PARP inhibition enhances tumor cell-intrinsic immunity in ERCC1-deficient non-small cell lung cancer

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The cyclic GMP-AMP synthase–stimulator of interferon genes (cGAS/STING) pathway detects cytosolic DNA to activate innate immune responses. Poly(ADP-ribose) polymerase inhibitors (PARPi) selectively target cancer cells with DNA repair deficiencies such as those caused by *BRCA1* mutations or *ERCC1* defects. Using isogenic cell lines and patient-derived samples, we showed that ERCC1-defective non-small cell lung cancer (NSCLC) cells exhibit an enhanced type I interferon transcriptomic signature, and that low ERCC1 expression correlates with increased lymphocytic infiltration. We demonstrated that clinical PARPi, including olaparib and rucaparib, have cell-autonomous immunomodulatory properties in ERCC1-defective NSCLC and BRCA1-defective triple-negative breast cancer (TNBC) cells. Mechanistically, PARPi generated cytoplasmic chromatin fragments with micronuclei characteristics; these were found to activate cGAS/STING, downstream type I interferon signaling and CCL5 secretion. Importantly, these effects were suppressed in *PARP1*-null TNBC cells, suggesting that this phenotype resulted from an on-target effect of PARPi on PARP1. PARPi also potentiated interferon-γ-induced PD-L1 expression in NSCLC cell lines and in fresh patient tumor cells; this effect was enhanced in ERCC1-deficient contexts. Our data provide the preclinical rationale for using PARPi as immunomodulatory agents in appropriately molecularly-selected populations.

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Abstract
The cyclic GMP-AMP synthase–stimulator of interferon genes (cGAS/STING) pathway detects cytosolic DNA to activate innate immune responses. Poly(ADP-ribose) polymerase inhibitors (PARPi) selectively target cancer cells with DNA repair deficiencies such as those caused by BRCA1 mutations or ERCC1 defects. Using isogenic cell lines and patient-derived samples, we showed that ERCC1-defective non-small cell lung cancer (NSCLC) cells exhibit an enhanced type I interferon transcriptomic signature, and that low ERCC1 expression correlates with increased lymphocytic infiltration. We demonstrated that clinical PARPi, including olaparib and rucaparib, have cell-autonomous immunomodulatory properties in ERCC1-defective NSCLC and BRCA1-defective triple-negative breast cancer (TNBC) cells. Mechanistically, PARPi generated cytoplasmic chromatin fragments with micronuclei characteristics; these were found to activate cGAS/STING, downstream type I interferon signaling and CCL5 secretion. Importantly, these effects were suppressed in PARP1-null TNBC cells, suggesting that this phenotype resulted from an on-target effect of PARPi on PARP1. PARPi also potentiated interferon-γ-induced PD-L1 expression in NSCLC cell lines and in fresh patient tumor cells; this effect was enhanced in ERCC1-deficient contexts. Our data provide the preclinical rationale for using PARPi as immunomodulatory agents in appropriately molecularly-selected populations.
Introduction

Immune checkpoint inhibitors (ICI) have revolutionized the prognosis of several aggressive cancers, notably non-small cell lung cancer (NSCLC). Very recent impressive results of large phase III trials in NSCLC have reported unprecedented improvements in overall survival and progression free survival when using anti-programmed death receptor 1 or -ligand 1 (anti-PD-(L)1) in first-line therapy (1, 2). Likewise, remarkable 5-year survival rates of 16% have recently been reported in this disease (3), highlighting the ability of these agents to provide long-term tumor control. Although these results are encouraging, they also reinforce the fact that still only a minority of patients receive long-term benefit. Better understanding of the determinants of response to ICI and identification of rationale combinations that would increase the proportion of patients benefiting from these therapies is therefore crucial.

Several factors have been associated with response to immunotherapy: tumor-related factors (e.g. cancer cells mutations), microenvironment-related factors (e.g. expression of immune checkpoints, lymphocytic infiltration or interferon signatures) and host-related factors (e.g. microbiome) (4). Defects in the DNA damage response (DDR) in cancer cells are key determinants of cancer immunogenicity. Indeed, DDR defects result in genomic instability and increased tumor mutational burden (TMB), which has been linked – at least in some cases – to better outcome upon ICI treatment (5). The best illustrations of this are probably mismatch-repair deficient tumors (6), and POLE/POLD1-mutated endometrial carcinoma and glioblastoma (7, 8), which are highly sensitive to ICI, likely due to their increased neoantigen repertoire. High TMB has also been correlated with better response to ICI in melanoma (9) and NSCLC (2, 10, 11). Other DNA repair defects, such as BRCA1/2 mutations have been found enriched in ICI responders (12). However, a simple correlation between DNA repair defects-induced genomic instability, TMB and response to ICI cannot be claimed (5), as tumor heterogeneity (13) and other determinants of response also play a role that, importantly, seems to be independent from TMB in response to ICI (14, 15).
Another interface between DDR and immunogenicity, which has recently generated particular attention in immuno-oncology, is the cyclic GMP-AMP synthase / stimulator of interferon genes (cGAS/STING) pathway (16). This pathway, involved in the sensing of foreign or damaged cytosolic DNA, triggers innate immune responses through the activation of a signaling cascade connecting the cytoplasmic DNA sensor cGAS, several signal transducers including STING and TBK1, and eventually transcription factors (mainly IRF3 and NFκB) that are collectively responsible for the induction of a type I interferon (IFN) response (16). Thus, processes that disrupt the nuclear DNA integrity and favor the translocation of DNA to the cytosol (either in a context of endogenous DNA repair deficiency, or through the use of exogenous DNA damaging agents), may activate cGAS/STING. For example, defects in homologous recombination genes (BRCA1/2 or FANCD2), and use of radiotherapy or S-phase-dependent chemotherapies have been associated with cell cycle-dependent formation of cytoplasmic chromatin fragments (CCF) and subsequent increase in STING signaling in breast cancer (17-20). Further, cGAS recently appeared to be an essential mediator of the anti-tumor effects of ICI (21), and identification of cGAS/STING activators has become an intense research area, with several ongoing phase 1 trials evaluating such molecules (22). As one of the main therapeutic challenges in immuno-oncology is currently to turn “cold, non-T-cell inflamed” tumors into “hot, T-cell inflamed” tumors, a better understanding of cellular contexts in which targeted therapies can activate the cGAS/STING pathway is key.

DDR-deficient cancers that present elevated genomic instability may represent a favorable environment for selective cGAS/STING activation. Excision repair cross-complementation group 1 (ERCC1) deficiency is the most frequent DDR defect in NSCLC and occurs in 30-50% of cases (23). The tumor suppressor protein BRCA1 is also frequently defective in triple-negative breast cancer (TNBC), either following germline mutation or somatic alteration (mutation or epigenetic silencing) (24, 25). Both ERCC1 and BRCA1 defects confer sensitivity to platinum-based therapy (26, 27) and PARP inhibitors (PARPi) (28, 29), and while PARPi have demonstrated their efficacy in advanced BRCA-deficient breast cancers (30), these
agents are also being clinically assessed in ERCC1-defective (platinum-sensitive) NSCLC (PIPSeN trial, NCT02679963). Therefore, ERCC1 deficiency represents an attractive candidate for harnessing cGAS/STING activation in NSCLC, where ICI have shown unprecedented efficacy, yet only in a small proportion of patients.

Here, we show that loss of ERCC1 in NSCLC leads to increased STING expression and constitutive activation of type I IFN signaling, which associates with enhanced T-cell infiltration in patient-derived samples. Using a unique combination of isogenic models of ERCC1-deficient NSCLC, BRCA1-deficient and PARP1-resistant TNBC, we find that multiple clinical PARPi generate cytosolic DNA in a cell cycle- and DDR defect-dependent fashion, as a result of an on-target effect of PARPi. This in turn activates cGAS/STING signaling and elicits specific tumor cells-intrinsic immune responses, including type I IFN response and CCL5 secretion. PARPi further synergize with IFNγ to induce cell-surface PD-L1 expression in NSCLC models, a phenotype that is specifically enhanced in ERCC1-deficient cells. Our data unveil an unexpected immunomodulatory potential of PARPi that could be therapeutically exploited to enhance ICI efficacy in ERCC1-deficient NSCLC patients.
Results

**ERCC1 deficiency in isogenic systems is associated with increased type I IFN signaling, cytokine signaling and lymphocytic infiltration in NSCLC.**

We hypothesized that the absence of key DNA repair tumor suppressor gene function, such as *ERCC1*, in tumor cells, might influence the molecular processes that control anti-tumor cell immune responses. To address this hypothesis in a relatively unbiased fashion, we first used RNA sequencing (RNA-Seq) to profile the transcriptome of isogenic ERCC1-defective and wildtype A549 NSCLC cells (31, 32). Briefly, this isogenic model was generated using zinc-finger targeting of *ERCC1*, and consists of one A549-ERCC1\textsuperscript{WT/WT} parental cell line, one *ERCC1*-heterozygous cell line (herein referred to as A549-ERCC1\textsuperscript{+/-}), and three *ERCC1*\textsuperscript{-/-} clones in which we reconfirmed no detectable levels of ERCC1 (*Figure 1A and 1B*), and that are characterized by exquisite sensitivity to cisplatin (31) – highlighting their clinical relevance (26) – and to PARPi (28) (*Supplementary Figure S1A*). Comparative transcriptomics of A549-ERCC1\textsuperscript{WT/WT} and one *ERCC1*\textsuperscript{+/-} clone (clone c216, referred to as A549-ERCC1\textsuperscript{+/-} below) revealed 1486 significantly differentially expressed genes (DEGs, *Figure 1C*). Consistent with the known functions of the ERCC1/XPF endonuclease in the resolution of stalled replication fork and subsequent appropriate cell cycle progression, Gene Set Enrichment Analysis (GSEA) using the REACTOME pathway database revealed a significant down-regulation of several DNA repair-, cell cycle-, and DNA replication-related pathways in A549-ERCC1\textsuperscript{+/-} cells (*Figure 1D, Supplementary Figure S1B*). This analysis also identified a significant enrichment of numerous immune-related pathways in A549-ERCC1\textsuperscript{+/-} cells – indeed, 24 immune-related pathways were found among the top 50 upregulated REACTOME terms (*Supplementary Figure S1C*) –, suggesting a role for ERCC1 in modulating the immune characteristics of NSCLC cells in a cell-autonomous fashion. Among these pathways, the most significantly enriched were type I and II IFN signaling, antigen presentation through class I major histocompatibility complex (MHC-I), innate immune system and cytokine signaling (*Figure 1D*). GSEA plots interpretation confirmed that type I IFN signaling was significantly enriched in ERCC1-deficient cells compared to isogenic wildtype cells (normalized enrichment
score (NES) = 2.11; false discovery rate (FDR) = 0.0057) (Figure 1E). Consistent with this enrichment, a refined analysis of the transcriptomic data also identified several IFNa/β/γ-inducible proteins as being upregulated in ERCC1-defective cells, including several members of the signal transducer and activator of transcription (STAT) family (Supplementary Figure S1D). Cytokine signaling was also found significantly enriched in the A549-ERCC1<sup>−−</sup> cell line (NES = 1.79; FDR = 0.0057) (Figure 1F), and consistently, we found an upregulation of several C-C-motif and C-X-C-motif chemokines in those cells (Supplementary Figure S1E). In particular, the chemotactic chemokines CCL2, CCL5, CXCL1, CXCL2, CXCL5, CXCL8 and CXCL10 were > 5-fold upregulated in A549-ERCC1<sup>−−</sup> cells (FDR < 0.05). Considering the critical immunogenic and lympho-attractant properties of these chemokines (33), this suggested that ERCC1 deficiency might contribute to the establishment of cancer-cell autonomous immunity in NSCLC cells. These findings were also observed in two independent A549-ERCC1<sup>−−</sup> clones (clone c295 and c375), which displayed similar significant enrichment in type I IFN signaling on RNA-Seq profiling (NES = 2.54, FDR = 0.0062 for clone 295 and NES = 2.79, FDR = 0.0063 for clone 375; Supplementary Figure S2A-D). As ERCC1-deficient NSCLC tumors still harbor residual levels of ERCC1, we sought to investigate which threshold of ERCC1 deficiency was sufficient to induce such type I IFN signature in vitro by using the ERCC1-heterozygous cell line of our isogenic model (A549-ERCC1<sup>++−</sup> cell line). Interestingly, type I IFN signature was also significantly enriched in this model, albeit to a lesser extent than in ERCC1<sup>−−</sup> clones (NES = 1.86; FDR = 0.0131; Supplementary Figure S2E and S2F). Whole exome sequencing of the isogenic clones did not identify mutations in any gene involved in immune signaling (28), suggesting the defect in ERCC1 as the most likely cause of the observed transcriptional dysregulation.

In other contexts, DDR defects have been linked to innate immune response and type I IFN signaling via cytosolic DNA sensing, notably involving the cGAS/STING pathway (16, 17). We hypothesized that loss of ERCC1 might modulate such signals. Although expression of STING, the major initiator of the cytosolic DNA sensing pathway, was almost undetectable by
western blotting in A549-ERCC1<sup>WT/WT</sup> and A549-ERCC1<sup>-/-</sup> cells, we found elevated STING protein expression in A549-ERCC1<sup>-/-</sup> cells (Figure 2A). Consistent with this, STING mRNA levels were > 2.5-fold increased in A549-ERCC1<sup>-/-</sup> cells (log<sub>2</sub> fold change (LFC) = 1.3769, FDR = 0.0009), compatible with a transcriptional mode of regulation. A similar upregulation of STING was also detected in the two other A549 ERCC1-deficient clones (Supplementary Figure S2G). In order to confirm that this observation was a primary effect of ERCC1 deficiency, we assessed STING expression in an isogenic cell line derived from the A549-ERCC1<sup>-/-</sup> clone in which the functional isoform of ERCC1 (isoform 202) had been stably reintroduced (herein referred to as “A549-ERCC1<sup>-/-</sup> + isoform 202”). Strikingly, re-expression of ERCC1 in this isogenic model led to loss of STING protein expression (Figure 2A), suggesting an ERCC1-dependent reversible modulation of STING expression. Of note, cGAS expression was also increased in A549-ERCC1<sup>-/-</sup> cells (LFC = 0.5336, FDR = 0.0098). As activation of cGAS/STING results in phosphorylation of TBK1, recruitment of IRF3 and eventually type I IFN genes expression (16), this suggested that the observed transcriptomic profile could be linked to STING activation in A549-ERCC1<sup>-/-</sup> cells.

In order to evaluate whether this enhanced cell-autonomous immune signaling could shape the tumor micro-environment, we estimated the extent of tumor-infiltrating lymphocytes (TILs) in a series of 55 human tumor samples derived from resected lung adenocarcinoma patients (stage I, II and IIIA). ERCC1 status in these tumors was evaluated by immunohistochemistry (IHC) as previously described (32) and TILs were assessed using a morphology-based coverage score. This analysis identified a statistically significant association between low ERCC1 expression and high levels of TILs ($P = 0.0265$, Mann-Whitney U test, Figure 2B and 2C).

Taken together, these data suggest that ERCC1 loss results in a constitutive and cell-autonomous increase of STING expression and type I IFN signaling in NSCLC models <em>in vitro</em>. 
These observations are consistent with enhanced tumor lymphocytic infiltration in vivo in human NSCLC adenocarcinoma samples.

**PARPi generate CCF in an ERCC1 defect- and cell cycle-dependent manner**

As previously reported (28), A549-ERCC1\(^{−/−}\) cells display enhanced sensitivity to PARPi when compared to A549-ERCC1\(^{WT/WT}\) cells, an effect that we reconfirmed in short-term survival assays (Figure 3A). We further hypothesized that PARPi, which do not only inhibit the catalytic activity of PARP1, but also trap PARP1 onto the DNA, causing stalled replication forks and subsequent double-strand breaks (DSBs) (34), might specifically favor the formation of CCF and in turn trigger cGAS/STING signaling in ERCC1-defective tumor cells.

To test this hypothesis, we exposed A549-ERCC1 isogenic cell lines to increasing concentrations of two different clinical PARPi, rucaparib and olaparib, and monitored the presence of CCF using immunofluorescence. For each PARPi, we selected concentrations surrounding the SF50 of the parental cell line (dose generating 50% cell survival in short-term assays, after 5 days of exposure to the drug); we therefore started with a dose corresponding to the SF80 of the wildtype cell line and applied a 2-fold increase at each subsequent dose to determine the final dose-range. Although the number of CCF in A549-ERCC1\(^{WT/WT}\), A549-ERCC1\(^{+/-}\), A549-ERCC1\(^{-/-}\) and A549-ERCC1\(^{-/-}\) + isoform 202 cells was not significantly different in the absence of PARPi (Supplementary Figure S3A), we observed a dose-dependent increase of CCF number upon PARPi exposure, an effect that was significantly enhanced in A549-ERCC1\(^{-/-}\) and A549-ERCC1\(^{+/-}\) models compared to A549-ERCC1\(^{WT/WT}\) cells (Figure 3B and 3C; > 6-fold difference at 10 μM olaparib, \(P=0.0016\); > 4-fold difference at 5 μM olaparib, \(P=0.03\); two-way ANOVA, post hoc Sidak’s test), but not in A549-ERCC1\(^{-/-}\) + isoform 202 cells. These findings were further validated in the independent H1975-ERCC1 isogenic NSCLC model: in these cells, ERCC1 loss caused increased CCF levels in the absence of treatment (Supplementary Figure S3A; 2.5-fold increase, \(P=0.0035\); Welch’s t-
test), a phenotype that was enhanced upon PARPi exposure (Supplementary Figure S3B and S3C).

BRCA1-defective tumor cells are also profoundly sensitive to PARPi (29). We therefore assessed whether the effects seen in ERCC1-defective cells could be reproduced in BRCA1-defective cells. To do this, we used recently described isogenic series derived from the BRCA1-mutant TNBC SUM149 cell line (35, 36). This isogenic series consists of three cell lines (Figure 4A): one BRCA1-mutant parental line (SUM149-BRCA1mut), one BRCA1-mutant reverted line which is PARPi-resistant following restoration of the native BRCA1 reading frame and functionality (35) (SUM149-BRCA1rev), and one SUM149 clone with PARPi resistance caused by loss of PARP1 (36) (SUM149-PARP1−/−). In the absence of PARPi, SUM149-BRCA1mut cells displayed a significantly higher number of CCF compared to SUM149-BRCA1rev cells (> 8-fold increase, P=0.0233, Welch’s t-test; Supplementary Figure S3A). Upon PARPi exposure, we observed a dose-dependent increase of CCF number in SUM149-BRCA1mut cells, but not in SUM149-PARP1−/− cells, confirming the on-target effect of PARPi in triggering this phenotype (Figure 4B and 4C); SUM149-BRCA1rev cells displayed an intermediate phenotype (Figure 4C), confirming that the generation of CCF was a BRCA1-dependent process. As PARPi have been reported to modulate histone PARylation and chromatin structure (37), thereby potentially promoting transcription of retro-elements, we further confirmed the presence of chromatin (i.e. histone-bound DNA as opposed to naked DNA) by assessing the presence of histone H3 in cytoplasmic fractions. This demonstrated increased H3 levels after PARPi exposure in SUM149-BRCA1mut cells, but not in SUM149-PARP1−/− cells, thus confirming respectively the presence of cytoplasmic chromatin and the on-target effect of PARPi (Figure 4D). Consistent with this finding, assessment of H3K27me3 by immunofluorescence in the A549-ERCC1 isogenic cells revealed a co-localization of this heterochromatin marker with CCF in PARPi-exposed cells (Supplementary Figure S3D).
As PARPi cause S-phase-dependent DNA damage, we hypothesized that the observed CCF might, at least in part, be micronuclei (19, 20). Micronuclei are signs of genomic or chromosomal instability which have two main characteristics: (i) their formation is cell cycle-dependent – as they arise during anaphase from lagging chromosomes or chromatid bridges following unresolved DNA lesions; (ii) they contain heterochromatin, initially surrounded by a fragile lamin B1 membrane that is eventually easily ruptured (19). We therefore first assessed the formation of CCF in the presence of PARPi and upon cell cycle blockade in A549-ERCC1WT/WT cells, using three different cell cycle inhibitors: (i) the CDK1 inhibitor (CDK1i) RO-3306; (ii) hydroxyurea and (iii) 5-fluorouracil. All three cell cycle inhibitors caused a complete abrogation of the PARPi-induced generation of CCF, supporting that their formation was cell cycle-dependent (Figure 4E; Supplementary Figure S3E and S3F). Similar observations were made in the SUM149-BRCA1mut cell line (Figure 4F). Immunofluorescence analysis of the co-staining of H3K27me3 and lamin B1 in the A549-ERCC1 isogenic model revealed some typical micronuclei structures (Supplementary Figure S3D), although the fragile lamin B1 envelope was not detectable around all Picogreen/H3K27me3 co-localizing foci. Automated quantification of micronuclei revealed baseline patterns similar to that of CCF (Supplementary Figure S3A), as well as a consistent dose-dependent formation of micronuclei upon PARPi exposure, both in ERCC1- and BRCA1-isogenic models (Supplementary Figure S3G and S3H). Consistent with our previous observations (Figure 3C and 4C), this effect was more pronounced in A549-ERCC1+ (Supplementary Figure S3G) and SUM149-BRCA1mut cells (Supplementary Figure S3H), and abrogated in the SUM149-PARP1+ model.

This data support the hypothesis that PARPi exposure promotes the accumulation of CCF, some of which have micronuclei characteristics. This phenotype results from an on-target effect of PARPi, is cell cycle-dependent and is enhanced in ERCC1-defective NSCLC cells and other PARPi-sensitive models such as BRCA1-defective TNBC.
PARPi-induced CCF activate cGAS/STING signaling in ERCC1-deficient NSCLC cells

To investigate the potential of PARPi-induced CCF to activate cGAS/STING signaling, we assessed the ability of cGAS to form foci through relocalization to CCF. Immunofluorescent staining of cGAS in A549-ERCC1 isogenic cell lines exposed to increasing concentrations of rucaparib or olaparib revealed a significant dose-dependent increase in the number of cytoplasmic cGAS foci in A549-ERCC1<sup>−/−</sup> and A549-ERCC1<sup>+/−</sup> cells, but not in A549-ERCC1<sup>WT/WT</sup> cells (Figure 5A and 5B; \( P=0.0122, P=0.0123 \) and \( P=0.1657 \) respectively; Kruskal-Wallis test). Similar results were obtained in the independent H1975-ERCC1 isogenic NSCLC model (Supplementary Figure S4A and S4B). Increased formation of cytoplasmic cGAS foci was also detected in SUM149-BRCA1<sub>mut</sub> cells after PARPi exposure, at a higher level than in SUM149-BRCA1<sub>rev</sub> cells (1.4-fold difference at 20 μM olaparib, \( P=0.0033 \); two-way ANOVA post hoc Sidak’s test) (Figure 5C and 5D). More importantly, no increase in cytoplasmic cGAS foci was detected in the PARPi-resistant SUM149-PARP1<sup>−/−</sup> cell line, supporting that the minimal levels of CCF measured in this cell line did not trigger cGAS relocalization. Analysis of cGAS staining intensity within CCF revealed a significant increase in cGAS foci/CCF co-localization upon PARPi exposure in both NSCLC ERCC1 isogenic models (Figure 5E and Supplementary Figure S4C; \( P < 0.0001 \), Kruskal-Wallis test), thus confirming that cGAS relocalized to CCF following PARPi exposure.

We then investigated whether the detection of CCF by cGAS could activate STING signaling. Analysis of phosphorylated TBK1 (pTBK1, a key downstream effector of STING signaling) revealed a dose-dependent increase upon PARPi exposure in A549-ERCC1<sup>−/−</sup> and A549-ERCC1<sup>+/−</sup> models, but not A549-ERCC1<sup>WT/WT</sup> cells (Figure 6A, Supplementary Figure S4D). Consistent results were observed in the H1975-ERCC1 isogenic model, where an increase of pTBK1 was detected upon PARPi exposure, which was more pronounced in the H1975-ERCC1<sup>−/−</sup> cells (Figure 6B and Supplementary Figure S4E). This suggested the ERCC1-dependency of PARPi-induced TBK1 phosphorylation. Furthermore, while silencing of either cGAS or STING only moderately decreased PARPi-induced TBK1 phosphorylation in A549-
ERCC1<sup>WT</sup> cells (Supplementary Figure S4F), simultaneous silencing of both sensors allowed complete abrogation of pTBK1, as observed in H1975-ERCC1<sup>WT/WT</sup> cells (Figure 6C). We then evaluated pTBK1 in the SUM149-BRCA1 isogenic TNBC model. Consistent with results obtained in the NSCLC models, we found a dose-dependent increase of pTBK1 upon PARPi exposure in SUM149-BRCA1<sub>mut</sub> but not SUM149-BRCA1<sub>rev</sub> cells (Supplementary Figure S4G). A corresponding increase in phosphorylation of several transcription factors involved in the final steps of STING signaling cascade, including p65-NFκB, IRF3 and IRF7, confirmed the activation of the pathway in SUM149-BRCA1<sub>mut</sub> cells. (Supplementary Figure S4G).

Because CCF can also be detected by alternative STING-independent pattern recognition receptors (PRR), namely the Toll-like receptors (TLR) and RIG-1-like receptors (RLR) pathways, we monitored the activation of those pathways after PARPi exposure. No activation of the TLR or RLR effectors could be detected upon PARPi exposure, (Supplementary Figure S4H and S4I), suggesting that the detection of CCF following PARP inhibition is relatively specific to the cGAS/STING pathway.

Because our previous observations showed cell cycle-dependency of CCF formation (Figure 4E and 4F), we monitored TBK1 phosphorylation upon cell cycle blockade by CDK1i. Although CDK1i itself appeared to increase baseline pTBK1, we could detect an abrogation of the previously observed increase in TBK1 phosphorylation upon PARPi exposure (Figure 6D). Collectively, these results support the notion that PARPi-induced CCF activate cGAS/STING signaling, and that this effect relies on the presence of specific DNA repair defects.

**PARPi promote secretion of CCL5 and activate type I IFN signaling**

Increased production of the chemotactic chemokine CCL5 has been previously reported in the context of DDR deficiency and following treatment with S-phase dependent DNA damaging agents or ionizing radiation (17, 19, 20). We therefore hypothesized that PARPi might enhance CCL5 production, especially in ERCC1-deficient cells. RT-qPCR analyses of CCL5 mRNAs
revealed a dose-dependent activation of CCL5 transcription in both A549-ERCC1<sup>+/−</sup> and A549-ERCC1<sup>WT/WT</sup> cells following PARPi exposure (Figure 7A), while baseline expression levels were 12-fold higher in A549-ERCC1<sup>+/−</sup> cells compared to A549-ERCC1<sup>WT/WT</sup> cells (Figure 7B). Consistent with these observations, quantification of CCL5 protein concentration in cell supernatants using enzyme-linked immunosorbent assay (ELISA) revealed a similar dose-dependent increase of CCL5 secretion upon PARPi exposure in A549-ERCC1<sup>+/−</sup> but not A549-ERCC1<sup>WT/WT</sup>, A549-ERCC1<sup>+/+</sup> or A549-ERCC1<sup>+/−</sup> + isoform 202 cells (Figure 7C). Depletion of STING and cGAS by siRNA resulted in a suppression of CCL5 transcription and secretion, suggesting the involvement of cGAS/STING in this process (Figure 7A-C). Similarly, SUM149-BRCA1<sub>mut</sub> cells, but not SUM149-BRCA1<sub>rev</sub> cells, displayed enhanced expression and secretion of CCL5 following treatment with olaparib, which was reduced upon co-depletion of STING and cGAS (Supplementary Figure S5A and S5B).

We next evaluated whether the production of other major chemokines was upregulated by PARPi exposure. Although a significant increase in IFNB1 transcription was observed in SUM149-BRCA1<sub>mut</sub> cells exposed to olaparib (Supplementary Figure S5C), no increase in the corresponding secreted protein could be detected in cell supernatants (Supplementary Figure S5D). Similarly, no secretion of IFNγ or TNFα could be detected after olaparib exposure in this cell line (Supplementary Figure S5E).

To comprehensively characterize cell-autonomous immunomodulation induced by PARPi, we performed RNA-Seq on A549-ERCC1<sup>WT/WT</sup> and A549-ERCC1<sup>+/−</sup> cells exposed to talazoparib, the most potent and specific clinical PARPi. This demonstrated a significant upregulation of a number of genes involved in type I IFN signaling (Figure 7D and 7E) which was associated with a significant enrichment of the type I IFN signature in A549-ERCC1<sup>+/−</sup> cells (NES = 2.12; FDR = 0.0018). Although this upregulation was observed in both A549-ERCC1<sup>+/−</sup> and A549-ERCC1<sup>WT/WT</sup> cells, the corresponding enrichment was more modest in wildtype A549 cells (NES = 1.64; FDR = 0.0314), highlighting the role of ERCC1 deficiency in potentiating this
phenotype. This data support the hypothesis that PARPi specifically activate, in a cell-autonomous fashion in NSCLC cells, a type I IFN immune response that is enhanced in the context of ERCC1 deficiency, and can be linked to the secretion of CCL5 in our models.

**PARPi synergize with IFNγ to induce cell-surface PD-L1 expression**

PD-L1 is a major immune checkpoint protein expressed by tumor cells to evade immunosurveillance. PD-L1 induction is classically triggered by IFNγ which mediates the activation of type II IFN signaling cascade (38), but recent data suggest that platinum-based chemotherapies (39) as well as some PARPi (40) can also transiently enhance PD-L1 expression. Although the underlying mechanisms explaining these effects are largely unknown, activation of PD-L1 expression following S-phase-specific DNA damage has been associated with cGAS/STING recruitment (17). Because our previous data suggested mostly activation of type I IFN signaling through cGAS/STING, we questioned whether PARPi could synergize with IFNγ to induce PD-L1 expression, and whether ERCC1 status would influence this modulation. We exposed A549-ERCC1 isogenic and H1975-ERCC1 isogenic cell lines to PARPi and/or IFNγ, and monitored cell-surface PD-L1 expression. Consistent with the published literature (40), we observed a significant dose-dependent upregulation of PD-L1 expression after treatment with eitherrucaparib, talazoparib or niraparib as monotherapy in both models (**Figure 8A and 8B**; **Supplementary Figure S6A**). Interestingly, co-treatment of PARPi plus IFNγ was synergistic and could potentiate the induction of PD-L1 expression (**Figure 8A and 8B**). This induction was more profound in A549-ERCC1−/− and H1975-ERCC1−/− models when compared to their ERCC1-proficient counterparts, both at cell-surface and at the transcriptional level (**Figure 8C and 8D**). To assess the specificity of the observed effect, we evaluated the membrane expression of Toll-like receptor 4, an immune-related cell-surface marker which has been described to be positively correlated with PD-L1 expression in NSCLC samples (41). No TLR4 induction could be detected under similar conditions, suggesting that PD-L1 induction may be relatively specific (**Supplementary Figure S6B**). We finally confirmed that the observed PD-L1 induction resulted from an on-target effect of PARPi
using the SUM149-PARP1 isogenic model: although a significant synergy between talazoparib and IFNγ could be observed in the parental SUM149-BRCA1\textsubscript{mut} cells, this effect was completely abrogated in SUM149-PARP1\textsuperscript{−/−} cells, where only IFNγ could induce cell-surface PD-L1 (Figure 8E).

To explore this interaction between PARPi and type II IFN signaling, we assessed whether PARPi could modulate the activation of signal transducer and activator of transcription 1 (STAT1), a key protein in the IFNγ signaling pathway which mediates PD-L1 induction. Although exposure to PARPi monotherapy did not induce STAT1 phosphorylation, an enhanced STAT1 phosphorylation was detected following combination treatment of PARPi plus IFNγ, compared to IFNγ alone (Supplementary Figure S6C). Interestingly, this effect was abrogated upon cell cycle blockade with the CDK1i RO-3306, suggesting that progression through the cell cycle is required for the potentiation of STAT signaling by PARPi.

We next sought to assess whether these mechanisms would also operate in patients and exposed fresh cells from a patient’s NSCLC pleural effusion sample to PARPi and IFNγ. After selection of EpCAM+ cells, we quantified PD-L1 cell-surface expression by flow cytometry. This revealed a similar PD-L1 induction upon PARPi exposure, which was again potentiated by IFNγ, consistent with our in vitro findings (Figure 9A).

As PARPi have a dual mechanism of action (i.e. inhibition of the PARylation catalytic activity of PARP1 and PARP1 trapping), we also sought to evaluate whether PARylation levels or PARP1 expression would correlate with PD-L1 expression in human tumor samples. We performed immunostaining of PAR, PARP1 and PD-L1 in a series of 49 resected stage I/II NSCLC samples. No correlation was found between PARP1 expression and PD-L1 staining (Supplementary Figure S6E and S6F). A significant inverse correlation was found between PAR and PD-L1 levels on tumor cells (mean PARylation H-score of 100 in PD-L1-low vs 60 in PD-L1-high tumor cells; \(P = 0.003\); Mann-Whitney \(U\) test; Figure 9B and 9C) but not PD-L1
expression on immune cells (Supplementary Figure S6D), consistent with a previous report in breast cancer specimens (40).

A proposed model to explain cGAS/STING activation following PARPi exposure in tumor cells with ERCC1-deficiency

Although several scenarios may explain our findings, we propose the following model which is consistent with our observations (Figure 10): ERCC1^{WT/WT} cells adequately repair endogenous DNA lesions. PARPi exposure causes DNA damage, mostly initiated by PARP1 itself trapped onto the DNA at sites of spontaneous single-strand breaks. Trapped-PARP1 generates stalled replication forks and subsequent DSBs during DNA replication. In ERCC1^{WT/WT} cells, trapped-PARP1 lesions can be adequately excised, a process in which ERCC1 is thought to play a major role (28). Following excision, repair of DSBs occurs through homologous recombination (HR), and replication restarts. Residual unrepaired lesions cause minimal formation of CCF, which are insufficient to trigger cGAS/STING signaling (Figure 10A-D). By contrast, ERCC1^{-/-} cells are exposed to increased levels of endogenous DNA damage, which are further enhanced upon PARPi exposure. Trapped-PARP1 lesions cannot be appropriately resolved in the absence of ERCC1 (28), which prevents the subsequent processing of DSBs through HR (Figure 10E-G). This results in the accumulation of stalled replication forks, subsequent DSBs and unrepaired DNA lesions which eventually causes increased micronuclei formation and CCF generation (Figure 10G). These are detected by cGAS, which activates the STING/pTBK1 cascade and results in the transcription of type I IFN genes, including CCL5 (Figure 10H).
Discussion

DNA repair deficiency is a hallmark of cancer which has been therapeutically exploited for more than fifty years using DNA damaging cytotoxic chemotherapies, and more recently with the advent of targeted DNA repair inhibitors, such as PARPi, using synthetic lethality. Here, we show that multiple clinical PARPi can trigger the activation of innate immune pathways in a cell-autonomous fashion in vitro. By studying multiple isogenic models of DDR deficiency or PARPi resistance, and using fresh and archived tumor samples, we find that PARPi can elicit a cGAS/STING/pTBK1/pIRF3/type I IFN response in cancer cells. Importantly, this response is exacerbated in ERCC1-deficient NSCLC contexts. We further show that ERCC1 deficiency and PARPi are synergistic with IFNγ in inducing PD-L1 expression (Figure 8 and 9), thereby providing a rationale for combination strategies between PARPi and anti-PD-(L)1 therapies in appropriately molecularly-selected populations.

Despite significant improvements in outcome brought by the advent of anti-PD-(L)1 therapies, NSCLC still represents the leading cause of cancer-related death. Only a minority of patients currently benefit from ICI, and strategies to turn “cold” tumors into “hot” tumors are actively investigated. Interestingly, high TILs levels correlate with improved survival following neoadjuvant therapy in TNBC (42) – an observation that was also made in some NSCLC series in the adjuvant setting (43). In line with our findings, preclinical data evaluating rucaparib in combination with anti-PD-(L)1 in syngeneic models of ovarian cancer have suggested a possible role for PARPi in promoting tumor lymphocytic infiltration, and increased benefit compared to anti-PD-(L)1 monotherapies in BRCA1-mutated tumors (44). Our observation that PARPi trigger cGAS/STING signaling (Figure 5 and 6) and favor secretion of lympho-attractant chemokines such as CCL5 in ERCC1-deficient NSCLC (Figure 7) suggests that PARPi could be used as immunomodulatory agents to trigger lymphocytic infiltration in this histotype. Conversely, adding anti-PD-(L)1 therapies to PARPi might allow overcoming immune escape mechanisms resulting from PARPi-induced PD-L1 upregulation on tumor cells. Importantly, recent data from the large randomized double-bind phase III study Keynote
189 evaluating the combination of pembrolizumab and platinum-based chemotherapy in first-line metastatic NSCLC have reported impressive benefits in progression-free and overall survival (HR for death = 0.49; 95% CI, 0.38 to 0.64; P<0.001) (1). These results were practice-changing, setting up the frame for recommending anti-PD-(L)1 therapy in combination with a platinum-based doublet chemotherapy in first-line advanced NSCLC. Interestingly and contrary to previous studies evaluating anti-PD-(L)1 monotherapy, this benefit was observed across all subgroups and was independent from baseline PD-L1 tumor expression score, suggesting that other tumor characteristics – such as the DDR status – might play a role in response to this combination. Because platinum-sensitivity is a relevant phenotypical biomarker of sensitivity to PARPi (27), this suggests that PARPi – which are much better tolerated and do not have severe bone marrow toxicity – might represent an interesting alternative or complement (in the maintenance setting) to platinum-based chemotherapy in combination with anti-PD-(L)1 agents in NSCLC. The development of clinical trials addressing this question is underway.

TMB is an important determinant of response to ICI (9, 10). While various DDR defects have been shown to critically contribute to the accumulation of mutations in the tumor genome, only some of them have proven their predictive value as biomarkers of response to anti-PD-(L)1 therapy (6, 45). ERCC1 deficiency has been associated with increased mutation frequency and genomic instability in mice (46), an observation that is consistent with the known roles of ERCC1 in processes that ensure chromosomal stability and maintenance of genome integrity, such as the interstrand crosslink DNA repair pathway (47) and the resolution of Holliday junction (48). However, no formal studies have yet been conducted to assess whether ERCC1 status might influence TMB in NSCLC. This would be best addressed by interrogating large datasets (e.g. TCGA) for an inverse correlation between ERCC1 isoform 202 expression and TMB. Furthermore, whether ERCC1 status itself represents an independent predictive biomarker of response to anti-PD-(L)1 in NSCLC is currently unknown and deserves further investigation.
Although our study focused on ERCC1 – the most frequent DDR deficiency in NSCLC –, we can hypothesize that our findings may be applicable to other DDR defects detected at a lower frequency in NSCLC. For example, BRCA1, BRCA2, ATM or MSH2 (mutated in 5%, 6%, 9% and 3% of NSCLC cases, respectively (49)) have also been associated with PARPi sensitivity and type I IFN signature (50-53). Interestingly, Teo et al. recently reported that somatic DDR alterations that were associated with improved clinical outcomes in platinum-treated patients with advanced urothelial carcinoma (54), also correlated with longer progression-free survival and overall survival upon anti-PD-(L)1 therapy (15). Authors reported that 25% of patients presented tumors with alterations in DDR genes, the most commonly altered genes being ATM, POLE, BRCA2, ERCC2, FANCA and MSH6. Overall, these observations suggest that the interplay between DDR deficiency, platinum- or PARPi-sensitivity and anti-cancer immune response operates in several tumor types and can involve multiple DDR defects.

cGAS is a very potent sensor of almost any form of cytoplasmic DNA. It can detect free DNA, arising from reverse-transcription of endogenous retroviruses (55) or mitochondrial DNA instability (56), and micronuclei, which arise from genomic instability and display chromatin-like structures, sometimes surrounded by a fragile lamin B1 membrane (19). Our data provide evidence that clinical PARPi can induce cytosolic DNA (Figure 3 and 4), which is at least in part in the form of micronuclei, and subsequently activates cGAS/STING signaling (Figure 5 and 6). In particular, our observation that these effects are abrogated in PARP1-null cells supports a direct on-target effect of these drugs, which is mostly mediated by their PARP-trapping potential (36). Furthermore, our use of several specific isogenic models demonstrates that this effect is enhanced in several DNA repair-deficient contexts, notably ERCC1- and BRCA1-deficiency. This extends and is consistent with previous publications, which reported activation of cGAS/STING by micronuclei either following ionizing radiation (19, 20) or S-phase-specific chemotherapies (17, 18), and further described PARPi-mediated induction of IFN signals (notably increased STAT1 phosphorylation) in BRCA1-deficient contexts (20). Importantly, our observation that the immune phenotype of the ERCC1-heterozygous model
closely resembles that of the ERCC1<sup>+</sup> clones suggests that our findings may be clinically relevant for NSCLC, where some residual expression of ERCC1 is usually retained.

In conclusion, we provide a scientific rationale for assessing PARPi in combination with anti-PD-(L)1 therapy in molecularly-selected DNA repair-deficient populations. While several clinical trials are currently combining PARPi with anti-PD-(L)1 in BRCA1/2-mutant breast or ovarian cancers, other histologies, such as NSCLC in which anti-PD-(L)1 therapies have already shown impressive results, might also benefit from such association. Basket clinical trials will be launched soon to explore this question.
Materials and Methods

Study design

The aim of this study was to assess the immunomodulatory potential of PARPi, their ability to trigger specific cancer cells-associated immune responses, and their potential to enhance cancer cells immunogenicity through cell-autonomous mechanisms. Our pre-specified hypothesis was that PARPi might have intrinsic immunomodulatory properties capable of activating immune signaling in a cell-autonomous fashion in cancer cells, specifically in those harboring DDR defects. Hence, we used a unique combination of in vitro isogenic systems of DDR-deficient and PARPi-sensitive or -resistant NSCLC and TNBC. All laboratory experiments were performed in at least three independent biological replicates to allow appropriate statistical calculations, using multiple well-characterized models and several PARPi evaluated in dose-response at clinically relevant concentrations. RNA-Seq was performed to generate at least 20M reads per sample. For each condition, three biological replicates were included in the RNA-Seq analysis. Most significant findings were assessed in retrospectively analyzed archived patient tumor samples as well as one fresh prospectively collected pleural effusion.

Cell lines

SUM149, A549 and H1975 cells were obtained from ATCC. The generation of SUM149-BRCA1rev and SUM149-PARP1 - secondary mutant cell lines was performed using CRISPR-Cas9 site directed mutagenesis, as previously described (35, 36). The secondary mutant cell lines A549-ERCC1- and H1975-ERCC1- were generated using zinc finger nuclease gene targeting, as described previously (31). SUM149 cells were cultured in Ham's F12 nutrient mixture with 10% fetal bovine serum (FBS), 1µg/mL insulin and 500ng/mL hydrocortisone. A549 and H1975 cells were cultured respectively with high glucose Dulbecco’s Modified Eagle’s medium and Roswell Park Memorial Institute 1640 medium with 10% fetal bovine serum. The cells were cultured under physiological conditions at 37°C and 5% CO₂. All cell lines were short tandem repeat typed (STR typed) using StemElite ID (Promega, Madison,
WI, USA) to confirm identity prior to the study, and verified for mycoplasma contamination every 2 months using MycoAlert (Lonza, Basel, Switzerland).

**Drugs and chemicals**

PARPi olaparib (AZD-2281, Astra Zeneca), rucaparib (PF-01367338, Clovis Oncology), and talazoparib (BMN-673, Pfizer) were purchased from Selleck Chemicals (Houston, TX, USA). Niraparib (MK-4827, Tesaro) was obtained from MedChemexpress (Monmouth Junction, NJ, USA). Inhibitor stock solutions were prepared in DMSO and stored in aliquots at -80°C. Hydroxyurea (HU), 5-Fluorouracil (5FU) and the CDK1 inhibitor RO-3306 were obtained from Sigma-Aldrich (Gillingham, UK). Interferon gamma-1b (IMUKIN, Boehringer Ingelheim) was purchased from Boehringer (Ingelheim am Rhein, Germany).

**Immunofluorescence and image analysis**

Immunofluorescence assays were performed in 96-well plates. Cells were fixed in 4% paraformaldehyde (PFA) in PBS during 20 min at RT, washed twice with PBS, and permeabilized with 0.5% Triton X-100 in PBS for 10 min. After two additional washes, cells were blocked with 2% BSA, 2% FBS in PBS (IFF) for 1 h at RT. Cells were then incubated with primary antibodies in IFF at 4°C overnight. The cells were then washed three times with PBS, each for 10 min, followed by incubation with Alexa Fluor 594-conjugated rabbit and Alexa Fluor 647-conjugated mouse secondary antibodies (Thermo Fisher Scientific), 1 μg/ml DAPI and 1:400 PicoGreen (Thermo Fisher Scientific) in IFF for 1 h at RT. After that, cells were washed again three times with PBS, and 100 μL PBS was finally added to each well prior to imaging. Plates were imaged using an Operetta high-content imaging system (PerkinElmer). Quantification of the number of CCF, micronuclei, and cGAS foci was performed under identical microscopy settings between samples, using the Columbus image analysis system (PerkinElmer). 25 randomly selected fields containing over 200 cells were analysed within three individual replicates for each sample.
**Secreted cytokines detection**

Detection of secreted cytokines in cell supernatants was performed through ELISA detection. Culture media were collected after 72 h culture, and cell numbers counted for normalization. The media were dispensed in 96-well V-bottomed plates and centrifuged at 500g for 5 min to pellet cells and debris. The resulting supernatants were used for ELISA detection with the following kits: Human CCL5 ELISA MAX™ Deluxe kit (#440806), Human IFNγ ELISA MAX™ Standard kit (#430103) and Human TNFα ELISA MAX™ Deluxe (#430206) from BioLegend; Human IFNβ ELISA kit (#41410-1) from PBL Assay Science (Piscataway, NJ, USA). Assays were performed in four replicates following the manufacturers’ protocols. Absorbance was evaluated using a Victor multilabel plate reader (PerkinElmer).

**Flow cytometry analyses**

Cells were detached using Versene solution (Thermo Fisher Scientific) and transferred to round-bottom fluorescence-activated cell sorter (FACS) tubes. After 5 min centrifugation at 500g, the cells were washed with PBS and incubated at 4°C for 30 min with primary antibodies, diluted in 5% BSA in PBS. The supernatants were removed after centrifugation, and the cells were washed again twice with PBS. The pellets were finally diluted in 250 μL PBS containing 1 µg/ml propidium iodide (PI) prior to analysis. Cell-surface expression of PD-L1 and TLR-4 was quantified and analysed by flow cytometry on an LSR-II flow cytometer (BD Biosciences). PI was used as a viability marker. Data analysis was performed using the FlowJo software package.

**Statistical analyses**

No statistical methods were used to predetermine sample size. The experiments were not randomized. The investigators were not blinded to allocation during experiments and outcome assessment. All bar graphs show mean values with error bars (standard deviation, SD); 95% confidence intervals were used, and significance was considered when * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$; ns, not significant.
Study approval
No specific approval was required for this study as no experiments were conducted in animals or humans. Informed consent was obtained from patients for the collection and study of pleural effusion samples.

Data availability
All RNA-seq data sets generated as part of this study are publicly available at the European Nucleotide Archive (https://www.ebi.ac.uk/ena), under the accession number PRJEB30090.

Additional Material and Methods are available in Supplementary Material.
Authors’ contribution

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References


Figure 1. Loss of ERCC1 results in increased type I IFN and cytokine signaling in NSCLC models in vitro. A, Schematic of the generation of ERCC1-deficient clones from the parental NSCLC cell line A549. Full procedures are detailed in Friboulet et al. (31) B, Western blot showing expression of ERCC1 in the parental (ERCC1WT/WT), heterozygous (ERCC1+/−), and ERCC1-knock-out clones (c216, c295 and c375). C, Heatmap displaying all significantly DEGs in A549-ERCC1−/− cells compared with A549-ERCC1WT/WT, determined by RNA-Seq. N = 3; Heatmap scale is a Z score. Threshold for ERCC1+− Top upregulated pathways
ERCC1−/− Top downregulated pathways
differential expression was $|\text{LFC}| > 1$ and threshold for significance was FDR < 0.05. D, GSEA of REACTOME pathways in A549-ERCC1\textsuperscript{−/−} compared with A549-ERCC1\textsuperscript{WT/WT} cells. Red, top 10 upregulated REACTOME pathways in A549-ERCC1\textsuperscript{−/−} cells; Yellow, top 10 downregulated REACTOME pathways A549-ERCC1\textsuperscript{−/−} cells. All pathways displayed are FDR < 0.05. AP Folding*: Antigen Presentation Folding Assembly; Capped Intron*: Capped Intron Containing Pre-mRNA. E, GSEA of the REACTOME pathway Interferon Alpha Beta Signaling, and associated heatmap showing the genes of the pathway, ranked by FDR. $N = 3$; Heatmap scale is a Z score. Purple, significantly DEGs with FDR < 0.05 and $|\text{LFC}| > 1$; Grey, non-significantly DEGs. F, GSEA of the REACTOME pathway Cytokine Signaling in Immune System, and associated heatmap showing the genes of the pathway, ranked by FDR. $N = 3$; Heatmap scale is a Z score. Purple, significantly DEGs with FDR < 0.05 and $|\text{LFC}| > 1$; Grey, non-significantly DEGs. Abbreviations: DEGs, Differentially Expressed Genes; FDR, False Discovery Rate; GSEA, Gene Set Enrichment Analysis; LFC, $\log_2$ (fold-change); NES, Normalized Enrichment Score; RNA-Seq, RNA sequencing.
Figure 2. Loss of ERCC1 associates with increased STING expression in vitro and enhanced lymphocytic infiltration in human NSCLC samples. A, Western blot illustrating ERCC1 and STING expression in A549-ERCC1<sup>WT/WT</sup>, A549-ERCC1<sup>+/−</sup>, A549-ERCC1<sup>−/−</sup> and A549-ERCC1<sup>−/−</sup> + isoform 202 isogenic cell lines. B, Scatter box plots of ERCC1 protein expression (assessed by IHC staining) and the percentage of TILs (assessed through morphology) in a series of resected human NSCLC adenocarcinoma samples (n = 55). Mann-Whitney U test. C, Representative images of ERCC1 and Haematoxylin-Eosin stainings in two surgical specimens of resected lung adenocarcinoma. Case A displays low ERCC1 staining in tumor cells and high stromal TIL density; Case B displays high ERCC1 staining in tumor cells and low stromal TIL density. Scale bar: 50 μm. Abbreviations: IHC, Immunohistochemistry; SD, Standard Deviation; TILs, Tumor-Infiltrating Lymphocytes.
Figure 3. PARPi generate cytoplasmic chromatin fragments in an ERCC1-dependent manner in NSCLC cells. 

A, Assessment of Ola cytotoxicity in A549-ERCC1^{WT/WT} vs A549-ERCC1^{+/+}, A549-ERCC1^{+/-} and A549-ERCC1^{-/-} + isoform 202 cell lines. Cells were treated with a dose range of Ola and continuously exposed to the drug for 5 days. Shown are dose-response curves displaying surviving fractions; Mean ± SD, N = 4.

B, Representative immunofluorescence images of DMSO-, Ruca- and Ola-exposed A549-ERCC1^{WT/WT} and A549-ERCC1^{+/-} cells. Blue, DAPI; Red, α-Tubulin; Green, PicoGreen. Cells were exposed to 15 μM Ruca or 40 μM Ola during 72h. White arrows, CCF; Yellow arrows, micronuclei. Scale bar, 20 μm.

C, Automated quantification of CCF in A549-ERCC1 isogenic cells exposed to increasing doses of Ruca or Ola (μM). Shown are CCF number per cell normalized to DMSO. Mean ± SD, N = 3, Kruskal-Wallis test and post hoc Dunn’s test, relative to DMSO control.
Results shown are representative of 2 experiments performed with similar results. Abbreviations: Ola, olaparib; Ruca, rucaparib. SD, Standard Deviation.
Figure 4. PARPi generate cytoplasmic chromatin fragments in a DNA repair defect- and cell cycle-dependent manner. A, Schematic of the generation of BRCA1-revertant and PARP1-knocked-out cell lines from the parental BRCA1-mutant SUM149 TNBC cell line. B, Representative immunofluorescence images of DMSO-, Ruca- and Ola-exposed SUM149-BRCA1mut and SUM149-PARP1rev cells. Blue, DAPI; Red, α-Tubulin; Green, PicoGreen. Cells were exposed to 6 μM Ruca, 10 μM Ola or DMSO (vehicle) during 72h. White arrows, CCF; Yellow arrows, micronuclei. Scale bar, 20 μm. C, Automated quantification of CCF in SUM149-BRCA1mut, SUM149-BRCA1rev and SUM149-PARP1rev cells exposed to increasing doses of Ruca or Ola (μM). Shown are CCF number per cell normalized to DMSO. Mean ± SD, N = 3, Kruskal-Wallis test and post hoc Dunn’s test, relative to DMSO control. Results shown are representative of 2 experiments performed with similar results. D,
Western blot of Histone H3 in the nuclear and cytoplasmic fractions of SUM149-BRCA1\textsubscript{mut} and SUM149-PARP1\textsuperscript{−/−} cells exposed to PARPi during 48h. β-tubulin and lamin B1 are used as fractions purity controls. E, F, Automated quantification of CCF in A549-ERCC1\textsuperscript{WT/WT} (E) and SUM149-BRCA1\textsubscript{mut} (F) cells exposed to increasing doses of Ruca or Ola (μM) in the presence or absence of the cell cycle blocker CDK1i RO-3306. Shown are CCF number per cell normalized to DMSO. Mean ± SD, N = 3, Kruskal-Wallis test and post hoc Dunn’s test, relative to DMSO control. Abbreviations: CDK1i, CDK1 inhibitor; Ola, olaparib; Ruca, rucaparib. SD, Standard Deviation.
Figure 5. PARPi-induced cytoplasmic chromatin fragments are detected by cGAS. A, Representative immunofluorescence images of DMSO-, Ruca- and Ola-exposed A549-ERCC1^{WT/WT} and A549-ERCC1^{-/-} cells. Green, PicoGreen; Orange, cGAS. Cells were exposed to 15 µM Ruca or 40 µM Ola during 72h. White arrows, CCF; Yellow arrows, micronuclei. Scale bar, 20 µm. Images corresponding to the DMSO condition originate from the same image field as those displayed in Figure 3B. B, Automated quantification of cytoplasmic cGAS foci in A549-ERCC1 isogenic cells exposed to increasing doses of Ruca or Ola (µM). Shown are cytoplasmic cGAS foci number per cell normalized to DMSO. Mean ± SD, N = 3, Kruskal-Wallis test and post hoc Dunn’s test, relative to DMSO control.
Results shown are representative of 2 experiments performed with similar results. C, Representative immunofluorescence images of DMSO-, Ruca- and Ola-exposed SUM149-BRCA1\textsubscript{mut} and SUM149-PARP1\textsuperscript{+} cells. Green, PicoGreen; Orange, cGAS. Cells were exposed to 6 µM Ruca, 10 µM Ola or DMSO (vehicle) during 72h. White arrows, CCF; Yellow arrows, micronuclei. Scale bar, 20 µm. D, Automated quantification of cytoplasmic cGAS foci in SUM149-BRCA1\textsubscript{mut}, SUM149-BRCA1\textsubscript{rev} and SUM149-PARP1\textsuperscript{+} cells exposed to increasing doses of Ruca or Ola (µM). Shown are cytoplasmic cGAS foci number per cell normalized to DMSO. Mean ± SD, N = 3, Kruskal-Wallis test and post hoc Dunn’s test, relative to DMSO control. Results shown are representative of 2 experiments performed with similar results. E, Scatter box plots displaying cGAS foci intensity for each co-localizing CCF foci in A549-ERCC1\textsuperscript{WT/WT} and A549-ERCC1\textsuperscript{+} cells exposed to DMSO (vehicle), 15 µM Ruca or 40 µM Ola. N = 3, Kruskal-Wallis test and post hoc Dunn’s test. Abbreviations: Ola, olaparib; Ruca, rucaparib. SD, Standard Deviation.
Figure 6. cGAS-mediated detection of PARPi-induced cytoplasmic chromatin fragments activates STING signaling in an ERCC1-dependent manner. A, B, Western blot of phospho-TBK1 in A549-ERCC1WT/WT and A549-ERCC1-/- cells (A) or H1975-ERCC1WT/WT and H1975-ERCC1-/- (B) upon PARPi exposure. Cells were exposed for 48h to DMSO (vehicle) and a dose range of Ola (A), or DMSO, 25 µM Ruca and 40 µM Ola (B). Lysates were probed with the indicated antibodies. C, Western blot of phospho-TBK1 in DMSO- or Ruca-treated H1975-ERCC1WT/WT cells in the context of siRNA silencing of cGAS/STING. Cells were transfected with siCTRL, siSTING, sicGAS or siSTING+sicGAS, exposed to DMSO (vehicle) or 25 µM Ruca, and lysates were probed with the indicated antibodies. D, Western blot of phospho-TBK1 in DMSO- or Ola-treated H1975-ERCC1WT/WT cells upon cell cycle blockade. Cells were exposed to DMSO, 20 µM or 80 µM Ola in the presence or absence of the cell cycle blocker CDK1i RO-3306. Lysates were probed with the indicated antibodies. Bar plot: pTBK1/TBK1 intensity was measured for each condition and normalized to DMSO. Mean ± SD, N = 3, Kruskal-Wallis test and post hoc Dunn’s test, relative to DMSO control. Abbreviations: CDK1i, CDK1 inhibitor; Ola, olaparib; Ruca, rucaparib. SD, Standard Deviation.
Figure 7. PARPi induce secretion of the chemotactic chemokine CCL5 in a cGAS/STING-dependent manner, and activate type I IFN signaling. A, RT-qPCR analysis of RNA isolated from...
Ola-exposed A549-ERCC1\textsuperscript{WT/WT} and A549-ERCC1\textsuperscript{-/-} cells, in the presence or absence of cGAS/STING silencing by siRNA. Cells were transfected with siCTRL or sicGAS + siSTING and treated for 72h with DMSO or a dose range of Ola (µM). CCL5 mRNAs were analyzed relative to GAPDH. Box-and-whisker plots show arbitrary units of gene expression, normalized to DMSO-treated control. Boxes indicate median, lower and upper quartiles; Whiskers indicate the 5\textsuperscript{th} to 95\textsuperscript{th} percentile range; \(N = 12\), Kruskal-Wallis test and \textit{post hoc} Dunn's test, relative to DMSO control. B, RT-qPCR analysis of RNA isolated from A549-ERCC1\textsuperscript{WT/WT} and A549-ERCC1\textsuperscript{-/-} cells, in the presence or absence of cGAS/STING silencing by siRNA. Cells were transfected with siCTRL or sicGAS + siSTING. CCL5 mRNAs were analyzed relative to GAPDH. Shown are arbitrary units of gene expression, normalized to A549-ERCC1\textsuperscript{WT/WT} DMSO-treated control. Mean ± SD, \(N = 4\), two-way ANOVA test. C, Quantitative analysis of CCL5 secretion in A549-ERCC1 isogenic cells supernatants upon Ola exposure, in the presence or absence of cGAS/STING silencing by siRNA. Cells were transfected with siCTRL or sicGAS + siSTING and treated for 72h with DMSO or a dose range of Ola (µM). Supernatants were collected and analysed by ELISA for detection of CCL5. Box-and-whisker plots show CCL5 concentrations. Boxes indicate median, lower and upper quartiles; Whiskers indicate the 5\textsuperscript{th} to 95\textsuperscript{th} percentile range; \(N = 4\), Kruskal-Wallis test and \textit{post hoc} Dunn’s test, relative to DMSO control. D, E, GSEA of the REACTOME pathway Interferon Alpha Beta Signaling in Talazoparib vs DMSO-treated A549-ERCC1\textsuperscript{WT/WT} cells (D) or A549-ERCC1\textsuperscript{-/-} cells (E). A heatmap displaying the genes of the pathway is shown below. \(N = 3\); Heatmap scale is a Z score. Purple, significantly DEGs with FDR < 0.05 and |LFC| > 1; Green, significantly DEGs with FDR < 0.05 and |LFC| > 0.58; Grey, non-significantly DEGs. Abbreviations: DEGs, Differentially Expressed Genes; ELISA, Enzyme-Linked Immunosorbent Assay; LFC, \(\log_2\) (fold-change); Ola, olaparib; Talazo, talazoparib; SD, Standard Deviation.
Figure 8. ERCC1-deficiency and PARPi exposure potentiate IFNγ-induced cell-surface PD-L1 expression. A, Quantification of PD-L1 cell surface expression by flow cytometry in H1975-ERCC1WT/WT and A549-ERCC1WT/WT cells upon PARPi and IFNγ exposure. Cells were treated for 48h with DMSO, 15 µM Ruca, 3 µM Talazo, 10 µM Nira, and/or 500 U/mL IFNγ. MFI ± SD normalized to IFNγ; N = 4, two-way ANOVA and post hoc Tukey’s test. B, Corresponding flow cytometry histograms; shown is the percentage of PD-L1-positive cells. C, Quantification of PD-L1 cell surface expression by flow cytometry in A549-ERCC1WT/WT, A549-ERCC1−/−, H1975-ERCC1WT/WT and H1975-ERCC1−/− cells treated for 48h with DMSO, 3 µM Talazo, and/or 500 U/mL IFNγ. MFI ± SD; N = 3, two-way ANOVA and post hoc Tukey’s test. D, RT-qPCR analysis of RNA isolated from A549-ERCC1WT/WT
and A549-ERCC1<sup>WT</sup> cells exposed to PARPi and/or IFNγ. Cells were treated for 48h with DMSO, 3 μM Talazo or 13.5 μM Ruca, and/or 500 U/mL IFNγ. PD-L1 mRNAs were analyzed relative to GAPDH (to control for cDNA quantity). Shown are arbitrary units of gene expression, normalized to A549-ERCC1<sup>WT/WT</sup> DMSO-treated control. Mean ± SD, N = 3, two-way ANOVA and post hoc Tukey’s test.

E, Quantification of PD-L1 cell surface expression by flow cytometry in SUM149-BRCA1<sub>mut</sub> and SUM149-PARP1<sup>−</sup> cells treated for 48h with DMSO, 3 μM Talazo, and/or 500 U/mL IFNγ. MFI ± SD; N = 3, two-way ANOVA and post hoc Tukey’s test. Corresponding flow cytometry histograms are displayed to the right. Abbreviations: MFI, Mean Fluorescence Intensity; Nira, niraparib; Ola, olaparib; Ruca, rucaparib; SD, Standard Deviation; Talazo, talazoparib.
Figure 9. PARPi induce PD-L1 expression in patient-derived NSCLC cells and high PD-L1 expression associates with low PARylation in human NSCLC samples. A, Quantification of PD-L1 cell surface expression by flow cytometry in the EpCam-positive cells of a pleural effusion sample upon PARPi and/or IFNγ exposure. Cells were treated in vitro for 48h with DMSO, 10 µM Nira, 500 U/mL IFNγ or both. MFI of a single staining is shown. B, Scatter box plot depicting PARylation levels and tumor cell expression of PD-L1 (as assessed by IHC staining) in a series of resected stage I/II NSCLC (invasive adenocarcinomas and squamous cell carcinomas) samples (n = 49). Mann-Whitney U test. C, Representative images of PAR and PD-L1 IHC stainings in surgical specimens of NSCLC. Case A displays low PD-L1 staining in tumor cells and high PARylation levels; Case B displays high PD-L1 staining in tumor cells and low PARylation levels. Scale bar, 50 µm. Abbreviations: IHC, immunohistochemistry; Nira, niraparib.
Figure 10. Model of ERCC1 defect-dependent activation of cGAS/STING following PARPi exposure. A, ERCC1WT/WT cells have a functional DNA damage response and adequately maintain genome integrity. B, Upon PARPi exposure, exogenous DNA damage is triggered, mostly initiated by PARP1 itself trapped onto the DNA at sites of spontaneous SSBs. C, During the S-phase of the cell cycle, trapped PARP1 generates lesions which prevent the progression of replication forks, leading to fork stalling and subsequent formation of DSBs. In ERCC1WT/WT cells, most trapped-PARP1 lesions are removed, which enables the processing of DSBs through HR and eventually allows replication to restart. Residual inadequately repaired lesions cause moderate formation of CCF. D, The low levels of CCF generated are unable to trigger the pTBK1/IRF3/NFκB signaling cascade or subsequent transcription of type I IFN genes; PD-L1 is moderately induced. E, ERCC1−/− cells are exposed to increased endogenous DNA damage following the loss of ERCC1. This generates minimal levels of CCF. F, Upon PARPi exposure, ERCC1−/− cells are subjected to an additional exogenous source of DNA damage. G, During the S-phase of the cell cycle, trapped-PARP1 generates lesions which prevent the progression of replication forks, leading to forks stalling and subsequent formation of
DSBs. In the absence of ERCC1, trapped-PARP1 lesions cannot be adequately resolved, which triggers increased DSBs and eventually generates high levels of CCF. H, CCF are detected by cGAS and, due to the enhanced expression of STING in ERCC1<sup>−/−</sup> cells, these efficiently activate cGAS/STING signaling. Activated STING homodimer phosphorylates TBK1, which in turn phosphorylates IRF3 and NFκB; this triggers their translocation into the nucleus and results in the transcription of type I IFN genes: CCL5 and other type I IFN cytokines are secreted. Higher PD-L1 expression is induced at the cell surface. Abbreviations: CCF, Cytoplasmic Chromatin Fragments; DSB, Double-Strand Break; HR, Homologous recombination; IFN, interferon; SSB, Single-Strand-Break.