



## Research Paper

# Upregulated LINE-1 Activity in the Fanconi Anemia Cancer Susceptibility Syndrome Leads to Spontaneous Pro-inflammatory Cytokine Production



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## ARTICLE INFO

## Article history:

Received 8 January 2016

Received in revised form 8 April 2016

Accepted 5 May 2016

Available online 6 May 2016

## Keywords:

Inflammation

DNA damage

SLX4 complex

endogenous retroelement

Interferon

Fanconi Anemia

cGAS-STING

Innate immune sensing

Cytoplasmic DNA

## ABSTRACT

Fanconi Anemia (FA) is a genetic disorder characterized by elevated cancer susceptibility and pro-inflammatory cytokine production. Using SLX4<sup>FANCP</sup> deficiency as a working model, we questioned the trigger for chronic inflammation in FA. We found that absence of SLX4 caused cytoplasmic DNA accumulation, including sequences deriving from active Long Interspersed Element-1 (LINE-1), triggering the cGAS-STING pathway to elicit interferon (IFN) expression. In agreement, absence of SLX4 leads to upregulated LINE-1 retrotransposition. Importantly, similar results were obtained with the FANCD2 upstream activator of SLX4. Furthermore, treatment of FA cells with the Tenofovir reverse transcriptase inhibitor (RTi), that prevents endogenous retrotransposition, decreased both accumulation of cytoplasmic DNA and pro-inflammatory signaling. Collectively, our data suggest a contribution of endogenous RT activities to the generation of immunogenic cytoplasmic nucleic acids responsible for inflammation in FA. The additional observation that RTi decreased pro-inflammatory cytokine production induced by DNA replication stress-inducing drugs further demonstrates the contribution of endogenous RTs to sustaining chronic inflammation. Altogether, our data open perspectives in the prevention of adverse effects of chronic inflammation in tumorigenesis.

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## 1. Introduction

Fanconi Anemia (FA) is a rare autosomal recessive genetic disorder caused by bi-allelic mutations in any of the 17 identified FA complementation group genes that compose the FA DNA repair pathway (Wang and Smogorzewska, 2015). FA is characterized by elevated pro-inflammatory cytokine levels, heightened cancer susceptibility, severe developmental defects and progressive bone marrow failure (Bogliolo and Surralles, 2015). Recent work has shown that the SLX4<sup>FANCP</sup> effector of the FA DNA repair pathway, is directly involved in regulating pathogen-induced cytokine production (Laguette et al., 2014). Indeed, SLX4 assembles several proteins involved in DNA metabolism, into a complex (SLX4com) involved in the repair of double strand breaks (Matos and West, 2014), but also likely acts on incoming pathogen-associated nucleic acids to favor their clearance (Laguette et al., 2014) preventing innate immune sensing. Furthermore, spontaneous production of pro-inflammatory cytokines by FA cells (Du et al., 2014; Dufour et al., 2003; Laguette et al., 2014) also raises the hypothesis

that this DNA repair pathway may be required for the processing of immunogenic endogenous nucleic acids.

Accordingly, besides their role in DNA repair, a direct role of DNA damage response (DDR) proteins in the control of spontaneous and pathogen-induced pro-inflammatory cytokine production has recently emerged (Bregnard et al., 2014; Hartlova et al., 2015). It has been shown that DDR proteins can either detect non-canonical endogenous and pathogen-derived nucleic acids to trigger the production of pro-inflammatory cytokines, including antiviral interferons (IFN) (Ferguson et al., 2012; Kondo et al., 2013; Zhang et al., 2011), or promote their degradation and prevent recognition by the innate immune system (Laguette et al., 2014; Stetson et al., 2008; Yan et al., 2010). In agreement, persistent genomic lesions have been linked to elevated pro-inflammatory cytokine production (Bald et al., 2014; Brzostek-Racine et al., 2011).

Recent reports also show that cancer cells present with cytoplasmic nucleic acids that trigger the production of type I IFN (Hartlova et al., 2015; Koo et al., 2015; Shen et al., 2015). Recognition of these nucleic acids relies on pattern recognition receptors that converge on adaptor proteins to ensure activation of transcription factors such as NF-κB and interferon regulatory factor (IRF) 3 and IRF7 to produce IFN and additional pro-inflammatory cytokines (Paludan, 2015). Detection of cytoplasmic DNA leads to the activation of the adaptor protein stimulator of

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interferon genes (STING) upon activation of sensor proteins like Cyclic GMP-AMP synthase (cGAS) or Gamma-interferon-inducible protein ifi-16 (IFI16) (Ishikawa and Barber, 2008; Ishikawa et al., 2009), while extracellular nucleic acids are detected by Toll-Like Receptors (TLRs) that reside on intracellular organelles such as endosomes, leading to the activation of myeloid differentiation primary response gene 88 (MYD88) or TIR-domain-containing adaptor-inducing interferon- $\beta$  (TRIF) (Rakoff-Nahoum and Medzhitov, 2009). While the origin of endogenous immunogenic nucleic acids is still debated, there are indications that they are generated in the nucleus (Hartlova et al., 2015; Yang et al., 2007) and are, at least in part, comprised of DNA repair by-products (Rigby et al., 2014; Shen et al., 2015). Interestingly, absence of DDR proteins can lead to upregulated retrotransposition (Stetson et al., 2008), which constitutes a potential source of immunogenic endogenous nucleic acids. Indeed, a subfamily of Long INterspersed Element-1 (LINE-1), retains the ability to retrotranspose autonomously and re-insert at distant genomic locations (Babushok and Kazazian, 2007; Kazazian et al., 1988). In addition, de-repression of LINE-1 elements has been documented in a range of inflammatory and autoimmune diseases (Volkman and Stetson, 2014) associated with mutations in genes involved in the metabolism of nucleic acids, including genomic lesions and incoming pathogen-derived nucleic acids (Gasior et al., 2008; Stetson et al., 2008). However, how upregulated LINE-1 activity may lead to induction and persistence of pro-inflammatory signals remains to be investigated.

Here, we wished to identify the trigger for spontaneous pro-inflammatory cytokine production in FA. We used SLX4 deficiency as a working model, because we previously identified a role for SLX4-associated nuclease activities in repressing HIV-mediated pro-inflammatory signaling (Laguette et al., 2014). We reveal that SLX4-deficient cells present cytoplasmic DNA that is recognized by the cGAS-STING pathway to activate IFN signaling. We further establish that these cytoplasmic nucleic acids are, at least in part, generated by upregulated LINE-1 activity. Accordingly, treatment with Tenofovir, a nucleoside analogue reverse-transcriptase (RT) inhibitor that can prevent endogenous RT activities, leads to decreased cytoplasmic LINE-1-derived nucleic acids and pro-inflammatory cytokine levels. Finally, we also show that treatment with Tenofovir prevents chemotherapy-induced inflammation. Altogether, by establishing a link between upregulated endogenous RT activities and spontaneous pro-inflammatory cytokine production in SLX4 deficiency, our findings open perspectives in preventing pervasive pro-inflammatory signaling in the FA cancer susceptibility syndrome and chemotherapy.

## 2. Materials and Methods

For detailed experimental procedures see Supplementary material.

### 2.1. Cell Lines and Plasmids

RA3331- and PD20-derived cell lines were kind gifts from Dr. Smogorzewska and Dr. Rosselli respectively. The A549 IFN-responsive cell line (Chen et al., 2010) was obtained from Dr. Goodbourn.

pCEP-UB\_LRE3\_GFPi (LINE-1 GFP), pAD2TE1 (LINE-1 NEO RT+) and pAD135 (LINE-1 NEO RT-) were gifts from Dr. Gilbert (Doucet et al., 2010; Wei et al., 2001). pYX017 (LINE-1 LUC RT+) and pYX015 (LINE-1 LUC ORF1mut) were kindly provided by Dr. A. n (Xie et al., 2011).

### 2.2. Cytoplasmic and Nuclear DNA Extraction and Analysis

All steps were performed at 4 °C except otherwise mentioned. Cytoplasmic and nuclear fractions were extracted from equal amounts of cells. Cells were lysed in low salt lysis buffer (100 mM NaCl; 10% glycerol; 0.1% Triton; 20 mM Tris [pH 7.5]; 2 mM MgCl<sub>2</sub>; 0.5 mM EDTA; 0.2 mM PMSF; 10 mM  $\beta$ -mercaptoethanol) for 20 min on wheel,

followed by 10 min centrifugation at 4000 rpm. The supernatant, corresponding to the cytoplasmic fraction, was collected while the remaining pellet was further extracted with high salt lysis buffer (340 mM NaCl; 10% glycerol; 0.1% Triton; 20 mM Tris [pH 7.5]; 2 mM MgCl<sub>2</sub>; 0.5 mM EDTA; 0.2 mM PMSF; 10 mM  $\beta$ -mercaptoethanol) for 30 min on wheel, followed by 30 min centrifugation at 13,200 rpm. The supernatant, corresponding to the soluble nuclear fraction, was collected. Cytoplasmic and nuclear fractions were assessed by WB and further treated or not with RNase cocktail (RNase A 5 U/ml, RNase T1 200 U/ml; Ambion) for 30 min at 37 °C and Proteinase K (200  $\mu$ g/ml; Ambion) for 2 h at 50 °C prior to DNA extraction using Phenol/Chloroform/Isoamyl pH 8 (12/12/1).

For qPCR analysis using LINE-1-specific primers, results were normalized on Cytochrome B levels and Genomic DNA contamination. Active LINE-1 sequences were identified using primers described by Boissinot et al. (2000). To sequence cytosolic DNA from RA3331 cells, PCR products were cloned into the TOPO Blunt Vector (ThermoFisher Scientific). Analysis of sequences was performed using a consensus LINE-1 sequence (m80343 clone; NCBI).

For radiolabeling experiments, the DNA was dephosphorylated using rSAP (NEB) prior to labeling with  $\gamma$ -<sup>32</sup>P ATP for 30 min at 37 °C using the T4 Polynucleotide kinase (NEB). Subsequent S1 Nuclease, dsDNase and RNaseH (ThermoFisher Scientific) treatments were performed following the manufacturer's protocol. Unbound radiolabeled nucleotides were removed using Illustra Microspin G-50 Columns (GE Healthcare) prior to resolution on 5% acrylamide, 0.5% Tris Borate Ethylamide gel and autoradiography.

Southern Blot analysis using a LINE-1-specific probe was performed following resolution on 1.3% agarose gels. The probe was generated using the pYX017 plasmid and labeled using the Prime-a-Gene Labeling System (Promega) according to the manufacturer's protocol.

### 2.3. RNA Extraction and RT-qPCR

RNA was extracted using Trizol (Invitrogen) and treated with TURBO DNase (Ambion) according to the manufacturers' protocol. Reverse transcription (SuperSriptIII reverse transcriptase; Invitrogen) and qPCR (QIAGEN) using specific primers were performed using standard protocols.

### 2.4. Immunoprecipitation

293T cells were transfected with indicated plasmids using the calcium phosphate method. Forty-eight hours post-transfection, cells were lysed in 10 cell pellet volumes of buffer (20 mM Tris-HCl [pH 7.5], 0.5 mM EDTA, 150 mM NaCl, 10 mM KCl, 0.5% Triton, 1.5 mM MgCl<sub>2</sub>, 10% glycerol, 10 mM; 0.2 mM PMSF; 10 mM  $\beta$ -mercaptoethanol) 30 min at 4 °C on wheel, followed by 30 min of centrifugation at 13,200 rpm. Whole-cell extracts were incubated with anti-FLAG beads (Sigma-Aldrich) and immunoprecipitates eluted using an excess of FLAG peptide followed by WB or DNA content analysis. For DNA-IP, DNA from input and eluates were extracted using Phenol/Chloroform/Isoamyl pH 8 (12/12/1). qPCR analysis was performed using specific primers and data normalized for input DNA. Disruption of protein/nucleic acid interactions was achieved by preparing whole cell extracts in the presence of 0.1 mg/ml Ethidium Bromide (Sigma-Aldrich) and 0.1 U/ $\mu$ l DNaseI (Sigma-Aldrich).

### 2.5. Retrotransposition Assay

After siRNA transfection or SLX4 overexpression, cells were transfected with LINE-1 reporter plasmids using the FuGene-6 transfection reagent (Promega). In cases where neomycin reporter plasmids were used, cells were grown in the presence of G418 (600  $\mu$ g/ml) for 18 days prior to fixation with 3.2% paraformaldehyde and crystal violet staining. For dual luciferase retrotransposition assays, luminescence

was measured 6 days post-transfection using the Dual-Luciferase Reporter Assay System (Promega), following the manufacturer's instructions.

## 2.6. Analysis of Interferon Secretion

RA3331<sup>SLX4</sup> cells were grown for 48 h in the presence of conditioned medium from RA3331 and RA3331<sup>SLX4</sup>, pre-incubated or not with IFN $\alpha$ , IFN $\beta$  (pbl assay science) and IFN $\gamma$  neutralizing antibody (Abcam) for 2 h. A549 cells were treated with conditioned medium from RA3331, RA3331<sup>SLX4</sup> or RA3331<sup>SLX4 $\Delta$ SAP</sup> for 24 h. IFN response was measured by RT-qPCR.

## 2.7. Immunofluorescence

Cells were seeded on fibronectin-coated coverslips (Sigma-Aldrich). Fixation was performed in 80% methanol followed by 1 h blocking in PBS, 0.1% Tween, 1% BSA. ssDNA was visualized using anti-ssDNA (Abcam) at 1:50 dilution in PBS, 0.1% Tween, 1% BSA followed by incubation with anti-mouse Alexa 488-conjugated secondary antibody (Life Technologies). When indicated, S1 Nuclease digestion was performed before ssDNA staining. Nuclei were stained with DAPI and images were collected on Leica DM6000 or Zeiss Aptom microscope.

## 3. Results

### 3.1. Accumulation of Cytoplasmic ssDNA Leads to Interferon Production in SLX4 Deficiency

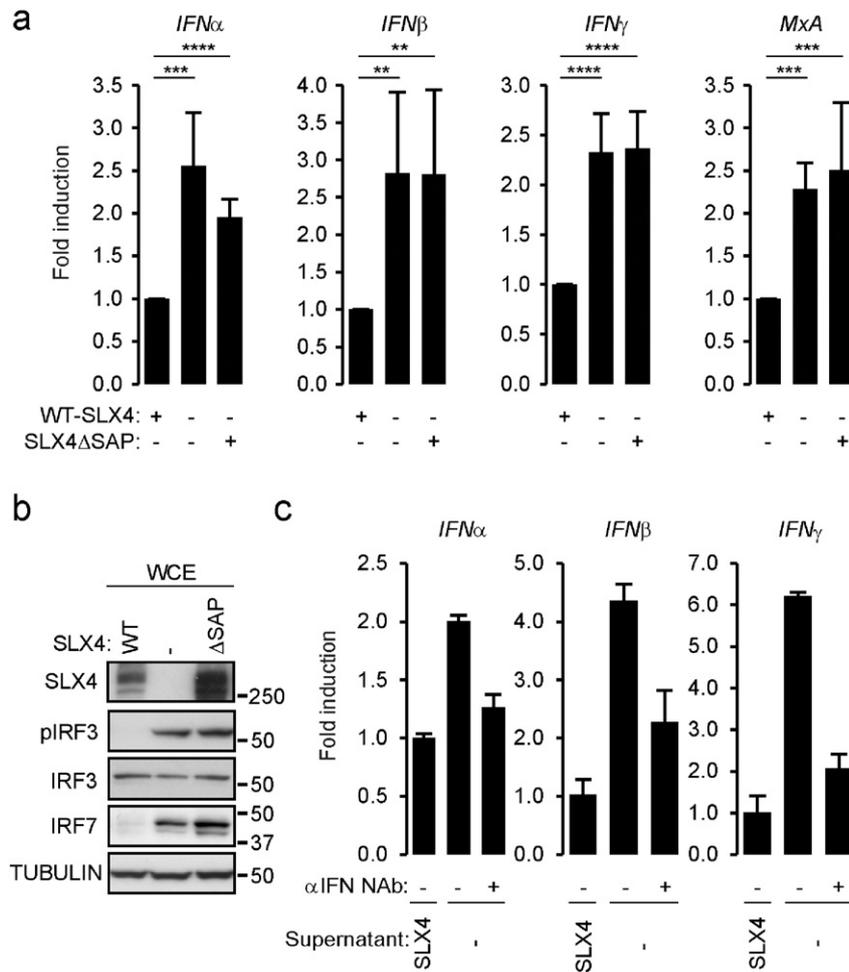
We previously reported (Laguet et al., 2014) that absence of SLX4 and of MUS81 both lead to upregulated type I IFN (IFN $\alpha$  and IFN $\beta$ ) and IFN stimulated gene (MxA) mRNA expression. Here, we wished to determine whether the control of spontaneous pro-inflammatory cytokine production by SLX4 and MUS81 results from independent activities or a coordinated activity within the SLX4com. To this aim, we used the previously described cell lines derived from SLX4-deficient FA patient cells, complemented with either an empty vector (RA3331), a WT-SLX4 allele (RA3331<sup>SLX4</sup>), or a SLX4 allele harboring a deletion of the SAP domain (SLX4 $\Delta$ SAP) responsible for interaction with MUS81-EME1 (RA3331<sup>SLX4 $\Delta$ SAP</sup>) (Kim et al., 2013). This SLX4 construct is impaired in its ability to interact with MUS81-EME1 but can still bind other SLX4 partners, including ERCC4 (Fig. S1A and (Kim et al., 2013)). Accordingly, RA3331<sup>SLX4 $\Delta$ SAP</sup> cells present elevated sensitivity to drugs that cause replication fork collapse, but not to DNA crosslinking agents such as Mitomycin C (MMC) (Kim et al., 2013). Measuring type I, type II IFN and MxA mRNA levels by RT-qPCR showed spontaneous production in RA3331 cells that was prevented by expression of SLX4, but not of SLX4 $\Delta$ SAP (Fig. 1A). Additional Interferon Stimulated Genes (ISGs) were also found to be upregulated in RA3331, and RA3331<sup>SLX4 $\Delta$ SAP</sup> (Fig. S1B). Of note, WT SLX4 expression was more efficient at decreasing the expression of some tested ISGs, indicating a potential contribution of additional SLX4 domains in the control of pro-inflammatory signals. Consistently, analysis of protein levels of IRF7, IRF3 and of the phosphorylated active form of IRF3 (pIRF3) by Western Blot (WB) reveals an accumulation of IRF7 and pIRF3 in RA3331 and RA3331<sup>SLX4 $\Delta$ SAP</sup> as compared to RA3331<sup>SLX4</sup> (Fig. 1B). To investigate whether upregulation of IFN mRNA expression results in *bona fide* secretion of cytokines, experiments were performed where RA3331<sup>SLX4</sup> cells treated with conditioned media from RA3331 displayed increased type I and type II IFN mRNA levels as compared to cells treated with media collected from RA3331<sup>SLX4</sup> (Fig. 1C). In addition, pre-incubating conditioned medium from RA3331 with human neutralizing antibodies against IFN $\alpha$ , IFN $\beta$  and IFN $\gamma$  prevented the measured increase of IFN mRNA (Fig. 1C). This is further confirmed by cultivating the A549 IFN-responsive cell line in the presence of conditioned medium collected from RA3331, RA3331<sup>SLX4</sup>, and RA3331<sup>SLX4 $\Delta$ SAP</sup> showing that

conditioned media from RA3331 and RA3331<sup>SLX4 $\Delta$ SAP</sup> but not RA3331<sup>SLX4</sup> induced upregulation of MxA mRNA levels (Fig. S1C). This, together with previous observation that MUS81 depletion leads to upregulation of pro-inflammatory cytokines (Laguet et al., 2014), strongly suggests that recruitment of MUS81-EME1 to SLX4 is required for SLX4com-mediated repression of pro-inflammatory pathways.

Because the SLX4com processes HIV-derived nucleic acids (Laguet et al., 2014), preventing its innate immune sensing, and absence of SLX4 leads to activation of pattern recognition pathways (Fig. S1B), we hypothesized that SLX4-associated nuclease activities may be involved in processing endogenous nucleic acids to avoid spontaneous pro-inflammatory cytokine production. Consequently, absence of SLX4 should lead to accumulation of cytoplasmic immunogenic nucleic acids. To test this hypothesis, we performed immunofluorescence staining of RA3331, RA3331<sup>SLX4</sup> and RA3331<sup>SLX4 $\Delta$ SAP</sup> cells using a ssDNA-specific antibody (Fig. 2A–B). Enrichment in cytoplasmic staining was observed in RA3331 and RA3331<sup>SLX4 $\Delta$ SAP</sup> cells but not in RA3331<sup>SLX4</sup> cells. Additionally, absence of MUS81 in mouse embryonic fibroblasts (MEF<sup>MUS81 $^{-/-}$</sup> ) also led to accumulation of cytosolic ssDNA as compared to WT MEFs (Fig. S2A). This shows that lack of SLX4 results in accumulation of cytosolic ssDNA and highlights the requirement of MUS81 binding to SLX4 for cytosolic DNA clearance. Of note, treatment with the S1 Nuclease that preferentially digests ssDNA, caused a net decrease of the observed cytoplasmic staining (Fig. S2B).

Since it has been previously reported that the cytoplasm of cancer cells contains a mix of ssDNA, dsDNA and DNA:RNA hybrids (Koo et al., 2015; Shen et al., 2015), we questioned the nature of the nucleic acids present in the cytoplasm of SLX4-deficient cells. To this aim,  $\gamma$ P32-labeling of cytoplasmic DNA from RA3331, RA3331<sup>SLX4</sup> and RA3331<sup>SLX4 $\Delta$ SAP</sup> cells was performed (Fig. 2C, left panel). Cytoplasmic and nuclear extracts were analyzed by WB to check for nuclear contamination of cytoplasmic fraction (Fig. S2C). Autoradiography revealed increased presence of nucleic acids in the cytoplasmic fraction of RA3331 and RA3331<sup>SLX4 $\Delta$ SAP</sup> as compared to RA3331<sup>SLX4</sup> (Fig. 2C, right panel). Importantly, treatment of the material obtained from RA3331 and RA3331<sup>SLX4 $\Delta$ SAP</sup> cells with S1 Nuclease leads to reduction of the signal (Fig. 2C and S2D) confirming the presence of ssDNA in the cytoplasm of SLX4-deficient cells. Similarly, treatment with dsDNase or RNaseH, led to decreased levels of cytoplasmic nucleic acid in RA3331 and RA3331<sup>SLX4 $\Delta$ SAP</sup> (Fig. S2E and S2F), indicating that the cytosol of SLX4-deficient cells contains dsDNA, ssDNA and DNA:RNA hybrids and that SLX4-MUS81 interaction is required to prevent their accumulation. Of note, it has been reported that immunogenic nucleic acids present in the cytoplasm of cancer cells are generated in the nucleus (Hartlova et al., 2015; Yang et al., 2007). Interestingly, radiolabeling experiments of DNA extracted from the soluble nuclear fraction of RA3331, RA3331<sup>SLX4</sup> and RA3331<sup>SLX4 $\Delta$ SAP</sup>, followed by digestion with S1 Nuclease (Fig. S2G) shows the presence of ssDNA in the nuclear fraction of RA3331 and RA3331<sup>SLX4 $\Delta$ SAP</sup>, but not RA3331<sup>SLX4</sup>.

To investigate whether the DNA species present in the cytoplasm of RA3331 cells are responsible for IFN production, RA3331 cells were engineered to stably express an inducible short hairpin RNA (shRNA) targeting *STING* (*TMEM173*). To assess the contribution of extracellular nucleic acids to the signaling pathway triggered in the absence of SLX4, we also included shRNAs targeting *MYD88* (Fig. 2D–F). As a control, RA3331 cells expressing an inducible shRNA targeting the *Luciferase* gene were also engineered. Following 6 days of shRNA induction, IRF3 phosphorylation status was assessed in whole cell extracts (WCE) (Fig. 2D), while knockdown efficiency (Fig. 2E), MxA and additional IFN-inducible genes mRNA levels (Fig. 2F) were measured by RT-qPCR. Knockdown of *STING* but not *MYD88* led to decreased pIRF3 and IFN signaling (Fig. 2D–F). This indicates that cytoplasmic DNA present in SLX4 deficient cells is sensed through the *STING* pathway to induce IFN production. To investigate whether previously identified nucleic acid sensors of the *STING* pathway are required for pro-inflammatory signaling in SLX4 deficiency, we engineered cells where cGAS or



**Fig. 1.** The SAP MUS81-EME1-interacting domain of SLX4 is required for repressing inflammatory signals. (A) IFN $\alpha$ , IFN $\beta$ , IFN $\gamma$  and MxA mRNA levels were measured by RT-qPCR in RA3331 cells complemented either with WT-SLX4 (RA3331<sup>SLX4</sup>) or SLX4 $\Delta$ SAP (RA3331<sup>SLX4 $\Delta$ SAP</sup>). Graphs present means ( $\pm$ SD) of triplicate measurement of 6 independent experiments expressed as fold change in mRNA expression relative to RA3331<sup>SLX4</sup>. \*\*\*\*:  $p < 0.001$ ; \*\*\*:  $p < 0.005$ ; \*\*:  $p < 0.01$ . (B) WCE from RA3331, RA3331<sup>SLX4</sup> and RA3331<sup>SLX4 $\Delta$ SAP</sup> were analyzed by WB using indicated antibodies. (C) IFN $\alpha$ , IFN $\beta$  and IFN $\gamma$  mRNA levels were measured in RA3331<sup>SLX4</sup> treated with conditioned medium from RA3331<sup>SLX4</sup> or RA3331 pre-incubated or not with neutralizing antibody to human IFN $\alpha$ , IFN $\beta$  and IFN $\gamma$ . Graph represents mean ( $\pm$ SD) mRNA levels of one representative experiment relative to cells treated with conditioned medium from RA3331<sup>SLX4</sup>. See also Fig. S1.

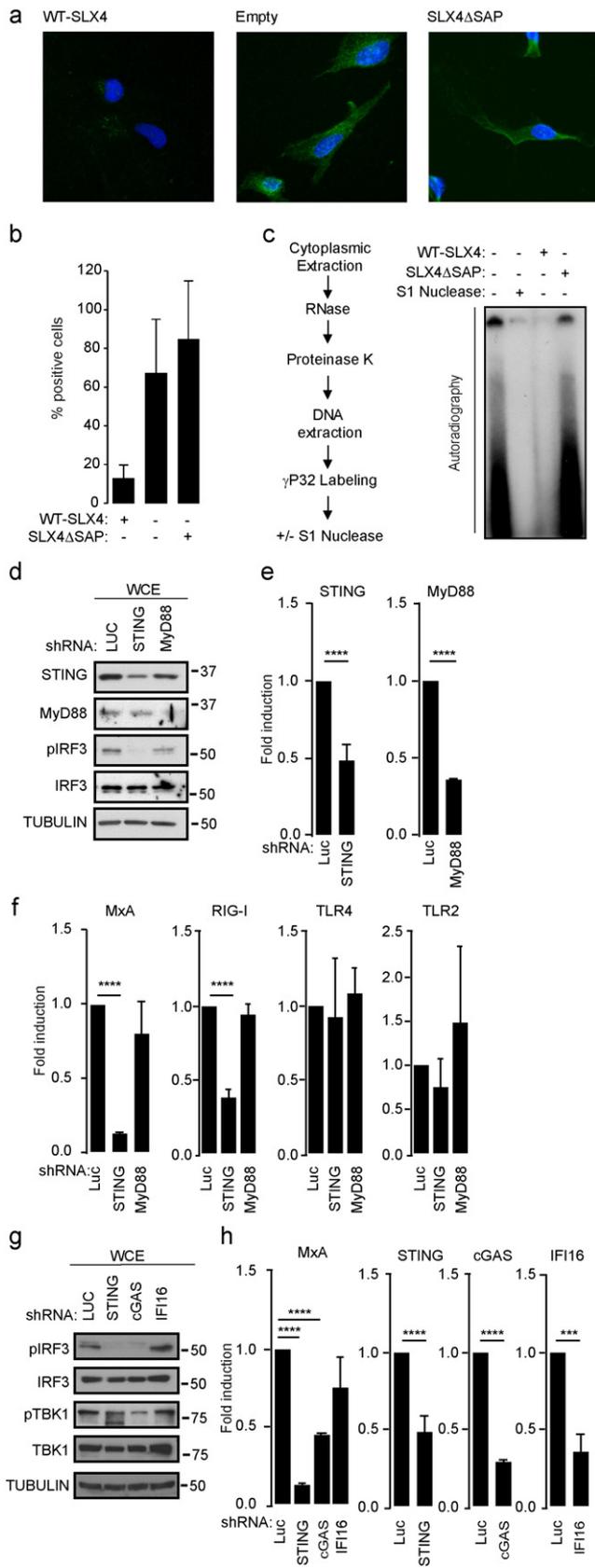
IFI16 (Dempsey and Bowie, 2015) silencing is inducible and measured the levels of pIRF3 and phosphorylated active TBK1 kinase (pTBK1), known to be recruited by STING, by WB and of MxA by RT-qPCR (Fig. 2G–H). Knockdown efficiency was assessed by RT-qPCR (Fig. 2H). Silencing cGAS but not IFI16 led to decreased pro-inflammatory signaling (Fig. 2G) and MxA induction (2H), highlighting a role for the cGAS-STING pathway in detecting cytosolic nucleic acids in SLX4-deficient cells. However, because of poor TRIF knockdown (not shown), we cannot exclude a contribution of endosomal nucleic acids in the activation of the inflammatory signal observed in SLX4-deficient cells.

### 3.2. The SLX4 Complex Interacts With the LINE-1 Retrotransposition Complex

We have shown that absence of SLX4 leads to HIV-derived DNA accumulation (Laguetta et al., 2014), suggesting that SLX4-associated endonucleases are involved in processing the latter. This is reminiscent of what has been reported for the TREX1 exonuclease (Yan et al., 2010). Because TREX1 inhibits LINE-1 retrotransposition and cell-intrinsic initiation of autoimmunity (Stetson et al., 2008), we hypothesized that absence of SLX4 may similarly lead to LINE-1 deregulation, which could account for subsequent cytoplasmic DNA accumulation. To test this hypothesis, DNA was extracted from the cytoplasmic fraction of RA3331 and RA3331<sup>SLX4</sup> (Fig. S3A). LINE-1 DNA was either quantified by qPCR

(Fig. 3A) or visualized by Southern Blot (Fig. 3B). We thereby observed an increase of LINE-1 DNA in the cytoplasmic fraction of RA3331 as compared to RA3331<sup>SLX4</sup> (Fig. 3A–B). Interestingly, this observation is concomitant to an increase of LINE-1 mRNA as measured by RT-qPCR (Fig. S3B), suggesting that this DNA accumulation is linked to increased LINE-1 activity. Indeed, qPCR performed using previously described primers (Boissinot et al., 2000) that allow the amplification of active LINE-1 (Fig. 3C) and sequencing of obtained PCR products (Fig. S3C) confirmed the presence of DNA originating from the Ta-1 subfamily of active LINE-1 elements.

Because SLX4 regulates LINE-1-derived cytoplasmic DNA levels, we next asked whether the SLX4com interacts with the LINE-1 retrotransposition complex and whether this interaction is DNA-dependent. LINE-1 expresses a 6 kb bicistronic mRNA encoding two proteins that are essential for retrotransposition: the ORF1p RNA binding protein and the ORF2p protein that possess endonuclease and RT activities. During LINE-1 retrotransposition, ORF1p and ORF2p bind to LINE-1 RNA, forming a ribonucleoprotein particle competent for the reverse transcription of LINE-1 RNA into dsDNA that may integrate into genomic DNA. Using a previously described LINE-1 reporter construct (LINE-1-NEO) where the ORF1p protein is C-terminally T7-tagged (Fig. 3D) (Doucet et al., 2010), we assessed the interaction between SLX4com and ORF1p. LINE-1-NEO was co-expressed with N-terminally FLAG- and HA-tagged SLX4 (F/H-SLX4) or SLX4 $\Delta$ SAP (F/H-SLX4 $\Delta$ SAP) in

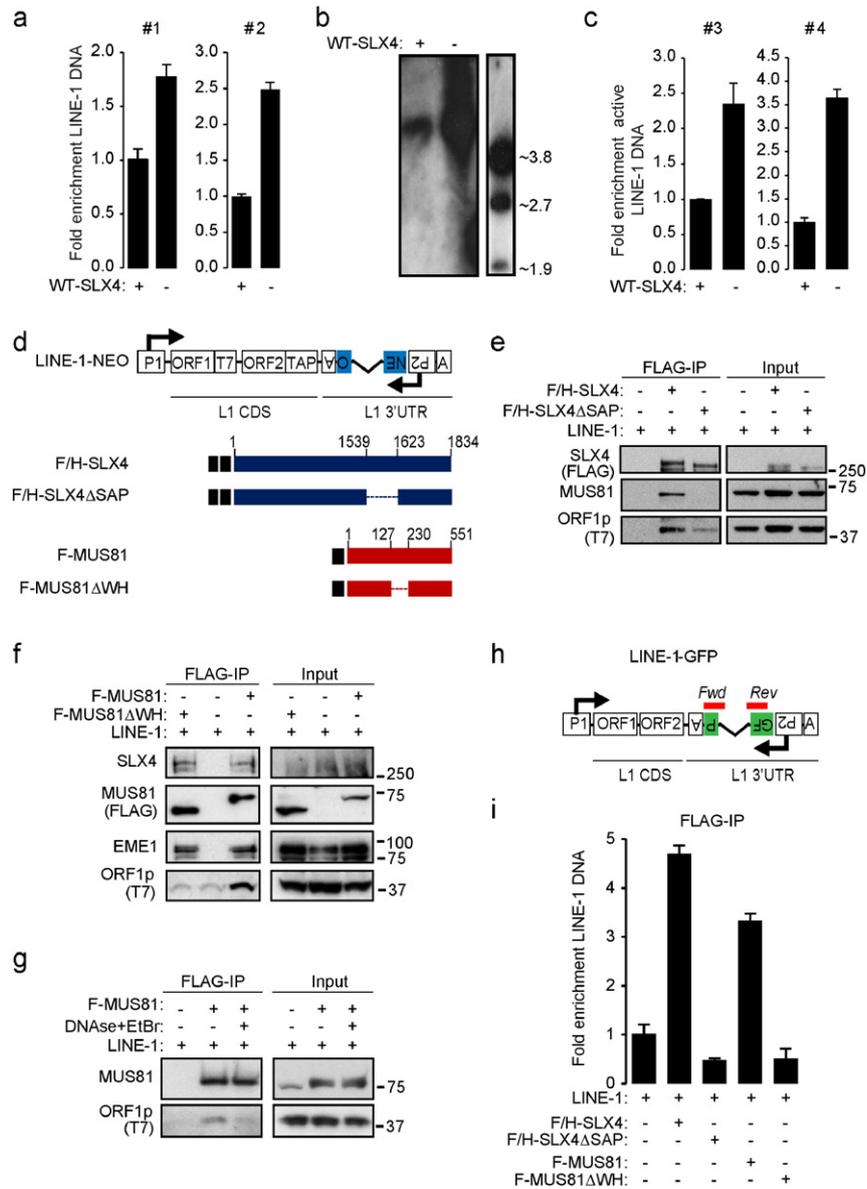


SLX4 $\Delta$ SAP immunoprecipitation. This shows that SLX4 interacts with ORF1p through its SAP domain. To explore a potential role of MUS81-EME1 in this interaction, FLAG-tagged MUS81 (F-MUS81) (Fig. 3F) was co-expressed with the LINE-1-NEO reporter plasmid in 293T cells. WCE were subjected to FLAG-immunoprecipitation and the presence of ORF1p was analyzed by WB. Fig. 3F shows that SLX4, EME1 and ORF1p were recovered in F-MUS81 immunoprecipitation. To evaluate the contribution of MUS81-DNA binding domain for interaction with ORF1p, we used the MUS81 $\Delta$ WH mutant (Fadden et al., 2013) that harbors a deletion of its nucleic acid-binding domain (Fig. 3D). While F-MUS81 $\Delta$ WH and F-MUS81 displayed similar ability to interact with SLX4 and EME1, interaction of F-MUS81 $\Delta$ WH with ORF1p was impaired (Fig. 3F), showing that interaction of SLX4com with LINE-1 depends on MUS81 DNA-binding domain. Interestingly, when F-MUS81 immunoprecipitation was performed in the presence of DNaseI and Ethidium Bromide (EtBr) that disrupt nucleic acid/protein interactions, MUS81 and ORF1p interaction was lost (Fig. 3G). Altogether, these experiments show that SLX4com binding to ORF1p is DNA-dependent and requires MUS81 DNA-binding domain.

We next wished to assess the binding of LINE-1-derived DNA to the SLX4com. To this aim, we used the LINE-1-GFP reporter plasmid (Fig. 3H and (Coufal et al., 2009)). In this construct, a *green fluorescent protein* (*gfp*) gene is inserted in the 3' untranslated region (UTR) of LINE-1 in antisense orientation relative to LINE-1 expression, under the control of an independent promoter. The sequence of the *gfp* is disrupted by an intronic sequence and GFP expression can only be measured when the retroelement has undergone a full cycle of retrotransposition, involving transcription, splicing, reverse transcription and integration. We designed qPCR primers that flank the intronic sequence to discriminate spliced (136 bp) from unspliced LINE-1-derived DNA (Fig. 3H). This construct was co-expressed in 293T cells together with F/H-SLX4, F/H-SLX4 $\Delta$ SAP, F-MUS81 or F-MUS81 $\Delta$ WH prior to whole cell extraction and FLAG-immunoprecipitation coupled to peptide elution. Efficient immunoprecipitation of target proteins was verified by WB (Fig. S3D). Eluted material was subjected to DNA extraction followed by qPCR. We observed that LINE-1 DNA was efficiently co-immunoprecipitated with F/H-SLX4 and F-MUS81, but not with SLX4 $\Delta$ SAP and MUS81 $\Delta$ WH (Fig. 3I). We verified that the amplified LINE-1 fragment was indeed 136 bp and that similar levels of LINE-1 input material was used (Fig. S3E–F). Altogether, our observations strongly suggest that the SLX4com binds to LINE-1 reverse-transcribed DNA via interaction with the MUS81-EME1 endonuclease module.

**Fig. 2.** Cytoplasmic accumulation of ssDNA is responsible for pro-inflammatory signals in SLX4 deficiency. (A) Immunofluorescence analysis was performed on RA3331, RA3331<sup>SLX4</sup> and RA3331<sup>SLX4 $\Delta$ SAP</sup> using ssDNA-specific antibody and DAPI nuclear staining. Images are representative of at least 4 independent experiments. (B) Quantification of staining observed in A. (C) Left panel: experimental scheme. Briefly, cytoplasmic extraction was performed on RA3331, RA3331<sup>SLX4</sup> and RA3331<sup>SLX4 $\Delta$ SAP</sup> followed by RNase cocktail (RNase A and RNase T1) and proteinase K treatments prior to DNA extraction and radiolabeling. DNA extracted from RA3331 cells was subjected or not to a subsequent S1 Nuclease treatment prior to separation on acrylamide gel and autoradiography. Right panel: autoradiography of a representative experiment. (D) WCE from RA3331 expressing *Luciferase*-, *MYD88*-, or *STING*-targeting inducible shRNAs were analyzed by WB using indicated antibodies. (E) Graphs present RT-qPCR quantification of knock-down efficiency, measured using specific primers, as mean ( $\pm$ SD) mRNA expression relative to cells expressing *Luciferase*-targeting shRNA from 3 independent experiments. (F) *MxA*, *RIG-I*, *TLR4* and *TLR2* mRNA levels were measured in cells described in D. Graphs present means ( $\pm$ SD) of 3 independent experiments as fold increase mRNA expression relative to cells expressing a *Luciferase* targeting shRNA. (G) WCE from RA3331 expressing *Luciferase*-, *STING*-, *cGAS*- or *IFI16*-targeting inducible shRNAs were analyzed by WB using indicated antibodies. (H) *MxA*, *STING*-, *cGAS*- and *IFI16* mRNA levels were measured in cells described in G. Graphs present means ( $\pm$ SD) of 3 independent experiments expressed as fold change in mRNA expression relative to cells expressing a *Luciferase* targeting shRNA. \*\*\*\*:  $p < 0.001$ ; \*\*\*:  $p < 0.005$ ; \*\*:  $p < 0.01$ . See also Fig. S2.

293T cells (Fig. 3E). WCE were subjected to FLAG-immunoprecipitation. Input and FLAG-eluted material were analyzed by WB. As shown in Fig. 3E, ORF1p was efficiently recovered in F/H-SLX4, but not in F/H-



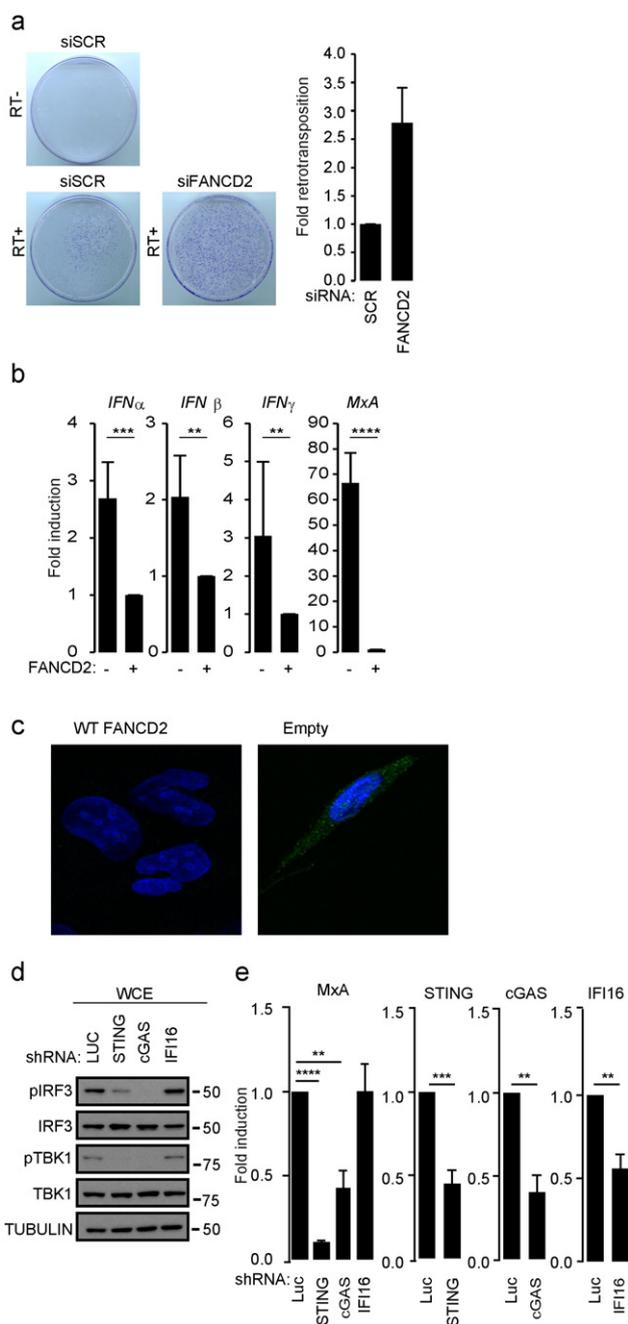
**Fig. 3.** The SLX4com regulates cytoplasmic accumulation of LINE-1 DNA. (A) LINE-1 DNA was measured by qPCR in the cytoplasmic fraction of RA3331 or RA3331<sup>SLX4</sup>. #1: primers targeting the 5' of ORF1. #2: primers targeting the 3' of ORF2. (B) Cytoplasmic DNA extracted from RA3331 or RA3331<sup>SLX4</sup> were analyzed by Southern Blot using LINE-1 specific probe. The right panel shows the molecular weight ladder. (C) Same as in A, except that primers allowing the amplification of active LINE-1 elements were used. #3 and #4: primer pairs targeting the 3' UTR sequence of LINE-1 Ta-1 sub-family. (D) Constructs used in E-G and I. (E) FLAG/HA-WT-SLX4 or FLAG/HA-SLX4ΔSAP were FLAG-immunoprecipitated from 293T cells in the presence of a LINE-1-expressing plasmid. Bound material was peptide-eluted. Input and eluates were analyzed by WB using indicated antibodies. (F) FLAG-WT-MUS81 or FLAG-MUS81ΔWH were FLAG-immunoprecipitated from 293T cells in the presence of a LINE-1-expressing plasmid. Bound material was peptide-eluted. Input and eluates were analyzed as in E. (G) FLAG-WT-MUS81 was purified as in E in the presence or absence of 0.1 mg/ml EtBr and 0.1 U/μl DNaseI. Input and eluates were analyzed as in E. (H) LINE-1 construct used in I. Primers are indicated in red. (I) FLAG/HA-WT-SLX4, FLAG/HA-SLX4ΔSAP, FLAG-WT-MUS81 or FLAG-MUS81ΔWH were purified as in E prior to DNA extraction. Samples were analyzed by qPCR using primers indicated in H. See also Fig. S3.

### 3.3. The SLX4 Complex Negatively Regulates LINE-1 Retrotransposition

We next wished to investigate the consequences of SLX4com binding to LINE-1 retrotransposition complexes. To this aim, the LINE-1-NEO reporter construct (Fig. 3D) was transfected in HeLa cells expressing empty vector, WT-SLX4 or SLX4ΔSAP. LINE-1-NEO is similar to the LINE-1-GFP construct, except that the *gfp* sequence is replaced by a *neo*mycin resistance gene and neomycin resistance arises when the retroelement has undergone a full retrotransposition cycle. As negative control, a LINE-1 mutant construct unable to perform reverse transcription (RT-) was also included in the assay. Crystal violet staining of neomycin-resistant colonies showed that expression of WT-SLX4, but not of SLX4ΔSAP (Fig. S4A), decreases the number of colonies as compared to cells where empty vector was expressed (Fig. 4A). This shows that SLX4

is a negative regulator of LINE-1 retrotransposition, a function that requires its SAP domain. We next performed similar experiments except that we silenced SLX4 or MUS81 using small interfering RNA (siRNA) prior to transfection of the LINE-1-NEO reporter construct (Fig. 4B and S4B-C). We observed that absence of SLX4 or MUS81 both lead to increased LINE-1 retrotransposition (Fig. 4B). Finally, to confirm these observations, we performed retrotransposition assays in RA3331 cell lines. We used a previously described highly sensitive retrotransposition dual Luciferase reporter system (Xie et al., 2011) where a *Firefly luciferase* (*Fluc*) gene, disrupted by an intronic sequence, is inserted in the 3' untranslated region (UTR) of LINE-1 in antisense orientation relative to LINE-1 expression, under the control of an independent promoter and a *Renilla luciferase* (*RLuc*) cassette is inserted in the same backbone to allow for normalization (Fig. 4C). As negative control, a mutant





**Fig. 5.** Absence of FANCD2 leads to upregulated STING-dependent IFN production. (A) Retrotransposition was measured and results presented as in Fig. 4A except that cells were treated with scrambled siRNA or siRNA targeting FANCD2. (B) IFN $\alpha$ , IFN $\beta$ , IFN $\gamma$  and MxA mRNA levels were measured in FANCD2 deficient cells and on their WT-FANCD2 complemented counterparts. Graphs present means ( $\pm$ SD) from triplicate measurement of 6 independent experiments expressed as fold mRNA expression relative to FANCD2 proficient cells. (C) Immunofluorescence was performed on FANCD2 deficient or proficient cells as in Fig. 2A. (D) WCE from PD20 expressing *Luciferase*, *STING*, *cGAS* or *IFI16*-targeting inducible shRNAs were analyzed by WB using indicated antibodies. (E) MxA, STING, cGAS and IFI16 mRNA levels were measured in cells described in D. Graphs present means ( $\pm$ SD) of 4 independent experiments expressed as fold change in mRNA expression relative to cells expressing *Luciferase* targeting shRNA. \*\*\*\*:  $p < 0.001$ ; \*\*\*:  $p < 0.005$ ; \*\*:  $p < 0.01$ . See also Fig. S5.

phosphorylation (Fig. 6F and S6B–C). We also verified that TenoF alone did not significantly affect pCHK1 and SLX4 expression levels (Fig. S6D–E). This implies that TenoF inhibits activation of pro-inflammatory pathways triggered by tested DNA damage inducing drugs, independently from their ability to cause DNA lesions. Of note, we also observed that TenoF treatment weakly affected DNA crosslink- and cytokine-

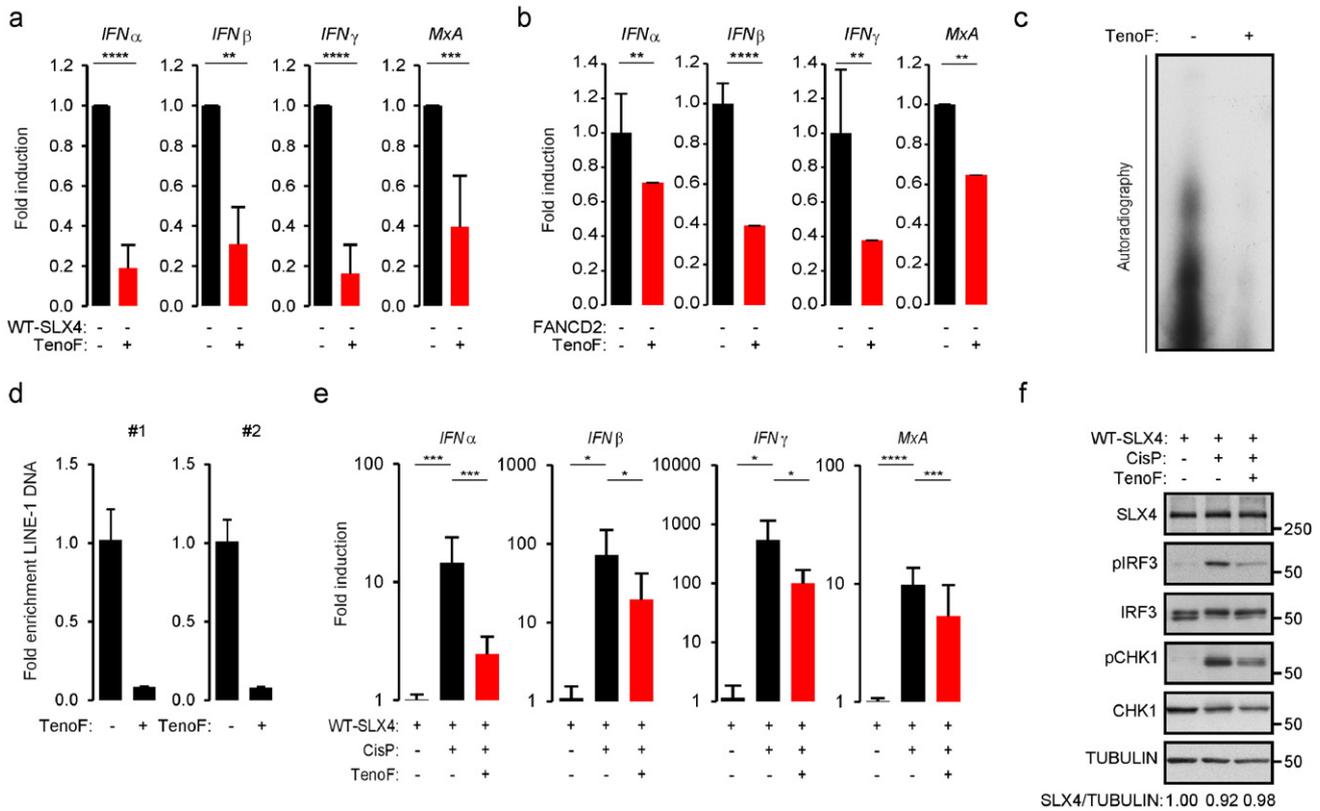
associated cell death of RA3331 cells (Fig. S6F–G). Thus, altogether, our observations strongly suggest that DNA damaging agents cause activation of pro-inflammatory pathways through endogenous reverse transcriptase activity including that of LINE-1.

#### 4. Discussion

Chronic inflammation that fosters a favorable environment for tumor initiation can be generated by both cell-extrinsic and cell-intrinsic factors. The latter include inability to repair genomic lesions (Rodier et al., 2009; Zheng et al., 2007). Indeed, recent work has provided evidence for the implication of DDR pathways in the regulation of spontaneous and pathogen-induced innate immune responses (Bregnard et al., 2014). However, there is as of today poor understanding of the underlying molecular mechanisms. Here we used FA as a model cancer susceptibility syndrome to investigate the origins of chronic inflammation. We show that elevated pro-inflammatory cytokine production in the absence of a functional FA DNA repair pathway results, at least in part, from upregulated LINE-1 activity. In this model, we observed that absence of SLX4 leads to upregulated LINE-1 retrotransposition, accumulation of immunogenic cytoplasmic DNA comprising active LINE-1-derived sequences and concomitant up-regulated pro-inflammatory cytokine production. This is further supported by the witnessed physical interaction between the SLX4com and the LINE-1 retrotransposition complex and decreased pro-inflammatory cytokine production upon RT inhibitor treatment. In addition, in SLX4-deficient cells, LINE-1 transcription and retrotransposition are increased and the nucleoplasm contains immunogenic nucleic acids that may subsequently be exported to the cytoplasm and recognized by the cGAS-STING pathway to induce pro-inflammatory cytokine production (Fig. 7). Requirement of cGAS for activation of pro-inflammatory pathways in these cells suggests that dsDNA species are involved in the signaling cascade. However, since we also observed the accumulation of ssDNA and DNA:RNA hybrids, one can speculate that such nucleic acid species may either be recognized by the same pathway, or by additional yet to be identified pathways. Moreover, additional transposable elements detected by the cGAS-STING pathway may induce IFN production in SLX4 deficiency (Zeng et al., 2014). Interestingly, it has also been reported that tumor-derived DNA can activate the STING pathway in innate immune cells within the tumor microenvironment (Woo et al., 2014) and that a STING-dependent mechanism is involved in the induction of an adaptive immune response following radiation (Deng et al., 2014). It would thus be interesting to investigate the influence of SLX4-deficient cells-derived DNA on adaptive immune responses.

FA presents with progressive bone marrow failure, increased cancer susceptibility and elevated pro-inflammatory cytokine production. FA-associated bone marrow failure has been shown to, at least in part, result from exhaustion of chronic cytokine-stimulation of bone marrow-resident stem cells (Garaycochea and Patel, 2014). While deregulated cytokine production contributes to these bone marrow-related hematological dysfunctions, it is possible that persistent secretion of IFN can also contribute to the advent of malignancies in FA. Here we show that SLX4 and its upstream activator FANCD2 directly repress LINE-1 retrotransposition and prevent accumulation of cytoplasmic nucleic acids. This suggests that the FA DNA repair pathway as a whole may be involved in regulating LINE-1 activity and subsequent pro-inflammatory cytokine production. This further implies that upregulated LINE-1 retrotransposition occurs in FA patients and that LINE-1 activity may play an important role in the advent of clinical manifestations of FA. This is particularly important because of the pivotal role of FANCD2 in the activation of the FA DNA repair pathway.

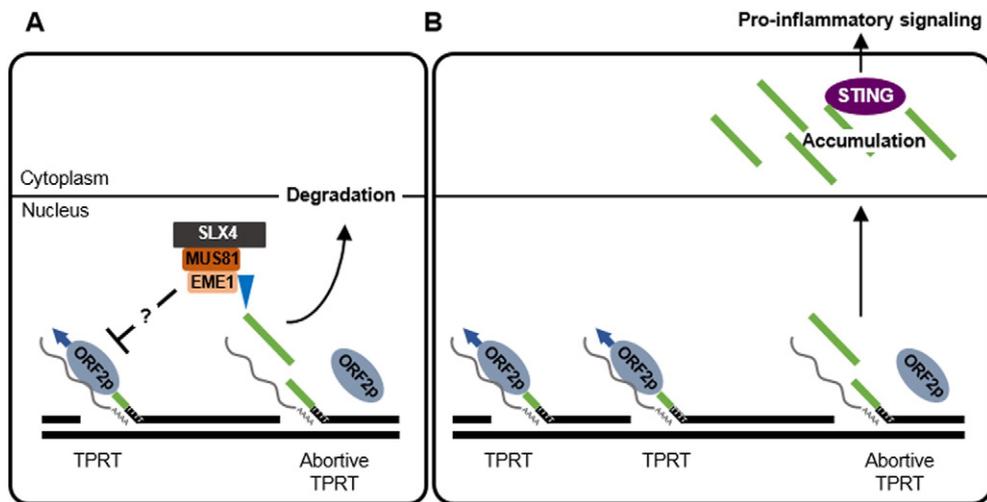
We also show that recruitment of MUS81-EME1 is required for SLX4com-mediated control of pro-inflammatory signaling. This suggests that, similar to what was previously reported for HIV biology (Laguette et al., 2014), the SLX4com likely represses LINE-1 retrotransposition through the endonucleolytic action of MUS81-



**Fig. 6.** Upregulated retrotransposition leads to pro-inflammatory signaling. (A) IFN $\alpha$ , IFN $\beta$ , IFN $\gamma$  and MxA mRNA levels were measured in RA3331 cells following 48 h treatment with 125  $\mu$ M TenoF. Graphs present means ( $\pm$ SD) from triplicate measurement of 6 independent experiments expressed as fold change in mRNA expression relative to RA3331. \*\*\*\*:  $p < 0.001$ ; \*\*\*:  $p < 0.005$ ; \*\*:  $p < 0.01$ . (B) IFN $\alpha$ , IFN $\beta$ , IFN $\gamma$  and MxA mRNA levels were measured in FANCD2-deficient cells following 48 h treatment with 5  $\mu$ M TenoF. Graphs present means ( $\pm$ SD) from triplicate measurement of 6 independent experiments expressed as fold change in mRNA expression relative to untreated cells. \*\*\*\*:  $p < 0.001$ ; \*\*\*:  $p < 0.005$ ; \*\*:  $p < 0.01$ . (C) Cytoplasmic DNA was prepared from RA3331 cells following 8 h treatment with 125  $\mu$ M TenoF, radiolabeled and analyzed by autoradiography. (D) DNA was prepared as described in C and analyzed by qPCR using primers described in Fig. 3A. Graph presents data from a representative experiment. (E) IFN $\alpha$ , IFN $\beta$ , IFN $\gamma$  and MxA mRNA levels were measured in RA3331<sup>SLX4</sup> treated or not with 125  $\mu$ M TenoF 24 h prior to treatment with 10  $\mu$ M Cisplatin. Graphs present means ( $\pm$ SD) from 3 independent experiments as fold change in mRNA expression, relative to untreated cells. \*\*\*\*:  $p < 0.001$ ; \*\*\*:  $p < 0.005$ ; \*\*:  $p < 0.01$ ; \*:  $p < 0.05$ . (F) WCE from cells treated as in E were analyzed by WB. See also Fig. S6.

EME1. Importantly, for the latter to acquire full endonuclease activity within the SLX4com, PLK1-dependent EME1 hyperphosphorylation is required, which occurs around the G2/M boundary. It would thus be interesting to investigate whether the control of endogenous retroelements mobility and the ability to repress pro-inflammatory

cytokine production depend on the cell cycle status. Additional post-translational modifications, such as SUMOylation and PARYlation, were recently shown to be required for SLX4 activity by ensuring its localization to sites of DNA damage (Gonzalez-Prieto et al., 2015; Guervilly et al., 2015; Ouyang et al., 2015). Requirement for such post-



**Fig. 7.** Inhibition of LINE-1 retrotransposition by the SLX4com prevents pro-inflammatory signaling. (A) The SLX4com inhibits LINE-1 retrotransposition by preventing accumulation of reverse transcribed LINE-1 DNA. LINE-1-derived DNA are likely subsequently degraded. (B) In the absence of the SLX4com, by-products of LINE-1 reverse-transcription accumulate in the cytoplasm. These are recognized by the cGAS-STING pathway to activate pro-inflammatory signaling. Grey lines represents: LINE-1 RNA; green lines: LINE-1 reverse-transcribed DNA.

translational modifications in the recognition and processing of potential immunogenic nucleic acids remains to be investigated.

Interestingly, it has been previously reported that the bone marrow of FA patients is compromised because of accumulation of DNA damage in hematopoietic stem cells (Ceccaldi et al., 2012). Our findings imply that this may also be linked to upregulated LINE-1 activity. Indeed, LINE-1 mobility is mostly repressed in cells through 5' UTR methylation (Woodcock et al., 1997; Yu et al., 2001) and stem cells have a hypomethylated genome that allows enhanced LINE-1 activity (Wissing et al., 2012). Thus, absence of the FA pathway may also allow for pro-tumorigenic LINE-1 re-insertion events, especially in the light of the LINE-1 DNA deriving from the most active LINE-1 subfamily accumulating in the cytoplasm of SLX4-deficient cells. This raises the possibility that treatment with RT inhibitors, which inhibit the accumulation of immunogenic nucleic acids responsible for initiating pro-inflammatory cytokines production, may delay or prevent the onset of bone marrow failure in FA. In support, immunotherapy aiming at neutralizing a single pro-inflammatory cytokine (TNF $\alpha$ ) has shown promising positive effects in patients (Mehta et al., 2012; Miehsler et al., 2010). Accordingly, we observed that treatment with TenoF weakly decreased the cell death of SLX4-deficient cells following treatment with TNF $\alpha$  or DNA damaging drugs. Of note, bone marrow failure is a clinical trait shared by additional familial cancer susceptibility syndromes (Parikh and Bessler, 2012) that could also be linked to deregulated endogenous RT.

Finally, the key role played by chronic inflammation in the progression of cancer is highlighted by the fact that anti-inflammatory drugs when administered to patients can reduce the risk of progression to metastasis (Markowitz, 2007). One interesting finding from our work is that treatment with TenoF prevents inflammation caused by DNA-damaging agents such as those used in chemotherapy. This is of particular importance because it has been reported that treatment with chemotherapy may generate a pro-inflammatory environment that would select cells that are resistant to treatment (de Visser and Jonkers, 2009; Koti et al., 2015). Our data confirm the previous observation that certain DNA-damage inducing drugs can cause LINE-1 reactivation (Rudin and Thompson, 2001) and enforce the contribution of endogenous RT activities to inflammation. Furthermore, we observed a weak but reproducible decrease of pCHK1 levels in cells treated with RT inhibitors in the presence of DNA-damaging drugs. This is in agreement with previous reports of LINE-1 activity posing a mutagenic threat to the genome because of their ability to integrate at distant genomic locations. Thus, altogether our in vitro observations suggest that combination of chemotherapy with RT inhibitors could prevent the establishment of an inflammatory tumor microenvironment that would favor chemoresistance and decrease the risk of genomic instability. Thus, our study may open perspectives for the treatment of FA patients but also for reducing adverse effects of chemotherapy.

### Conflicts of Interest

We declare no conflict of interest.

### Author Contributions

All authors performed experiments. CB, JG, MB and NL discussed and interpreted obtained results. CB and JG equally contributed to the design and performing of experiments. CB and JG assembled the figures. MB and NL wrote the manuscript.

### Acknowledgments

We thank Dr. Smogorzewska for the gift of RA3331 cell lines, Dr. Rosselli for PD20 cells, Dr. N. Gilbert for LINE-1 constructs, and Dr. W. An for the LINE-1-LUC construct. We thank Drs. Dufourt, Cristofari and Gilbert for fruitful discussions. We thank members of the Molecular Basis of Cancer-Related Inflammation laboratory for critical reading of

the manuscript. Work in NL laboratory was supported by the ERC (637763), ANRS (109560) and by "la Fondation ARC pour la Recherche sur le Cancer" (PJA20141201605). Work in MB laboratory was supported by ERC (250333). CB was supported by ERC (250333) followed by an ANRS fellowship (14065). JG and SD were supported by the ERC (637763).

### Appendix A. Supplementary Data

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.ebiom.2016.05.005>.

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