R-loops trigger the release of cytoplasmic ssDNAs leading to chronic inflammation upon DNA damage

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How DNA damage leads to chronic inflammation and tissue degeneration with aging remains to be fully resolved. Here, we show that DNA damage leads to cellular senescence, fibrosis, loss-of-tissue architecture, and chronic pancreatitis in mice with an inborn defect in the excision repair cross complementation group 1 (Ercc1) gene. We find that DNA damage-driven R-loops causally contribute to the active release and buildup of single-stranded DNAs (ssDNAs) in the cytoplasm of cells triggering a viral-like immune response in progeroid and naturally aged pancreata. To reduce the proinflammatory load, we developed an extracellular vesicle (EV)-based strategy to deliver recombinant S1 or ribonuclease H nucleases in inflamed Ercc1−/− pancreatic cells. Treatment of Ercc1−/− animals with the EV-delivered nuclease cargo eliminates DNA damage-induced R-loops and cytoplasmic ssDNAs alleviating chronic inflammation. Thus, DNA damage-driven ssDNAs causally contribute to tissue degeneration, Ercc1−/− paving the way for novel rationalized intervention strategies against age-related chronic inflammation.

INTRODUCTION  
The great majority of premature aging-like (progeroid) syndromes are associated with inborn defects in genome maintenance, supporting the notion that DNA damage drives aging and causally contributes to age-related diseases (1–3). To counteract DNA damage, mammalian cells rely on a series of partially overlapping DNA repair systems to remove DNA lesions that may block transcription or DNA replication. For bulky helix-distorting damage, such as the main ultraviolet (UV)–induced lesions, the principal repair mechanism is the evolutionarily conserved nucleotide excision repair (NER) pathway (4, 5). NER operates via a “cut and patch” type of mechanism involving ~30 proteins that recognize and remove helical distortions throughout the genome, i.e., global genome NER, or selectively from the transcribed strand of active genes, i.e., transcription-coupled NER (6, 7). In humans, defects in NER are causally linked to mutagenesis and cancer initiation as in the cancer-prone syndrome xeroderma pigmentosum (XP; complementation groups XP-A to XP-G) or to developmental and neuronal abnormalities as seen in a heterogenous group of premature aging-like (progeroid) syndromes, including the Cockayne syndrome (affected genes: Csa and Csb), trichothiodystrophy (affected genes: Xpb and Xpd), or the XP-ERCC1 syndrome (XFE; affected genes: Ercc1 and Xpf) (2). We and others have recently shown that DNA damage–driven inflammation contributes substantially in NER progeria and age-related degenerative diseases, but the mechanisms remain unresolved (8–11). Xeroderma pigmentosum F - excision repair cross complementation group (XPF-ERCC1) is a heterodimeric, structure-specific endonuclease complex required for lesion excision in NER (4, 5) that plays an analogous role in the repair of highly cytotoxic DNA interstrand cross-links (ICLs) (12).

RESULTS  
Persistent DNA damage associates with chronic pancreatitis in Ercc1−/− mice  
DNA damage causally contributes to aging, as exemplified by the growing list of progeroid DNA repair–deficient animals with age-related metabolic and endocrine defects (1, 10, 14–16). To gain insight into the causal contribution of DNA damage in tissue-degenerative changes in the pancreas, we studied the pancreata of 15-day-old DNA repair–deficient Ercc1−/− and age-matched wild-type (wt) littermate control mice. Scanning and transmission electron micrographs (SEM and TEM) reveal loss-of-tissue architecture and fibrosis with frequent and significantly denser collagen fibrils, observed in the vicinity of stellate cells in Ercc1−/− pancreata (Fig. 1A) but not in the pancreata of the NER-defective Csbhm/m, Xpa−/−, or Xpc−/−, known to manifest a milder phenotype, or the wt corresponding control animals (fig. S1A). Fibrosis in Ercc1−/− pancreata...
Fig. 1. Pancreatic fibrosis and inflammation in Ercc1−/− mice. (A) SEM (left) and TEM (right) of collagen (arrows) in wt and Ercc1−/− (Er1−/−) pancreas. N, nucleus; col, collagen; AC, acinar cell; SC, stellate cell. (B) Mmp9, Col1a, and Timp2 mRNA levels in Er1−/− and wt pancreata. (C) Infiltrated leukocytes (arrows) in wt and Er1−/− pancreata (SEM). (D) Immunostaining of CD45- and MAC1-positive cells infiltrating wt and Er1−/− pancreata (n = 3). (E) Ccl2, Il6, Cxcl10, Tnfs, and Il1β mRNA levels in Er1−/− compared to wt pancreata (n = 3). (F) Plasma amylase levels [international units per liter (IU/liter)] in wt and Er1−/− mice. (G) Immunostaining of α-SMA and VCAM1 in wt and Er1−/− pancreata (n = 3). (H) α-SMA protein levels in wt and Er1−/− whole-cell pancreas extracts (n = 4). (I) Immunostaining of γH2AX and pATM in wt and Er1−/− PPCs (n = 3). (K) γH2AX, pATM, and FANCI protein levels in of wt and Er1−/− whole-cell pancreas extracts (n ≥ 3). In (B) and (E), the red dashed lines depict the wt levels. The graphs in (D) and (G) represent the mean fluorescence intensity (MFI) per cubic micrometer of tissue; in (I) and (J), the percentage of positive-stained cells; and in (H) and (K), the β-tubulin (β-TUB)–normalized protein expression levels (n.e.l.) in Er1−/− pancreata compared to wt controls. All tissues and cells are derived from P15 mice. Scale bars, 5 μm. Error bars indicate SEM among n ≥ 3 replicates.
is accompanied by an increase in the mRNA levels of collagen 1a (Col1a) and matrix metalloproteinase 9 (Mmp-9) genes and no changes in the mRNA levels of the tissue inhibitor of metalloproteinases 2 (Timp2) gene (Fig. 1B). Likewise, we find a significant increase in the mRNA levels of Col1a and Mmp-9 genes in the pancreata of severely proliferative P15 Csb/mXpc/− double-mutant (DKO) mice.

Consistently, our analysis revealed normative mRNA levels for the Timp2 gene in the DKO or single-mutant pancreata (fig. S1B). Further SEM studies, as well as immunostaining against CD45 (for leukocytes) or MAC1 (for macrophages), and quantitative polymerase chain reaction (qPCR) analysis reveal the presence of lymphocytic gene in the DKO or single-mutant pancreata (fig. S1B). Fur-

We find that the pancreatic tissue amylase levels are comparable to wt controls. Amylase, a pancreatic enzyme that catalyzes the hydrolysis of starch into smaller carbohydrate molecules, creata compared to wt controls. However, we find a higher number of annexin V (+)/propidium iodide (PI) (+) cells in Ercc1−/− PPCs (Fig. 2F), as evidenced by the higher number of 53BP1 foci that colocalized with telomeres in Ercc1−/− PPCs (Fig. 2G), while Tert mRNA levels are comparable to wt controls (fig. S2C). Consistent with previous findings, further analysis of Ercc1−/− PPCs revealed that P15 PPCs do not accumulate telo-

ErbB2 and pSTING (phosphorylated signal transducer and transcrip-

The ERCC1-XPF defect triggers a viral-like immune response associated with the accumulation of cytoplasmic ssDNA moieties

To assess the senescence-associated gene expression changes trig-

Further analysis within the overrepresented biological processes re-

3 of 17
Fig. 2. Er1−/− pancreatic cells present senescent, but not apoptotic, characteristics. (A) TUNEL in wt and Ercc1−/− (Er1−/−) PPCs (n = 3). (B) Immunostaining of cleaved (cl.) caspase 3 in wt and Er1−/− PPCs (n = 5). (C) Cleaved caspase 3 (casp3) in wt and Er1−/− pancreas whole-cell extracts. UV-irradiated (20 J/m²) wt BMDM protein extracts were used as positive control (+) (n = 3). (D) Bcl2, Casp8, Bad, and Bcl_xl mRNA levels in Er1−/− compared to wt pancreata (n = 3). (E) P21 mRNA levels in Er1−/− compared to wt pancreata (n = 5). (F) Telomere length in wt and Er1−/− pancreata compared to a single-copy gene (Rarb2). The graphs depict the standard curves for telomere (Tel) and Rarb (left) and the relative telomere to Rarb telomere/single copy gene (T/S) ratio in wt and Er1−/− pancreata (right, n = 4). ct, cycle threshold. (G) Immunofluorescence in situ hybridization of 53BP1 with telomeric DNA (TelG) in wt and Er1−/− PPCs. Arrows denote TIFs on telomeres. The graph depicts the mean percentage of cells containing ≥2 TIFs ± SD, n = 3. (H) Lipofuscin staining, as depicted by GL13 binding (SenTraGor), in wt and Er1−/− pancreata (n = 3). (I) SA-β-gal assay in wt and Er1−/− PPCs (n = 3 technical replicates). (J) Immunostaining of cytoplasmic HMGB1 in wt and Er1−/− PPCs (white arrows; n = 3). In (D) and (E), the red dashed lines represent the wt levels. The graphs in (H) and (I) depict the percentage of positive-stained cells and, in (J), cytoplasm-positive–stained cells. Tissues and cells are derived from P15 mice. Scale bars, 5 μm. Error bars indicate SEM among n ≥ 3 replicates.
Fig. 3. Ercc1 deletion drives a viral-like response and cytoplasmic ssDNA accumulation. (A) HMGB1 in whole-cell extracts (w.c.e.) and in the culture media of wt and Er1−/− PPCs (n = 4). (B) Overrepresented biological processes (Gene Ontology) of the differentially expressed genes in Er1−/− compared to wt pancreata (meta–false discovery rate (FDR) ≤ 0.01, fold change ≥ ±1.5, n = 3). Processes with up-regulated genes (green) and down-regulated genes (red). (C) Heatmaps of representative genes associated with inflammation, leukocyte migration, and extracellular matrix (ECM) organization up-regulated in Er1−/− (1 to 3) compared to wt (1 to 3) pancreata. (D) mRNA levels of selected overrepresented genes in wt and Er1−/− pancreata. (E) mRNA levels of overrepresented Ifnα signature genes in wt and Er1−/− pancreata. (F) Phosphorylated STING (pSTING) and (G) phosphorylated STAT1 (pSTAT1) in whole-cell extracts of wt and Er1−/− pancreata (n = 3 to 4). (H) Immunostaining of ssDNA in wt and Er1−/− pancreas, (I) liver, and (J) kidney (n = 3). The graphs in (A), (F), and (G) represent the β-TUB n.e.l. of proteins in wt and Er1−/− pancreata and, in (H) to (J), the MFI per cubic micrometer of tissue. In (D) and (E), the red dashed lines represent the wt levels. Tissues and cells are derived from P15 mice. Scale bars, 40 μm. Error bars indicate SEM among n ≥ 3 replicates.
The observed IFN response and the increase in the mRNA levels of several genes associated with nucleic acid sensing, i.e., *Sting*, *Irf1*, *Ddx58*, *Ddx60*, and *Tlr3* (fig. S3B) (8), prompted us to test for the presence of DNA fragments in the cytoplasm of *Ercc1−/−* PPCs. Immunofluorescence (IF) studies with antibodies that are preferentially reactive against ssDNA or double-stranded DNA (dsDNA) revealed the accumulation of cytoplasmic ssDNA moieties in the pancreas (Fig. 3H), liver (Fig. 3I), and kidney (Fig. 3J) of P15 *Ercc1−/−* animals as well as in *Ercc1−/−* PPCs (fig. S3C) and in bone marrow–derived macrophages (BMDM) (Fig. S3D). We find no evidence of cytoplasmic dsDNAs in *Ercc1−/−* PPCs (fig. S3E) or in the kidney (fig. S3F) and liver (fig. S3G) of *Ercc1−/−* mice. Further IF studies revealed that ssDNAs also accrue in the cytosol of the NER-defective Csbb/m and Xpc−/− pancreatic cells, albeit to a lesser extent (fig. S3H). Notably, we find that cytoplasmic ssDNAs also accumulate in the pancreata and livers of the 24-month-old naturally aged mice (Fig. 4, A and B) followed by an increase in the mRNA levels of proinflammatory and IFN response genes, i.e., *Sting*, *Ifitm1*, *Mx1*, *Ift11*, *Cc22*, *Iilb*, *Tnfa*, *Cxxl10*, and *Colia* (Fig. 4C); the latter is consistent with the immune response against cytosolic DNA fragments (8). To further confirm the presence of cytoplasmic ssDNAs in *Ercc1−/−* PPCs, cells were treated with mung bean S1 endonuclease, which specifically degrades ssDNA, leaving the duplex DNA intact (29). Treatment with the S1 nuclease led to the complete removal of ssDNAs from the cytoplasm of *Ercc1−/−* PPCs (Fig. 4D).

**DNA damage–induced R-loops trigger the accumulation of cytoplasmic ssDNAs**

Owing to the DNA repair defect in *Ercc1−/−* pancreata, we reasoned that the presence of irreparable DNA lesions causally contribute to the generation of ssDNAs in the cytoplasm of *Ercc1−/−* PPCs. In support, treatment of wt PPCs with the potent genotoxin mitomycin (MMC), which generates DNA ICLs, or with UV-C irradiation (15 J/m²) led to the rapid accumulation of ssDNAs in the cytoplasm (fig. S4, A and B). Inactivation of ATM kinase with KU-55933 inhibitor or of ataxia-telangiectasia and Rad3-related (ATR) kinase with NU6027 had no detectable impact on cytoplasmic ssDNAs in MMC- or UV-treated cells (fig. S4, A and B; as shown), indicating that the accumulation of cytoplasmic ssDNAs is driven by DNA damage itself and it does not require canonical DDR signaling. To further establish that nuclear DNA damage causally contributes to the release of ssDNAs in the cytoplasm, we isolated PPCs from cyclobutane pyrimidine dimer (CPD)–photolyase transgenic mice that ubiquitously express the marsupial CPD-specific photolyase transgene (30). The CPD photolyase can directly revert UV-induced CPDs into undamaged bases using visible light energy in a reaction known as photoreactivation. Exposure of CPD-photolyase transgenic PPCs to UV irradiation (15 J/m²) and subsequent incubation in the dark (CPD photolyase is inactive and UV-induced CPDs persist) led to the accumulation of ssDNA moieties in the cytoplasm of these cells (Fig. 4E; as indicated). ssDNAs were absent when UV-treated, CPD-photolyase transgenic PPCs were also exposed to the photoreactivating light (photolyase is active and UV-induced CPDs are repaired) (Fig. 4E; as indicated). Further studies revealed that treatment of wt PPCs with Illudin S, a fungal sesquiterpene known to induce transcription-blocking DNA adducts, recapitulates the accumulation of cytoplasmic ssDNAs seen in *Ercc1−/−* PPCs or in MMC- and UV-treated PPCs (Fig. 4F), indicating that irreparable DNA lesions interfering with transcription may underlie the observed release of ssDNAs in the cytoplasm of PPCs. Persistent DNA lesions often hamper the progression of RNA polymerase II on actively transcribed genes, leading to the gradual buildup of R-loops in cells. In line, confocal microscopy studies revealed the accumulation of R-loops in *Ercc1−/−* and Illudin S–treated wt PPCs (Fig. 4G). Detection of RNA by the S9.6 antibody was recently shown to generate artifacts when imaging RNA:DNA hybrids (31). To exclude that the S9.6 IF signal observed arises from dsRNA rather than RNA:DNA hybrids, we transfected PPCs with the RNA:DNA hybrid–specific nuclease, RNase H; the latter removed RNA-DNA hybrids, leading to the substantial decrease of R-loops in these cells (Fig. 4G; as indicated). Next, we sought to test whether DNA damage–driven R-loops causally contribute to the release of ssDNA moieties in the cytoplasm of Illudin S–treated and *Ercc1−/−* PPCs. Consistently, we find that ssDNAs are removed from the cytoplasm of Illudin S–treated or *Ercc1−/−* PPCs when these cells are transfected with RNase H to eliminate R-loops (Fig. 5A). Likewise, R-loops accumulate significantly in MMC-treated PPCs (fig. S4C), and RNase H–mediated removal of R-loops leads to the substantial elimination of cytoplasmic ssDNAs in MMC-treated PPCs (fig. S4D). Consistent with the accumulation of cytoplasmic ssDNAs in the 24-month-old pancreata and livers, we find that R-loops also accumulate in the cell nuclei of pancreata, livers, and kidneys of the 24-month-old naturally aged mice compared to 2-month-old young adult animals (Fig. 5, B and C, and fig. S4E). In addition to the nuclear accumulation of R-loop structures, we find that RNA:DNA hybrids also accumulate in the cytoplasmic pancreatic cells in 24-month-old kidneys and livers. However, this fraction is mostly colocalized with mitochondria (32). The S9.6 IF signal was efficiently removed by RNase H treatment but remained unaffected upon treatment with ribonucleases RNase A or T1, indicating that the signal is not derived from dsRNA or ssRNA moieties, respectively (fig. S4F). Cytosolic ssDNAs also accumulate in serum-starved or hydroxyurea-treated *Ercc1−/−* PPCs [1% fetal bovine serum (FBS); fig. S5, A and B], suggesting that DNA damage–associated ssDNAs can accumulate in the cytoplasm of these cells independently of DNA replication.

To further explore the origin of cytoplasmic ssDNAs, we treated wt pancreatic cells with *trans*-retinoic acid (tRA) and Illudin S, to induce transcription of tRA-responsive genes, in the presence of transcription-blocking DNA lesions. We then performed qPCR on ssDNA fragments isolated from untreated and tRA/Illudin S–treated PPCs, where the second strand was synthesized with the Klenow fragment of DNA polymerase I (fig. S5C). Our analysis revealed the presence of ssDNAs originating from the tRA-inducible genes *Rarb* and *Strat6* (33) in tRA/Illudin S–treated PPCs compared to untreated controls, indicating that cytoplasmic ssDNAs are generated by cotranscriptional R-loops. The β-actin gene was used as a control because it is a tRA-nonresponsive gene that was previously shown to accumulate R-loops (34, 35). Next, we explored the possibility of long interspersed nuclear element 1 (LINE1) derepression by assessing LINE1 transcripts in wt and *Ercc1−/−* pancreata, as well as in untreated and tRA/Illudin S–treated PPCs (36). qPCR analysis revealed no evidence to support the presence of LINE1 open reading frame 1 (ORF1), ORF2, 5′ untranslated region (5′UTR), and 3′UTR transcripts (fig. SSD).

To further investigate how R-loops lead to the generation of ssDNAs, we used a DNA-RNA immunoprecipitation (D RIP) approach coupled to Western blotting, to isolate RNA:DNA hybrids from wt and *Ercc1−/−* pancreata, in the presence or absence of RNase H. In
Fig. 4. DNA damage–induced cytoplasmic ssDNA accumulation is instigated by R-loops. (A) Immunostaining of ssDNA in 2-month (2m)– and 24-month (24m)–old wt pancreata (n = 3). (B) IF detection of ssDNA in 2 month (2m)– and 24 month (24m)–old wt livers (n = 3). (C) mRNA levels of selected genes in 24-month-old wt pancreata. The red dashed line represents the levels in 2-month-old wt pancreata (n = 4 to 5). (D) Immunostaining of ssDNA (arrows) in wt and Ercc1−/− (Er1−/−) PPCs with or without S1 nuclease treatment (n = 3). (E) Immunostaining of cyclobutane-pyrimidine dimers (CPD) (left; red) or ssDNA (right; green) in untreated (untr.) CPD− transgenic PPCs, UV-irradiated (15 J/m²) and kept in the dark or under white light (1.5 hours of incubation; n = 3). The graphs depict the percentage of CPD-positive–stained cells and the ssDNA MFI per cell, respectively. (F) Immunostaining of ssDNA (arrows) in untreated and Illudin S–treated (IllS) (30 ng/ml; 3 hours) wt PPCs (n = 4). (G) Immunostaining of R-loops (S9.6 antibody) in Er1−/− and untreated or Illudin S–treated (IllS) (30 ng/ml; 3 hours) wt PPCs in the absence or presence of transfected recombinant RNase H (n = 3). The graphs in (A) and (B) depict the MFI per cubic micrometer of tissue; in (D) and (F), the MFI per cell; and in (G), the MFI per nucleus. Unless otherwise stated, tissues and cells are derived from P15 mice. Scale bars, 5 μm; in (A) and (B), 40 μm. Error bars indicate SEM among n ≥ 3 replicates.
Fig. 5. RPA mediates the nucleocytoplasmic transport of ssDNA moieties. (A) Immunostaining of ssDNA in Ercc1−/− (Er1−/−) and untreated or Illudin S–treated wt PPCs in the absence or presence of transfected recombinant E. coli RNase H (n = 3 to 4). (B) Immunostaining of R-loops, by means of S9.6 antibody staining, in 2- and 24-month-old naturally aged pancreata in the absence or presence of transfected recombinant RNase H (n = 3). (C) S9.6 immunostaining of R-loops in 2- and 24-month-old livers in the absence or presence of transfected recombinant RNase H (n = 3). (D) S9.6 immunoprecipitation (DRIP) followed by Western blotting for XPG, XPF, RNase H1 (RNH1), and DNA topoisomerase 1 (TOP1) in wt and Er1−/− pancreatic nuclear extracts with and without RNase H (RH) treatment (n = 6). (E) Immunostaining of ssDNA (green) in Er1−/−, untreated, MMC- (10 μg/ml; 4 hours), and Illudin S–treated (30 ng/ml; 3 hours) wt PPCs, cultured with or without the nuclear export inhibitor leptomycin B (LMB; 40 nM). Image inlays show the nuclear ssDNA signal (green) (n = 3). The graphs depict the MFI per cell in (A), per nucleus in (B) and (C), and per cytoplasm in (E). Unless otherwise stated, all tissues and cells are derived from P15 mice. Scale bars, 5 μm. Error bars indicate SEM among n ≥ 3 replicates.
agreement with previous findings, this approach revealed that XPG (xeroderma pigmentosum G) and XPF nucleases are bound together with DNA topoisomerase 1 (TOP1) to R-loops in wt and Ercc1−/− pancreatic cells (Fig. 5D and fig. S5E; as indicated) (33, 37, 38). Our finding that, in Ercc1−/− cells, the XPF DRIP signals are lower than those of XPG supports previous observations indicating that XPF is unstable in the absence of ERCC1 (39, 40). The latter makes XPF the limiting factor during the endonuclease-mediated resolution of R-loops in Ercc1−/− cells. Consistent with the increased amount of RNA:DNA hybrids in Ercc1−/− deficient pancreata (Fig. 4G), we also find higher levels of RNase H1 bound to R-loops in Ercc1−/− cells compared to wt controls (Fig. 5D and fig. S5E; as indicated).

ssDNAs are released into the cytoplasm via an active nucleocytoplasmic transport mechanism

Microinjected containing whole or fragmented chromosomes may passively diffuse into the cytoplasm when the nuclear envelope breaks down in mitotically dividing cells (41). The lack of 4,6-diamidino-2-phenylindole (DAPI)—stained chromatin fragments in the cytoplasm of Ercc1−/− PPCs along with the presence of cytoplasmic ssDNAs in hydroxyurea-treated Ercc1−/− PPCs or in Ercc1−/− livers and kidneys, where the great majority of cells typically do not divide, prompted us to test whether ssDNAs are actively released in the cytoplasm of Ercc1−/− cells. Analysis of nucleocytoplasmic protein shuttling is greatly facilitated by the use of leptomycin B (LMB), an inhibitor of CRM1-dependent nuclear export that does not interfere with protein transport into the nucleus. We find that treatment of Ercc1−/− PPCs and MMC- or Illudin S–treated wt PPCs with LMB leads to the marked decrease of ssDNAs in the cytoplasm that concurrently accumulate in the nuclei of these cells (Fig. 5E and fig. S5F). This finding suggests that cytoplasmic ssDNAs are of nuclear origin and that they are released into the cytoplasm of DNA repair–deficient cells via an active nucleocytoplasmic transport mechanism. Replication protein A (RPA) binds preferentially to ssDNA during DNA replication, repair, or recombination in eukaryotic cells (42). Western blotting of nuclear and cytoplasmic protein extracts in wt and Ercc1−/− pancreata revealed that RPA70, the DNA binding subunit of RPA (from now on referred to as RPA), is located both in the nucleus and the cytoplasm of Ercc1−/− deficient PPCs (Fig. 6A). We find that RPA binds cytoplasmic ssDNAs in Ercc1−/− PPCs and that the RPA-ssDNA interaction is abolished when Ercc1−/− PPC cytoplasmic protein extracts are treated with the mung bean S1 nuclease (Fig. 6B). Consistently, we find higher levels of RPA bound to ssDNA in the cytoplasm of MMC-treated compared to wt controls (Fig. 5D and fig. S5E; as indicated).

An EV-based nucleic cargo eradicates cytoplasmic ssDNAs and the proinflammatory response in Ercc1−/− pancreata

Cells respond to cytosolic DNA introduced into the cytoplasm by triggering an innate immune response. We, therefore, reasoned that the removal of ssDNAs from the cytoplasm of Ercc1−/− PPCs would be beneficial for reducing the proinflammatory load in inflamed cells, thereby improving the outcome of chronic pancreatitis in Ercc1−/− mice. To test this, we first transiently transfected recombinant mung bean S1 nucleases into MMC-treated wt PPCs and corresponding untreated control cells. We find that mung bean S1–mediated degradation of MMC-induced ssDNAs (Fig. 6E) prevents the nuclear translocation of nuclear factor κB (NF-κB), a potent regulator of the innate immune response against foreign pathogens (Fig. 6F) (44). Moreover, the nuclease-mediated removal of cytosolic ssDNAs lowers the Ifna miRNA levels compared to those observed in untreated wt cells (Fig. 6G). Thus, removal of cytosolic ssDNAs substantially dampens the proinflammatory response seen in Ercc1−/− PPCs.

EVs are lipid bilayer–delimited particles that are produced in the endosomal compartment of most eukaryotic cells and are important drivers of intercellular communication. EVs are released into the bloodstream and are known to discharge their content into far distant recipient cells and tissues to exert multiple physiological stimuli (45). First, we used an EV–based strategy to deliver recombinant S1 nuclease to remove the cytoplasmic ssDNAs in Ercc1−/− PPCs. We find that treatment with naive (i.e., not loaded) or mung bean S1 nuclease–loaded EVs substantially diminishes the accumulated ssDNAs in the cytoplasm of Ercc1−/− PPCs (Fig. 7A). Next, we embarked on a physiological application by injecting intraperitoneally P15 Ercc1−/− mice with naive or mung bean S1 nuclease–loaded EVs for five consecutive days (Fig. 7B). We find that treatment of Ercc1−/− mice with EV–based S1 nuclease cargo leads to the substantial removal of cytoplasmic ssDNAs (Fig. 7C). To test that cytoplasmic ssDNAs in Ercc1−/− pancreata causally contribute to the observed IFNγ response (Figs. 3E and 7C), we next prepared National Institutes of Health (NIH) 3T3–derived EVs carrying the cytoplasmic nucleic acid fraction from wt (EVswt cyt) or Ercc1−/− (EVsErcc1 cyt)
pancreata. Consistently, we find a substantial increase in the Ifna mRNA levels of PPCs treated for 24 hours with EVs Ercc1 cyto but not with EVs wt cyto. Ifna mRNA levels substantially decrease when PPCs are treated with EVs Ercc1 cyto carrying cytoplasmic DNA already pretreated with the mung bean S1 nuclease but not with the RNase A (Fig. 7D; as indicated). Our finding that DNA damage–driven R-loops causally contribute to the release of cytoplasmic ssDNAs in Ercc1−/− pancreata prompted us to test whether an EV-mediated...

Fig. 6. In vitro and in vivo effect of ssDNA abrogation in Er1−/− pancreata. (A) RPA70 in nuclear and cytoplasmic extracts from wt and Er1−/− pancreata (n = 4). (B) Immunoprecipitation (IP) using anti-ssDNA in untreated or S1 nuclease–treated cytoplasmic extracts from Er1−/− pancreata for RPA70. The input (INP) and flow through (FTH) are 1/20 of the extract used (n = 3). (C) RPA70 in whole-cell extracts of wt and Er1−/− PPCs, untreated, or incubated with scrambled (sictrl) or siRNA against Rpa70 (sicrlRPA70) (n = 3). (D) Immunostaining of ssDNA in wt and Er1−/− PPCs, untreated, or incubated with scrambled (sictrl) or siRNA against Rpa70 (sicrlRPA70) (n = 3). (E) Immunostaining of ssDNA upon recombinant S1 nuclease transfection in untreated and MMC-treated (10 μg/ml; 4 hours) wt PPCs (n = 3). (F) Immunostaining of NF-κB upon recombinant S1 nuclease transfection in untreated and MMC-treated (10 μg/ml; 4 hours) wt PPCs (n = 3). (G) Ifna mRNA levels in MMC-treated wt PPCs, untreated, or transfected with scrambled (sictrl) or siRNA against Rpa70 (sicrlRPA70) (n = 4). The graphs in (A) and (C) represent the β-TUB– or fibrillarin (FBL)–normalized expression of RPA70 levels (n.e.l.) in Er1−/− compared to corresponding wt controls, as indicated. The graphs in (D) to (F) depict the MFI per cell. All tissues and cells are derived from P15 mice. Scale bars, 5 μm. Error bars indicate SEM among n ≥ 3 replicates.
delivery of recombinant RNase H could remove R-loops and the accumulated cytosolic ssDNAs in Ercc1\(^{-/-}\) pancreata. Intraperitoneal injections of P15 Ercc1\(^{-/-}\) mice with naive or recombinant Escherichia coli RNase H–loaded EVs for two consecutive days revealed the substantial removal of R-loops (Fig. 7E), leading to a decrease in cytoplasmic ssDNAs (Fig. 7F) in Ercc1\(^{-/-}\) pancreata. We found that treatment of Ercc1\(^{-/-}\) mice with EVs carrying RNase H together with S1 nuclease (RH + S1) has an additive effect on the removal of...
DISCUSSION

Whereas the links between genome instability and enhanced cancer predisposition are well established, less is known about how DNA damage leads to the premature onset of age-related pathological features in man and accompanied animal models with mutations in DNA repair genes. Here, we provide evidence that persistent DNA damage triggers the formation of R-loops, leading to the accumulation of cytoplasmic ssDNA moieties in Ercc1−/− pancreata or cells exposed to genotoxins. In turn, the presence of ssDNA fragments in the cytoplasm stimulates a proinflammatory response, leading to the premature onset of chronic pancreatitis and fibrosis in progeroid Ercc1−/− mice. In this work, cytosolic ssDNAs also accrue in inflamed naturally aged pancreata where fibrosis and chronic pancreatitis are known to be frequently observed with aging (46). Treatment with EVs carrying RNase H or S1 nuclease in mice can eliminate cytoplasmic ssDNAs, reducing the proinflammatory load in Ercc1−/− PPCs and pancreata. The latter has important ramifications for the development of efficient rationalized intervention strategies against chronic inflammation in NER progeria and likely also with aging (Fig. 7I).

MATERIALS AND METHODS

Animal models

The generation and characterization of NER-deficient mice have been previously described (13, 58). Animals were kept on a regular diet and housed at the Institute of Molecular Biology and Biotechnology (IMBB) animal house, which operates in compliance with the “Animal Welfare Act” of the Greek government, using the “Guide for the Care and Use of Laboratory Animals” as its standard. As required by the Greek law, formal permission to generate and use genetically modified animals was obtained from the responsible local and national authorities. All animal studies were approved by an independent Animal Ethical Committee at the Foundation of Research and Technology - Hellas (FORTH).

Primary cells and treatments

PPCs were isolated from P15 animals. Briefly, pancreata were excised, minced, and incubated in collagenase type IV (2.5 mg/ml) at
37°C for 15 min. After centrifugation, cells were resuspended and cultured in standard medium containing Dulbecco’s modified Eagle’s medium supplemented with 10% FBS, streptomycin (50 μg/ml), penicillin (50 U/ml) (Sigma-Aldrich), and 2 mM 1-glutamine (Gibco) for 2 days. In BMDM cell cultures, the medium was supplemented with 30% L929-conditioned culture media for 5 days. For subsequent experiments, cells were rinsed with phosphate-buffered saline (PBS); exposed to UV-C irradiation (10 to 20 J/m²), MMC (10 μg/ml, 4 hours; AppliChem), Illudin S (30 ng/ml, 3 hours; Santa Cruz Biotechnology), and hydroxyurea (650 μM, 16 hours); and cultured at 37°C before subsequent experiments. Preincubation with ATM inhibitor (10 μM; Millipore), ATRi (10 μM; Millipore), and LMB (40 nM; Merck) started 1 hour before genotoxic treatments.

**Protein transfection and EV injections**

For the protein transfection experiments (Pierce Protein Transfection Reagent, Thermo Fisher Scientific), 10 U of recombinant mung bean S1 nuclease (100 U/μl; Thermo Fisher Scientific) or RNase H (5 U/μl; New England Biolabs) was used according to the manufacturer’s instructions. For the RPA70 knockdown, 20 pmol of a stealth oligo was used (MSS 229016, Thermo Fisher Scientific). EVs were transfected with Lipofectamine 2000 (Invitrogen) and subsequently seeded in 6-well or 24-well plates, according to the manufacturer’s protocol. As a nontargeting control, the AllStars negative (Qiagen) was used. For protein cell extracts, tissues were digested with collagenase (2.5 mg/ml) at 37°C for 15 min. Collagenase was neutralized with 10% FBS, and samples were further washed with 1× PBS/1% bovine serum albumin (BSA). For the delivery of EVs, neutralized with 10% FBS, and samples were further washed with PBS, and the lipofuscin-positive cells were counted in random fields in triplicate wells. For SenTraGor staining, cells were treated according to the manufacturer’s instructions (Arriani Pharmaceuticals, AR8850020). Briefly, fixed differentiated PPCs were washed with 50 and 70% EtOH incubated with SenTraqor reagent at RT, washed in EtOH, and incubated with primary anti-biotin antibody and fluorochrome-labeled secondary antibody. Cells were washed in PBS, and the lipofuscin-positive cells were counted in random fields in each of the six individual repeats using Fiji software.

**Immunofluorescence**

For IF experiments of mouse tissues, minced tissues were fixed in 4% formaldehyde, permeabilized with 0.5% Triton X-100, and blocked with 1% BSA. After overnight incubation with primary antibodies, secondary fluorescent antibodies were added, and DAPI was used for nuclear counterstaining. Samples were visualized with an SP8 TCS laser scanning confocal microscope (Leica), and scanned sections were analyzed using the Fiji (ImageJ) software. Mouse livers, kidneys, and pancreata were embedded in optimal cutting temperature (OCT) compound, before frozen sectioning on a Cryotome (CM 1850, permeabilization, blocking, and immunostaining as described above. For the SA–β-gal activity, the β-gal assay kit was used (Abcam Inc., ab65351) according to the manufacturer’s instructions. Briefly, Er1−/− and wt PPCs were fixed, washed with PBS, and stained in β-gal fixative solution at 37°C until β-gal staining became visible in either experimental or control plates (3 hours). Cells were washed in PBS, and β-gal–positive cells were counted in random fields in triplicate wells. For SenTraqor staining, cells were treated according to the manufacturer’s instructions (Arriani Pharmaceuticals, AR8850020). Briefly, fixed differentiated PPCs were washed with 50 and 70% EtOH incubated with SenTraqor reagent at RT, washed in EtOH, and incubated with primary anti-biotin antibody and fluorochrome-labeled secondary antibody. Cells were washed in PBS, and the lipofuscin-positive cells were counted in random fields in each of the six individual repeats using Fiji software.

**SDS–polyacrylamide gel electrophoresis**

For SDS–polyacrylamide gel electrophoresis (PAGE) analysis, cells were pelleted and tissues from Er1−/− and wt animals were homogenized in sucrose buffer [0.32 M sucrose, 15 mM Hepes-KOH, 60 mM KCl, 2 mM EDTA, 0.5 mM EGTA, 0.5% BSA, 0.1% NP-40, and protease inhibitors (pH 7.9)]. Cell pellets were washed three times with 1× PBS. Cell pellets were then resuspended in NP-40 lysis buffer [10 mM tris-HCl (pH 7.9), 10 mM NaCl, 3 mM MgCl2, 0.5% NP-40, and protease inhibitors] and incubated for 10 min at 4°C. After centrifugation, the supernatant was kept as the cytoplasmic fraction, and pellets were resuspended in high-salt extraction buffer [10 mM Hepes-KOH (pH 7.9), 380 mM KCl, 3 mM MgCl2, 0.2 mM EDTA, 20% glycerol, and protease inhibitors] and incubated for 60 min at 4°C. The supernatant after centrifugation was kept as the nuclear fraction. For whole-cell extract preparations, cell pellets were resuspended in 150 mM NaCl, 50 mM tris (pH 7.5), 5% glycerol, 1% NP-40, and 1 mM MgCl2 and incubated on ice for 20 min.

**Antibodies**

Antibodies against FANCI (H102; IF: 1/50), amylase (G-10; WB: 1/500, IF: 1/100), goat anti-rat immunoglobulin G (IgG)–CFL 647 (sc-362293; IF: 1/1000), translocase of outer membrane, subunit 20 (TOM20) (sc-17764; IF: 1/100), XPF (sc-398032; WB: 1/500), and donkey anti-goat IgG–horseradish peroxidase (HRP) (sc-2020; WB: 1/5000) were from Santa Cruz Biotechnology. Antibodies against ssDNA (MAB3299; IF: 1/50; 15 μg/5 μg of protein), dsDNA (MAB1293; IF: 1/100), S9.6 (MABE1095; IF: 1/100), DRIP (8 μg/5 μg of DNA), γH2AX (50–636; IF: 1/12,000), goat anti-rabbit IgG-HRP (AP132P; WB: 1/10,000), and goat anti-mouse IgG-HRP (AP124P; WB: 1/10,000) were from Millipore. α-SMA (ab5694; WB: 1/500, IF: 1/50), HMGB1 (ab18256; WB: 1/1000, IF: 1/150), γH2AX (ab22551; WB: 1/500), GAPDH (glyceraldehyde phosphate dehydrogenase) (ab8245; WB: 1/4000), fibrilin (FBL) (ab5821; WB:
enzyme per reaction for 1 hour at 37°C in the dark. Then, they were washed for 30 min and mixed with 45 μl of TUNEL mix and 5 μl of hydro, washed for 30 min, and permeabilized in 0.1% Triton X-100 TUNEL assay was used, and data were analyzed using the FlowJo software (Tree Star). Buffer was added to each sample. A FACSCalibur (BD Biosciences) 15 min at RT in the dark. Four hundred microliters of 1× binding buffer was added per sample. The cells were vortexed gently and incubated for 5 min, and 10°C for forever. For the master mix, 15 mM tris-Cl and 0.05% calcium chloride. Afterward, the tissue was fixed for 2 hours with 2% paraformaldehyde (PFA)–2% glutaraldehyde (pH 7.42) with 0.1% osmium tetroxide in sodium cacodylate buffer, and samples were processed for DNA extraction. After phenol purification, the DNA was precipitated with 2.5 volumes of ice-cold 100% EtOH and 200 mM NaCl at −20°C overnight. DNA quantity was measured with nanodrop and diluted at a final concentration of 35 ng/μl. The PCR program for the telomeric amplification is as follows: 95°C for 10 min, (95°C for 15 s, 54°C for 2 min, and 72°C for 7 min) × 22 cycles, and 72°C for 7 min; and for the single-copy gene (Rarb): 95°C for 10 min, (95°C for 15 s, 58°C for 1 min, and 72°C for 30 s) × 30 cycles, 72°C for 5 min, and 10°C for forever. For the master mix, 15 mM tris-Cl (pH 8), 50 mM KCl, 2 mM MgCl₂, 0.2 mM dNTPs (deoxyribonucleotide triphosphate), 5 mM dithiothreitol, 1% DMSO, 5× Sybr Green, and 1.25 U of Platinum Taq. The standard curve was prepared with the use of serial dilutions of a DNA sample of known quantity. The primer sequences used in qPCR are, for TelC-telG, GGTGGTTTGAGG- GTGAGGGTGAG (forward) and GTGAGGGTGAGGGTC- CCGACTATCCCTATCCCTATCCCTATCCCTATCCCTATCCCTA (reverse) and, for Rarb, CCTGAGTCGAAGGACACT (forward) and CCTGAGTCGACAGGACACT (reverse).

**Telomere length measurement**

The measurements of telomere length in pancreata were conducted as described previously (60). The pancreata were lysed, and nuclei were processed for DNA extraction. After phenol purification, the DNA was precipitated with 2.5 volumes of ice-cold 100% EtOH and 200 mM NaCl at −20°C overnight. DNA quantity was measured with nanodrop and diluted at a final concentration of 35 ng/μl. The PCR program for the telomeric amplification is as follows: 95°C for 10 min, (95°C for 15 s, 54°C for 2 min, and 72°C for 7 min) × 22 cycles, and 72°C for 7 min; and for the single-copy gene (Rarb): 95°C for 10 min, (95°C for 15 s, 58°C for 1 min, and 72°C for 30 s) × 30 cycles, 72°C for 5 min, and 10°C for forever. For the master mix, 15 mM tris-Cl (pH 8), 50 mM KCl, 2 mM MgCl₂, 0.2 mM dNTPs (deoxyribonucleotide triphosphate), 5 mM dithiothreitol, 1% DMSO, 5× Sybr Green, and 1.25 U of Platinum Taq. The standard curve was prepared with the use of serial dilutions of a DNA sample of known quantity. The primer sequences used in qPCR are, for TelC-telG, GGTGGTTTGAGG- GTGAGGGTGAG (forward) and GTGAGGGTGAGGGTC- CCGACTATCCCTATCCCTATCCCTATCCCTATCCCTA (reverse) and, for Rarb, CCTGAGTCGAAGGACACT (forward) and CCTGAGTCGACAGGACACT (reverse).

**Immunofluorescence in situ hybridization**

PCCs were seeded on coverslips and fixed in 4% PFA/1× PBS for 10 min at 4°C. Immunostaining for 3BP1 was performed as described above; cells were then washed with 1× PBS and fixed again in 4% FA/1× PBS for 10 min at RT. After dehydration of the cells in increasing concentration of ethanol, 250 mM peanut agglutinin telomeric probe (TelC-Cy3; F1002) was added to the coverslip, in hybridization buffer [20 mM tris (pH 7.4), 70% formamide, salmon sperm DNA (0.1 μg/ml), and 2× standard saline citrate (SSC)]. The cells were denatured at 85°C for 10 min and then left at RT for 2 hours. Then, cells were washed in prewarmed 2× SSC/0.1% Tween 20, and nuclei were counterstained with DAPI.

**Metaphase chromosome spreads**

For the metaphase chromosome studies in wt and Er1−/− pancreatic cells, cells were arrested in colcemid (0.1 μg/ml) for 20 hours, harvested by trypsinization, incubated for 15 min at 37°C in 75 mM KCl, and fixed in freshly made methanol/acetic acid (3:1). Cells were dropped onto wet slides and air-dried overnight in a chemical hood. Fluorescence in situ hybridization was performed as described above.

**RNA-seq and qPCR**

Total RNA was isolated from wt and Er1−/− pancreata with the total RNA isolation kit (Qiagen) as described by the manufacturer. For RNA-seq, libraries were prepared using an Illumina TruSeq mRNA stranded sample preparation kit. Library preparation started with 1 μg of total RNA. After poly-A selection (using poly-T oligo-attached magnetic beads), mRNA was purified and fragmented using divalent cations under elevated temperature. The RNA fragments underwent reverse transcription using random primers. This is followed by second-strand cDNA synthesis with DNA polymerase I and RNase H. After end repair and A-tailing, indexing adapters were ligated. The products were then purified and amplified (14 PCR

**Annexin V–PI FACS analysis**

Pancreata from 15-day-old Er1−/− and wt (n = 3) were minced and digested in 1× PBS/1% BSA/0.1% Na3/collagenase (2.5 mg/ml). Homogenized tissue was further washed twice with ice-cold PBS and passed through a 100 μM wire mesh. The cells (10⁶/ml) were re-suspended in 1× binding buffer. Cells (10⁵) were used for the staining, and 5 μl of annexin V–fluorescein isothiocyanate and 5 μl of PI were added per sample. The cells were vortexed gently and incubated for 15 min at RT in the dark. Four hundred microliters of 1× binding buffer was added to each sample. A FACSCalibur (BD Biosciences) was used, and data were analyzed using the FlowJo software (Tree Star).

**TUNEL assay**

The pancreatic cells or tissue were fixed for 30 min in 4% formaldehyde, washed for 30 min, and permeabilized in 0.1% Triton X-100 or 0.1% Na citrate for 2 or 8 min, respectively. The samples were washed for 30 min and mixed with 45 μl of TUNEL mix and 5 μl of enzyme per reaction for 1 hour at 37°C in the dark. Then, they were washed with PBS and counterstained for 10 min with DAPI.
cycles) to create the final cDNA libraries. After library validation and quantification (Agilent 2100 Bioanalyzer), equimolar amounts of library were pooled. The pool was quantified by using the Qubit KAPA Library Quantification Kit and the Applied Biosystems 7900HT Sequence Detection System. The pool was sequenced by using an S2 flow cell on the Illumina NovaSeq6000 sequencer and the 2x100nt protocol. qPCR was performed with a Bio-Rad 1000-series thermal cycler according to the instructions of the manufacturer (Bio-Rad) as previously described (61). In case of the qPCR for the intronless Ifna gene, the RNA samples were treated with DNase (Promega). The generation of specific PCR products was confirmed by melting curve analysis and gel electrophoresis. Each primer pair was tested with a logarithmic dilution of a cDNA mix to generate a linear standard curve [crossing point (CP) plotted versus log of template concentration], which was used to calculate the primer pair efficiency ($E = 10^{(-1/slope)}$). Hypoxanthine guanine phosphoribosyltransferase concentration, which was used to calculate the primer pair efficiency ($E = 10^{(-1/slope)}$). Hypoxanthine guanine phosphoribosyltransferase concentration, which was used to calculate the primer pair efficiency ($E = 10^{(-1/slope)}$). Hypoxanthine guanine phosphoribosyltransferase concentration, which was used to calculate the primer pair efficiency ($E = 10^{(-1/slope)}$). Hypoxanthine guanine phosphoribosyltransferase concentration, which was used to calculate the primer pair efficiency ($E = 10^{(-1/slope)}$). Hypoxanthine guanine phosphoribosyltransferase concentration, which was used to calculate the primer pair efficiency ($E = 10^{(-1/slope)}$). Hypoxanthine guanine phosphoribosyltransferase concentration, which was used to calculate the primer pair efficiency ($E = 10^{(-1/slope)}$). Hypoxanthine guanine phosphoribosyltransferase concentration, which was used to calculate the primer pair efficiency ($E = 10^{(-1/slope)}$). Hypoxanthine guanine phosphoribosyltransferase concentration, which was used to calculate the primer pair efficiency ($E = 10^{(-1/slope)}$). Hypoxanthine guanine phosphoribosyltransferase concentration, which was used to calculate the primer pair efficiency ($E = 10^{(-1/slope)}$). Hypoxanthine guanine phosphoribosyltransferase concentration, which was used to calculate the primer pair efficiency ($E = 10^{(-1/slope)}$). Hypoxanthine guanine phosphoribosyltransferase concentration, which was used to calculate the primer pair efficiency ($E = 10^{(-1/slope)}$).

The genes tested by qPCR are as follows: Il6 (forward: AGTTGCTCTTGGGAGACTGA; reverse: CAGAAATGGCATTGGCAACAC), Cc12 (forward: TGATCCCAATGATGCGCTGAG; reverse: ATGTCCTGACACCTTCTCCT), Cxcl10 (forward: ATGACGGGACCTGAGAATG; reverse: CATTGTGCAATGCTTCAACA), Col1a (forward: CCTCACTGGATCCCTGCACA; reverse: TCACGTCCAGATTTCAACGAG), Mmp9 (forward: GTCTGGATAAGGAG; reverse: CACTGCAGGAGGTCGTAGG), Timp2 (forward: CGAGTTTATCTACCGCCC; reverse: CAGGTCTCTTCTCCTGAGG), Bcl2 (forward: TCGCAGAGAGTCCACTCAG; reverse: ATGCGGCTTCAAGGTACTCAG), Casp8 (forward: GGCCTCCTACTCTAGCTGCA; reverse: TGTGTTCTTGTTCGCTGAAG), Bad (forward: CTTGCCGGAGCTTCGCTGAG; reverse: TCGCATTGCTGAGTCTGCT), Bclxl (forward: AGGGCTTATGGCTGCTGAAG; reverse: GTGCAAGAGCCTTGCAGGA; P21 (forward: TTGCACCTGGTCTGAGG; reverse: TCTGGGCTGAGTGATGAGTA), Sting (forward: AAAATCTGCCGCTTATT; reverse: TGGGAGAGGCTGATCCAT), Ifn2 (forward: CCAAGTTCTGCTGAGT; reverse: AGGCAAGTGGACTGACT), Il1b (forward: GCCCATCTCCCTGTAGCAT; reverse: TTGTGCTTGTCTGGCTTCC), Tert (forward: TCAAGTGTCTGGCTTCTCATTCT; reverse: GAGATGTGATGGTCTGCTGCT), Barb (forward: GAGTTAGAGTCTTTTGTGGTT; reverse: TACCACCACACTATCAACAAA), Strats (forward: ATATGGTGCGGCGTGTAGT; reverse: TCTTGCAGTGTTCCTGACC), B-actin (forward: TAGGGTGCGGTGGGT; reverse: GGTGCTTACTAAGTCCT), L1 ORF1 (forward: ATCTGTCTCCAGGTGCTC; reverse: AGTGTGGTCGTCCTGTAGT), L1 ORF2 (forward: GCTTGGATGAGTCTGAGGA; reverse: TCTGTCAGAGTACGCAGGAG), L1 5'UTR (forward: CTGCCCTGAGAGACAG; reverse: AGTGTGGTCGTCCTGTAGT), and L1 3'UTR (forward: AGCCAAATGGATGACCTGG; reverse: AGAAGGGGTCATGTGCTC).

### Data analysis

For RNA-seq, the data were downloaded as FASTQ files, and their quality was checked with FASTQC, a quality control tool for high-throughput sequence data: www.bioinformatics.babraham.ac.uk/projects/fastqc. A warning for overrepresenting sequences related to hydrolytic enzymes was ignored because of the physiology of the pancreas. The data were aligned to mm10 genome assembly available from the University of California Santa Cruz via Bowtie2. The differentially expressed genes were identified with metaseqR. Count normalization was performed on the basis of edgeR algorithm. Statistical analysis was performed with DESeq2, edgeR, and NOIseq algorithms. Differentially expressed genes found by all three algorithms were used for downstream analysis. Significant overrepresentation of pathways and gene networks was determined by Gene Ontology (http://geneontology.org/) and Kyoto Encyclopedia of Genes and Genomes pathways (www.genome.jp/kegg/pathway.html). Data analysis was performed with the PANTHER Classification System using the overrepresentation test. The $P$ values were determined by the Fisher's exact test, and fold enrichment is referred to the observed over the expected number within the reference list (Mus musculus) that map to the annotation data category. For all analyses, unless otherwise indicated, $P$ values were determined by two-tailed Student's t test, and the significance is set at $P \leq 0.05$.

### DRIP assay

DRIP analysis was based on the protocol by Goulielmaki et al. (33). Briefly, DRIP was performed without a cross-linking step. Nuclei were isolated using 0.5% NP-40 buffer and resuspended in TE buffer supplemented with 0.5% SDS and 100 mg of proteinase K. Genomic DNA was isolated after the addition of potassium acetate (1 M) and DNA was isolated after the addition of potassium acetate (1 M) and DNA was isolated after the addition of potassium acetate (1 M) and DNA was isolated after the addition of potassium acetate (1 M) and DNA was isolated after the addition of potassium acetate (1 M). Isoform specific PCR was performed with primers specific for each isoform.

### ssDNA immunoprecipitation assay

For coimmunoprecipitation assay, untreated or MMC-treated PPCs from wt mice or pancreata from wt and Er1−/− mice were mixed in 1% formaldehyde at RT for 2.5 min, and cells were lysed using the

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Chatzidoukaki et al., Sci. Adv. 7, eabj5769 (2021)  19 November 2021

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[15 of 17]
NP-40 lysis buffer [10 mM tris-HCl (pH 7.9), 10 mM NaCl, 3 mM MgCl$_2$, 0.5% NP-40, and protease inhibitors]. Cytoplasmic lysates (1.5 to 3 mg) were diluted threefold by adding ice-cold HENG buffer [10 mM Hepes-KOH (pH 7.9), 1.5 mM MgCl$_2$, 0.25 mM EDTA, and 20% glycerol] and precipitated with 5 to 10 µg of anti-siDNA antibody (MAB3299) o/n at 4°C followed by incubation for 3 hours with protein G Sepharose beads (Millipore). Immunoprecipitates were washed five times [10 mM Hepes-KOH (pH 7.9), 300 mM KCl, 0.3% NP-40, 1.5 mM MgCl$_2$, 0.25 mM EDTA, 20% glycerol, and protease inhibitors], eluted, and resolved on 8 to 12% SDS-PAGE.

**REFERENCES AND NOTES**

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**RESEARCH ARTICLE**

1. Chatzidoukaki et al., Sci. Adv. 7, eabj5769 (2021) | November 2021

Chatzidoukaki et al., Sci. Adv. 7, eabj5769 (2021) | November 2021

16 of 17
and complementing activities of ERCC4, ERCC11 and xeroderma pigmentosum group F.

40. M. Biggerstaff, D. E. Szymkowski, R. D. Wood, Co-correction of the ERCC1, ERCC4 and xeroderma pigmentosum group F DNA repair defects in vitro. EMBO J. 12, 3685–3692 (1993).

41. S. M. Harding, J. L. Benci, J. Irianto, D. E. Discher, A. J. Minn, R. A. Greenberg, Mitotic progression following DNA damage enables pattern recognition within micronuclei. Nature 548, 466–470 (2017).

42. M. S. Wold, Replication protein A: A heterotrimeric, single-stranded DNA-binding protein required for eukaryotic DNA metabolism. Annu. Rev. Biochem. 66, 61–92 (1997).

43. L. I. Toledo, M. Altmeyer, M. B. Rask, C. Lukas, L. K. Povlsen, S. Bekker-Jensen, N. Mailand, J. Bartek, J. Lukas, ATR prohibits replication catastrophe by preventing global exhaustion of RPA. Cell 153, 1088–1103 (2013).

44. D. Baltimore, Discovering NF-kappaB. Cold Spring Horb. Perspect. Biol. 1, a00026 (2009).

45. U. Sterzenbach, U. Putz, L. H. Low, J. Silke, S. S. Tan, J. Howitt, Engineered exosomes as vehicles for biologically active proteins. Mol. Ther. 25, 1269–1278 (2017).

46. J. M. Lohr, N. Panic, M. Vujasinovic, C. S. Verbeke, The ageing pancreas: A systematic review of the evidence and analysis of the consequences. J. Intern. Med. 283, 446–460 (2018).

47. K. Skourti-Stathaki, N. J. Proudfoot, N. Gromak, Human senataxin resolves RNA/DNA hybrids formed at transcriptional pause sites to promote Xrn2-dependent termination. Mol. Cell 711–721 (2009).

48. X. Li, J. L. Manley, Inactivation of the SR protein splicing factor ASF/SF2 results in genomic instability. Cell 122, 365–378 (2005).

49. P. Huertas, A. Aguilera, Cotranscriptionally formed DNA:RNA hybrids mediate transcription-coupled nucleotide excision repair factors promote R-loop-induced genome instability. Mol. Cell 56, 777–785 (2014).

50. J. Sollier, C. T. Stork, M. L. Garcia-Rubio, R. D. Paulesen, A. Aguilera, K. A. Cintrich, RNA polymerase III regulates cytosolic RNA:DNA hybrids in mice with Cockayne syndrome. EMBO J. 35, 831–844 (2016).

51. F. Coquel, M. J. Silva, H. Techer, K. Zadorozhny, S. Sharma, J. Nieminuszczy, C. Mettling, K. J. Mackenzie, P. Carroll, L. Lettice, Z. Tarnauskaite, K. Reddy, F. Dix, A. Revuelta, E. A. Farkash, E. T. Luning Prak, DNA damage and L1 retrotransposition. Nat. Genet. 43, 180 (2011).

52. K. Skourti-Stathaki, N. J. Proudfoot, N. Gromak, Human senataxin resolves RNA/DNA hybrids formed at transcriptional pause sites to promote Xrn2-dependent termination. Mol. Cell 794–805 (2011).

53. K. Skourti-Stathaki, N. J. Proudfoot, N. Gromak, Human senataxin resolves RNA/DNA hybrids formed at transcriptional pause sites to promote Xrn2-dependent termination. Mol. Cell 711–721 (2009).

54. E. Abbondati, R. E. Rigby, B. Rabe, F. Kilanowski, G. Grimes, A. Fluteau, P. S. Devenney, G. A. Garinis, ERCC1-XPF cooperates with CTCF and cofacilin to facilitate the developmental silencing of imprinted genes. Nat. Cell Biol. 19, 421–432 (2017).

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R-loops trigger the release of cytoplasmic ssDNAs leading to chronic inflammation upon DNA damage

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