EtOH}, 7 \%$ Acetic acid) including $0.5 \mathrm{mg} / \mathrm{mL}$ Brilliant blue R 250 (Sigma) 8 days after MMC treatment and counted.

## DNA fiber analysis

Cells were pulse labelled with $25 \mu \mathrm{M}$ IdU (Sigma) for 20 min , washed with pre-heated medium and PBS, then pulse labeled with $50 \mu \mathrm{M}$ CIdU (Sigma) for 20 min. In Fig. 6 and Extended data Fig. 8a, cells were pre-treated for 3 h with $50 \mu \mathrm{M}$ cordycepin (Sigma) and cordycepin was maintained during the labelling procedure. In Extended data Fig. 8b, cells were treated for 3 h with $1 \mu \mathrm{M}$ triptolide (Sigma) and triptolide was maintained during the labelling procedure. Cells were harvested by trypsinization and resuspended in ice-cold PBS at 500,000 cells per ml. $2 \mu \mathrm{~L}$ of the cell suspension ( 1,000 cells) were spotted on microscope glass slides and lyzed in $7 \mu \mathrm{~L}$ of spreading buffer ( 200 mM Tris-HCI [pH 7.5], 50 mM EDTA [pH8.0], 10\% SDS). Slides were tilted to $15^{\circ}$ in order to spread the DNA molecules. After fixation with a mixture of methanol and acetic acid (3:1), air-dried slides were incubated twice in $\mathrm{H}_{2} \mathrm{O}$ for 5 min followed by denaturation in 2.5 M HCl for 1 hr . Slides were washed in PBS and blocked for 1 h in blocking buffer ( $1 \%$ BSA, $0.1 \%$ Tween20 in PBS). IdU, CIdU and total DNA were immunodetected with the following procedure: (1) mouse anti-BrdU (clone B44 347580, Becton Dickinson, 1/20, IdU) + rat antiBrdU (clone BU1/75 AbC117-7513, Abcys, 1/100, CldU) for 1 h at room temperature, (2) goat anti-mouse AF594 (A11032) + goat anti-rat AF488 (A11006) for 30 min at $37^{\circ} \mathrm{C}$, (3) mouse antisingle stranded DNA (clone 16-19 MAB3034, Ozyme, 1/100) for 45 min at $37^{\circ} \mathrm{C}$, (4) rabbit antimouse AF350 (A21062) for 20 min at $37^{\circ} \mathrm{C}$ and (5) goat anti-rabbit AF350 (A11046) for 20 min at $37^{\circ} \mathrm{C}$. All AlexaFluor-coupled antibodies were from Molecular probes and used at $1 / 100$ dilution. All antibodies were diluted in blocking buffer. Samples were mounted in fluorescent mounting medium (DAKO). Images were acquired on an Axio Imager Z1 microscope using the Axio Vision software (Zeiss). Images were analyzed with the Axio Vision software. Broken replication tracks, established by total DNA counterstaining, were excluded from the analysis.

For each intact individual fork (i.e. one IdU tract flanked by a CldU track), the fork ratio was calculated as the ratio between $\max (I d U, C I d U)$ and $\min (I d U, C l d U)$.

## Immunofluorescence

siRNA-treated HeLa Flp-In TREX or U2OS Flp-In TREX cells expressing Flag-HA-SLX4 or YFP-SLX4 were cultured on coverslips. Cells were washed with CSK buffer ( $100 \mathrm{mM} \mathrm{NaCl}, 300$ mM sucrose, 10 mM PIPES [pH 7.0], 3 mM MgCl 2 ) and pre-extracted in CSK buffer containing $0.5 \%$ TritonX-100 for 3 min at $4^{\circ} \mathrm{C}$ then fixed in $4 \%$ paraformaldehyde/PBS. For Extended data Fig. 1b, HeLa FIp-In TREX cells expressing YFP-SLX4 were washed with PBS and fixed in $4 \%$ paraformaldehyde/PBS then permeabilized in $0.5 \%$ TritonX-100/PBS for 15 min . After PBS wash, cells were incubated in blocking buffer ( $3 \%$ BSA/PBS) for 30 min then immunostained with primary antibodies in blocking buffer for 2 hrs at room temperature or overnight at $4^{\circ} \mathrm{C}$. Cells were washed three times with blocking buffer and stained with secondary antibodies in blocking buffer for 2 hrs at room temperature. After three times of PBS wash, coverslips were mounted in fluorescent mounting medium supplemented with DAPI (Vector Laboratory or Dako). Images were acquired with LSM-880 microscope using Zen software (Zeiss) (Fig. 4a) or with Axio Imager Z1 and Z2 microscope using Axio Vision software (Zeiss) (Extended data Fig. 1c). Images were analyzed with ImageJ software.

## In situ Proximity ligation assay (PLA)

For PLA, cells were seeded on fibronectin-coated glass coverslips. PLA between SLX4, RTEL1 or FANCD2 and RNA polymerase II were performed as described previously ${ }^{34}$. Cells were fixed with $4 \%$ paraformaldehyde/PBS and permeabilized in $0.5 \%$ TritonX-100/PBS for 5 min . PLA was performed with the Duolink PLA In situ Green kit (Sigma) according to manufacturer's instructions. The following couples of primary antibodies were used: mouse anti-HA [16B12] (to detected SLX4, 1/1000) + rabbit anti-RNA polymerase II ab5095 (1/900); mouse anti-RNA polymerase II MMS-129R (1/600) + rabbit anti-FANCD2 ab2187 (1/1000) or rabbit anti-RTEL1 (1/500). When indicated, HA counterstaining was performed by incubating the coverslips with goat anti-mouse AF594 after the PLA procedure.
PLA between SLX4 and nascent DNA was performed as described previously ${ }^{66}$, with minor modifications. Briefly, cells were pulse labeled with $30 \mu \mathrm{M}$ EdU for 7 min , pre-extracted with CSK100 buffer ( $100 \mathrm{mM} \mathrm{NaCl}, 300 \mathrm{mM}$ sucrose, 10 mM PIPES $[\mathrm{pH} 6.8], 3 \mathrm{mM} \mathrm{MgCl} 2,1 \mathrm{mM}$ EGTA, $0.5 \%$ TritonX-100, protease inhibitors) for 5 min at $4^{\circ} \mathrm{C}$ and fixed. Biotin-azide (Molecular Probes) was conjugated to EdU by click chemistry. PLA was performed using mouse anti-HA [16B12] (1/1000) and rabbit anti-biotin (A150-109A, Bethyl, 1/3000) antibodies or mouse anti-biotin (1/3000) and rabbit anti-RTEL1 (1/500) antibodies. PLA was followed by EdU counterstaining.

## Bioinformatic and structural analyses

Homolog Sequences of RTEL1 and SLX4 were retrieved using PSI-BLAST searches ${ }^{67}$ against the RefSeq database. Multiple sequence alignments were calculated using MAFFT (e-insi mode) ${ }^{68}$ and represented using Jalview ${ }^{69}$. Secondary structures (H for helix, E for extended) and disorder status (d) were predicted by PSIPRED ${ }^{70}$ and SPOTD ${ }^{71}$ algorithms, respectively. 3d models of the HD1 and HD2 harmonin domains of human RTEL1 in complex with the helix of human SLX4 were generated by template-based docking using the alignments calculated by hhpred $^{72}$ and the RosettaCM ${ }^{73}$ protocol for comparative modeling. As a structural template the structure (PDB code:4yl6) of the complex between the harmonin domain of CCM2 and the MEKK3 helix ${ }^{74}$ was used as it was the best hhpred hit obtained from an X-ray structure (hhpred proba of $98.2 \%$ and $97.8 \%$ for HD1 and HD2, respectively). HD1 and HD2 share $21 \%$ and $17 \%$ sequence identity with CCM2, respectively, while SLX4 shares significant similarities with MEKK3 sequence, which were used to align both ligands sequences.

## Next generation sequencing of clinical samples

Targeted NGS was applied to a custom-made panel of 494 "cancer-associated" genes selected for their involvement in cancers (CCP-V8 panel). The DNA libraries of all coding exons and intron-exon boundaries of all genes using the HaloPlex Target Enrichment System (Agilent, Santa Clara, CA, USA) were done as described ${ }^{75}$. Sequencing was done using the $2 \times 150$-bp paired-end technology on the Illumina Nextseq500 platform according to the manufacturer's instructions (Illumina, San Diego, CA, USA). To identify somatic mutations only, germline DNAs from normal counterpart samples (blood lymphocytes of corresponding patients) were similarly sequenced. Samples were biopsied metastases from patients enrolled in the prospective PERMED-01 trial (NCT02342158) ${ }^{76}$. Samples were sequenced at an average depth greater than 700x for the targeted regions. PERMED 1069 was a liver metastasis of gastric adenocarcinoma; the SLX4 NM_032444 exon8 c.T1853c p.L618P variant had an allelic frequency of $2.30 \%$ (the ratio of malignant cells was $30 \%$ ). PERMED 1115 was a lung metastasis of chondroblastic osteosarcoma; the SLX4 NM_032444 exon8 c.A1841G p.D614G variant had an allelic frequency of $6.80 \%$ (the ratio of malignant cells was $90 \%$ ).

## Mass spectroscopy and data analysis methods are presented as a Supplementary Note.

## Statistics

GraphPad Prism 6 software was used for statistics. Statistical comparisons of replication track length and fork ratio distributions were assessed with the non-parametric two-tailed Mann-

Whitney test. IF and PLA experiments were tested with the non-parametric two-tailed KruskalWallis followed by Dunn's multiple comparisons. Complete results of statistics tests are shown in the Source Data files.

## DATA AVAILABILITY

The data supporting the findings of this study are available with the online article. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE [1] partner repository with the datasets identifiers PXD012426 and PXD012425. Next Generation sequence data (PERMED 1069 = isolate P1, PERMED 1115 = isolate P2) were deposited in the Sequence Read Archive (SRA) under the BioProject PRJNA611917 and can be retrieved at https://www.ncbi.nIm.nih.gov/Traces/study/?acc=PRJNA611917

Reporting summary. Further information on experimental design is available in the Nature Research Reporting Summary linked to this paper.

## METHODS-ONLY REFERENCES

64. Le Guen, T. et al. Human RTEL1 deficiency causes Hoyeraal-Hreidarsson syndrome with short telomeres and genome instability. Hum Mol Genet 22, 3239-3249 (2013).
65. Buck, D. et al. Cernunnos, a novel nonhomologous end-joining factor, is mutated in human immunodeficiency with microcephaly. Cell 124, 287-299 (2006).
66. Despras, E. et al. Rad18-dependent SUMOylation of human specialized DNA polymerase eta is required to prevent under-replicated DNA. Nat Commun 7, 13326 (2016).
67. Altschul, S. F. et al. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res 25, 3389-3402 (1997).
68. Katoh, K. \& Standley, D. M. MAFFT multiple sequence alignment software version 7: improvements in performance and usability. Mol. Biol. Evol. 30, 772-780 (2013).
69. Waterhouse, A. M., Procter, J. B., Martin, D. M. A., Clamp, M. \& Barton, G. J. Jalview Version 2--a multiple sequence alignment editor and analysis workbench. Bioinformatics 25, 1189-1191 (2009).
70. Jones, D. T. Protein secondary structure prediction based on position-specific scoring matrices. J Mol Biol 292, 195-202 (1999).
71. Hanson, J., Yang, Y., Paliwal, K. \& Zhou, Y. Improving protein disorder prediction by deep bidirectional long short-term memory recurrent neural networks. Bioinformatics 33, 685-692 (2017).
72. Zimmermann, L. et al. A Completely Reimplemented MPI Bioinformatics Toolkit with a New HHpred Server at its Core. J Mol Biol 430, 2237-2243 (2018).
73. Song, Y. et al. High-resolution comparative modeling with RosettaCM. Structure 21, 1735-1742 (2013).
74. Wang, X. et al. Structural Insights into the Molecular Recognition between Cerebral Cavernous Malformation 2 and Mitogen-Activated Protein Kinase Kinase Kinase 3. Structure 23, 1087-1096 (2015).
75. Collette, Y. et al. Drug response profiling can predict response to ponatinib in a patient with $\mathrm{t}(1 ; 9)(\mathrm{q} 24 ; \mathrm{q} 34)$-associated B-cell acute lymphoblastic leukemia. Blood Cancer J 5, e292 (2015).
76. Chanez, B. et al. De novo metastatic small cell carcinoma of the prostate with BRCA2 mutation: report of a successful precision medicine management with PARP inhibitors. JCO Precision Oncology, 2:1-8. doi.org/10.1200/PO.18.00083 (2018)

Figure 1


Figure 2


Figure 3
a




C

| siRNA $\downarrow$ | siRNA | seeding | EdU fix $\downarrow \downarrow$ | ation |
| :---: | :---: | :---: | :---: | :---: |
| D1 | D2 | D3 | D4 | EdU counterstaining DAPI |
| + DOX |  |  |  | (or HA staining) |



(n)


d



Figure 4
a


Figure 5


Figure 6
a


b

(n) $\quad \frac{(278)(348)}{\text { WT }} \frac{(370)(228)}{\text { D614G }} \frac{(391)(353)}{\text { L618P }}$





## Extended Data Figure 1




## Extended Data Figure 2

|  |  | PSM Number (Spectral count) |  |  |  |  |  |  |  |
| :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: |
|  |  | ATP. |  |  |  | ATP+ |  |  |  |
|  |  | YFP-SLX4 WT |  | FITO |  | YFP-SLX4 WT |  | FITO |  |
|  |  | Run1 | Run2 | Run1 | Run2 | Run1 | Run2 | Run1 | Run2 |
| Accession | Description | PSM | PSM | PSM | PSM | PSM | PSM | PSM | PSM |
| Q81Y92 | SLX4 | 423 | 415 |  |  | 363 | 353 |  |  |
| Q9BQ83 | SLX1 | 17 | 18 |  |  | 18 | 15 |  |  |
| Q92889 | XPF | 83 | 72 |  |  | 71 | 69 |  |  |
| P07992 | ERCC1 | 28 | 24 |  |  | 26 | 23 |  |  |
| Q96NY9 | MUS81 | 20 | 17 |  |  | 14 | 15 |  |  |
| P43246 | MSH2 | 31 | 29 |  |  | 28 | 25 |  |  |
| Q15554 | TRF2 | 11 | 8 |  |  | 7 | 8 |  |  |
| Q96AY2 | EME1 | 17 | 17 |  |  | 12 | 11 |  |  |
| Q5VYV7 | SLX4IP | 9 | 8 |  |  | 6 | 5 |  |  |
| Q9NZ71 | RTEL1 | 7 | 6 |  |  | 2 | 2 |  |  |

## Extended Data Figure 3

|  |  | PSM Number (Spectral count) |  |  |  |  |  |  |  |  |  |  |  |
| :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: |
|  |  | YFP-SLX4 WT |  |  | YFP-SLX4 D614G |  |  | YFP-SLX4 L618P |  |  | FIT0 |  |  |
|  |  | Run1 | Run2 | Run3 | Run1 | Run2 | Run3 | Run1 | Run2 | Run3 | Run1 | Run2 | Run3 |
| Accession | Description | PSM | PSM | PSM | PSM | PSM | PSM | PSM | PSM | PSM | PSM | PSM | PSM |
| Q8IY92 | SLX4 | 278 | 306 | 311 | 225 | 239 | 243 | 232 | 245 | 247 | 2 | 3 | 2 |
| Q9NZ71 | RTEL1 | 13 | 12 | 14 |  |  |  |  |  |  |  |  |  |
| P81605 | Dermcidin | 3 | 4 | 3 |  |  |  |  |  |  |  |  |  |
| P12273 | Prolactin-inducible protein | 3 | 3 | 3 |  |  |  |  |  |  |  |  |  |

## Extended Data Figure 4

a
HD1 extension required for interaction
HD1 RTEL1
with SLX4 helix

Sec. Struct. pred. (PSIPRED) Disorder pred. (SPOTD) Homo sapiens (NP 057518) 886-1158 Miniopterus natalensis (XP 016057738) 889-116 Bos taurus (XP 024856143) 889-1158 Ratus novegicus (NP 001178786) 886-1157 Mus musculus (XP 006500700) 886-1140 Corvus cornix (XP 019139906) 890-1169 Gallus gallus (XP 004947187) 895-1172
Alligator mississippiensis (XP 006273127) 894-1181 Xenopus laevis (XP 018090505) 893-1140 Danio rerio (NP 001013328) 898-1151 Oryzias latipes (XP 011471289) 899-1139 Takifugu rubripes (XP 011619108) 887-1130 Nematostella vectensis (XP 001631451) 912-1082 Hydra vulgaris (XP 012564281) 907-1015 Drosophila melanogaster (NP 572254) 895-985 Anopheles gambiae (XP 311162) 895-98 Schistosoma haematobium (XP 012795483) 989-1081


HD2 RTEL1

Sec. Struct. pred. (PSIPRED) Disorder pred. (SPOTD) Homo sapiens (NP 057518) 886-1158 Equus cabalus (XP 023482708) 903-1178 Miniopterus natalensis (XP 016057738) 889-1
Bos taurus (XP 024856143) 889-1158 Bos taurus (XP 024856143 ) $889-1158$ Mus musculus (XP 006500700) 886-1140 Corvus comix (XP 019139906) 890-1169 Gallus gallus (XP 004947187) 895-1172 Alligator mississippiensis (XP 006273127) 894-1181 Xenopus laevis (XP 018090505) 893-1140 Danio rento NP 01013328 830-1151 Takifugu rubripes (XP 011619108) 887-1130 Nematostella vectensis (XP 001631451) 912-1082 Hydra vulgaris (XP 012564281) 907-1015 Drosophila melanogaster (NP 572254) 895-985 Apis mellifera (XP 026302051) 899-989 Anopheles gambiae (XP 311162) 895-985
Schistosoma haematobium (XP 012795483) 989-1081

b


B: Beads
Ft: Flow through

C

d


YFP pull down


## Extended Data Figure 5



## Extended Data Figure 6


b

c



[^0]:    ${ }^{\text {s}}$ These authors contributed equally to this work
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